PHYTOREMEDIATION POTENTIAL OF *CANNABIS SATIVA* L. GROWN ON METALLIFEROUS SOIL AND METAL RECOVERY

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Environmental Science

Institute of Environmental Sciences and Engineering
School of Civil and Environmental Engineering
National University of Sciences and Technology
Islamabad, Pakistan
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(2024)

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Dedication

All my achievements are dedicated to my beloved parents for their endless love, support, and encouragement.

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Afsheen Fatima

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ABSTRACT

Heavy metals in soil, especially at higher concentrations near industrial zones, present potential hazards to human well-being and agricultural productivity. In this study, the ultimate goal was to examine *Cannabis sativa* L. potential in the remediation of metalliferous soils using phytoremediation techniques. The research included growing *Cannabis sativa* L. seeds using two different soil compositions. The research findings demonstrate a significant decrease in many plant growth indicators within Soil Type 2 compared to Soil Type 1. The study examined variations in photosynthetic parameters under both soil types. The relative chlorophyll content exhibited a reduction of 17% under Soil Type 1. The PhiNPQt and PhiNO decreased 47% and 97%, while Phi2 demonstrated a decline of 33% under Soil Type 2. The study detected variations in the enzymatic responses of antioxidants influenced by the presence of heavy metals. The quantity of hydrogen peroxide (H_2O_2) in plants grown in Soil Type 2 showed a reduction of 42%. There was a significant increase in levels of superoxide dismutase (SOD; 20%), catalase (CAT; 27%), and peroxidase (POD; 24%), respectively. The root part of the plants significantly had a higher concentration of heavy metals. Consequently, the recorded values for BCF, TF, and EC_f were below factor 1, suggesting limited translocation and bioconcentration inside the plant. The study used residual plant biomass in a new green synthesis technique. For this purpose, zinc oxide (ZnO) nanoparticles were made using the waste material. Thus, this research presents a valuable methodology for extracting potentially toxic metals and the possible role that *Cannabis sativa* L. plays in solving the soil contamination problem via phytoremediation in regions affected by heavy metal contamination

CHAPTER 1

INTRODUCTION

Industrial applications extensively use heavy metals, although their prolific production and utilization carry a considerable cost: harmful consequences for human health and the extensive agricultural lands responsible for global food production (Idris et al., 2023). As industries in 2023 depend on sophisticated technologies for sustainability, the importance and urgency of this issue have escalated. Battery cells, solar panels, and electric vehicle batteries exemplify renewable technologies that greatly depend on the utilization of potentially harmful metals (Ahmed et al., 2022). Concurrently, the never-ending increase in population, especially in underdeveloped regions, necessitates the need for more fertile lands for crop cultivation, which also continues to grow.

The residue of metals from the extraction and refining of metal-rich ores, along with the fallouts from power lines, urban waste, fertilizers, pesticides, and sewage, has rendered vast tracts of land unfit for crop cultivation (Din et al., 2023). It is essential to remember that metals do not undergo decomposition but instead experience changes in oxidation states or organic complex transformations (Maqsood et al., 2022). Remediation strategies for polluted soils are often costly and inflict further environmental harm. Researchers have tested multiple techniques to find a reliable solution to remediate the contaminated soils. These approaches include soil burial, chemical treatment to immobilize metals, and using acidic solutions to eliminate metals from excavated soil, substituting the purified soil residue.

The use of phytoremediation for soil pollution cleaning has gained widespread acceptance due to its practicality, cost-effectiveness, and ecological viability. The pollutants can be restricted, removed, or neutralized using plants of various species tolerant to abiotic stresses (Shen et al., 2022). Numerous plants are recognized for their capacity to accumulate these metal pollutants. However, the extent of this capacity exhibits significant variations. Many plants that accumulate metals are mainly characterized as small shrubs with limited root depth, necessitating specific cultivation techniques to ensure optimal growth and development. To effectively remediate the soil, cleaning the top layer is insufficient; deeper soil layers must also be free from pollutants. Hemp is particularly advantageous in this respect, with a deep subsurface root system that facilitates the extraction of metallic elements from the soil, and its commercial viability further enhances its appeal. Hence, hemp can serve as a commercially profitable crop for phytoremediation.

1.1. Cannabis: History and Uses

Cannabis sativa L. is a plant species widely recognized for its multiple uses throughout civilizations, such as hemp or marijuana (Vīsković et al., 2023). Initially, it gained prominence for its versatile applications in producing textiles, including rope, clothing, paper, and ship sails (Zimniewska, 2022), evident in historical records from ancient societies in the Middle East and Asia (Aloo et al., 2022). The earliest use of *Cannabis sativa* L. dates back to 13,000 - 2,000 B.C., with archaeological evidence from sites in China and Taiwan showing its utilization in pottery decoration, rope, and clothing production (Siracusa et al., 2023). Nomadic tribes significantly spread *Cannabis sativa* L. across Asia and eventually to Neolithic Europe (Mechoulam, 2019).

While initially gathered from natural habitats, *Cannabis sativa* L. underwent domestication due to agricultural advancements (Clarke & Merlin, 2016). Historical sources suggest intentional plant cultivation for about 8,500 years, with hemp extensively cultivated in the western hemisphere for grain and fiber (Zhang et al., 2018). Jativa, Spain, had the first paper manufactured using hemp in 1150 (Charitos, 2021).

The global dissemination of Cannabis is closely linked to human migrations, enabling its integration into various cultures and civilizations. Hemp was a crucial player in the Americas' maritime endeavors. It was used in sail and rope production for ships crossing the Atlantic in 1492 (Rull et al., 2022). In the last eight decades, the historical significance of *Cannabis sativa* L. has been overshadowed by conflicts, particularly with the United States' classification of its active compound (i.e., THC) as a schedule 1 drug, leading to bans in other countries (Rubens, 2014). However, the negative perception of possible negative uses of Cannabis has changed with the growing recognition of its various upbeat benefits. While textile manufacture remains relevant, the modern popularity of Cannabis is primarily attributed to its biochemical properties.

1.2. Types of Cannabis

Cannabis, part of the Cannabaceae family, encompasses three species: *C. sativa* var. sativa, indica, and ruderalis, each with distinct physiological traits influenced by geography (Jin et al., 2020). A robust plant, hemp endures various conditions, flourishing particularly near high-moisture regions. Different cultivation techniques yield varied plant characteristics. Increased planting density results in longer stems and more fiber, whereas broader spacing promotes branching and a higher flower and seed yield (Tanney et al., 2021). Hemp's resilience to pests and diseases minimizes the need for chemical treatments, mainly due to aromatic terpenoids and cannabinoids produced by trichomes (Arey et al., 2022). The distinction between hemp and marijuana lies in the THC concentration; hemp is characterized by its low THC content, often measuring less than 0.3%., while marijuana may contain up to 20% (Khajuria et al., 2020; Bozman et al., 2022).

1.3. Medicinal Characteristics

C. sSativa's various therapeutic attributes are increasingly recognized in the medical field. Compounds from *Cannabis sativa* have been demonstrated to soothe and remedy a wide range of ailments and conditions, such as reducing ocular tension associated with glaucoma and curbing epileptic seizures. Central to its therapeutic applications are cannabinoids, compounds found in *C. sativa*. Till now, more than 125 cannabinoids have been differentiated from C. sativa, with THC and CBD being the major well-understood (Radwan et al., 2021). These cannabinoids, a diverse collection of phenolic terpenoid compounds, offer protective benefits to the plant, safeguarding it against UV radiation at elevated altitudes and serving as a deterrent to herbivores.

Notably, these cannabinoids can interact with the mammalian endocannabinoid system. Moreover, the G protein-coupled receptors CB1 and CB2 are essential for humans. These receptors facilitate all of the manifestations of therapeutic and psychoactive effects in the consumer. These receptors play a substantial part in interacting with mammalian endocannabinoids, particularly N-arachidonoylethanolamine (anandamide) (Finn et al., 2021). Anandamide, a vital endogenous cannabinoid, functions in the peripheral and central nervous systems. Among other functions, anandamide is crucial in early embryonic development, neural creation, and feeding control, and it has demonstrated inhibition of breast cancer cell growth (Mock et al., 2022).

1.4. Heavy Metal Contamination in Soils

An increasing demand for available land has increased in parallel with the development of societies and economies since the beginning of the 21st century (Vardhan et al., 2019). Factors such as production activities, fossil fuel combustion, metal mining, non-standard use of pesticides and fertilizers in agriculture, and daily wastewater discharge have led to environmental harm, imbalance in land ecology, and an annual increase in soil pollution, contributing to land degradation (Zhang & Wang, 2020). The main factors contributing to land degradation are erosion, desertification, salinization, pollution, and reduced fertility (Mohamed et al., 2019). Heavy metal contamination has become of utmost importance due to its enduring persistence over extended periods, irreversibility, limited transfer amount, severe toxicity, complexity, and ecological impacts, making it the most pressing issue in soil pollution (Azimi et al., 2017).

1.5. The Role of Phytoremediation

It is a method for remediation and restoration of polluted environments where plants are at the center of the strategy. Plants and microorganisms can degrade, accumulate, and stabilize pollutants within the surrounding environment (Shah & Daverey, 2020). This includes various types of plants, such as grasses, shrubs, and trees. Those above "green technology" can reduce the number of secondary byproducts generated, concurrently eradicating soil contaminants, including heavy metals and organic pollutants. This method is characterized by its user-friendly nature, feasibility, affordability, aesthetically pleasing design, and overall positive reception.

Phytoextraction and phytoaccumulation are terms used to describe the biological processes by which plants absorb and retain heavy metals inside their tissues for future use. Phytovolatilization, conversely, pertains to the emission of these metals into the atmosphere. At the same time, phytostabilization denotes the process of immobilizing these metals in the growth medium, especially near the roots (phytostabilization) (Shen et al., 2022).

Phytoextraction is when plants absorb hazardous metals with water and vital nutrients. Phytoaccumulation refers to the mechanism through which a plant absorbs a heavy metal, precipitates it, and sequesters it in its aerial components (Corzo et al., 2020).

Plants can turn metals volatile and let them out in the open environment through stomatal cells (phytovolatilization) (Babu et al., 2021). This is only possible for a few metals (i.e., Hg, Se, and As) and amenable target elements for phytovolatilization (Naikoo et al., 2020) The technique of phytostabilization can decrease bioavailability. Absorption of metals is significantly aided by roots, subsequently causing them to become immobilized.

1.6. Use of Hemp for Phytoremediation

Hemp is recognized for its rapid growth and expansive, deep root system (Placido & Lee, 2022). This plant is resilient and can thrive in diverse soil conditions and a broad range of climates (Rehman et al., 2021). Research indicates its robust tolerance to metals and capability to absorb and store them in various plant parts without harming itself (Yin et al., 2022). During the process of phytoremediation, it has been shown that contaminants tend to accumulate inside various plant structures, including the root system, stems, and leaves (Kafle et al., 2022). Hence, the stems possess potential use in the construction industry, paper production, textile manufacturing, and as a source of biofuel. In contrast, the leaves are often left unharvested for consumption or use in products for personal use. (Shmaefsky, 2020).

Hemp's remediation effectiveness is well-documented, with successful usage for decontaminating soil heavily polluted by the 1986 Chornobyl nuclear disaster since 1998 (Placido & Lee, 2022). In addition, in 2008, it was used in an agricultural region in Italy to solve the problem of dioxin and some more pollutants that the proximity of a steel plant had introduced. (Valentukeviciene et al., 2022). *Dioxins* are hazardous chemicals that have been linked to cancer, congenital disabilities, weakened immune systems, and altered hormone levels. Once the dioxin-contaminated plant material has been cleaned up, it may be used to generate electricity. Hemp fibers are also being studied as a filter for mitigating metals from contaminated water (Tofan et al., 2020), expanding their remediation capabilities beyond soil.

1.7. Green Synthesis of Nanoparticles from Waste Material

The pursuit of "Sustainable development" is closely associated with "green chemistry" based on research within the past 15 years (Chen et al., 2020). The chemical industry places significant emphasis on addressing pollution concerns and minimizing the excessive utilization of natural resources, making sustainable development a critical priority within this sector (Sheldon, 2012). There is a prevalent association made by the general public between the term "chemical" and concepts such as "hazard" or "toxic" (Anastas & Eghbali, 2010). This connection is rooted in a preexisting perception that the field of chemistry is inherently perilous. Thus, it is essential to use a green approach to synthesizing nanoparticles.

Using traditional chemical methods can often be excessively expensive and entails using hazardous and toxic substances, which give rise to various environmental issues (Duan et al., 2015). The biosynthetic methodology is viable for biomedical applications where microorganisms and plants are used in the synthesis process. This technique offers several advantages: safety, biocompatibility, and environmental friendliness (Brar et al., 2022). Various plant components, including leaves, fruits, roots, stems, and seeds, in synthesizing nanoparticles, have been attributed to phytochemicals in their extract. These phytochemicals stabilize and reduce agents, as Kharissova et al. (2013) noted.

1.8. Scope of this Thesis

This thesis explores the potential of hemp for phytoremediation, given its various inherent qualities like substantial biomass, quick growth and development, extensive root systems, and adaptability to diverse conditions. Focusing on hemp's capacity to remediate heavy metals, this research scrutinizes plant tolerance, performance, and root-and-shoot accumulation of these toxic elements under controlled conditions. The research aims to augment the knowledge of hemp's phytoremediation capabilities for various pollutants, hoping to employ hemp to restore polluted industrial sites while yielding valuable by-products.

1.9. Objectives of the Study

This research seeks to accomplish three primary goals:

1. Evaluation of *Cannabis sativa* L. phytoremediation potential grown on metalliferous soil.

2. To analyze the plant growth and antioxidant responses of Cannabis plants under multiple metal stress.
3. Metal recovery by using a green synthesis approach.

CHAPTER 2

LITERATURE REVIEW

2.1. Heavy Metal Contamination in Soil

The levels of environmental pollution have experienced a significant increase in recent years, culminating in hazardous conditions in specific regions. Heavy metals and metalloids pose significant risks as pollutants due to their ability to harm living organisms extensively (Jjemba, 2004). We cannot expect heavy metals to degrade naturally or become less or non-toxic (Gupta et al., 2003). Some heavy metals may benefit natural systems, but their concentration needs to be low; however, this differs for As, Pb, and Cd.

According to the study conducted by Puttaiah and Kiran (2008), the presence of essential metals in high concentrations has been shown to have adverse repercussions. Zinc and iron oxides have the potential to induce irritation of the skin and mucous membranes, as well as gastrointestinal discomfort and emesis. A significant body of research exists that establishes a connection between exposure to most toxic heavy metals and elevated susceptibility to cardiovascular disease, leukemia, and cancer, as reported by Drasch et al. (2005). In contrast, magnesium (Mg) and cobalt (Co) have divergent correlations, with magnesium being associated with hypertension and cobalt being associated with anemia. Among other diseases, Asthma, and bronchitis/emphysema are two respiratory issues linked to heavy metal exposure (Pope, 2000; Wiwatanadate & Liwsrisakun, 2011). Additionally, cardiovascular disease has also been associated with heavy metal exposure, as indicated by studies conducted by multiple researchers (Schwartz, 2001; Miller et al., 2007).

The prevalence of heavy metal contamination in several environmental compartments in Pakistan's ever-increasing and uncontrolled population increase has resulted in the expansion of industry and other anthropogenic activities. The components include many environmental mediums, such as air, soils, groundwater, surface water, wastewater, and urban traffic dust. Likewise, several health disorders in the U.S.A. have been linked with exposure to toxic heavy metals (Haq et al., 2012). There is more evidence in the form of reports showing an increase in heavy metal concentrations in food items and various region's agricultural soils in Pakistan (Azizullah et al., 2011; Waseem et al., 2014). The presence of these toxic heavy metals in food items is above permissible limits. Many primary and secondary studies conducted thus far have primarily concentrated on quantifying detrimental metal concentrations, with limited consideration given to remedial measures. Thus, there is a need to focus on finding suitable remediation techniques to solve this serious environmental problem.

2.2. Sources of Pollution: Heavy Metals in Pakistan's Soil

2.2.1. Agricultural Activities

Heavy metals come from all types of pesticides, fertilizers, and everything else used in agriculture, including groundwater and sewage sludge. These significant contributors to this problem often go unnoticed. In addition, the application of animal excrement as a fertilizer has been linked with heightened concentrations of cobalt (Co). Likewise, it may also lead to an increase in zinc (Zn), manganese (Mn), and copper (Cu), as reported by Verkleji (1993). Contrarily, prior research has shown that using sewage as a fertilizer often leads to heightened contamination of heavy metals. Pakistan is not exempt from the prevailing trend since pesticides and chemical fertilizers have been identified as significant contributors to global pollution (Khan et al., 2013). The utilization of agrochemicals in agricultural practices significantly contributes to water pollution, as these substances are frequently applied to fields in conjunction with irrigation water.

Consequently, they permeate through the soil and ultimately find their way into adjacent water bodies. When these agrochemicals are applied or released into the air through volatilization, they both similarly contribute to air pollution. Moreover, there is an increasing trend in the deposition of toxic heavy metals in agricultural land and water bodies.

2.2.2. Industrial Effluents

Various forms of industrial waste release toxic metals into the surrounding environment. Previous research conducted by Sial et al. (2006) and the World Wildlife Fund (WWF, 2007) has shown a correlation between the petrochemical, fabric, medicinal products, ceramics, food, oil and steel mill, leather tanning, sugar, and fertilizer industries in Pakistan and elevated levels of pollution. The presence of metal contamination has been associated with the discharge of effluents and sewage sludge originating from many industrial sectors (Nagajyoti et al., 2010), including but not limited to textiles, plastics, paper, microelectronics, and wood preservatives. There are variations in how coal-fired, petroleum-fired, and nuclear power plants release deleterious metals into the surrounding environment. Verkleji (1993) posits that the presence of high-tension power lines has the potential to augment the release of several detrimental elements, like selenium (Se), boron (B), and more.

In Pakistan, significant contributors to heavy metal contamination are textile, metal fabrication, refinery, petrochemical, food processing, and paper and pulp industries. These enterprises generate substantial quantities of wastewater, which has been linked to an increasing contamination of the environment. As mentioned above, As, Cd, Cu, Cd, Pb, and Zn are only some metals and metalloids in this wastewater effluent (Ullah et al., 2009). In Pakistan, a total of 6,634 industries have been officially recognized. However, Sial et al. (2006) stated that a mere 1228 of these industries can be classified as significant contributors to pollution. According to the findings of Murtaza and Zia (2012), a mere eight out of the 388 cities in Pakistan were equipped with wastewater treatment facilities. Furthermore, their research revealed that only three of these facilities were fully operational. The cities above encompass Islamabad, Karachi, Faisalabad, and Lahore.

