INFLUENCE OF CADMIUM EXPOSURE ON INDUSTRIAL HEMP GROWN FOR CANNABINOID PRODUCTION (*CANNABIS SATIVA* L.)

by

AMANDA OLBRICK MARABESI

(Under the Direction of Timothy W. Coolong)

ABSTRACT

Industrial hemp (*Cannabis sativa* L.) has demonstrated promise for phytoremediation purposes due to its deep roots, large biomass, and ability to survive exposure to relatively high levels of heavy metals. However, little research has been conducted to determine the impact of heavy metal accumulation in hemp grown for flower production for medicinal use. In particular, cadmium (Cd) accumulation in smokable plants, such as tobacco and hemp, is concerning for human health due to the harmful effects of Cd inhalation, which has been associated with serious health conditions and is classified as a Group 1 carcinogen. In the present dissertation, we determined the potential for Cd accumulation in industrial hemp varieties grown for cannabinoid production. Three distinct studies were conducted, aiming to elucidate the influence of Cd on *C. sativa*. Results from these studies suggest that the hemp variety 'Purple Tiger' exposed to up to 10 $mg \cdot L^{-1}$ Cd in hydroponic solutions were able to uptake Cd from the solution and translocate it to aboveground tissues, although roots were the primary site for Cd accumulation. Tolerance mechanisms may include upregulating expression of multiple ATPase HMA transporter genes in the root tissue. These transporters may likely be involved in Cd uptake in the roots via regulating its transport and sequestration, and xylem loading for long distance transport of Cd to shoot, leaf,

and flower tissues. Furthermore, Cd was mainly localized in leaf epidermis, spongy mesophyll, and trichomes, and in root rhizodermis, cortex, and pericycle. Nutrient partitioning and Cd uptake, translocation, and accumulation in day-length sensitive and day neutral hemp varieties to assess distinctions in Cd accumulation and distribution among plant tissues in hemp varieties with different growth and flowering habits. While Cd concentrations that were available to plants used in these studies are greater than what would be generally encountered on agricultural soils, our results indicate that hemp plants have the potential to accumulate Cd in aboveground tissues, although this is impacted by variety.

INDEX WORDS: accumulation, cadmium, cannabinoid, cannabis indica, cannabis ruderalis, cannabis sativa, heavy metal, hemp, hydroponic, metal transporter genes.

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by

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DEDICATION

I dedicate this dissertation to my parents, Adriana and Mario, and my sister, Marina, who have supported me unconditionally along this journey; to my grandparents, Magdalena and Francisco, who have inspired me to pursue this degree; and to my husband, Jacob, who has been there for me at all times.

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CHAPTER 1

INTRODUCTION

Hemp (*Cannabis sativa* L.) is an ancient crop widely cultivated for centuries for its many applications (Ranalli, 1999). The primary distinction between hemp and marijuana is the concentration of tetrahydrocannabinol (THC) in the plant. Tetrahydrocannabinol and cannabidiol (CBD) are two of the more than 100 cannabinoids found in cannabis plants (ElSohly & Gul, 2014). High-THC cannabis (marijuana) is well known for its psychoactive effects, with production intended for recreational and medicinal purposes. Low-THC cannabis (< 0.3%) can be grown legally as industrial hemp for fiber to be used in the textile and construction industries, seeds (oil), and most commonly for its CBD content, which has medicinal uses, such as the treatment of anxiety and seizures (National Academies of Sciences, Engineering, and Medicine, 2017).

In the United States (U.S.), with the passage of the 2018 Farm Bill, industrial hemp production became federally legal. Although legalized by the federal government, each state was tasked with creating its guidelines for production. Significant regulations for industrial hemp production must be followed to stay in compliance with current laws. In addition to legal barriers, hemp growers face many production challenges. Increasing yield and optimizing cannabinoid content while reducing production costs are two concerns.

A potential issue with industrial hemp production for medicinal purposes is the potential for heavy metal contamination of agricultural soils. Humans may be exposed to heavy metals in any number of ways – via food (e.g. rice grown in contaminated flooded soils), cosmetics,

medicinal products, ingesting dust from contaminated soils, as well as habits such as smoking. For instance, cadmium (Cd) exposure has been associated with smoking tobacco, especially counterfeit cigarettes in developing markets (Akesson & Chaney, 2019). Nonetheless, consumers are less likely to perceive the harms of cannabis smoke to the same extent as those caused by smoking tobacco (Gauvin et al., 2018).