2.2.3. Pollution from Natural Sources

A combination of natural and artificial processes influences the discharge of toxic metals into the environment. Parent constituents inside the Earth's crust are the main culprit when releasing such toxic metals in water bodies and land areas. The subsurface geological composition and weathering mechanisms contribute to variations in metal concentrations within the soil.

Aside from widely known toxic metals like As, Pb, and Hg, elements like chromium (Cr), manganese (Mn), and tin (Sn) are also widely present in geological parent materials and have the responsibility for the observed escalation in heavy metal concentrations within the immediate vicinity of their operations (Kafayatullah et al., 2001). According to Nagajyoti et al. (2020), pollutant metals can also originate from forest fires and marine ecosystems, which emit aerosols into the atmosphere. Plants have been shown to contribute to environmental metal pollution via the leaching processes occurring in their stems and leaves.

Multiple sources of such pollution can be categorized into vehicular emissions, trash from urban areas, effluents released by industries, and more (Xia et al., 2011). The frequency of hazardous metal pollution in Pakistan is attributed to a diverse range of human and natural activities, including the discharge of industrial effluents and pollutants, agricultural methods, residential waste disposal, atmospheric deposition, vehicular emissions, metals processing, and other human pursuits, in conjunction with a multitude of natural phenomena, together contribute to the deterioration of the environment.

2.2.4. Pollution from Domestic Sources

Toxic metals are frequently present in household and municipal wastewater, leading to substantial environmental contamination. Based on a study by Beede and Bloom (1995), the global production of domestic and municipal solid waste exceeded 1.3 billion metric tons in 1990. However, a significant challenge municipal organizations face in developing countries is the limited availability of resources and expertise to address this issue effectively. Numerous nations have realized that existing waste management strategies must be improved to fulfill their objectives for sustainable development (Qdais, 2007).

According to a study conducted by the researcher, the disposal of various wastes (i.e., household and municipal) in Pakistan has the potential to adversely affect several components of the local infrastructure, including sewers, drainage systems, water bodies, neighboring farms, and indoor septic tanks (Murtaza & Zia, 2012). The absence of adequate treatment systems nationwide results in the lack of wastewater treatment, except in Karachi and Islamabad, where only a portion of the wastewater undergoes treatment before discharge. Even assuming all treatment facilities across the nation were operating at maximum capacity, a mere 8% of urban municipal and residential wastewater would have undergone treatment. The sedimentation ponds at most of these facilities exhibit inefficiency, leading to an approximate treatment rate of 1 percent (Murtaza & Zia, 2012).

2.2.5. Emissions from Vehicles

When examining the factors contributing to pollution, notably air pollution, automobiles are often identified as a significant contributor (Li et al., 2001). The scarcity of resources in Pakistan presents challenges in identifying the specific factors contributing to urban air pollution. However, prior research has identified the road transport sector as the primary contributor to this issue (Qadir, 2002). Studies undertaken by reputable authorities (i.e., WHO, EPA) have consistently shown that automobile air pollution has exceeded regulation limits over the last twenty years (Qadir, 2002).

Metropolitan regions have a higher prevalence of heavy metals, mainly attributed to the elevated vehicular density and the consequent emission of exhaust, which then permeates the soil near the source. Researchers conducted a study that revealed the presence of heightened amounts of heavy metals (i.e., Pb, Zn, Ni, and Cu) in air and soil samples collected near roadways (Parveen et al., 2012). Tires, diesel engines, and aerosols are significant sources of air pollution resulting from vehicular transportation. Moreover, the widespread use of lubricants is also a cause of concern regarding vehicular emissions (Nagajyoti et al., 2010).

2.3. Possible Soil Heavy Metal Remediation Methods

There have been many suggestions to deal with the soil pollution affecting agricultural lands and water bodies. Even with the many techniques, they can be classified into two groups: in-situ and ex-situ (Gomes et al., 2016). Implementing strategies to address pollutants in soil without physically transferring the contaminated material from its initial site is called in situ. On the other hand, contaminated soil is excavated and relocated to several sites to perform ex-situ cleanup. In situ, remediation offers potential advantages in terms of technical, economic, and environmental aspects, which are not present in ex situ remediation, as highlighted by Song et al. (2017). Nevertheless, according to (Mulligan et al., 2001), other factors such as site characteristics, types of pollutants targeted for removal, the concentration of contaminants, and the intended future use of the affected medium all influence decision-making when selecting the right strategy. Researchers can use several procedures to achieve remediation, including chemical, psychological, and biological approaches.

2.3.1. Chemical Methods

The potential use of chemical remediation to eradicate pollutants is discussed by Song et al. (2017). This experimental protocol involves using specific reagents, processes, and chemical principles. According to Jankaite and Vasarevicius (2005), the fundamental remediation procedures include solidification, vitrification, soil washing, and electrokinetics. The solidification/stabilization approach is the most popular, where the problematic soil is combined with various chemicals or materials. *Solidification* is a physical phenomenon characterized by the confinement of contaminants inside a solid matrix. This matrix is created via the use of various agents that work to bind the pollutants, and it includes materials like asphalt and thermoplastic materials.

On the contrary, stabilization involves the utilization of special chemicals that have properties to make contaminants immobile. According to Hodson et al. (2000), Bonemeal, a finely ground substance, can easily attach itself to metals and results in immobile metal phosphate compounds. Previous studies have demonstrated the efficacy of various waste resources, including lime, eggshell, calcined oyster shell, waste mussel shell, and calcined cockle shell, in the immobilization of metals in polluted soils and the enhancement of soil quality (Lim et al., 2013; Soares et al., 2015; Otero et al., 2015; Islam et al., 2017).

According to Yao et al. (2012), a recommended approach for stabilizing and solidifying soil that has been polluted involves the use of glass-forming precursors. To induce the transition of this chemical into a liquid state, it is subjected to elevated temperatures ranging from 1400 to 2000 degrees Celsius. Subsequently, the liquid is subjected to a cooling process, forming a glass material characterized by a uniform surface texture and absence of interior architectural features. According to Navarro (2012), using a glass matrix to immobilize heavy metals may be attributed to two fundamental interactions: chemical bonding and encapsulation. The predominant method used for immobilization is elevated temperatures during the vitrification process. According to Guo et al. (2006), using effective additives in the vitrification procedure can enhance the encapsulation of pollutants and decrease the probability of leaching. The right strategy for the remediation process of pollution removal entails using water or a suitable washing solution to purify the soil. Numerous academic investigations have yielded empirical data on the effectiveness of specific washing agents in facilitating remediation (Maity et al., 2013). It includes organic acid, surfactants, and chelating agents (Sun et al., 2011; Jiang et al., 2011; Kim et al., 2013).

Ethylenediaminetetraacetic acid (EDTA) can be used for remediation (Lestan et al., 2008). According to Qiao et al. (2017), EDTA has many favorable characteristics, such as its restricted biodegradability, little impact on soil microorganisms and enzyme activity, and easily accessible recycling methods. Electrokinetic (EK) remediation is another way of remediation. Several other processes have also been highlighted (Jankaite & Vasarevicius, 2005), including the use of electricity for various processes (i.e., electromigration and electrophoresis). In addition, electrolysis can also be utilized for remediation purposes. Moreover, chelating compounds represent an additional approach for enhancing the efficacy of electrokinetic (EK) systems in environments characterized by high soil pollution levels.

Chelating agents can influence electrokinetic (EK) efficiency (Song et al., 2016). Several heavy methods were investigated by Song et al. (2016) in order to assess their degree of mobility. Moreover, several scholars have endeavored to implement a cohesive fusion of flushing and electrokinetic (EK) methodologies. The objective of this method is to mitigate the issue of delicate particulate matter, which has the potential to impede the effectiveness of this technology. Moreover, using a pump in the EK flushing technique leads to an improved removal efficiency of heavy metal ions (Co^{2+} and Cs^{+}) (Kim et al., 2008). Likewise, another study examined the removal efficiency of Pb using two-stage electrokinetic (EK) (Ng et al., 2014). On the other hand, uranium pollution removal effectiveness was tested by (Kim et al., 2015) by using industrial-scale washing-electrokinetic (EK) separation. The research done by Li et al. (2016) showed that using electrochemical flushing as a remediation method for soil polluted with chromium led to improved effectiveness of the cleaning process.

2.3.2. Physical Methods

Yao et al. (2012) suggested that physical solutions (i.e., soil replacement, isolation, etc.) can effectively mitigate soil degradation. Soil replacement comprises diverse techniques, including surface capping, landfilling, and encapsulation, which entail the deposition of substantial volumes of uncontaminated soil onto pre-existing contaminated soil. The soil removal process is deemed suitable for addressing the issue of severely polluted soil in cases where the affected area is limited, as it involves significant labor and financial resources. Consequently, it can be utilized to deal with contaminated sites and ultimately improve the environment. Barrier walls surrounding a contaminated region can be constructed to confine the pollutants within its boundaries. In various containment systems, impermeable materials like concrete, steel, bentonite, and grout are often used as physical barriers. These materials are utilized in several configurations, including capping, vertical, and horizontal arrangements. Soil isolation or confinement is not often considered a remediation method, and it has shown efficacy in mitigating the leaching of metals into groundwater via drainage (Jankaite & Vasarevicius, 2005).

The use of subterranean heating in thermal treatment techniques is an effective means to eliminate soil pollution, owing to the fugitive characteristics shown by the pollutants. According to Song et al. (2017), the predominant heating techniques, like electrical resistive heating, are the best. Moreover, other techniques can also be used, for instance, conductive and RF heating. While previous studies have shown the effective removal of high vapor pressure pollutants such as Hg using this method (Chang & Yen, 2006), it is essential to note that it may induce significant changes in the characteristics of the selected soil. Moreover, subjecting soil to a thermal treatment at a temperature of 600°C to eliminate mercury (Hg) resulted in notable alterations in its mineralogical composition and physicochemical characteristics (Roh et al., 2000). According to Huang et al. (2011), it is recommended to use acid washing or chemical extraction techniques for remediation before conducting heat decontamination operations. It is essential to remember this as metals during thermal treatment potentially undermine the removal of mercury (Hg). The transformation of heavy metal compounds in manganese or iron oxides into organic-matter-bound forms can be achieved through thermal treatment at 550°C. Moreover, there can also be acid-extractable and many other residual forms. Furthermore, Huang et al. (2011) observed a decline in chromium, copper, and nickel mobilization and extractability after treatment. This finding suggests that the effectiveness of subsequent decontamination measures may be impacted.

2.3.3. Biological Methods

Bioremediation is a methodology in soil ecosystem remediation that explicitly targets mitigating heavy metal contamination. The approach proposed by Ayangbenro and Babalola (2017) elucidates the use of biological systems inherent in plants and microbes to eliminate, neutralize, or immobilize deleterious pollutants effectively. Bioremediation is a viable and ecologically sound approach to eliminating heavy metals, offering both environmental sustainability and economic feasibility. This technique opposes traditional chemical and physical procedures, which are frequently associated with high expenses and limited effectiveness, particularly in situations where metal concentrations could be higher. Additionally, these conventional methods often produce Substantial quantities of hazardous sludge (Ojuederie & Babalola, 2017). Another study discovered that the expenses associated with remediating one acre of soil polluted with Pb via bioremediation were much lower compared to traditional approaches (i.e., excavation) (Blaylock et al., 1997). The cost reductions achieved by bioremediation ranged from 50% to 65%. Microbes, plants, or a symbiotic relationship between these two organisms may facilitate bioremediation procedures that can lead to remediation. Microorganisms, including bacteria, fungi, and yeast, can effectively remedy heavy metals (Coelho et al., 2015). Numerous microorganisms have undergone comprehensive investigation due to their prevalent use in heavy metal bioremediation. Many bacteria can be used in the remediation process, for instance, *Pseudomonas putida* and *Sporosarcina ginsengisoli* (Achal et al., 2012; Balamurugan et al., 2014)

When it comes to microbial bioremediation, it is generally agreed that using multiple types of bacteria in the remediation process is better than only one. Moreover, scientists have researched heavy metal remediation using bacteria (Kang et al., 2016). In contrast to cultures consisting of a single strain, bacterial combinations exhibited more excellent resistance to heavy metals and enhanced efficacy in pollution remediation. Various methods are already employed for remediation, including precipitation and biosorption (Ojuederie & Babalola, 2017). The combined use of two approaches (i.e., plants and bacteria) to remediate polluted soils has been shown to be more effective and expedient (Vangronsveld et al., 2009).

The findings indicate that *mycorrhizae* can use several mechanisms to influence the alteration of trace metals within the *rhizosphere*. According to Hristozkova et al. (2017), root exudates have the potential to undergo acidification, immobilization, and modification. Additionally, hyphae may be sequestered, or chemical precipitation may occur. The use of *mycorrhizae* has been extensively employed in various remediation processes. Moreover, it was shown that *arbuscular mycorrhizal* fungi obtained from contaminated soils exhibited greater metal toxicity tolerance than those obtained from uncontaminated soils (Cornejo et al., 2013). Bhalerao (2013) observed that using phytostabilization techniques, such as incorporating natural arbuscular mycorrhizal fungus, yielded significantly superior results compared to applying laboratory stains. This observation suggests that the technique above can serve as a viable biotechnological instrument to rehabilitate ecosystems that have undergone degradation.

2.3.4. Phytoremediation

Pollution mitigation using various plants within their indigenous environments is termed phytoremediation (Ali et al., 2013). Moreover, Chibuike and Obiora (2014) recommend that contaminants be distributed extensively over the root zone. Kong and Glick (2017) assert that phytoremediation encompasses diverse mechanisms. These mechanisms can focus on degradation, volatilization, stabilizing the contaminant, filtering it, or extracting it from the growth medium. For instance, the extraction approach through plants can be effectively utilized to take out toxic metals from the growth medium through plant roots. Ultimately, the toxic metals stay in roots and move to upper parts like leaves (Bhargava et al., 2012).

On the other hand, sequestration through the use of plants employs a specific technique called phytofilteration (Dixit et al., 2015). The techniques included in this category consist of rhizofiltration, blastofiltration, and caulofiltration. The primary objective of phytostabilization is to mitigate the mobility and bioavailability of environmental pollutants, as Radziemska et al. (2018) reported. In addition, the volatilization of contaminants can also be achieved through the utilization of plants in the remediation process, where they go through various chemical changes and finally turn volatile and released into the air or collected in a controlled environment. Plants engage in a phenomenon known as phytodegradation, wherein they absorb metal pollutants from the

soil and subsequently undergo metabolic processes to transform or break down these contaminants (Kong & Glick, 2017).
Desirable characteristics in phytoremediation plant species include rapid growth, a well-developed root system, substantial biomass, resistance to elevated metal concentrations, and significant potential for metal accumulation (Jabeen & Iqbal, 2009). Mellem et al. (2012) report that hyperaccumulators possess significant potential for detoxification due to their exceptional capacity for absorption and efficient transport system from roots to shoots, which are inherently endowed with a tolerance of extremely high levels. Brooks et al. (1977) published the classification of plants as hyperaccumulators. It started with the Ni-accumulating plant, as he observed it accumulating in plant leaves at concentrations exceeding 1000 mg/kg dry weight.
Hyperaccumulating plants are characterized by their unique and unmatched accumulation ability, which crosses certain threshold levels of certain metals in their above-ground.e., shoots) within locations polluted with metals, as Baker and Brooks (1989) specified. There are many examples of such hyperaccumulation cases, such as the ability of plants to accumulate 10,000 mg/kg of metals like Mn and Zn according to their dry weight. Such similar examples can also be seen in heavy metals (oid) like As and Cd, where the plants accumulate 100 mg/kg of the dry weight of the said metals. Moreover, there are around 300,000 vascular plants, and a small fraction of 0.2% demonstrates the characteristic of hyperaccumulation (Ent et al., 2013). The discovery of more than 500 species as hyperaccumulators of one or more metals may be attributed to their inherent extracting capabilities. Likewise, many plant species can hyperaccumulate, with these being the majority of hyperaccumulators (Pollard et al., 2014).
AbovegrouAbovegroundare is the primary storage point of many plant species, and they do it without suffering any negative consequences. However, the suitability of most phytoaccumulators for practical use in field phytoremediation must be improved because of their restricted biomass and poor development rates (Rajkumar et al., 2009). Biotechnological methodologies have played an indisputable role in plants' enhanced tolerance to abiotic stress and hazardous metal uptake, encouraging hyperaccumulation (Mosa et al., 2016). Genetically engineered plants have been used to assist the process of phytoextraction via the utilization of metal transporters and improve sulfur metabolism

through augmentation of enzyme synthesis. More importantly, they are also helpful for chelators that detoxify metals (i.e., metallothioneins and phytochelatins). Plants used in such strategies take the heavy metals from the growth medium and store them in different parts (Kotrba et al., 2009). It can be used for various heavy metals (i.e., Pb, Cd, Cu, Hg, As, Se, etc.).

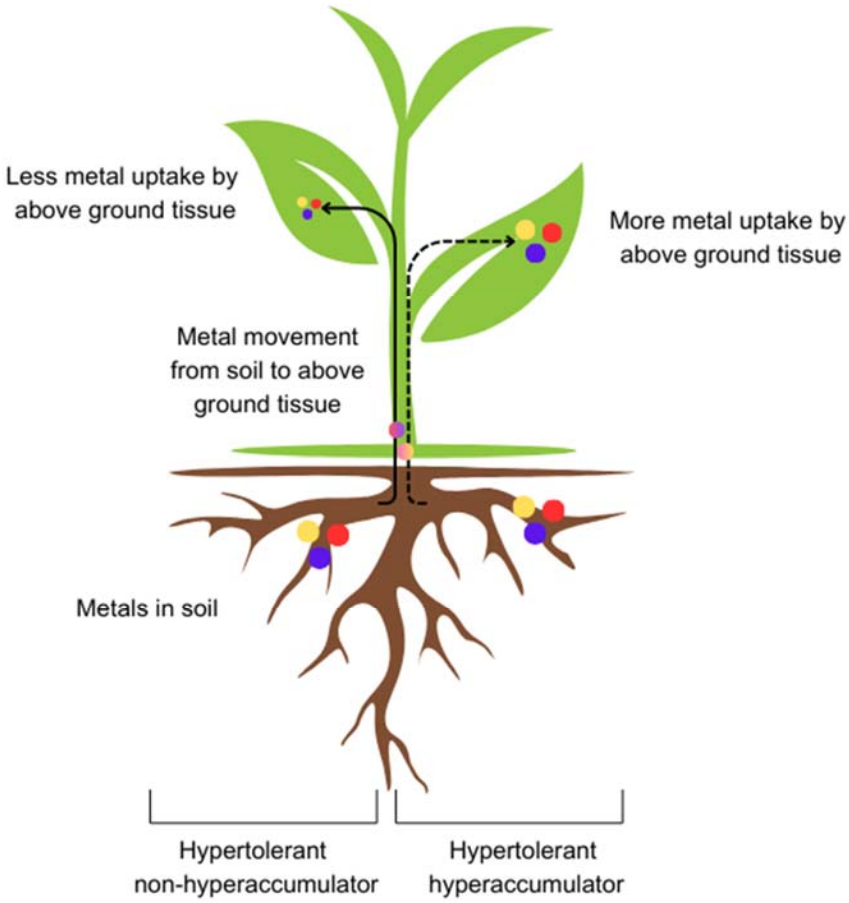

Figure 1: Metal uptake by hyperaccumulator and non-hyperaccumulator plant (Islam et al., 2021)

2.4. Mechanism of Adapting to Heavy Metal Contamination in Higher Plants

Many heavy metals affect plant cells due to their tonal properties, leading to decreased oxidative potential and disruption of various biomolecules, such as glutathione (GSH) (Vroblesky et al., 1992). The redox state of a plant cell can be augmented through the interaction between biomolecules, transition metals, and toxic metals. Moreover, specific noxious metallic elements can disrupt the intermolecular connections within plant proteins and the hereditary material found in living organisms, encompassing RNA and DNA.

According to Buescher et al. (2010), lower amounts of free metal ions may serve as a protective mechanism for plant cells, mitigating the physical harm of toxic metals. The optimization of ionic balance within a cellular system involves a multifaceted series of processes. These encompass the metal accumulation mechanisms, metal transportation into cellular compartments, the intricate interactions between various proteins and metals, and the synthesis of organic ligands, as discussed by Novo et al. (2014).

Choppala et al. (2014) have conducted research revealing the pivotal functions undertaken by specific transporter proteins in this intricate orchestration. These proteins facilitate the accumulation of metals and oversee the translocation of metals into the cellular milieu while mediating the essential interactions between metals and proteins.

Several transporters and metal-binding proteins have emerged within this framework as critical players. Among these, we find zinc (Zn) and iron (Fe) regulated transport proteins (referred to as ZIP transporters), as well as copper (Cu)-chaperone ATX1 proteins, metallothioneins (MTs), and phytochelatins (PCs), as elucidated in previous studies by Chaudhary et al. (2015).

Li et al. (2013) have contributed to our understanding by emphasizing the pivotal role of ZIP transporters in maintaining homeostasis. These transporters are crucial in ensuring the efficient absorption and conveyance of divalent metal ions within the cellular context.

Moreover, in the context of phytoremediation, the involvement of toxic metal ATPase genes can be attributed to their contribution to metal absorption, transport, and sequestration, as highlighted in the research conducted by Chaudhary et al. (2018). Cu-binding domains on protein molecules are postulated to serve a vital role in regulating intracellular Cu concentrations, primarily owing to their inherent capacity to form chelates with Cu, as suggested by Shin et al. (2012). Additionally, it is of noteworthy significance that a substantial level of sequence homology is observed across a range of antioxidant proteins, exemplified by the similarities between ATX1 and ATX2, as expounded upon in research by Shin et al. (2012). During the final phase, organic ligands are synthesized to exert control over plant genes through transcriptional and post-translational mechanisms. The use of molecular techniques in studying hypersensitive mutants of Arabidopsis thaliana has facilitated the discovery of genes implicated in synthesizing organic ligands inside plant tissue (Buescher et al., 2010).

2.4.1. Translocating and Accumulating Heavy Metals

Moreover, hyperaccumulator plant species display heightened metal uptake rates and are able to efficiently transport toxic metals from their root systems to their aerial portions. This proficiency stems from their capacity to sequester or convert noxious metals into diverse chemical compounds, thereby reducing the presence of toxic metals in their free ionic form. The unique traits enabling ion hyperaccumulation have prompted the development of enhanced ion transport tissues that capitalize on these attributes.

Within this context, the ZIP gene family plays a pivotal role, encompassing ZIP6 and ZIP9 in *A. halleri* and ZTN1 and ZTN2 in *T. caerulescens*. These genes encode transporters strategically localized within the plasma membrane. These transporters, present in hyperaccumulator species, facilitate the absorption of zinc (Zn), resulting in significantly higher Zn accumulation than non-hyperaccumulator counterparts. Certain plant species need to detoxify metals residing within the root cell cytoplasm, especially those contained in vacuolar structures, before their translocation and subsequent buildup in the aerial parts, as Rascio et al. (2011) discussed.

In the case of *T. caerulescens*, an exemplary hyperaccumulator, the translocation rate of Zn between roots and shoots is nearly twice as rapid as in plants with lower metal tolerance. Furthermore, *T. caerulescens* maintains a lower root Zn concentration, approximately 50-70% less than that of other plant species, as observed in studies by Lasat (1992) and Yang et al. (2006).

To maintain the desired physiological equilibrium within their tissues and counteract metal accumulation, hyperaccumulator plants employ diverse transporters, as outlined by Clemens et al. (1999). These include ATPases, ATP-binding cassettes (ABC), cation diffusion facilitators (CDF), cation exchangers (CAXs), copper transporters (COPTs), and ZIP transporters. Despite their selective permeability, hyperaccumulators possess the capacity to transport cadmium (Cd) and other potentially harmful metals across their membranes or channels. This underscores the critical role of transporters in facilitating the efficient internal transport of essential minerals, such as zinc (Zn), within plants, as Clemens et al. (1999) emphasized.

Further insights from Verret et al. (2004) illuminate that the HMA transporter family, particularly the P1B-ATPase subfamily, relies primarily on adenosine triphosphate (ATP) as its energy source for mediating the transmembrane transport of essential and toxic metallic elements. Notably, Verret et al. (2004) research suggests that transporters such as HMA4 and HMA5 are instrumental in facilitating the long-distance movement of metals, specifically from the plant's roots to its leaves.

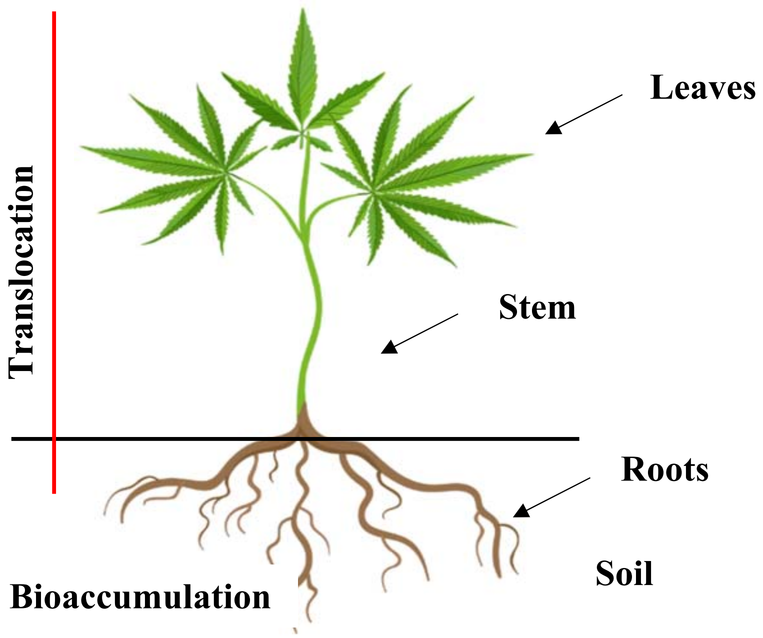

Figure 2: Translocation and Bioaccumulation of Heavy metals

2.4.2. Detoxification of Heavy Metals

Hyperaccumulating plants can neutralize various heavy metals, rendering them harmless to their leaves and stems. As highlighted in research by Küpper et al. (2000), the plant's cuticle, epidermis, and trichomes play critical roles in this detoxification process play critical roles in this detoxification process.

The initial step in the enzymatic metal detoxification process involves the extraction of organic ligands from the plant's metabolic region, as Sarwar et al. (2017) explained. Within plants, biomolecules such as phytochelatins (PCs), metallothioneins (MTs), and glutathione (GSH) are notable for their ability to produce thiols. Consequently, plants utilize these biomolecules as an effective means to eliminate harmful metals, as elucidated by Choppala et al. (2014). The complexes above are tolerance mechanisms, an essential part of the plant's defense mechanism. Within plant tissues, vacuoles house notable quantities of phytochelatins and heavy metal complexes (PC-HM). Vacuolar membranes are used to transport them and are provided by specialized transporters (i.e., HMT1) (Ortiz et al., 1995). It is worth noting that the mechanism observed in plants resembles the one identified in yeast. While glutathione (GSH) is renowned for its potent reducing properties, particularly in mitigating the impact of reactive oxygen species (ROS), HMT1 plays a vital role in eliminating toxic metals. According to Choppala et al. (2014), reactive oxygen species (ROS) are generated when any plant experiences stress above a specific limit. Glutathione (GSH) has also been associated with the synthesis of salicylic acid and the amelioration of toxicity caused by hydrogen peroxide and xenobiotics (Rouhier et al., 2008). GSH-HM complexation and impoundment in vacuoles is an additional detoxification mechanism regulated by GSH, which can potentially release the complexes into the apoplast (Li & Shuman, 1996).

Furthermore, metallothioneins produce MT-HM complexes, primarily consisting of cysteine and other chelating protein molecules with low molecular weight. The differentiation of the four metallothioneins (MTs), which exhibit unique tissue structural specialization and metal element selectivity, is attributed to cysteine deposits (Kotrba et al., 2009). Cd elimination is facilitated by two classes of chelators, namely MT1 and MT2b, out of the four identified classes (Zhou & Goldsbrough, 1994). Conversely, the fourth classification of metallothioneins (MTs) is crucial in the zinc (Zn) detoxification process. It demonstrates a superior ability for Zn storage compared to the earlier three categories within a specific timeframe (Milner et al., 2014).

2.4.3. Heavy Metal Tolerance in Plants

Regarding plant biology, genes affiliated with the cation diffusion facilitator family (CDF) assume the crucial role of sequestering toxic metals. This characteristic undergoes amplification within the plant's genetic makeup, as elucidated by Persans et al. (2001).

Metal transporter proteins (MTPs), categorized as CDF-like polymers, play a pivotal role in facilitating the translocation of metals across both the plasma membrane and tonoplast, as demonstrated by Van et al. in 2008. Notably, the CDF MTP1 protein significantly influences the process of hyper-accumulating zinc (Zn) and nickel (Ni) in the foliage of hyperaccumulating plant species.

Overexpression of the CDF gene, as reported by Persans et al. (2001), culminates in the accumulation of nickel and zinc within the vacuole of T. goes intense, as observed in the study. However, it is essential to recognize that organic ligands with higher molecular mass, such as phytochelatins, confront limitations in their efficacy in regulating the decontamination of hazardous metals. This limitation stems from the considerable energy expenditure associated with their production, exacerbated by the excessive presence of sulfur in the environment, as discussed by Schat et al. (2002).

The process of detoxifying toxic metals is subject to meticulous regulation, influenced by the presence of antioxidant enzymes. Research conducted by Van et al. (2008) exemplifies how antioxidant enzymes can effectively mitigate the oxidative stress induced by toxic metals in plants. Furthermore, the activation of genes in hyperaccumulating organisms enhances the production of glutathione (GSH), a crucial chemical used to detoxify toxic metals.

2.5. Phytoremediation Potential of *Cannabis sativa* L.

2.5.1. *Cannabis sativa* L.: An Overview

The Cannabaceae family, encompassing *Cannabis sativa* L.*,* is classified as a suborder within the Rosales taxonomic order (McPartland, 2018). While monoecious variations can exist, this particular plant is classified as dioecious, producing separate male and female flowers (Strzelczyk et al*.,* 2022). *Cannabis sativa* L. exhibits erect stems adorned with palmately compound leaves, and it typically attains a height ranging from 1 to 2 meters (ElSohly et al., 2017). Nevertheless, specific cultivars have the potential to reach a height of up to 5 meters. The plant's extensive fibrous root system enables it to thrive in diverse soil environments. Within the field of botany, a divergence in perspective arises regarding the classification of the genus Cannabis. Some botanists adopt a polytypic stance, recognizing the presence of several distinct species within the genus, specifically *Cannabis* *sativa*, *Cannabis indica*, and *Cannabis ruderalis*. In contrast, some adhere to a monotypic viewpoint, grouping all variations within the genus under subspecies or varieties of *Cannabis sativa* L, as discussed by Visković et al. (2023).

2.5.2. Historical Uses and Modern Applications

Cannabis sativa L. has been used for many purposes (Karche, 2019). Archaeological findings indicate that the plant was prevalent among ancient Chinese and Egyptians, who employed it for diverse medical, religious, and practical purposes (Bonini et al., 2018). These included its use as a fiber source for producing paper and textiles. Furthermore, the recreational utilization and cultural significance of Cannabis in numerous civilizations can be attributed to the chemical compound delta-9-tetrahydrocannabinol (THC), which is accountable for the plant's psychoactive properties (Sorrentino, 2021).

Advancements in scientific research and shifts in societal attitudes have facilitated a significant proliferation of applications for *Cannabis sativa* L. in contemporary times. Two distinctive cannabinoids (i.e., THC and CBD) are currently under investigation for their potential therapeutic use (Urits et al., 2020; Bonaccorso et al., 2019). Hemp refers to a specific kind of the cannabis plant, scientifically known as *Cannabis sativa* L., characterized by its deficient levels of tetrahydrocannabinol (THC), is extensively employed in various industrial sectors such as textile manufacturing, biofuel production, and construction material fabrication (Karche, 2019). The increasing recognition of hemp seeds in the health food sector can be attributed to their notable levels of protein and essential fatty acids (Leonard et al., 2020).

2.5.3. *Cannabis sativa* L. as Phytoremediator: Current Research Trends and Gaps

Considerable scholarly attention has been directed toward the utilization of *Cannabis sativa* L., especially industrial hemp, as a phytoremediation agent, as discussed by Golia et al. (2023). Phytoremediation, defined by Laghlimi et al. (2015), refers to harnessing plants to eliminate or mitigate detrimental substances within the environment. Growing evidence supports the potential of *Cannabis sativa* L. as an effective phytoremediator, owing to its extensive root system and resilience, enabling it to thrive across a broad spectrum of environmental conditions, as Rehman et al. (2021) noted.

Recent research has highlighted the effectiveness of *Cannabis sativa* L. in absorbing cadmium, lead, and nickel from contaminated soils, as demonstrated by Placido and Lee (2022). Additionally, according to Rheay et al. (2021), this plant species can break down organic pollutants like polycyclic aromatic hydrocarbons and agrochemicals. According to Wu et al. (2021), *Cannabis sativa* L. exhibits favorable characteristics for phytoremediation due to its significant biomass yield, rapid growth rate, and minimal maintenance needs.

Despite initial promising findings in using *Cannabis sativa* L., there are still significant knowledge deficiencies in phytoremediation (Vangronsveld et al., 2009). Firstly, it should be noted that our comprehension of the mechanisms through which this plant assimilates and expels various pollutants remains unknown (Dervash et al., 2023). It is imperative to comprehend these processes at the molecular and biochemical levels to enhance the efficacy of phytoremediation.

The fate of contaminants following their uptake by plants is currently under investigation. The extent to which *Cannabis sativa* L. can sequester heavy metals within its tissues remains unresolved, thus stopping their introduction into the food chain. When cultivating plants for commercial purposes, it is crucial to consider additional products, such as fiber or seeds, which may be obtained during stages of plant growth.

Cannabis sativa L. research as a phytoremediator has been conducted in controlled environments such as greenhouses or laboratories. A scarcity exists in the field of comprehensive field investigations that adequately consider the intricate nature of real-world contaminated regions, as well as the influence of variables such as soil composition, meteorological conditions, and microbial communities (Kuppusamy et al., 2016), concerning the results obtained from remediation endeavors.

The legal and regulatory constraints related to Cannabis cultivation pose significant obstacles to the implementation of large-scale field investigations and the execution of phytoremediation initiatives. The consequence of this phenomenon has led to an imbalanced focus on research conducted in regions where the cultivation of cannabis is legally permitted.

CHAPTER 3

MATERIALS AND METHODS

Cannabis sativa L. was tested for phytoremediation in two heavy metal-contaminated soils. A thorough research examined how soil affected morpho-physiological and biochemical Parameters. Furthermore, the production of nanoparticles as part of remediation was done in an eco-friendly manner using residual plant biomass.