Hemp has agronomic traits that could facilitate the accumulation of heavy metals such as a large and deep root system, rapid life cycle, and high biomass production (Citterio et al., 2003). As a result, companies producing hemp for floral material and subsequent cannabinoid extraction have expressed an interest in determining typical levels of heavy metals in hemp flowers. Studies have documented the ability of hemp to survive on soils with relatively high levels of heavy metals (Ahmad et al., 2015; Angelova et al., 2004; Citterio et al., 2003; Galic et al., 2019; Husain et al., 2019; Linger et al., 2002; Shi & Cai, 2009; Shi & Cai, 2010; Shi et al., 2012). However, these studies focused on fiber varieties for phytoremediation purposes and have not reported on heavy metal accumulation in hemp floral material.

Research Objectives

There is a dearth of research on heavy metal accumulation in industrial hemp grown for the medicinal market. Not only do the medicinal varieties differ from those grown for fiber and seed production, but the harvested portion of the plant, the unpollinated female flower, is also different. Therefore, in the present dissertation, we aimed to evaluate the potential for heavy metal accumulation in industrial hemp varieties grown for cannabinoid production. This dissertation aimed to elucidate the following research questions:

1. Does industrial hemp have the ability to accumulate Cd?

2. Is the accumulation restricted to the root system or is cadmium translocated to stems, leaves, and floral tissues?
3. How do the different varieties of hemp grown for cannabinoid production differ in the accumulation of Cd?
4. Does Cd accumulation interfere with cannabinoid synthesis?
5. How does Cd stress affect industrial hemp plant physiology?
6. What plant defense mechanisms are triggered under Cd stress?

Three distinct studies were conducted. Our first study aimed to evaluate the potential for Cd accumulation and its impact on growth and physiological responses related to photochemical efficiency and chlorophyll content, and transcript abundance of metal transporter genes in an industrial hemp variety grown specifically for cannabinoid production. The second study evaluated nutrient partitioning and Cd uptake and translocation in two day-length sensitive and two day-neutral hemp varieties to determine the impact of Cd exposure on plant growth and yield, nutrient partitioning, and cannabinoid concentrations in flowers. In the third study, our main objectives were to assess Cd-induced alterations at the cellular level in hemp roots and the ability of hemp to translocate Cd from the root system to the shoot, and to perform histochemical localization of Cd at the cellular level in roots and leaves.

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CHAPTER 2

LITERATURE REVIEW

Horticultural Hemp

Eurasia, more specifically Western Asia and Egypt, is considered the center of origin of *Cannabis* plants over 5000 years ago (Clarke & Merlin, 2016; ElSohly & Gul, 2016; Ranalli, 1999). Humans have cultivated *Cannabis* for its fiber, seeds, and psychoactive properties. For centuries, along the domestication process, ancestral wild populations of *Cannabis* have been subjected to selection pressures, giving rise to the multiple varieties that exist today (Clarke & Merlin, 2013).

Cannabis is a genus within the Cannabaceae family, with *Cannabis sativa* L. being the most common denomination associated with the plant. However, there is an ongoing debate on whether the *Cannabis* genus is polytypic. Some taxonomists suggest that there are three species within the genus, *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis*, which originated in different geographic regions and have distinct phenotypes. Other taxonomists have suggested that all *Cannabis* plants should be classified under *Cannabis sativa*.

Current industrial hemp breeding efforts target fiber, seed, or cannabinoid production, based on regional legislation and market potential (Cherney & Small, 2016; Clarke & Merlin, 2016). Some of the goals of breeding are: a) developing high fiber content varieties, preferably with a high cellulose to lignin ratio, b) developing varieties with different cannabinoid ratios to supply diverse markets, such as medicinal, recreational, cosmetic, etcetera, c) developing varieties

that produce seeds with improved nutritional value and greater oil content, and d) developing varieties that are day-neutral or minimally sensitive to day-length (Clarke & Merlin, 2016).

Propagation

Most hemp plants are dioecious, having distinct male and female plants, while monoecious plants are sometimes found in fiber varieties (Clarke & Merlin, 2016; Potter, 2014). Hemp may be propagated by seeds or vegetative cuttings. While planting seed is common for fiber and seed varieties of hemp it is less popular for hemp grown for cannabinoids. Hemp plants grown for cannabinoid production are almost always female. Because cannabinoids are highly synthesized in non-fertilized female flowers, also known as sinsemilla (Potter, 2014), pollen-bearing plants must be removed from the crop to avoid fertilization (Ranalli, 1999) and consequently a reduction in cannabinoid synthesis. Therefore, seeds must be feminized to be used as varieties for cannabinoid production. The process of producing feminized seeds consists of inducing the development of male flowers containing pollen sacs on genetically female plants; therefore, the viable pollen will carry only female genetics, being able to produce all-female seeds when crossed with another female plant. Chemical substances such as silver thiosulfate have been used via foliar spray to induce the development of male flowers in female *Cannabis* plants (Lubell & Brand, 2018).