3.1. Summary of Experimental Process

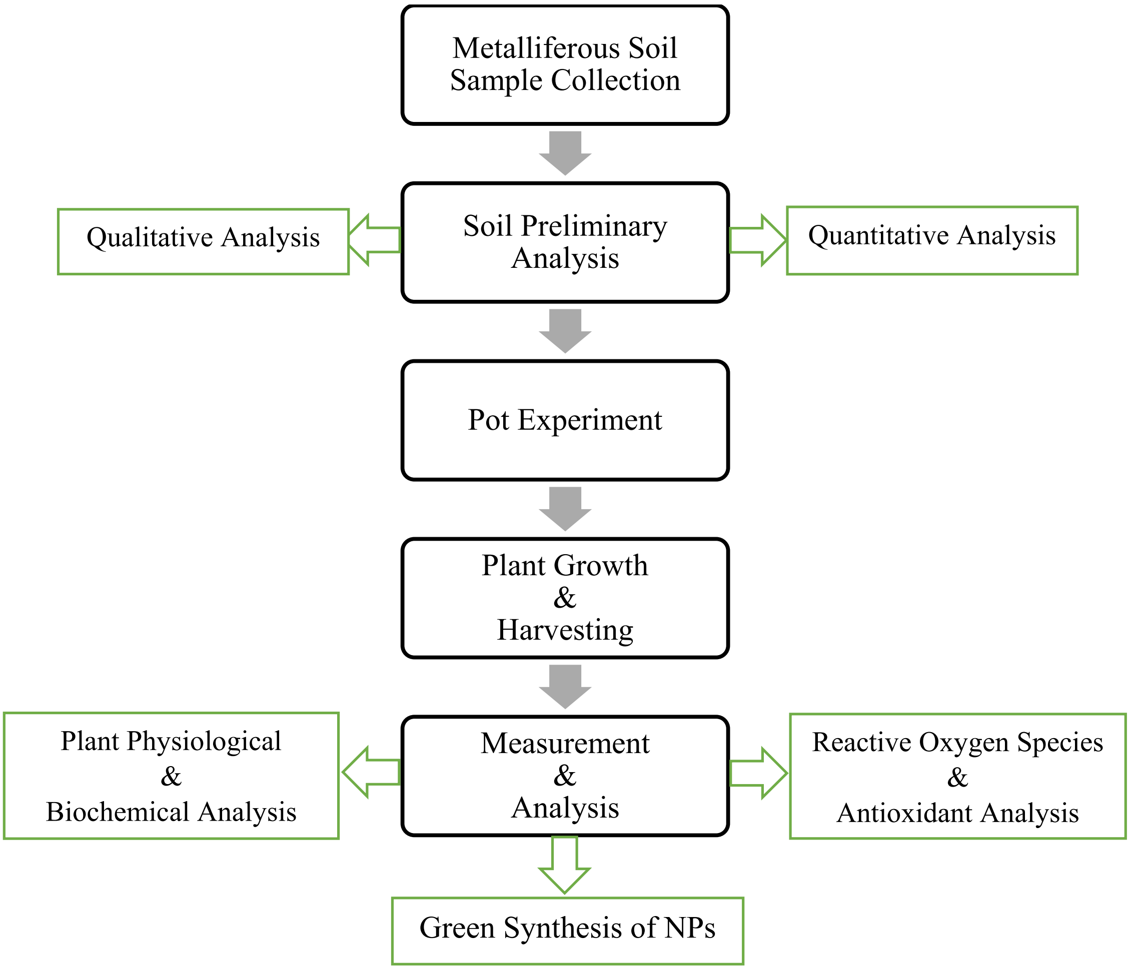

Figure 3: Schematic illustration of the experimental process

3.2. Soil collection site

Topsoil, within a 0-50 cm depth, was collected from a contaminated area near Industrial State Hattar. For experimental purposes, two distinct soil types were identified. The soil collected from the neighboring area of an industrial zone was labeled as Type 1, while the soil obtained from a nearby contaminated site was labeled as Type 2. These soil samples underwent a drying procedure to evaluate their physical and chemical characteristics and were subsequently sifted via a sieve measuring 2 mm.

3.3. Characterization of soil

Examined attributes of the soil encompassed its pH value, its capacity to conduct electricity (EC), its ability to retain water, levels of sodium present, the quantity of heavy metals, and the soil's texture.

3.3.1. Soil pH measurement

At the Environmental Biotechnology Laboratory, IESE, NUST, soil's pH was measured using a handheld pH meter. Initially, soil was collected to analyze pH values. Field-moist soil was weighed to 10 g and distributed into three cups for repeated analysis. Following the weighing process, the cups were sealed to prevent moisture evaporation. Each cup was then filled with 20 ml of deionized water using either a pipette or a graduated cylinder and subsequently sealed and shaken briefly. After removing the cap, the solution was allowed to acclimatize to the atmosphere for a minimum of 30 minutes. Later, pH values were noted for each sample (Tang et al., 2014).

3.3.2. Measuring soil electrical conductivity (EC)

Preparing samples for the electrical conductivity (EC) meter was the first step in measuring the electrical conductivity of soil. In order to completely dissolve the soil sample (25 g) in deionized water (100 ml), it was covered and left completely untouched for 30 minutes. An electrode was then suspended in the solution, and the EC was recorded as soon as the readings became stable (Meers et al., 2005). The concentration of ionized substances directly influences conductivity, indicating the water's capacity for transmitting electrical currents. Consequently, one can identify the total soluble salt content. EC is a valuable metric because of its high precision and simplicity in gauging (Nathan et al., 2004).

3.3.3. Determination of soil texture

The hydrometer was utilized to determine the soil texture (Groenendyk et al., 2015). The hydrometer's functionality relies on the particle size and settling velocities of silt and clay in a water column. The percentages of sand and silt were calculated by measuring the particle size and velocities of sand and silt in water. The USDA textural triangle was then employed to assess the soil class (Barman & Choudhury, 2020).

In the procedure, distilled water was poured into a soil sample (100 g) to get a solution for the experimental procedure. The mixture's water ratio to the dried soil's weight provided the soil texture value. The obtained percentage value was then used to get the exact soil texture by matching it with a USDA-approved triangle (shown below).

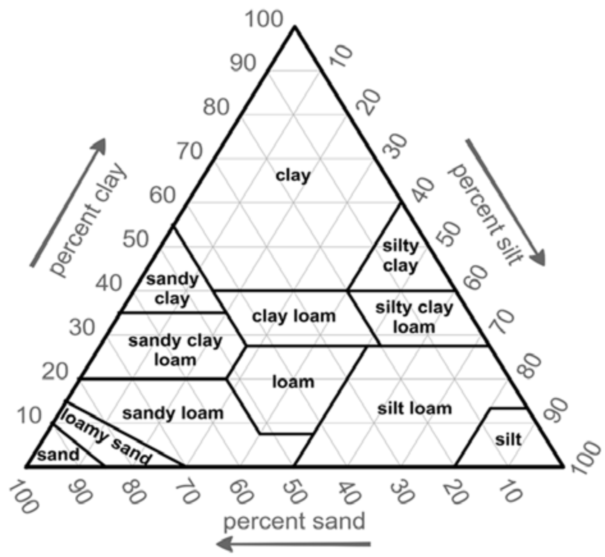

Figure 4: Soil texture triangle by USDA

3.3.4. Water holding capacity of soil

A weighing balance was used to measure a sample of soil (25 g), which was to be used in this experiment. A filter paper was taken, and the prepared sample was placed on it set into a funnel. Specifically, a specified quantity of distilled water (100 ml) was passed through the sample on the filter paper in the funnel. The filtrate from the soil was collected, and the volume of the filtrate was recorded (Horne and Scotter et al., 2016).

WHC = 100 mL Distilled Water – Filtrate in the Cylinder (mL)

3.3.5. Soil Sodium content

This soil analysis procedure, outlined by the OSU Extension Service Nutrient Management Guides, begins with preparing an ammonium acetate extraction solution. In a 1L volumetric flask, around 600 mL of deionized water is combined with 77.08 g of ammonium acetate, mixed thoroughly, and filled to volume. The solution, which was in a beaker, was used to determine the pH through a meter to ensure its value was 7.0. It is ensured by using acetic acid to lower the pH or ammonium hydroxide to increase the pH. Next, the soil sample, which was dried in air, was weighed to 2 g and put into a falcon tube of 50 mL (also called a polypropylene centrifuge tube). A CAL standard soil sample and a method blank (tube with no soil) are also prepared in the same way to serve as controls. 20 mL of the ammonium acetate solution was added to each tube, encompassing the CAL standard and method blank tubes. They were then agitated on a shaker for an hour. Following the shaking period, the mixtures are filtered using Whatman #1 filter paper and collection funnels. Finally, the cations present in the filtrate are measured using an atomic absorption spectrometer (AAS) (novAA 800D, Analytik Jena, Germany). This method allows for precisely identifying and quantifying cations in the soil samples (Normandin et al., 1988).

3.3.6. Heavy metal analysis

At the Wastewater laboratory of IESE, NUST in Islamabad, an atomic absorption spectrophotometer was utilized for heavy metal examination. This device, functioning based on the absorption or transmission of electromagnetic radiation relative to the exposed radiation's wavelength, was an atomic absorption spectrometer (AAS) (novAA 800D, Analytik Jena, Germany). The quantification of metals transpired via an air-acetylene flame. Lead (Pb), cadmium (Cd), Iron (Fe), and Zinc (Zn) were the four metals that were analyzed from the soil samples.

3.4. Experimental procedure

3.4.1. Plant selection

For the study of phytoremediation in specific soil areas, *Cannabis sativa* L. (Industrial Hemp) served as the chosen plant species. This species was selected because of its desired qualities for phytoremediation, including its hardiness, quick growth, deep-rootedness, and high biomass production.

3.4.2. Pot experiment

The study transpired in a regulated setting inside the wire house of the Institute of Environmental Sciences and Engineering, NUST, Islamabad. Pots were filled with equal measures (2.5 kg each) of Soil Type 1 and Soil Type 2. There were sixteen pots, i.e., eight for each soil type. The pots were further separated into two groups, i.e., light and dark groups.

(a)

(b)

Figure 5: Pot Experiment

Cannabis sativa L. seeds were procured from the University of Agriculture, Faisalabad. Subsequently, each pot received these seeds, which germinated after two days. In the beginning, irrigation supplied the necessary tap water to these seeds. The first-fortnight post-germination saw a half-strength Hoagland solution, succeeded by periodic applications of its full strength. The initial conditions for these plants encompassed temperatures ranging between 25-30 degrees Celsius and light durations spanning 14-16 hours. The surrounding humidity measured between 60-70%. The study employed a completely randomized design (CRD) with four replications for each treatment. All the pots were kept under sunlight for one month. Later, half of the pots were moved to dark conditions. Rectangular cardboard covered with a black sheet from the outside created dark conditions. It had wooden blocks at the bottom for airflow.

3.4.3. Plant harvesting

Over ten weeks, the plants were subjected to contaminated soil to assess their phytoremediation potential (Thamayanthi et al., 2013). The experiment was separated into two groups (i.e., light and dark). We wanted to compare the growth of plants in two different conditions. However, after one week, the plants kept under dark conditions started to wilt. Ultimately, they could not survive, but the plants kept under sunlight were doing well. The experiment was continued with the light group plants only as the data from the dark group was not adequate for comparison. Upon the completion of the exposure period, the plants were delicately extracted from the soil of each pot. Later, distilled water was used to rinse them. The plants underwent a concluding washing procedure using tap water to eliminate residual dirt particles. Subsequently, they were submerged in a solution consisting of 50% nitric acid for five minutes to eradicate any remaining contaminants of a heavy metal nature that may have been present on the surface of the roots. A second rinse with distilled water was performed, followed by blotting the plants with filter paper. Subsequently, the harvested plant samples were preserved for subsequent analysis.

(a) (b)

Figure 6: Ten weeks old Cannabis sativa* L. *plants before harvesting.

3.5. Plant growth parameters

Four plants were chosen from each group to assess the length of upper plant parts (i.e., root and shoot). Each measure was recorded using a centimeter scale, following the method described by Pokhrel and Dubey (2013). To enhance the dependability of the data, four duplicates were used for every group. To facilitate further examination, the plant shoot, root, and leaves were dried at a temperature of 75°C. Following a 48-hour drying period, the dry weights of the specimens were documented.

3.6. Plant physiological and biochemical parameters

3.6.1. Photosynthetic performance

The quantification of photosynthesis in hemp plants was conducted just before their harvest. A portable instrument known as the photosynq multispeq (Kuhlgert et al., 2016) was used to collect non-destructively phenotypic and environmental data from plants. The measurements included assessing the relative chlorophyll content, which indicates the greenness of the leaves.

Additionally, the quantum efficiency of light absorbed by the plant, denoted as Phi2, was determined. Furthermore, the quantum efficiency of light ingested by the plant but lost due to uncontrolled processes referred to as PhiNO, was also measured. Lastly, the quantum efficiency of light absorbed by the plant but lost as heat, known as PhiNPQ, was quantified. Simultaneously, measurements were obtained for the light intensity (PAR), temperature, and humidity, with a sample size of n=5.

Figure 7: Photosynq multispeq device

3.6.2. Membrane stability index and relative water contents

To ascertain the membrane stability index, the electrical conductivity of leaf leachate was evaluated in distilled water at 40°C and 100°C. The measurements were taken at specific time intervals of 30 and 10 minutes, respectively, following the methodology outlined by Sairam et al. (2002).

$$\boldsymbol{MSI}\ (\%) = \frac{(\boldsymbol{EC_1} - \boldsymbol{EC_0})}{(\boldsymbol{EC_2} - \boldsymbol{EC_0})}\ \boldsymbol{x}\ \mathbf{100}$$

Sairam et al. (2002) proposed the equation to calculate relative water content (RWC) in this study. This was achieved by collecting harvested leaf samples and weighing them at 0.5 g fresh weight (FW). After being submerged in 100 ml of distilled water for 4 hours, the leaf specimens' turgid weight (TW) was documented. Subsequently, the samples underwent a drying procedure in an oven maintained at a temperature of 70oC for 48 hours to determine their dry weight (DW).

$$\boldsymbol{RWC}\ (\%) = \frac{(\boldsymbol{Fresh\ Weight - Dry\ Weight})}{(\boldsymbol{Turgid\ Weight\ -\ Dry\ Weight})}\ \boldsymbol{x\ 100}$$

3.6.3. Leaf sample preparation for antioxidant and ROS analysis

The investigation included the isolation of the whole protein from plant leaves in order to examine enzyme activity. Leaf samples weighing about 200 mg were obtained from each plant species. Using liquid nitrogen, the specimens were ground into delicate dust. This dust was then combined with 1.2 ml of a cold protein extraction liquid. This liquid contained a potassium phosphate buffer, pH 7.8, at 0.2 mM concentration, and 0.1 mM of EDTA. The amalgamation occurred in a perpetually frozen mortar.

For 20 minutes, the samples were subjected to centrifugal forces at a velocity of 14,000 rpm and 4°C. The resultant upper layer was collected and placed in a 2 ml Eppendorf container. After another 12-minute centrifugation at 14,000 rpm and 4 °C, the residual mass was reintegrated with 0.8 ml of the extraction buffer (Elavarthi & Martin, 2010).

3.6.4. Superoxide Dismutase (SOD)

The buffer for Superoxide Dismutase (SOD) was formulated following the protocol provided by Beauchamp and Fridovich (1971). Each sample was allotted 3 ml of the prepared SOD buffer, to which 100 µL of riboflavin stock and sample (100 µL) extract were added. Later, they were put under a 40-watt fluorescent lamp and shaken for 30 minutes. During this period, the yellow hue in the test tubes transitioned to brown. A duplicate set of these samples was prepared and kept in darkness for the same duration.

A control sample (blank) was created by mixing 3 ml of SOD, 100 µL of riboflavin stock, and 100 µL of distilled water. On a UV/Vis spectrophotometer, the light absorbance of the two groups was assessed at 560 nm wavelength. The reading was noted for each sample, SOD activity was calculated by using a given formula, and the activity was expressed in Unit g^{-1} FW.

$$SOD\ Activity\ = \frac{\{(Ack - Ac)\ x\ V\}}{\{0.5\ x\ Ack\ x\ W\ x\ V_t\}}$$

Where,

Ac = Absorbance value in dark

Ack = Absorbance value in light

V = Volume of enzyme extract used (ml)

W = Fresh weight of the plant tissue (g)

Vt = Total volume of the reaction mixture (ml)

3.6.5. Catalysis activity

The analysis was performed based on the approach delineated by Aebi and Lester (1984). A 3 ml reaction mixture was prepared for each sample in individual test tubes. A 2 ml quantity of leaf extract was combined with 1 ml of a ten mM solution of hydrogen peroxide (H_2O_2) in an extraction medium composed of a 50 mM potassium phosphate buffer, pH 7.

The light absorbance for each specimen was recorded on a UV/Vis spectrophotometer at a designated 240 nm wavelength. A mathematical equation was employed to discern the enzymatic activity (Aebi & Lester, 1984), and activity was expressed in Unit g^{-1} FW.

$$CAT\ Activity = \frac{(\Delta A_{240}\ x\ V\ (ml)/a_{enz})}{(€_{mM}\ x\ W\ (ml))}$$

Where,

ΔA = Change in absorbance value

V = Volume of the reaction mixture (ml)

a = Amount of enzyme extract used (ml)

$€_{mM}$ = Absorption constant assumed to be 26.6

W = Fresh weight of the plant tissue (g)

3.6.6. Peroxide activity

Based on the procedures delineated by Hemeda and Klein (1990), the guaiacol peroxidase (POD) activity underwent scrutiny. The reaction mix included 200 µL of enzyme extract, a 50 mM phosphate buffer at pH 6, 15 mM of hydrogen peroxide (H_2O_2), and 12 mM guaiacol. For 90 seconds at 25°C, the mixture's light absorbance was monitored at a wavelength of 470 nm. The determined POD action was articulated as micromoles of guaiacol oxidized per Unit g^{-1} FW.

$$\boldsymbol{POD\ Activity = \frac{(\Delta A_{470}\ x\ V\ (ml)/a_{enz})}{(€_{mM}\ x\ W(ml))}}$$

Where,

ΔA = Change in absorbance value

V = Volume of the reaction mixture (ml)

a = Amount of enzyme extract used (ml)

$€_{mM}$ = Absorption constant assumed to be 26.6

W = Fresh weight of the plant tissue (g)

3.6.7. Hydrogen peroxide contents

By employing the method formulated by Islam et al. (2008), hydrogen peroxide (H_2O_2) concentrations in the leaf specimens were scrutinized. The method entails pulverizing a leaf sample weighing 0.5 g under liquid nitrogen and adding 0.1% trichloroacetic acid. This is followed by centrifuging the pulverized sample for 20 minutes at 12,000×g. Subsequently, a reactive mixture was formulated, integrating 1 mL of a 10 mM potassium phosphate buffer (pH 7.0), 1 mL of 2 M potassium iodide, and 1 mL derived from the plant extraction. Using a UV-Vis spectrophotometer, the samples' light absorption was noted at a wavelength of 390 nm.

3.7. Plant Sample Preparation for Heavy Metal Analysis

3.7.1. Preparing plant sample for analysis

Examining heavy metal content in the plant's root, shoot, and leaves post-harvest and oven-drying involves a wet digestion process. A total of 0.5 g of crushed plant matter, comprising roots and shoots, was used for digestion. The acid digestion process included the addition of 10 mL of concentrated Nitric Acid (HNO_3) and 4 mL of concentrated Hydrochloric Acid (HCl) to 0.5 g of plant material inside a 25 mL volumetric flask. In a fume hood, the flask was set on a hot plate, where it was gradually heated from an initial temperature of 50°C to a final one of 150°C, as Saifullah et al. (2010) described. The heating process was carried out on the hot plate until the sample turned translucent, signaling the complete digestion of all plant matter. After the solution turned colorless, it was removed from the hot plate and filtered using Whatman No. 42 filter paper. For heavy metal analysis, the filtered solution was diluted to a volume of 50 mL in a volumetric flask with distilled water and then stored at a temperature of 4 °C.

3.7.2. Heavy metals analysis

An atomic absorption spectrophotometer was used to perform the heavy metal analysis. The principle guiding this device is that when exposed to electromagnetic radiation, a substance can either absorb or emit radiation contingent on the wavelength. The samples were analyzed utilizing an atomic absorption spectrometer (AAS) model novAA 800D, produced by Analytik Jena, Germany. An air-acetylene flame was employed for metal measurement. The prepared plant samples were analyzed for heavy metals.

3.7.3. Use of Atomic Absorption Spectrophotometer

The novAA 800D model Atomic Absorption Spectrophotometry apparatus from Analytik Jena, Germany, was utilized to appraise concentrations of distinct heavy metals extracted from plant and soil samples. For sample formulation and ensuing examination, chemicals of analytical caliber boasting a superior spectroscopic purity of 99.9%, obtained from Merck Darmstadt, Germany, were employed.

All the analyses were performed using standard solutions generated by diluting certified standard solutions obtained from FlukaKamica, a reputable company based in Busch, Switzerland. These standard solutions had a concentration of 1000 mg/L for both components. In order to enhance the reliability and exactitude of the digestion process, we used blank reagents and standard reference plant materials (GBW-07602 (GSV1)) provided by the National Research Centre for Certified Reference Materials in China. For the assurance of data precision, every sample set underwent triplicate evaluations under controlled, optimal environments, yielding outcomes at a confidence interval of 95%.

3.7.4. Bioaccumulation factor

Three variables, namely the Enrichment Coefficient Factor (ECf), Bio Translocation Factor (TF), and Bioconcentration Factor (BCF) are instrumental in characterizing plant accumulation properties. The computation of the enrichment coefficient (ECf) entails dividing the cumulative heavy metal content found in the aerial components of plants by the cumulative heavy metal content present in the soil. The TF (translocation factor) may be determined by conducting a comparative analysis of the metal composition in the shoots and the soil (Aransiola et al., 2013; Zu et al., 2005). The TF indicates the plant's capacity to transport metals from its underground structures to its aboveground components. The phenomenon through which a plant can extract and accumulate hazardous metals from the soil is called its bioaccumulation capacity, often abbreviated as BCF.

The values were computed using suitable mathematical equations.

TF = Total metal in shoot / Total metal in root (Zu et al., 2005*)*

ECf = Total metal in shoot / Total metal in Soil (Zu et al., *2005)*

BCF = Total metal in root/ Total metal in soil (Yoon et al., *2006)*

3.8. Soil pH measurement after harvesting

After harvesting the plants, the pH of all eight pots was measured using a pH meter at the Environmental Biotechnology Laboratory, IESE, NUST, Islamabad. Each pot weighed 10 g of field moist soil in extraction cups. To prevent moisture loss, the cups were capped immediately after weighing.

Subsequently, each cup was filled with 20 ml of deionized water, capped, and briefly shaken using a pipet or graduated cylinder. The caps were removed to allow for proper equilibration with the atmosphere, and the solutions were left undisturbed for a minimum of 30 minutes. Afterward, the solution's pH was noted using a pH meter.

3.9. Synthesis of nanoparticles

3.9.1. Preparation of plant extract

Following a thorough rinsing with water to eliminate residual dust particles, the leaves of *Cannabis sativa* were subjected to a shade drying process, later converting them into a powdered form. The experiment introduced 5 grams of powdered leaves into a flask containing 100 ml of distilled water. The mixture was subjected to continuous stirring on a hot plate while being heated to a temperature range of 70 to 80 °C for 30 minutes. The filtration process included using filter paper to achieve the separation of solid particles from the liquid phase after the heat application to the mixture. After that, the filtrate was kept at a temperature of 4 °C for future use.

3.9.2. Synthesis of ZnO nanoparticles

The methodology used by Ramesh et al. included the production of zinc nanoparticles. A mixture of 50 ml of plant extract and 2 g of zinc nitrate hexahydrate ($Zn(NO_3)_2$ $6H_2O$) was subjected to continuous stirring while being heated within the temperature range of 70 to 80° C. The continuous rotational motion led to a noticeable shift in color, providing evidence for the formation of nanoparticles. Subsequently, the materials were subjected to heating, resulting in the formation of a paste. The paste underwent a thermal treatment for three hours at a temperature of 300°C inside a muffle furnace. The temperature caused the degradation of all components of the organic paste. The nanoparticles underwent calcination at 300°C, transforming their composition from zinc to zinc oxide. The dry powder obtained was subjected to extraction using a mixture of water and methanol, followed by preservation for further analysis.

(a) (b)

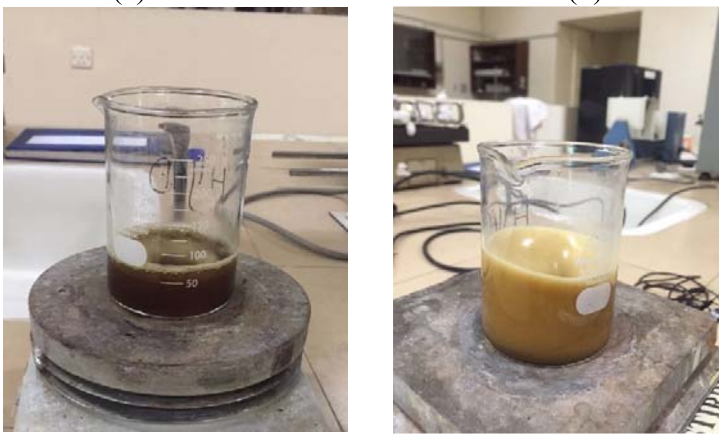

Figure 8: Visual confirmation of Zinc Oxide nanoparticles (ZnO)

3.10. Statistical analysis

The experimental treatments were duplicated four times, and the resulting data were subjected to analysis using the T-Test in the Statistical Package for the Social Sciences (SPSS) 16.0 program with a significance threshold of 0.05. Excel calculated the standard errors for each mean number representing a result. Comparative graphs were made using Microsoft Excel.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Soil Analysis

The initial investigation's outcomes, displayed in Table 1, reveal disparities in various physical parameters between soil type 1 and soil type 2. Post-plant harvest, it was noted that the soil pH across all pots fell within the favorable range of 7.6 to 8.0 for fostering plant growth.

Parameter	Values (Soil Type 1)	Values (Soil Type 2)
pH	8.0	7.6
Electrical Conductivity	1.15 (mS)	0.41 (mS)
Water Holding Capacity	693.2 mL	586.4 mL
Texture	Sandy Loam	Sandy Loam
Sodium Content	0.61 (g/L)	0.84 (g/L)
Cadmium (Cd)	10.1 ± 0.30 (mg/kg)	439 ± 0.36 ((mg/kg)
Iron (Fe)	21.5 ± 0.87 (mg/kg)	722.7 ± 1.73 (mg/kg)
Lead (Pb)	13.2 ± 0.21 (mg/kg)	708 ± 1.53 (mg/kg)
Zinc (Zn)	30.8 ± 0.1 (mg/kg)	260.5 ± 0.65 (mg/kg)

Table 1 The values of different parameters of Soil Type 1 and Soil Type 2

Soil Type 1 exhibited a slightly elevated EC compared to Soil Type 2, while the water retention capacity of Soil Type 1 surpassed that of Soil Type 2. Additionally, both soils shared an identical texture. The data illustrates that Soil Type 2 had more significant heavy metal contamination and a slight salinity. Similarly, both soil types exhibited mild salinity, with the principal contrast in their rich metal content. As indicated in Table 4.1, Soil Type 2 displayed higher levels of Cd, Fe, Pb, and Zn than Soil Type 1.

4.2. Plant Growth Parameters

4.2.1. Plant Height

Plant height was not significantly decreased under Soil Type 2 compared to Soil Type 1 (Figure 4.1). The mean size of plants grown under Soil Type 1 was 51 cm, while the height of plants grown under Soil Type 2 was 43 cm. Moreover, the plant height under Soil Type 2 decreased by 19% compared to Soil Type 1.

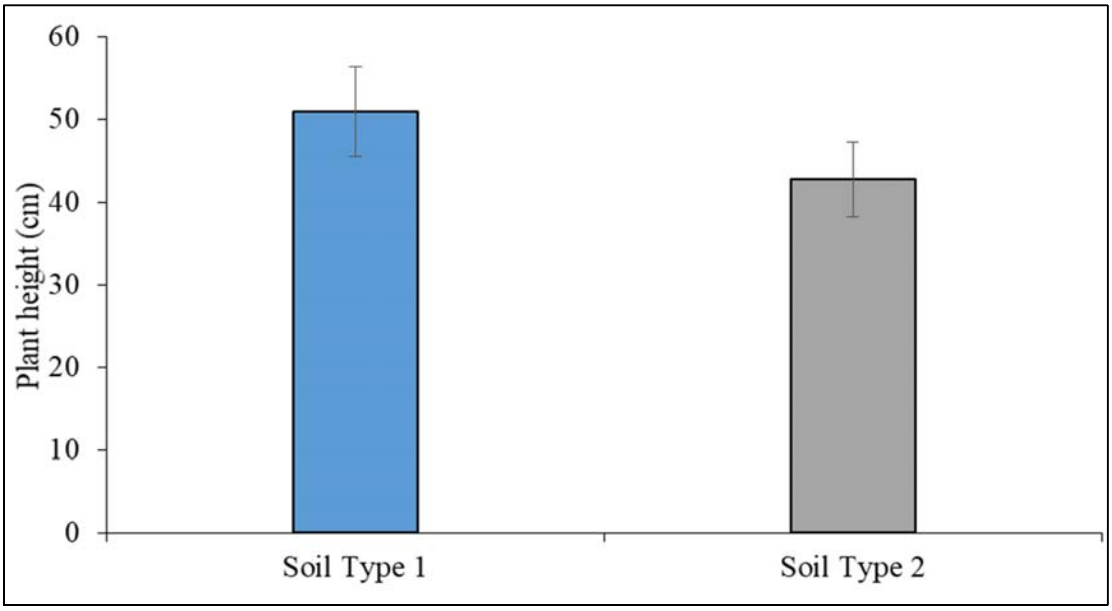

Figure 9: Effect of Soil Type 1 and Soil Type 2 on height of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant difference, while the bars with asterisk (*) show significant difference, leveling at $P \leq 0.05$.

4.2.2. Number of Leaves

The number of leaves under Soil Type 2 was not significantly decreased compared to Soil Type 1 (Figure 4.2). The mean number of leaves under Soil Type 1 was 23, while the number under Soil Type 2 was 20.5. Moreover, the number of leaves under Soil Type 2 decreased by 12% compared to Soil Type 1.

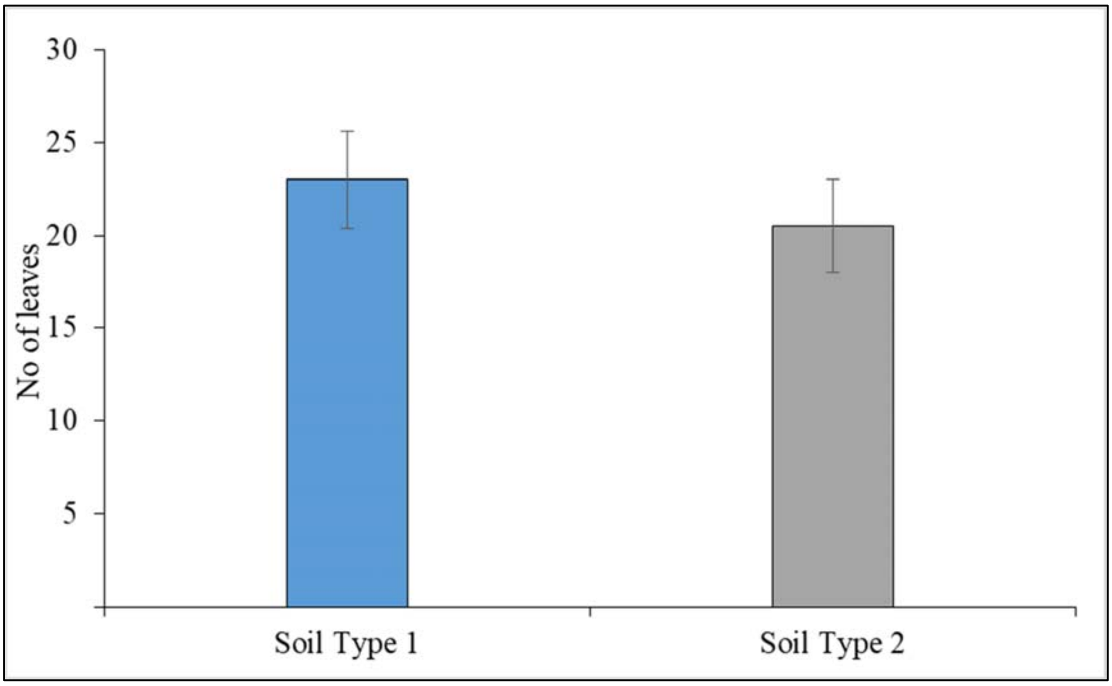

Figure 10: Effect of Soil Type 1 and Soil Type 2 on the number of leaves of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant difference, while the bars with asterisk (*) show significant difference, leveling at $P \leq 0.05$.

4.2.3. Shoot Fresh and Dry Weight

Shoot fresh weight significantly decreased under Soil Type 2 compared to Soil Type 1 (Figure 4.3). The mean shoot new weight under Soil Type 1 was 2.4 g, while the fresh weight under Soil Type 2 was 1.8 g. Moreover, the shoot fresh weight under Soil Type 2 decreased by 32% compared to Soil Type 1.

Shoot dry weight did not decrease significantly under Soil Type 2 compared to Soil Type 1 (Figure 4.3). The mean shoot dry weight under Soil Type 1 was 0.36 g, while the shoot dry weight under Soil Type 2 was 0.24 g. Furthermore, the shoot dry weight under Soil Type 2 decreased by 34% compared to Soil Type 1.

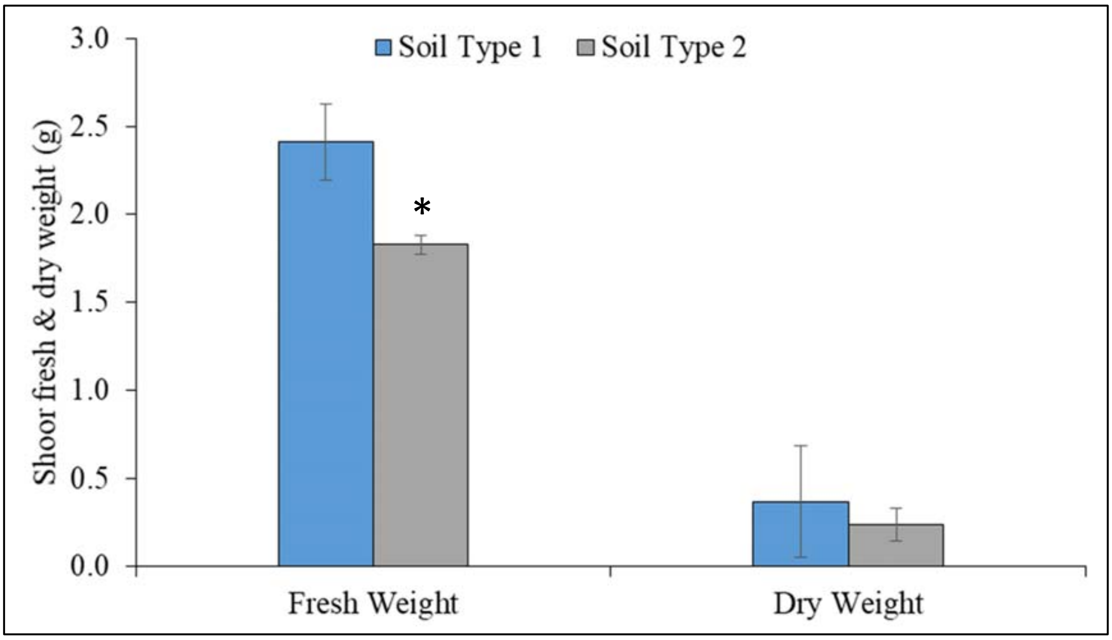

Figure 11: Effect of Soil Type 1 and Soil Type 2 on the shoot fresh and dry weight of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant difference, while the bars with asterisk (*) show significant difference, leveling at P ≤ 0.05.

4.2.4. Leaf fresh and dry weight

Leaf fresh weight was not significantly lower under Soil Type 2 than Soil Type 1 (Figure 4.4). The mean new leaf weight under Soil Type 1 was 2.1 g, while the fresh weight under Soil Type 2 was 1.8 g. Moreover, the leaf fresh weight under Soil Type 2 decreased by 15% compared to Soil Type 1.

On the other hand, leaf dry weight did not significantly decrease under Soil Type 2 compared to Soil Type 1 (Figure 4.4). The mean leaf dry weight under Soil Type 1 was 0.46 g, while the leaf dry weight under Soil Type 2 was 0.37 g. Moreover, the leaf dry weight under Soil Type 2 decreased by 24% compared to Soil Type 1.

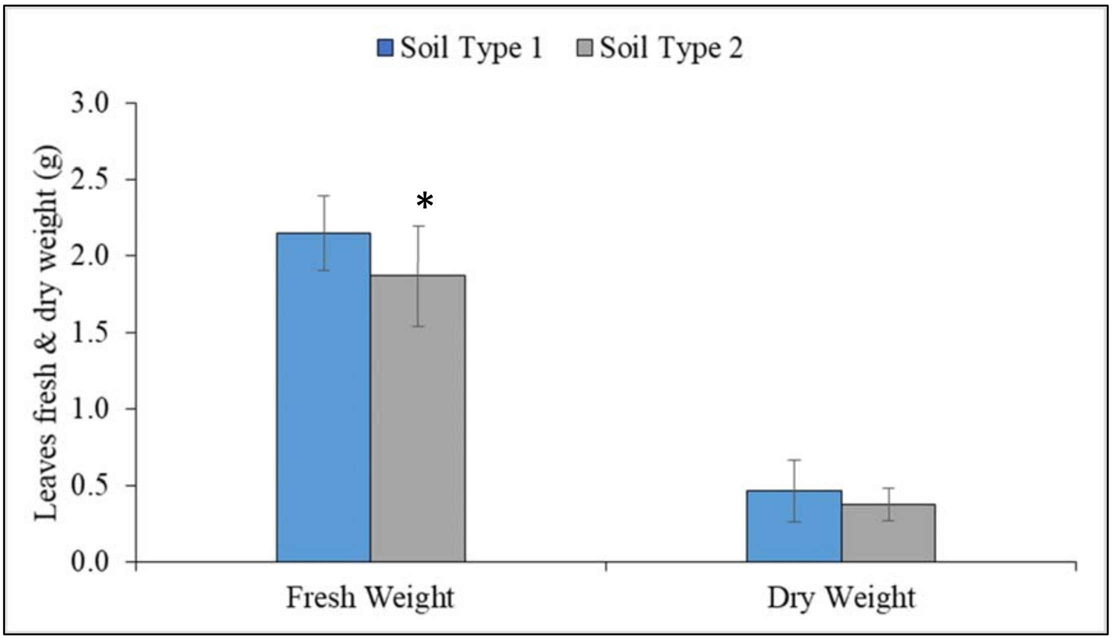

Figure 12: Effect of Soil Type 1 and Soil Type 2 on the leaf fresh and dry weight of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant difference, while the bars with asterisk (*) show significant difference, leveling at P ≤ 0.05.