Vegetative cuttings provide better control over plant sex since cuttings from a female stock plant will result in clones, i.e., female plants only. A challenge regarding vegetative cuttings is related to the loss of vigor and quality over time as multiple generations of cuttings may be taken from a single "mother" plant. In addition, the cost to maintain stock plants and take cuttings has increased expenses for growers. Tissue culture has also been used as a propagation method and is commonly used to create stock plants from which cuttings are taken. Tissue culture significantly

increases the production of quality plants, and the purchase of new tissue culture stock plants annually is encouraged to maintain plant quality and vigor (Sacirbey, 2019). Applications of hemp tissue culture include breeding for the selection of desired traits. For instance, hemp growers look for varieties with low THC, high CBD content, and high bast fiber content when the production is destined for the textile/construction industry. Tissue culture has not been widely used in the hemp industry; however, it presents an attractive and promising option for maintaining crop quality.

Photoperiod

Hemp plants have different light requirements for vegetative versus reproductive stages (Potter, 2014; Ranalli, 1999). While the vegetative stage is maintained by a photoperiod of 18 or more hours of light, day-length sensitive (DLS) plants typically initiate flower when exposed to 12 to 14 hours of uninterrupted dark (Higginbotham, 2019; Potter, 2014). Conversely, some varieties are not sensitive to day-length and have the reproductive stage regulated by physiological processes, a trait that comes from the *Cannabis ruderalis* species; these are called day neutral (DN) or colloquially referred to as *auto-flower* types (Potter, 2014). Despite the advantages of growing DN hemp, principally a lack of day-length manipulation, they are not easy to propagate through cuttings (Potter, 2014). Genotype determines how long the flowering stage should last until flowers are ready to be harvested (Potter, 2014).

Cannabinoids

Cannabis plants are rich in a wide range of secondary metabolites, including phenolic compounds, such as terpenes, and cannabinoids. Due to their popularity for medicinal and recreational purposes, cannabinoids are the most thoroughly researched phytochemicals in the hemp plant. Cannabinoid synthesis happens within secretory structures in the inflorescence called trichomes (Potter, 2014). Cannabinoid synthesis is initiated when the polyketide pathway and the

deoxyxylulose phosphate/methylerythritol phosphate (DOXP/MEP) pathway produce olivetolic acid and geranyl diphosphate (GPP), respectively (Flores Sanchez & Cerpoorte, 2008) (Fig. 1.2). When these two compounds are combined, they give rise to cannabigerolic acid (CBGA). CBGA is converted into cannabidiolic acid (CBDA) by cannabidiolic acid synthase and further into Δ9-tetrahydrocannabinolic acid (THCA) by Δ9-tetrahydrocannabinolic acid synthase. CBDA and THCA are the acidic forms of CBD and THC, respectively. CBDA and THCA have no psychoactive properties on their own. They must be converted to CBD and THC via decarboxylation, which can happen by exposure to high temperatures, during drying/storage and/or when smoked, to have any psychoactive effects on humans (de Meijer et al., 2003). Along with CBD and THC, several other cannabinoids, such as cannabigerol (CBG) and cannabichromene (CBC), are synthesized by cannabis plants in quantities that vary according to variety, environmental conditions, and agronomic practices.

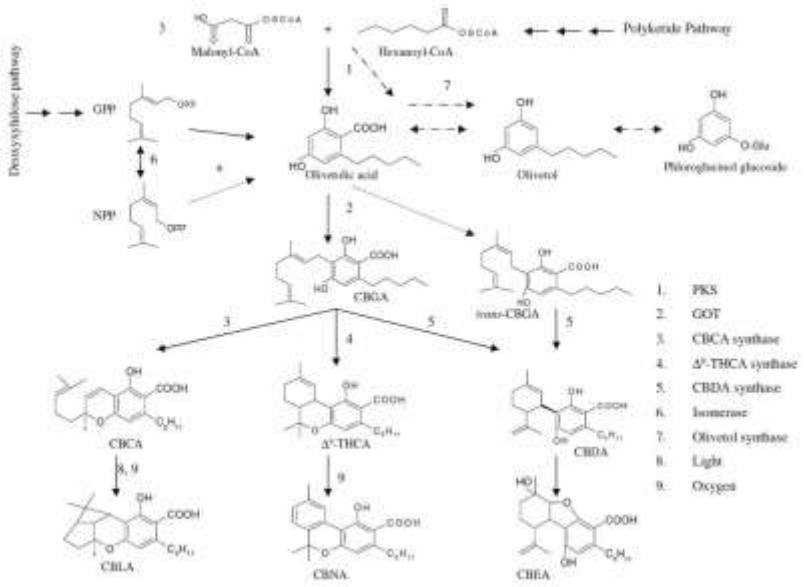

Figure 2.1. Cannabinoids biosynthesis pathways. *Note.* Reprinted from "Secondary metabolism in cannabis" by I. J. Flores-Sanchez & R. Verpoorte, 2008, *Phytochem Rev., 7*, p. 620.

Heavy Metals in Agricultural Soils

Heavy metals are naturally occurring elements that have a specific gravity greater than 5 $g \cdot cm^3$ (DalCorso, 2012; Mitra et al., 2014). Among the 53 existing heavy metals, some are considered essential or beneficial for plant growth and development, including iron (Fe), cobalt (Co), copper (Cu), molybdenum (Mo), manganese (Mn), nickel (Ni), and zinc (Zn). All of these elements are essential micronutrients and are toxic to plants at high concentrations. Non-essential heavy metals are not beneficial for plant growth and development and could be toxic at low concentrations; for example arsenic (As), cadmium (Cd), chromium (Cr) mercury (Hg), and lead (Pb) (DalCorso, 2012; Mitra et al., 2014).

Soils contaminated with heavy metals threaten crop production as metals can compete with essential nutrients for plant absorption (Zhuang et al., 2014). Additionally, some metals can negatively impact plant physiological processes, such as photosynthetic efficiency, and trigger oxidative stresses, accumulating reactive oxygen species (ROS) (Singh et al., 2016). These alterations could lead to a reduction in crop performance and yield.

Soil texture and pH also contribute to the availability of heavy metals in the environment (U.S. EPA, 2007). Soil texture is directly related to cation exchange capacity (CEC) and the general tendency is that CEC increases as the soil composition goes from sand (coarse) to clay (fine), even higher if organic matter is present. The higher the CEC, the greater the ability to retain cations in the soil particles, which could hinder plants' uptake and simultaneously avoid leaching. Soil pH directly influences the availability of elements to plants as the element form can change according to the pH. A soil pH between 5.5 and 6.5 is considered optimum for nutrient availability, although it varies according to distinct elements. In general, heavy metals are more available at lower pH.

Heavy metal contamination of agricultural soils is concerning when growing crops used for food or medicinal purposes, potentially harming human and animal health (DalCorso et al., 2013; Ismael et al., 2019). Heavy metals can be deposited on agricultural land by atmospheric deposition, especially near waste incinerators and metal smelting facilities, by the addition of fertilizers and non-treated biosolids, and could also be naturally present in the soil and mineralized over time (Chaney et al., 2010; Chaney & Baklanov, 2017).

Although the potential harms to ecosystems and human and animal health caused by heavy metals have been thoroughly investigated (Akesson & Chaney, 2019; Chaney, 2010; Chaney, 2012; Chaney, 2015; Gauvin et al., 2018), there are still inconsistencies regarding heavy metal thresholds for agricultural soils. The U.S. Environmental Protection Agency (EPA) has set regulatory limits on heavy metals applied to soils, including a) maximum concentration in sludge, b) annual pollutant loading rates, and c) cumulative pollutant loading rates (U.S. EPA, 1993). However, the U.S. EPA has set no thresholds for total or bioavailable concentrations of heavy metals in agricultural soils.

Data from the Georgia Environmental Protection Division (GA EPD), Cornell Waste Management Institute (WMI) (2011), and U.S. EPA are displayed in Table 2.1. The Ecological Soil Screening Levels (Eco-SSLs) (U.S. EPA, 2005) represent the concentration limits of contaminants used for identifying ecological risks associated with such contaminants. However, the Eco-SSLs do not serve as a parameter for soil remediation.

Table 2.1. Comparison of regulatory limits of soil heavy metals.

	Parts per million (ppm) in dry soil weight basis		
Heavy metal	GA EPD*	Cornell WMI**	Eco-SSL***
Arsenic	20	16	18

Cadmium	7.5	1.2-3	32
Chromium	100	-	-
Copper	920	50-120	70
Lead	270	100	120
Molybdenum	-	2	-
Nickel	510	30-60	38
Selenium	5.2	-	0.52
Zinc	5700	90-230	160

Note. *Limits established by GA EPD are regarding residential soils. **Values displayed as ranges indicate that limits will vary according to soil type (sandy<sandy loam to silt loam< silty clay to clay soil); limits for soils with pH 6 or above. ***Eco-SSL for terrestrial plants.