4.3. Physiological and biochemical parameters

4.3.1. Photosynthetic Performance

Relative chlorophyll was not significantly decreased under Soil Type 2 compared to Soil Type 1 (Figure 4.5). The mean relative chlorophyll under Soil Type 1 was 52, while under Soil Type 2, it was 45. Moreover, the relative chlorophyll under Soil Type 2 decreased by 17% compared to Soil Type 1.

On the other hand, the PhiNO was significantly decreased under Soil Type 2 compared to Soil Type 1 (Figure 4.6a). The mean PhiNO under Soil Type 1 was 0.26, while under Soil Type 2, it was 0.13. Moreover, the PhiNO under Soil Type 2 decreased by 97% compared to Soil Type 1.

On the other hand, the PhiNPQt was significantly decreased under Soil Type 1 compared to Soil Type 2 (Figure 4.6b). The mean PhiNPQt under Soil Type 1 was 0.43, while under

Soil Type 2, it was 0.64. Furthermore, the PhiNPQt under Soil Type 1 decreased by 47% compared to Soil Type 2.

Moreover, we noticed a significant decrease in Phi2 under Soil Type 2 compared to Soil Type 1 (Figure 4.6c). The mean Phi2 under Soil Type 1 was 0.30, while under Soil Type 2, it was 0.23. Moreover, the Phi2 under Soil Type 2 decreased by 33% compared to Soil Type 1.

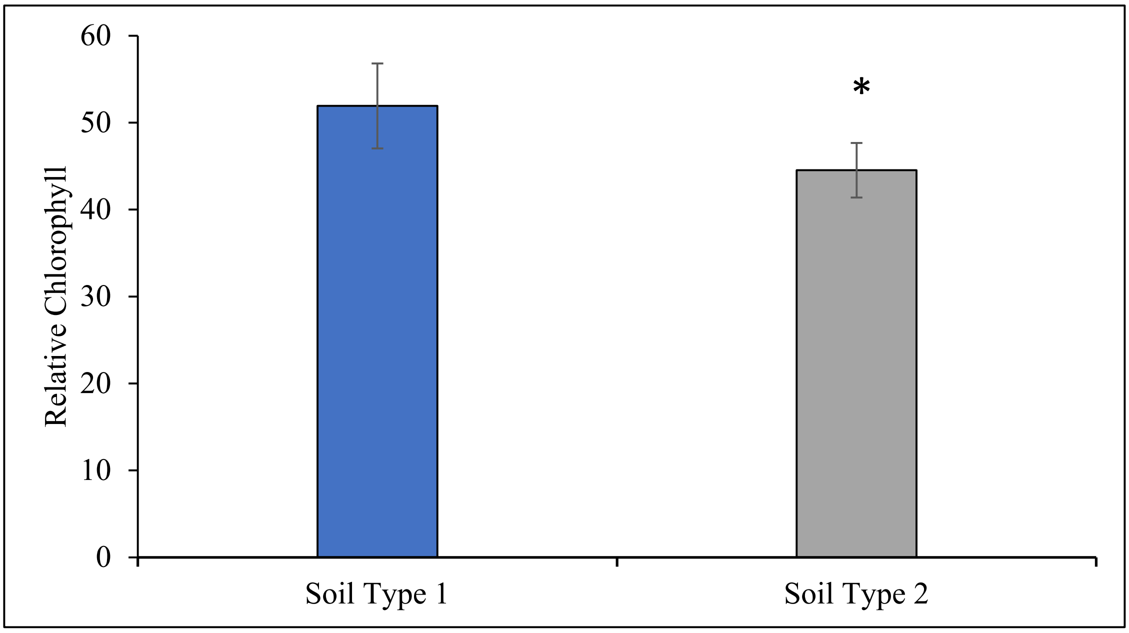

Figure 13: Effect of Soil Type 1 and Soil Type 2 on the chlorophyll content of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant difference, while the bars with asterisk (*) show significant difference, leveling at P ≤ 0.05.

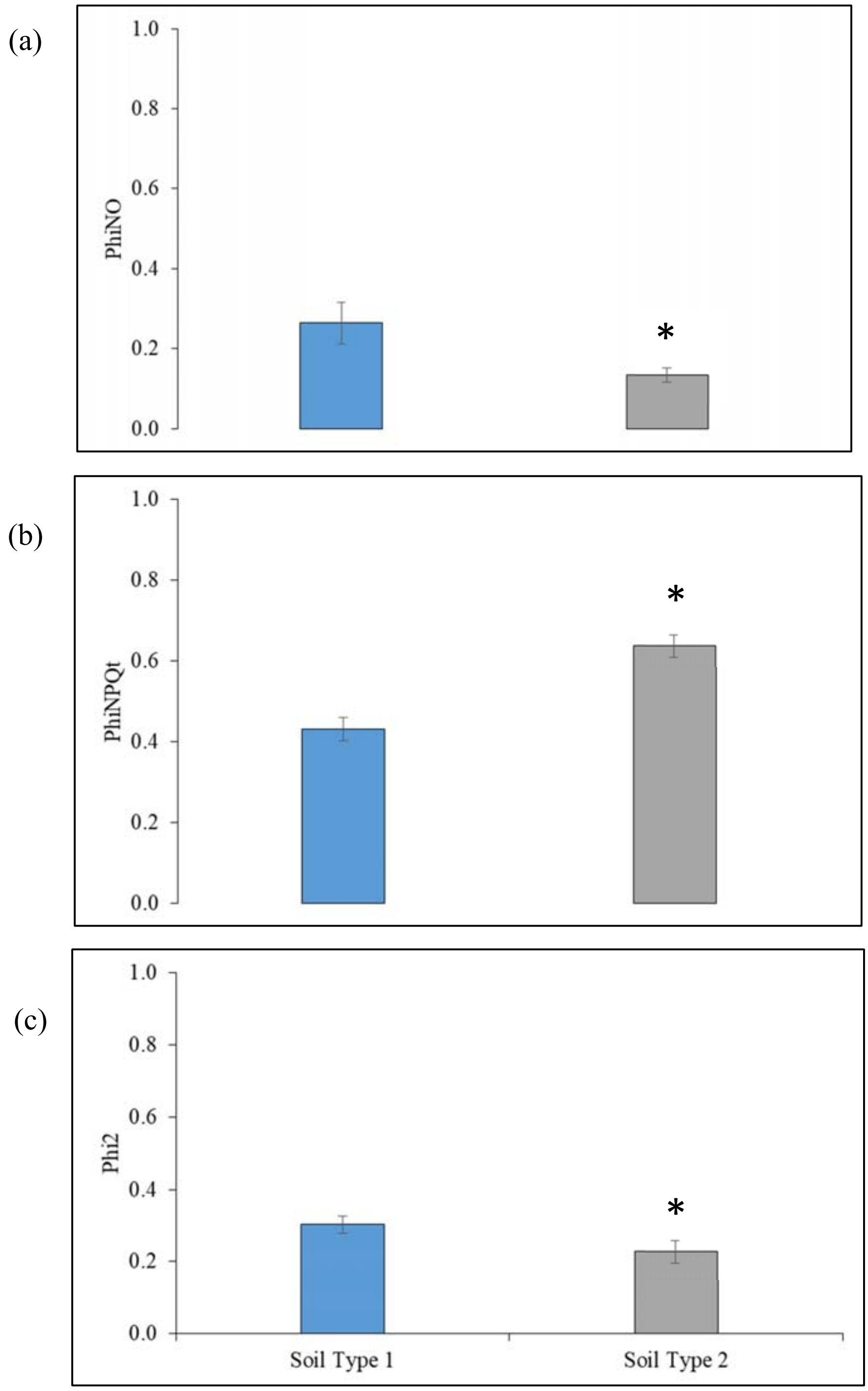

Figure 14: Effect of Soil Type 1 and Soil Type 2 on (a) PhiNPQt, (b) PhiNO, (c) Phi2 of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars with an asterisk (*) show a significant difference, leveling at $P \leq 0.05$.

4.3.2. Relative Water Contents

Relative water content was significantly decreased under Soil Type 2 compared to Soil Type 1 (Figure 4.6). The mean relative water content under Soil Type 1 was 88, while under Soil Type 2, 72. Moreover, the relative water content under Soil Type 2 decreased by 22% compared to Soil Type 1.

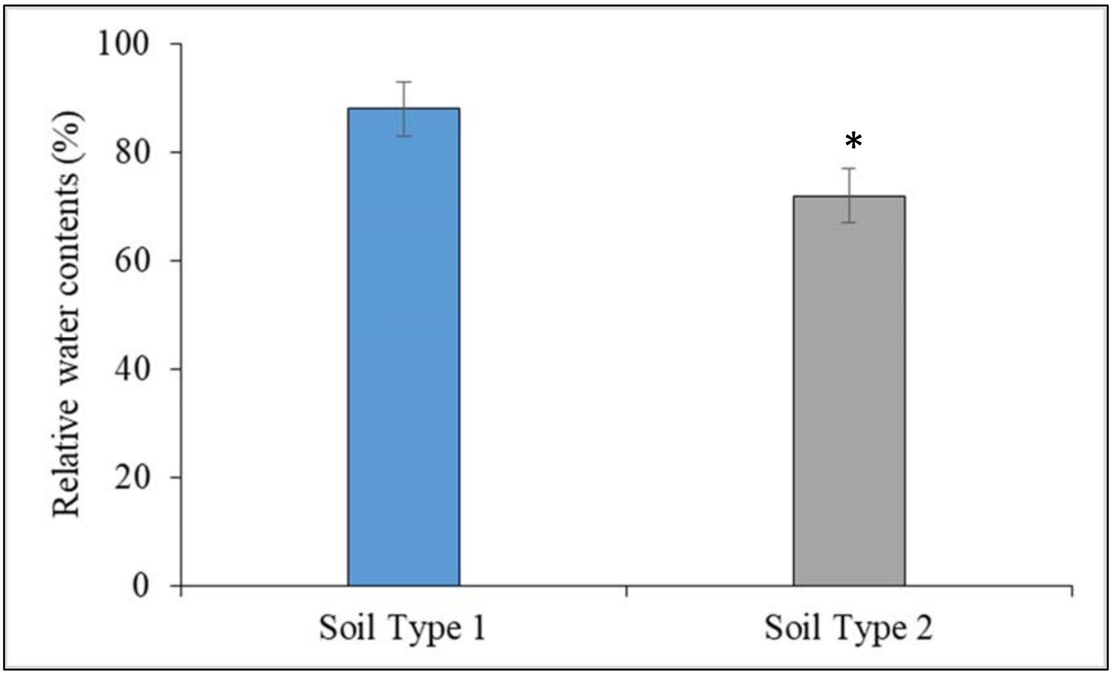

Figure 15: Effect of Soil Type 1 and Soil Type 2 on relative water contents of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars with an asterisk (*) show a significant difference, leveling at $P \leq 0.05$.

4.3.3. Membrane Stability Index

The membrane stability index was significantly decreased under Soil Type 2 compared to Soil Type 1 (Figure 4.7). The mean relative water content under Soil Type 1 was 94; under Soil Type 2, it was 66. Moreover, the relative chlorophyll under Soil Type 2 decreased by 42% compared to Soil Type 1.

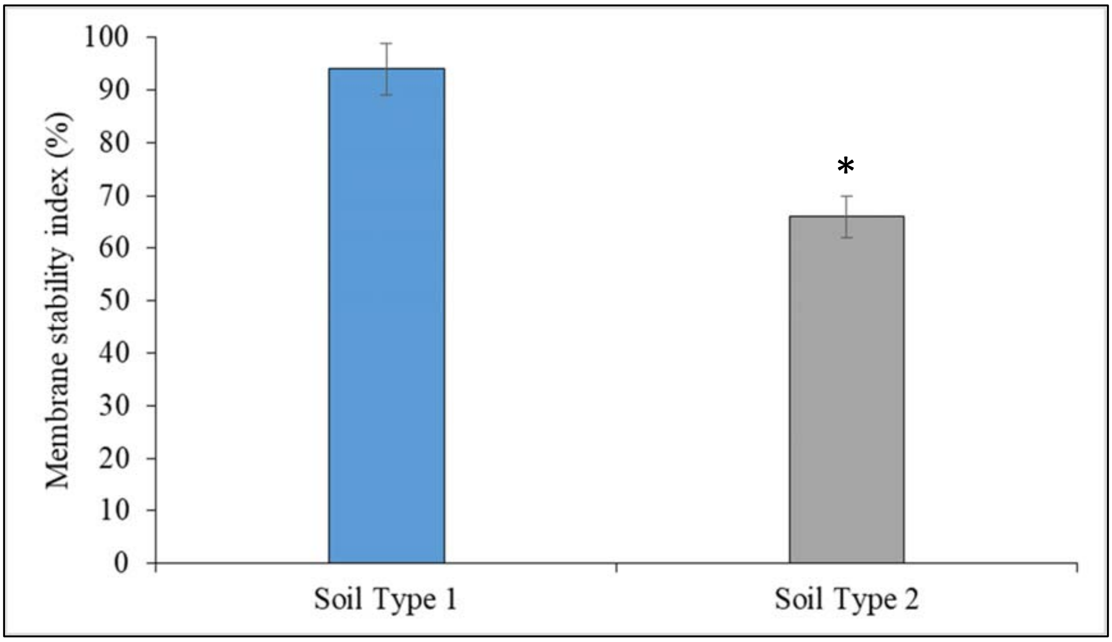

Figure 16: Effect of Soil Type 1 and Soil Type 2 on membrane stability index of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars with an asterisk (*) show a significant difference, leveling at P ≤ 0.05.

4.3.4. Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide was significantly increased under Soil Type 2 compared to Soil Type 1 (Figure 4.8). The mean H_2O_2 content under Soil Type 1 was 1.4 mmol g^{-1} FW, while under Soil Type 2, it was 2.4 mmol g^{-1} FW. Moreover, the H2O2 content of Soil Type 2 decreased by 42% compared to Soil Type 1.

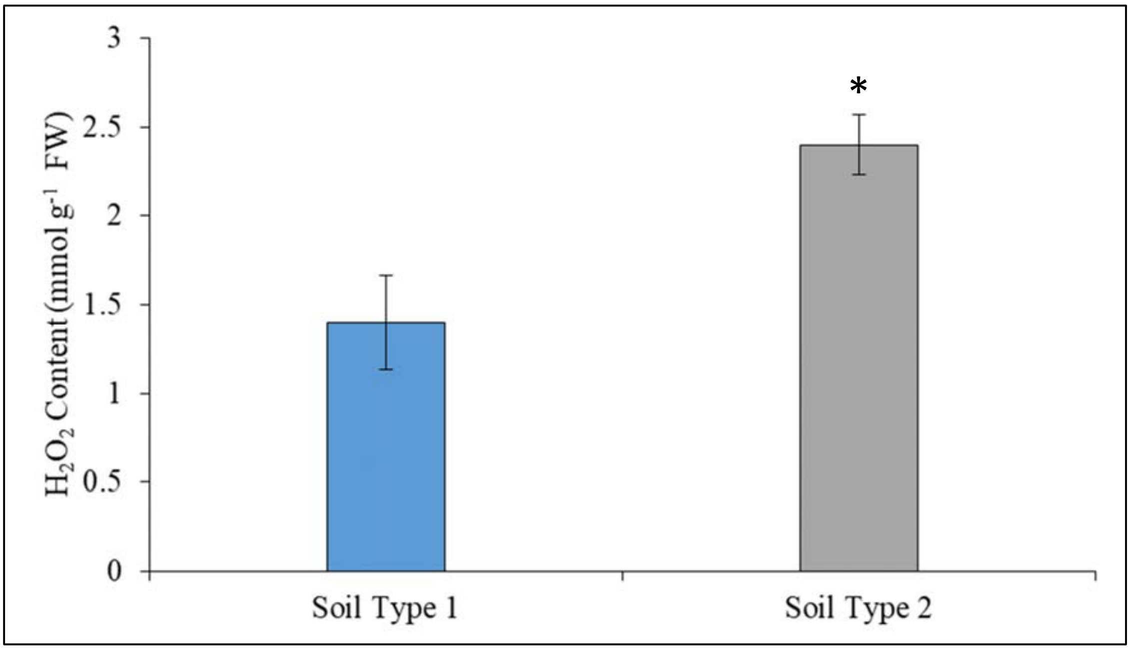

Figure 17: Effect of Soil Type 1 and Soil Type 2 on hydrogen peroxide of *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars with an asterisk (*) show a significant difference, leveling at $P \leq 0.05$.

4.3.5. Superoxide Dismutase (SOD)

Superoxide dismutase was significantly increased under Soil Type 2 compared to Soil Type 1 (Figure 4.9). The mean SOD activity under Soil Type 1 was 27 Unit g^{-1} FW, while under Soil Type 2, it was 34 Unit g^{-1} FW. Moreover, the SOD content of Soil Type 2 was increased by 20% as compared to Soil Type 1.