Phytoremediation

Phytoremediation techniques encompass several processes that aim to decontaminate soils and water from contaminants, such as metals and xenobiotics, using plants. It stems from the ability of some species of plants to establish themselves in otherwise phytotoxic environments (Chaney and Baklanov, 2017; Nesler and Furini, 2012). Although not all plants have the ability to accumulate metals, several species have been identified by several authors as natural hyperaccumulators of specific contaminants, including *Astragalus racemosus, Alyxia rubricaulis*, *Brassica juncea*, *Biscutella laevigata*, *Noccaea caerulescens*, *Phyllantus favieri*, *Phyllanthus serpentinus*, and *Pteris vittata* (Chaney and Baklanov, 2018; Yan et al., 2020).

According to Nesler and Furini (2012, p. 77), plants used for phytoremediation should ideally "(i) possess an intrinsic capacity to tolerate metals and to concentrate them in harvestable aerial tissues or to adsorb them on the root surface; (ii) grow rapidly and produce lots of biomass;

(iii) develop a deep and extensive root system; (iv) be widely distributed, allowing growth in many habitats; and (v) should be easy to cultivate and harvest." However, most natural hyperaccumulators are relatively rare, do not produce significant amounts of biomass, have fine-root traits, and have little economic value (Linger et al., 2002; Nesler and Furini, 2012; Yan et al., 2020).

Some indicators can be calculated to determine the accumulation potential of a plant species. For instance, the bioconcentration factor (BCF) is the ratio between the metal concentration in the plant tissues at harvest and the initial metal concentration in the soil or growing solution (Pachura et al., 2016). BCF values < 0.01 indicate no accumulation; values between 0.01 and 0.1 indicate low accumulation; values between 0.1 and 1.0 indicate medium accumulation; and values > 1.0 indicate high accumulation (Michałowski and Gołas, 2001, as cited in Pachura et al., 2016, p. 1474). The translocation factor (TF) is calculated as the ratio between the metal concentration in the shoot and the metal concentration in the root. Plant growth parameters can also be assessed to determine the tolerance index (TI), calculated as the ratio between growth in contaminated soil and growth in not contaminated soil.

Heavy Metal Accumulation in Hemp

There has been much speculation regarding *C. sativa* being a hyperaccumulator species. Theoretically, the plant has agronomic traits that would facilitate heavy metal extraction, such as a deep root system, fast life-cycle, and high biomass production (Citterio et al., 2003; Nesler & Furini, 2012). Indeed, several studies have documented the heavy metal accumulation potential by hemp, especially for fiber varieties (Ahmad et al., 2015; Angelova et al., 2004; Citterio et al., 2003; Galic et al., 2019; Husain et al., 2019; Linger et al., 2002, 2005; Shi & Cai, 2009; Shi & Cai, 2010; Shi et al., 2012).

There are currently no standards set by the U.S. Food and Drug Administration (FDA) or EPA for acceptable dietary intakes or for food crop concentrations of heavy metals. Therefore, researchers have relied on guidelines from the World Health Organization (WHO) or on limits established for the European Union. The U.S. Domestic Hemp Production Program established by the U.S. Department of Agriculture (USDA) does not mandate heavy metal testing for hemp plant materials and each state is responsible for implementing its hemp production guidelines. For instance, the California Bureau of Marijuana Control has established action levels for Cd, Pb, As, and Hg for three categories of *Cannabis* products (Table 2.2). Although these limits are not federally enforced, hemp producers and third-party testing laboratories have adopted resource like this as a guideline as there is a lack of information regarding permissible heavy metal concentrations in hemp products.

Table 2.2. Action levels for medical cannabis goods.

Heavy metal	Action level for medical edible cannabis products, suppositories, sublingual products, and other manufactured products (ppm)	Action level for all inhaled medical cannabis goods (ppm)	Action level for topical and transdermal medical cannabis goods (ppm)
Cadmium	0.5	0.2	5.0
Lead	0.5	0.5	10.0
Arsenic	1.5	0.2	3.0
Mercury	3.0	0.1	1.0

Note. Adapted from "Bureau of Marijuana Control Proposed Text of Regulations", Chapter 5: Testing Laboratories, p. 29-30.

Uptake and Transport Mechanisms of Heavy Metal Cations

Plants have defense mechanisms for reducing damage caused by heavy metals include accumulation and sequestration in vacuoles, excretion, and exclusion (Nikalje & Suprasanna, 2018). Such mechanisms vary according to plant species. In the present dissertation, we will focus on the ability of hemp plants to accumulate and sequester heavy metals to cope with heavy metal stress.