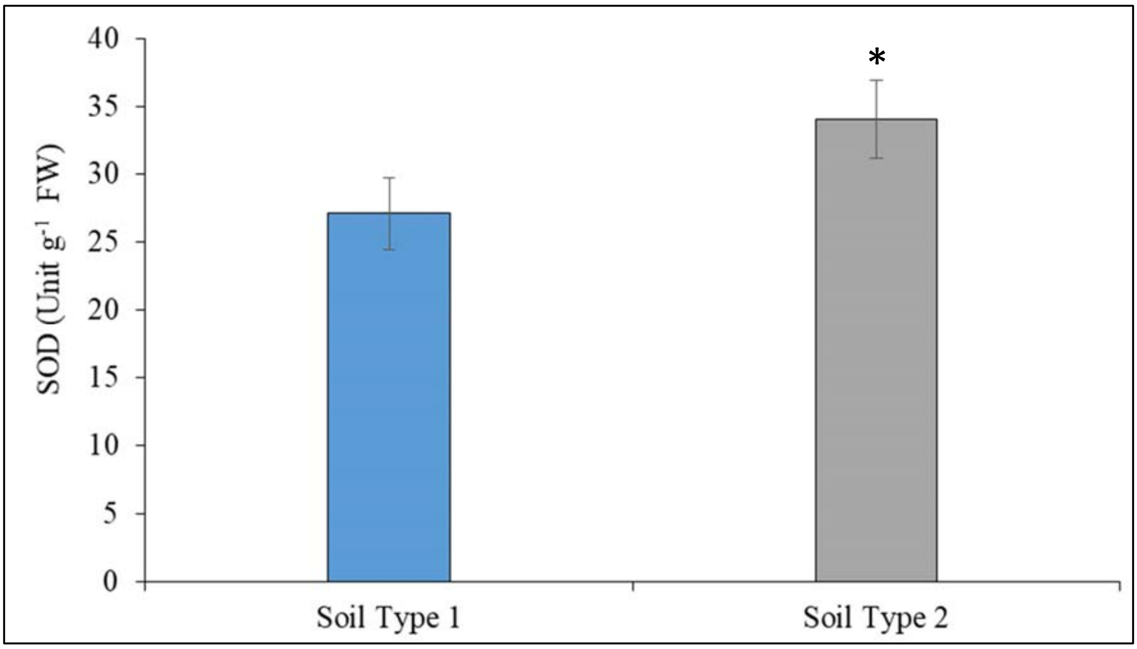

Figure 18: Effect of Soil Type 1 and Soil Type 2 on SOD activity in *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars with an asterisk (*) show a significant difference, leveling at $P \leq 0.05$.

4.3.6. Catalase (CAT)

Catalase was significantly increased under Soil Type 2 compared to Soil Type 1 (Figure 4.10). The mean CAT activity under Soil Type 1 was 0.12 Unit g^{-1} FW, while under Soil Type 2, it was 0.16 Unit g-1 FW. Moreover, the CAT content of Soil Type 2 increased by 27% compared to Soil Type 1.

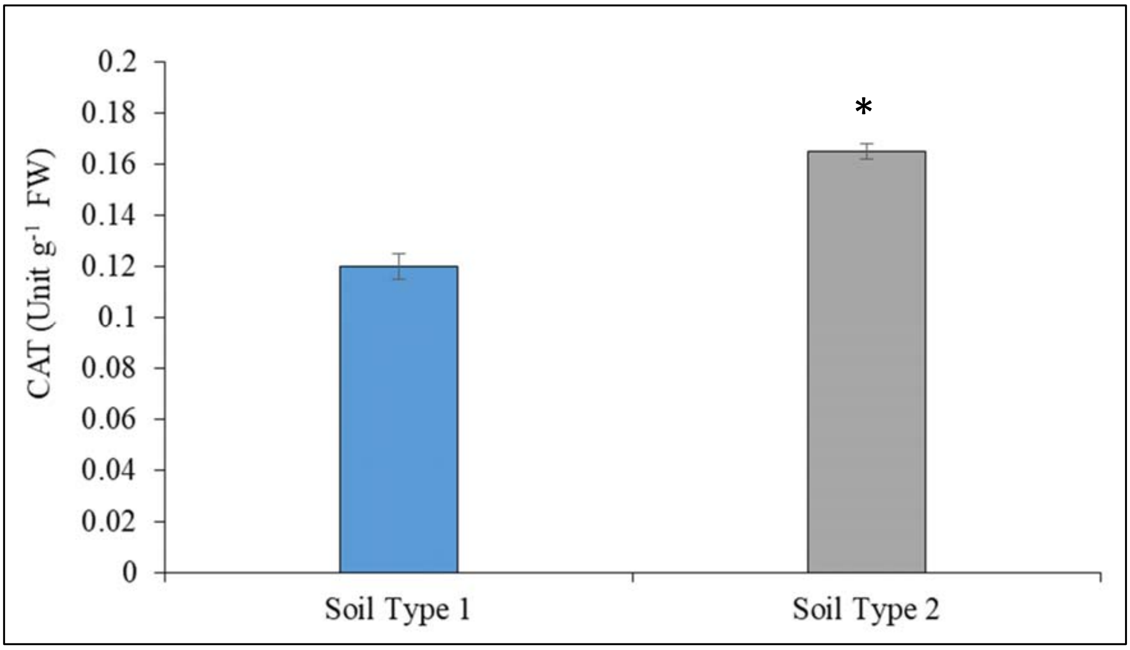

Figure 19: Effect of Soil Type 1 and Soil Type 2 on CAT activity in *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars with an asterisk (*) show a significant difference, leveling at $P \leq 0.05$.

4.3.7. Peroxidase (POD)

Peroxidase was significantly increased under Soil Type 2 compared to Soil Type 1 (Figure 4.11). The mean POD activity under Soil Type 1 was 0.06 Unit g^{-1} FW, while under Soil Type 2, it was 0.08 Unit g^{-1} FW. Moreover, the POD content of Soil Type 2 was increased by 24% compared to Soil Type 1.

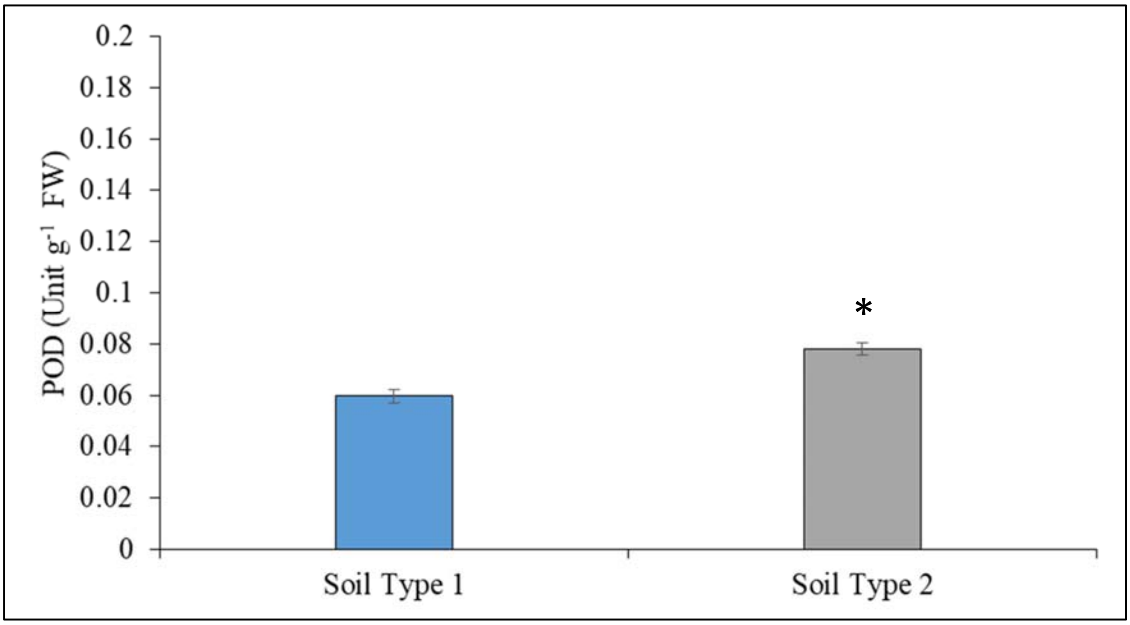

Figure 20: Effect of Soil Type 1 and Soil Type 2 on POD activity in *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars with an asterisk (*) show a significant difference, leveling at P ≤ 0.05.

4.3.8. Heavy metal in plant parts

In the context of Soil Type 2, it was shown that the roots exhibited a higher uptake of metals. The findings align with the outcomes of the soil heavy metal study, which indicated higher levels of contaminants in Soil Type 2. Moreover, the root tissues had the most significant heavy metals, whereas the shoots exhibited the lowest levels. In the case of Soil Type 2, the concentration of Cd in the roots was four times greater than in the nodes. Likewise, the root Fe concentration was three times higher than the shoot Fe concentration under Soil Type 2. Similarly, the Pb and Zn root concentrations were 5.2 and 3.6 times higher than the shoot Pb and Zn concentrations under Soil Type 2.

Parameter	**Shoots (mg/kg)**		**Roots (mg/kg)**	
Heavy Metal	**Soil Type 1**	**Soil Type 2**	**Soil Type 1**	**Soil Type 2**
Cd	0.60 ± 0.02	34.66 ± 1.00	1.51 ± 0.01	141.83 ± 1.62
Fe	1.86 ± 0.57	71.0667 ±1.49	7.23 ± 0.05	218.83 ± 1.62
Pb	1.30 ± 0.10	47.26 ± 0.50	2.13 ± 0.05	249.66 ± 1.52
Zn	3.33 ± 0.12	22.56 ± 1.55	9.13 ± 0.05	83.0 ± 1.00

Table 2: Concentration of heavy metals in shoot and roots.

Values presented here are mean of four replicates ± Standard Error ($P \leq 0.05$).

4.4. Bioconcentration Factor

Results show that *Cannabis sativa* L. had varied BCF values for tested heavy metals under Soil Types 1 and 2. The BCF values of Cd, Fe, Pb, and Zn under Soil Type 1 were 0.20, 0.42, 0.25, and 0.40, respectively. Likewise, the BCF values of Cd, Fe, Pb, and Zn under Soil Type 2 were 0.40, 0.44, 0.41, and 0.40, respectively. Moreover, the BCF value of Cd under Soil Type 1 significantly differed from Soil Type 2. Similarly, the BCF value of Pb under Soil Type 1 significantly differed from Soil Type 2. More importantly, all of the values were lower than 1. It means that the plant is not a metal accumulator.

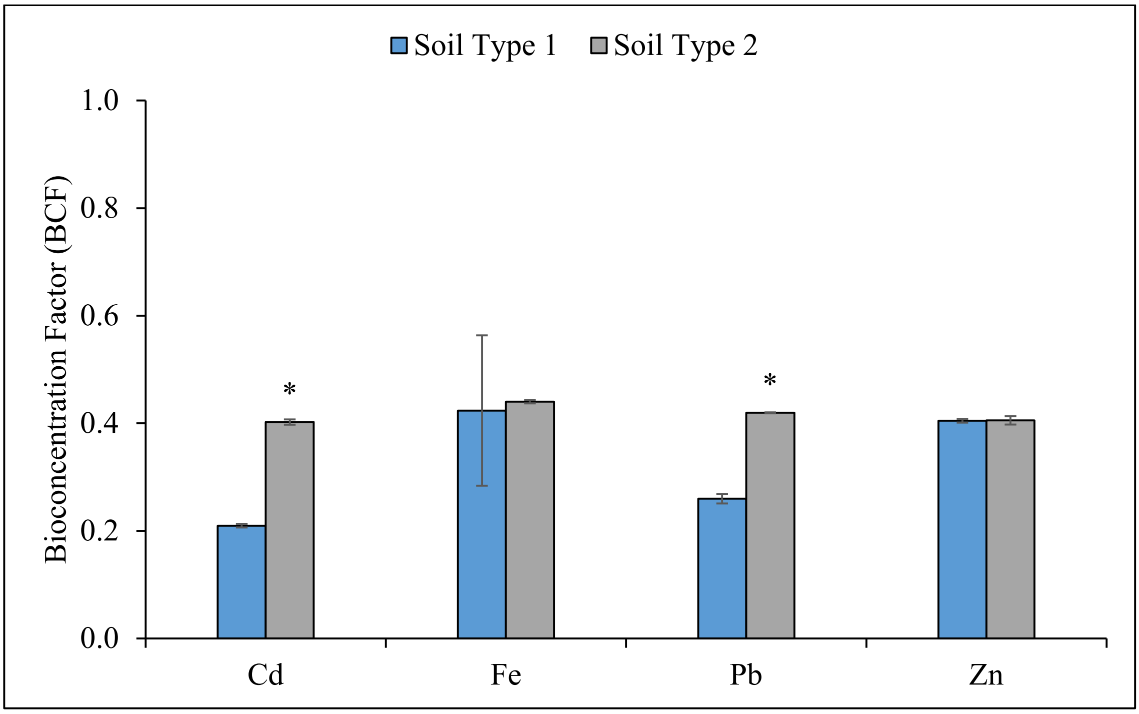

Figure 21: Bioconcentration Factor (BCF) of Soil Type 1 and Soil Type 2 in *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant difference, while the bars with asterisk (*) show significant difference, leveling at $P \leq 0.05$.

4.5. Translocation Factor

Results show that *Cannabis sativa* L. had varied TF values for tested heavy metals under Soil Types 1 and 2. The TF values of Cd, Fe, Pb, and Zn under Soil Type 1 were 0.24, 0.25, 0.60, and 0.36, respectively. Likewise, the TF values of Cd, Fe, Pb, and Zn under Soil Type 2 were 0.39, 0.32, 0.18, and 0.27, respectively. Moreover, the TF values of Cd and Fe under Soil Type 1 were significantly lower than Soil Type 2. However, the TF values of Pb and

Zn under Soil Type 1 were considerably higher than Soil Type 2. More importantly, all of the values were lower than 1. It means that the plant is not a metal accumulator.

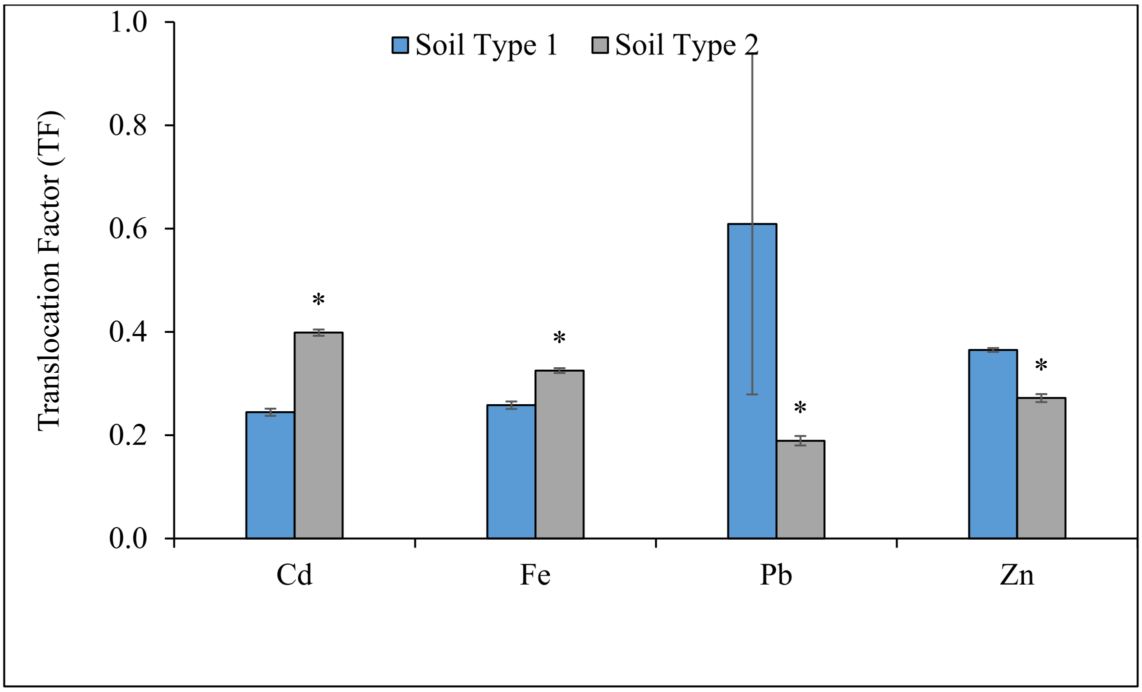

Figure 22: Translocation Factor (TCF) of Soil Type 1 and Soil Type 2 in *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant difference, while the bars with asterisk (*) show significant difference, leveling at P ≤ 0.05.

4.6. Enrichment Coefficient

Results show that *Cannabis sativa* L. had varied ECf values for tested heavy metals under Soil Types 1 and 2. The ECf values of Cd, Fe, Pb, and Zn under Soil Type 1 were 0.06, 0.09, 0.10, and 0.11, respectively. Likewise, the ECf values of Cd, Fe, Pb, and Zn under Soil Type 2 were 0.07, 0.09, 0.06, and 0.08, respectively. Moreover, the ECf values of Cd and Fe under Soil Type 1 were significantly lower than Soil Type 2. However, the TF values of Pb and Zn under Soil Type 1 were considerably higher than Soil Type 2. More importantly, all of the values were lower than 1. It means that the plant is not a metal accumulator.

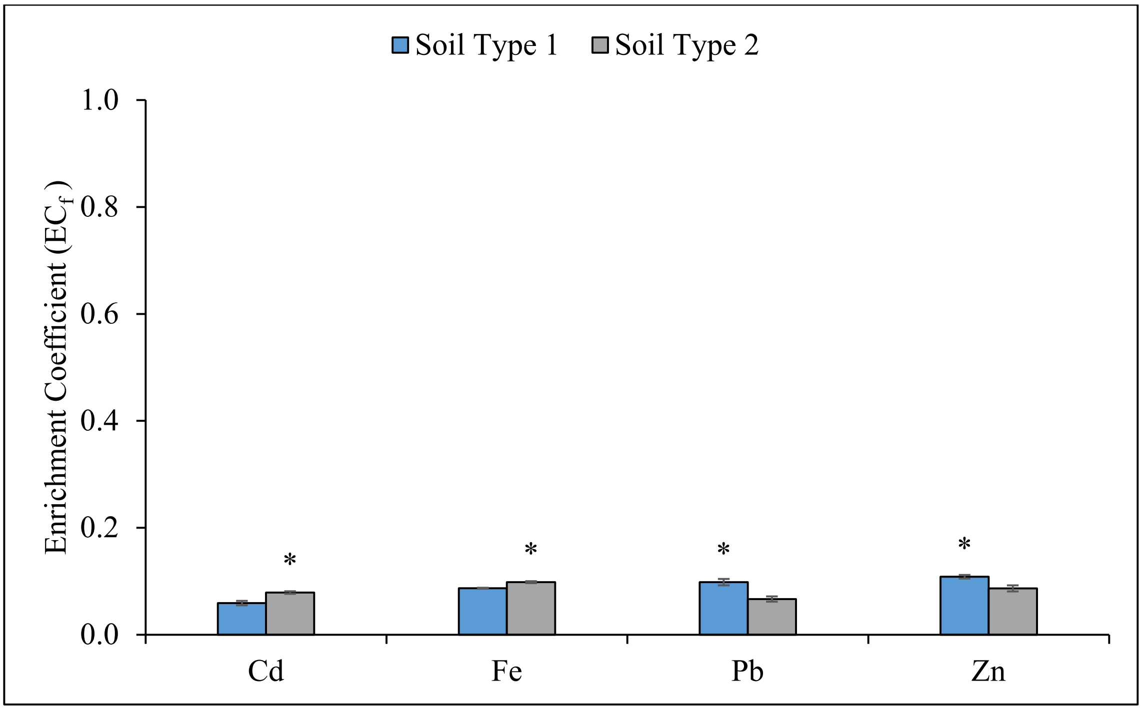

Figure 23: Enrichment Coefficient (ECf) of Soil Type 1 and Soil Type 2 in *Cannabis sativa* L.