The first step in heavy metal accumulation in plants is the uptake of ions by the roots. The rhizosphere is especially important in this process as it provides a convenient environment bioavailable forms of metals in terms of pH, redox potential, and presence of microorganisms (Ismael et al., 2019). The root apoplast adsorbs metal cations and subsequently can be taken up through the symplast until reaching the xylem (Yin et al., 2015). The accumulation process is followed by metal translocation to other plant tissues and sequestration into root vacuoles (Ismael et al., 2019). During this process, a number of mechanisms are triggered, for instance the synthesis of phytochelatins (PCs). Heavy metal ions form metal-thiolate bonds with PCs, forming complexes accumulated in plant vacuoles (Wong & Cobbett, 2009).

Several transport proteins families are involved in the transport of heavy metals (Wong & Cobbett, 2009; Ismael et al., 2019). Wong and Cobbett (2009) investigated the role of transporting ATPases such as *HMA2* and *HMA4* on Cd uptake and translocation in *Arabidopsis thaliana*. Their results showed that in double mutants *hma2*, *hma4* with loss of function of *HMA2* and *HMA4*, Cd concentration was increased in roots and decreased to less than 3% of the wild-type in shoots. This suggests the important role of *HMA2* and *HMA4* transporters in Cd translocation. The authors argued that more transporter proteins could be involved in Cd transport; however, to test this hypothesis, experiments should be conducted at phytotoxic Cd concentrations as an increased

concentration could up-regulate the expression of additional transporters. Furthermore, several transporter genes from YSL, NRAMP, and ZIP families were identified as crucial elements in Cd uptake and translocation (Ismael et al., 2019).

Cadmium Accumulation in Hemp

Food and medicinal crops grown in Cd-contaminated soil could be a potential source of Cd intake by humans. Some plant species can accumulate Cd at levels that harm human health while not experiencing phytotoxic effects. Therefore, even soils with permissible amounts of Cd could lead to the accumulation of dangerous concentrations of Cd in plant tissues, depending upon plant species (Ismael et al., 2019). Cd is known to cause serious health problems when ingested in amounts greater than the provisional tolerable monthly intake (PTMI) of 25 $\mu g \cdot kg^{-1}$ of body weight (Codex Alimentarius, 2019). The maximum allowable level of Cd in food crops varies from 0.05 to 0.2 ppm, including most cereal grains, fruits, and vegetables (Codex Alimentarius, 2019).

Agricultural land could have elevated Cd levels due to the addition of manure, phosphate fertilizers, non-treated biosolids, and atmospheric deposition (Chaney et al., 2010). Even in low levels in agricultural soils, Cd could be dangerous to human health due to soil-plant transfer of Cd (Ismael et al., 2019). Nevertheless, the EPA has set no standards for total or available Cd in agricultural soils, other than an allowance for a very high Cd cumulative pollutant loading rate (39 $kg \cdot ha^{-1}$) from sewage sludge (US EPA, 1993).

Ahmad et al. (2015) investigated Cd accumulation in *Cannabis* plants grown in soil contaminated with 100 $mg \cdot kg^{-1}$ Cd. Plants exposed to these high levels of Cd showed reduced plant growth. Most Cd accumulation ocurred in the roots, with little translocation to the stem and leaves. Cadmium accumulation in flowers was not quantified and plant variety was not described in the research. Shi & Cai (2009) grew the hemp variety ‘ym 12’ in pots containing a mixture of

sand and perlite contaminated with Cd at 0, 50, 100, and 200 $mg \cdot kg^{-1}$. Root growth was repressed at 200 $mg \cdot kg^{-1}$ but not in other treatments. Biomass accumulation was inversely proportional to Cd concentration in the substrate. Cd accumulation took place in the roots and translocation was reported as low.

Husain et al. (2019) compared hemp grown indoors to hemp grown outdoors utilizing contaminated mine-land soil (initial Cd = 0.34 $mg \cdot kg^{-1}$) and commercial greenhouse substrate (initial Cd < 0.25 $mg \cdot kg^{-1}$). Based on plant height measurements, no differences in plant growth were observed among treatments. Specifically for the variety Felina 32, suitable for fiber, seed, and CBD production, Cd accumulation was more than two times higher when grown indoors than outdoors (both in mine land soil). The authors suggested that metal transporters utilized by this variety might be more efficient when plants were grown at indoor temperatures.

Citterio et al. (2003) evaluated the fiber variety Fibranova for Cd tolerance. In their experiment, the substrate was contaminated with 27 $mg \cdot kg^{-1}$ (T1) and 82 $mg \cdot kg^{-1}$ (T2) Cd prior to seeding. There were no significant differences in plant growth between treatments, which could be explained by the physiological mechanisms activated when the plant is under stress, such as the production of high levels of phytochelatins and its precursor plant glutathione (GSH), which are antioxidant molecules. Results from plant tissue analysis (roots, stems, and leaves) when the plant had reached ideal concentrations of cannabinoids/biomass and was ready to be harvested, indicated that Cd accumulation occurred mainly in the roots. Interestingly, Cd translocation from roots to shoots was higher in T1 when compared to T2. The authors suggested that as the heavy metal concentration in the growing media increases, the rate of intracellular accumulation becomes lower compared to the biosorption of metals in the root system. Substrate analysis pre-germination and post-harvest have shown no significant differences in heavy metals concentration. According to

the authors, the hemp variety Fibranova is not a heavy metal hyperaccumulator, rather it can be classified as tolerant to high concentrations of Cd.

Huang et al. (2019) investigated alterations caused by Cd stress at the molecular level in two hemp fiber varieties, 'Yunma No.1' (Ym) and 'Neimengguxiaolidama' (Nx). Plants were grown hydroponically and followed two treatments: a) control solution containing essential nutrients and b) 100 μM $CdCl_2$ (= 11.2 $mg \cdot kg^{-1}$ Cd) plus nutrient solution as needed. In the treatment containing 100 μM $CdCl_2$, root growth, fresh weight, and Cd content (whole plant) in Ym were higher when compared to Nx. The accumulation of Cd-induced reactive oxygen species (ROS) was higher in Nx as observed in the 3,3′-diaminobenzidine (DAB) staining methodology analysis. Furthermore, the activities of the antioxidant enzymes superoxide dismutase (SOD) and peroxidase (POD) were decreased for both varieties; however, the decrease was more pronounced in Nx. These two enzymes confer plants with the ability to eliminate ROS, acting as an indicator of heavy metal tolerance. Synthesis of GSH, another antioxidant, was higher in Ym roots when compared to Nx roots. The presence of malondialdehyde (MDA), an indicator of cell damage by oxidative stress, was higher in Nx. Indeed, over five times more genes involved in plant defense processes to prevent Cd toxicity were expressed in Ym than in Nx. For instance, the expression of metal transporter genes, such as ZIP and ABC, was upregulated in Ym. These metal transporters play an important role in Cd tolerance as they can sequester Cd into vacuoles, preventing damage to the cells. Other genes that were upregulated in Ym included genes related to modifications in plant cell walls, such as the delivery of glycosyl hydrolases which contribute to the capture of Cd by the cell wall, thus, minimizing damage to the cells. Further results showed that genes encoding plant phytohormones such as ethylene, auxin, and gibberellins were upregulated in Ym when compared to Nx.

Influence of Heavy Metal Stress on Cannabinoid Content

Husain et al. (2019) measured gene expression of cannabinoid pathway genes and reported that cannabidiolic acid synthase (CBDAS) was expressed 18 times higher in plants grown with mine-land soil contaminated with heavy metals than when compared to commercial greenhouse substrate. However, no significant difference was found for tetrahydrocannabinolic acid synthase (THCAS) expression. These results suggest that heavy metal stress positively affect CBD synthesis, but that the overall impact on cannabinoids is variable. Furthermore, Citterio et al. (2003) analyzed THC content in the leaves of the fiber variety Fibranova after cultivation in a substrate contaminated with Cd, Cr, and Ni at increasing concentrations. They found that heavy metals in the substrate did not affect THC levels which remained below 0.3%.

Implications for Growers, Consumers, and Policy Makers

An increasing number of third-party testing laboratories are offering heavy metal testing for hemp products. Although heavy metal screening is not mandated by either the FDA or USDA, growers are inclined to pay for this service to increase product quality. Cadmium accumulation in smokable plants, such as tobacco and hemp, is concerning for human health due to the harmful effects of Cd inhalation, which affect body tissues such as lungs, kidneys, and liver (Akesson & Chaney, 2019; Gauvin et al., 2018; Ismael et al., 2019). However, the potential harms of cannabis smoke are not perceived as threatening by consumers to the same extent as those caused by smoking tobacco (Gauvin et al., 2018). Gauvin et al. (2018, p. 7) claimed that federal regulatory agencies must consider, among others, "the entourage environmental HM [heavy metal] contaminants that result from the basic chemo-attractive affinity of the marijuana [cannabis] plant itself when conducting public health risk assessments."