Error bars are the Standard Error (± SE) of four replicates. Bars without asterisk (*) show insignificant differences, while the bars with an asterisk (*) show significant differences, leveling at $P \leq 0.05$.

4.7. Zinc Oxide Nanoparticle Characterization

4.7.1. Energy Dispersive X-Ray (XRD) Analysis

Figure 4.13 illustrates the structural characteristics and phase purity of ZnO nanoparticles.

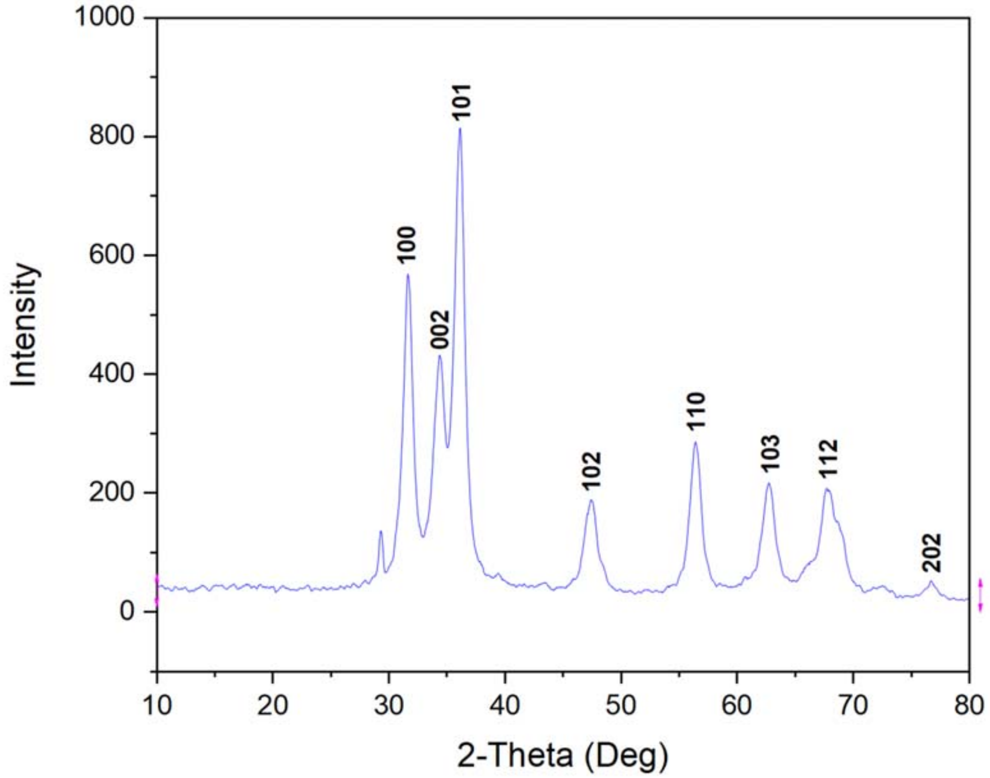

Figure 24: X-Ray diffraction (XRD) pattern of zinc oxide (ZnO) nanoparticles using *Cannabis sativa* L. leaf extract.

X-ray diffraction (XRD) diffractogram exhibits a complete correspondence with the hexagonal phase, namely the wurtzite structure. Furthermore, no additional steps or peaks indicating impurities are seen. The observation of distinct and pronounced diffraction peaks serves as empirical support for the presence of a highly ordered and structured crystalline arrangement. The sharp, intense diffraction peaks appear at about 2θ of 29.28, 31.63, 34.36, 36.12, 47.45, 56.40, 62.73, 67.73, 76.67 corresponding with those from (100), (002), (101), (102), (110), (103), (112), and (202) orientations, respectively.

4.7.2. Fourier Transform Infrared Spectroscopy

FTIR spectrum of ZnO NPs is shown in Figure 4.14.

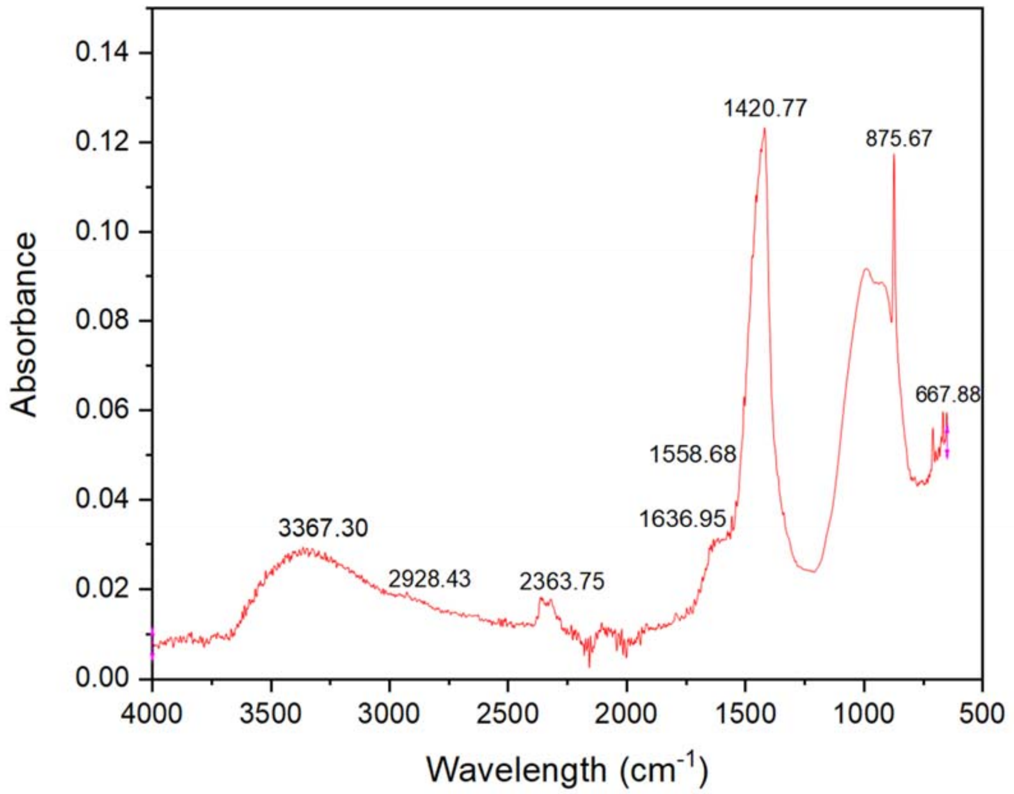

Figure 25: Fourier Transform Infrared Spectroscopy (FT-IR) pattern of zinc oxide (ZnO) nanoparticles using *Cannabis sativa* L. leaf extract.

Several vibration bands were seen in the spectra of the aqueous extract of leaves. The observed peaks at 3367.30 cm^{-1} correspond to O–H bonds stretching in alcohols. Similarly, the cliffs at 2928.43 cm^{-1} indicate the stretching of C–H bonds in alkanes. The mountains observed at 2363.75 cm^{-1} correspond to the asymmetric extension of C-O bonds. Furthermore, the peaks at 1636.95 cm^{-1} signify the attachment of C-C bonds in alkenes. The presence of peaks at 1558.68 cm^{-1} corresponds to nitro compounds' N– O stretching. Bands at 1420.77 indicate the stretching of C-C bonds in aromatic compounds. Additionally, the peak at 875.67 cm^{-1} corresponds to the extension of C–N bonds in amines, while the peaks at 667.88 cm^{-1} are associated with stretching C-C bonds in alkenes.

4.7.3. Scanning Electron Microscopy (SEM)

The analysis of synthesized nanoparticles' structural and morphological confirmation is often conducted by scanning electron microscopy (SEM) inspection. The scanning electron microscopy (SEM) images depicted individual zinc oxide nanoparticles (ZnONPs) and a certain quantity of aggregates, as seen in Figure 4.15. The diameter of the ZnO nanoparticles inside the cluster fell between the range of 20-30 nm. These nanoparticles exhibited a roughly spherical shape with flower-like morphology.

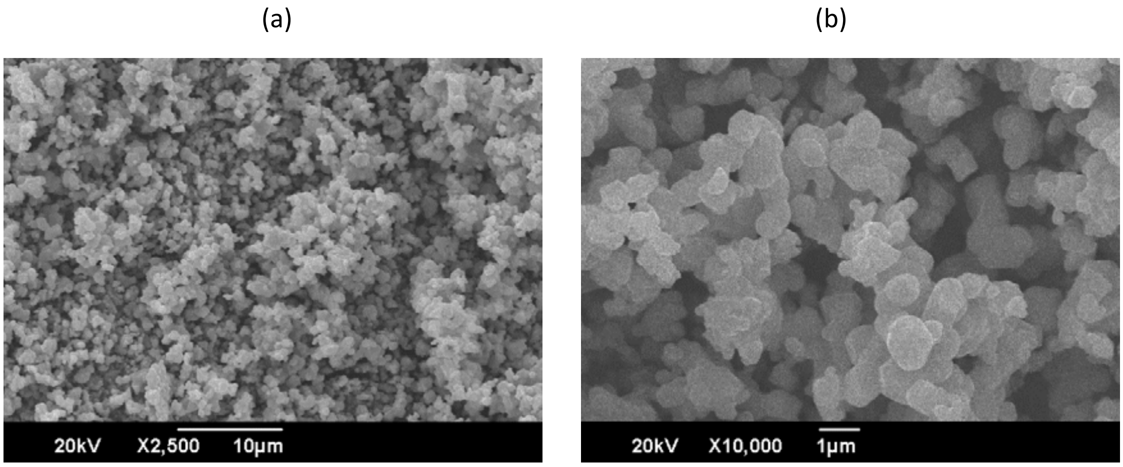

Figure 26: SEM micrographs at X2,500 and 10,000 magnifications

4.8. Discussion

Cannabis sativa L. plants exhibited noticeable adverse effects when exposed to heavy metals (HMs) in both soil types. The insufficient sunlight, crucial for photosynthesis, and the irreversible wilting observed in the experimental plants within just one month of their growth led to premature death before reaching maturity. Extensive research has investigated the detrimental impacts of various heavy metals, including cadmium (Cd), zinc (Zn), lead (Pb), and iron (Fe), on plant organisms (Linger et al., 2005; Picchi et al., 2022; Luyckx et al., 2023). The experimental findings underscored the harmful consequences of heavy metals on the biomass and yield of *Cannabis sativa* L. in the conducted studies. These repercussions encompassed reduced biomass accumulation, stunted growth, and decreased crop yield, which can be attributed to factors such as nutritional imbalances, hindered root hair formation, alterations in enzyme activity, and the suppression of chlorophyll production (Shahid et al., 2015).

Photosynthesis is the principal mechanism by which plants maintain their energy requirements, and the chlorophyll content in a plant serves as an indicator of its photosynthetic efficiency (Liu et al., 2015). The photosynthetic process is susceptible to disruption caused by heavy metals, leading to decreased chlorophyll production and other detrimental effects on plant health (Souri et al., 2019). Our study's results indicate that trace amounts of heavy metals did not considerably impact the chlorophyll levels in *Cannabis Sativa* L. This finding provides empirical support for the notion that Cannabis plants possess a degree of resistance against heavy metals (HMs) (Khan, 2020).

The antioxidant defense system is crucial in mitigating the detrimental impacts of heavy metals on the photosynthetic apparatus (Yang et al., 2021). Plants displaying resilience to heavy metal stress exhibit adaptive mechanisms that assist in preserving cellular integrity and physiological equilibrium. These robust plants can endure metal deposition without altering key gas exchange parameters like photosynthetic rate, transpiration rate, stomatal conductance, or water use efficiency (Zhang et al., 2014). Their antioxidative defense mechanisms substantially influence the durability of such plants. The heightened production of antioxidants such as glutathione and ascorbate in response to heavy metal-induced stress aids in alleviating the generation of reactive oxygen species (ROS) (Gill &

Tuteja, 2010). In such scenarios, this phenomenon contributes to maintaining membrane integrity, cellular structure, and overall physiological function (Ramos, 2018).

Regarding heavy metal distribution, the cannabis roots exhibited the highest heavy metal concentration, followed by the plant's stems and leaves. Our research findings align with prior scholarly investigations that have consistently reported elevated heavy metal levels in subterranean environments in contrast to surface-level surroundings (Magaji et al., 2018; Alam et al., 2019; Chitimus et al., 2023). The current study affirmed this alignment, demonstrating that BCF and TF values remained below unity across varying heavy metal concentrations. The observation of BCF and TF values below one suggests that Cannabis demonstrates phytoextraction rather than hyperaccumulation.

Hyperaccumulator plants exhibit a diminished capacity to retain heavy metals within their root systems, instead preferentially translocating these elements to their aboveground biomass (Hossain et al., 2023). The decreased TF value signifies a reduced movement of heavy metals (HMs) from the roots to the leaves and stems of the plant. Our findings suggest that Cannabis holds the potential for phytostabilization of heavy metal-contaminated sites due to its notably low bioconcentration factor (BCF) and transfer factor (TF) values. Several plant species have been categorized as heavy metal (HM) phytostabilizers based on their collective tendencies for metal accumulation (Mousavi et al., 2020; Da Silva et al., 2023; Ariyachandra et al., 2023).

Heavy metals disrupt cellular functions by impacting cellular metabolism, redox potential, and the generation of reactive oxygen species (ROS) (Riyazuddin et al., 2021; Mansoor et al., 2023). Plants experience oxidative stress due to an excess of reactive oxygen species (ROS), including hydroxyl (OH) or superoxide (O_2) radicals and hydrogen peroxide (H_2O_2) (Ali et al., 2019). In our study, cannabis plants exposed to heavy metals (HMs) exhibited elevated levels of hydrogen peroxide (H_2O_2) and oxidative stress (Parvez et al., 2020; Sharma et al., 2022; Ahmed et al., 2023). Enzymatic and non-enzymatic antioxidants protect plants from oxidative stress induced by reactive oxygen species (ROS) (Kapoor et al., 2019; Als & Tuten, 2022). The enzymes CAT, POD, and SOD collaboratively contribute to plants' response to oxidative stress. SOD, in particular, plays a vital role in neutralizing toxic superoxide (O2) radicals (Sharma et al., 2022). The concentration of heavy metals increased, leading to a rise in SOD activity in the experimental plants, a phenomenon supported by other studies (Abdelgawad et al., 2020; Mansoor et al., 2023). The activities of CAT, POD, and APX also increased in correlation with the rise in HM content in the soil. These enzymes aid in the breakdown of hydrogen peroxide (H2O2) into harmless water (H_2O) and oxygen (O_2) molecules (Fujita & Hasanuzzaman, 2022). Consistent with our research findings, various plants subjected to heavy metal stress exhibited increased levels of these enzymes (Abdelgawad et al., 2020; Saleem et al., 2022).

Using plant-derived byproducts for synthesizing nanoparticles (NPs) is gaining recognition as a valuable approach for sustainable waste management in phytoremediation (Meichtry et al., 2023). Zinc oxide (ZnO) nanoparticles were synthesized using the residue from this experiment. The synthesis was confirmed through X-ray diffraction (XRD), scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and similar techniques shown in Figures 1, 2, 3, and so on. This approach significantly reduces the risk of environmental contamination from the disposal of heavy metal-contaminated plants (Ankamwar et al., 2020). Nanoparticles are produced and developed within living plants to mitigate heavy metal penetration into groundwater and its subsequent ecological impact (Dikshit et al., 2021). Numerous studies support this approach's feasibility and safety (Oruc et al., 2019; Al Jabri et al., 2022; Priyadarshini et al., 2023). This technique embodies the circular economy concept of waste-to-value in environmental management and is eco-friendly. The nanoparticles generated through this method have potential applications in various fields, including medicine, owing to their antibacterial, anti-inflammatory, and other beneficial properties (Samuel et al., 2022). Hence, *Cannabis sativa* L. could potentially be a phytostabilization for heavily contaminated soils. It is undeniable that a deeper understanding of the mechanisms and processes through which *Cannabis sativa* L. interacts with and stabilizes heavy metals will undoubtedly yield novel findings and applications in the realm of environmental remediation. However, further research in this field is imperative for a comprehensive understanding.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

In a pot experiment, the heavy metal tolerance of *Cannabis sativa* L. was explored under industrial-contaminated soil. The plants produced the highest biomass and length under Soil Type 1 and decreased under Soil Type 2. Increased heavy metal concentration may affect the plant's physiological parameters. As a result, it influences physiological parameters like photosynthetic performance, ROS and antioxidant activity, and relative water contents. However, the photosynthetic parameters, including relative chlorophyll, PhiNO, Phi2, and PhiNPQt, showed varied responses. For instance, PhiNO and Phi2 decrease significantly under Soil Type 1 compared to Soil Type 2. However, relative chlorophyll was not significantly decreased, and PhiNPQT was increased significantly under Soil Type 2 as compared to Soil Type 1. Moreover, the substantial increase in reactive oxygen species levels and antioxidant enzyme activity observed serves as compelling evidence of the plant's reaction to abiotic stress induced by heavy metals. Notably, the BCF, TF, and ECF values remained below one, indicating that the plant does not possess hyperaccumulator traits for heavy metals in multi-contaminated soil. The plant exhibited a pattern of heavy metal accumulation in the following order: roots > shoots > leaves. Utilizing the plant material, nanoparticles were efficiently synthesized through an eco-friendly process. In conclusion, *Cannabis sativa* L. holds the potential for the phytoextraction of heavy metals in multi-contaminated industrial soil.

5.2. Recommendations

The following recommendations are derived from the results and deliberations of this experiment:

1. The use of *Cannabis sativa* L. in phytoextraction and nanoparticles synthesis holds promise for performing an economic analysis to evaluate its potential as a sustainable business model.

2. Considering the achievements in the environmentally friendly production of nanoparticles using plant-based materials, forthcoming investigations may concentrate on refining this procedure and broadening its utilization to encompass other types of heavy metals.
3. The relevance of comprehending the genetic makeup of *Cannabis sativa* L. with regard to its resilience becomes apparent when considering the advancements in the genomics field.

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