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CHAPTER 3

CADMIUM EXPOSURE IS ASSOCIATED WITH INCREASED TRANSCRIPT ABUNDANCE OF MULTIPLE HEAVY METAL ASSOCIATED TRANSPORTER GENES IN ROOTS OF HEMP (*CANNABIS SATIVA* L.)[1]

[1] Marabesi, A. O., Nambeesan, S. U., van Iersel, M. W., Lessl, J. T., & Coolong, T. W. (2023). *Frontiers in Plant Science, 14*:1183249. https://doi.org/10.3389/fpls.2023.1183249. Reprinted here with permission of the publisher.

Abstract

Industrial hemp (*Cannabis sativa* L.) has demonstrated promise for phytoremediation due to an extensive root system, large biomass, and ability to survive under relatively high levels of heavy metals. However, little research has been conducted to determine the impact of heavy metal uptake in hemp grown for medicinal use. This study evaluated the potential for cadmium (Cd) uptake and its impact on growth, physiological responses, and transcript expression of metal transporter genes in a hemp variety grown for flower production. The cultivar 'Purple Tiger' was exposed to 0, 2.5, 10, and 25 $mg \cdot L^{-1}$ Cd in a greenhouse hydroponic study in two independent experiments. Plants exposed to 25 $mg \cdot L^{-1}$ Cd displayed stunted plant growth characteristics, reduced photochemical efficiency, and premature senescence suggesting Cd toxicity. At the two lower concentrations of Cd (2.5 and 10 $mg \cdot L^{-1}$ Cd), plant height, biomass, and photochemical efficiency were not affected, with chlorophyll content index (CCI) being slightly lower at 10 $mg \cdot L^{-1}$ Cd, compared to 2.5 $mg \cdot L^{-1}$ Cd. There were no consistent differences between the two experiments in total cannabidiol (CDB) and tetrahydrocannabinol (THC) concentrations in flower tissues at 2.5 and 10 $mg \cdot L^{-1}$ Cd, compared to the control treatment. Root tissue accumulated the highest amount of Cd compared to other tissues for all the Cd treatments, suggesting preferential root sequestration of this heavy metal in hemp. Transcript abundance analysis of heavy metal-associated (HMA) transporter genes suggested that all seven members of this gene family are expressed in hemp, albeit with higher expression in the roots than in the leaves. In roots, *CsHMA3* was up-regulated at 45 and 68 d after treatment (DAT), and *CsHMA1*, *CsHMA*4, and *CsHMA*5 were upregulated only under long term Cd stress at 68 DAT, at 10 $mg \cdot L^{-1}$ Cd. Results suggest that expression of multiple HMA transporter genes in the root tissue may be upregulated in hemp exposed to 10 $mg \cdot L^{-1}$ Cd in a nutrient solution. These transporters could be involved in Cd uptake

in the roots *via* regulating its transport and sequestration, and xylem loading for long distance transport of Cd to shoot, leaf, and flower tissues.

Introduction

Hemp (*Cannabis sativa* L.) has been cultivated for centuries for feed, fiber, and medicinal purposes (Ranalli, 1999). In the United States (U.S.), production has been largely prohibited until the passage of the 2018 Agriculture Improvement Act (Farm Bill), which allowed for legal cultivation of industrial hemp [U.S. Department of Agriculture (USDA), 2019]. Industrial hemp is defined as *C. sativa* with a total delta-9 tetrahydrocannabinol (THC) concentration of less than 0.3% on a dry-weight basis. *C. sativa* plants with delta-9 THC concentrations above 0.3% are classified as marijuana and are federally prohibited in the U.S. Tetrahydrocannabinol and cannabidiol (CBD) are two of the more than 100 cannabinoids found in *C. sativa* plants, many of which have therapeutic uses such as the treatment of anxiety and seizures (ElSohly and Gul, 2014; National Academies of Sciences, Engineering, and Medicine (NASEM), 2017; USDA, 2019). Although legalized by the U.S. federal government, there are significant regulations for industrial hemp production that must be followed to stay in compliance with federal law. Further, processors often impose strict quality control standards for their products as they may be consumed as dietary supplements. Heavy metal accumulation in hemp biomass is an area of concern for processors and many have attempted to implement standards regarding maximum allowable levels of metals.

Previously, human exposure to cadmium (Cd), has been associated with smoking contaminated tobacco (*Nicotiana tabacum* L.), particularly counterfeit cigarettes sold in developing markets (Akesson & Chaney, 2019). Cadmium accumulation in smokable plants, such as tobacco and hemp, is concerning for human health due to the harmful effects of Cd inhalation, which has been associated with renal tubular dysfunction, osteomalacia, lung disease, and it is

classified as a Group 1 carcinogen (Akesson & Chaney, 2019; Gauvin et al., 2018; Ismael et al., 2019). Nonetheless, consumers are less likely to perceive the harms of *C. sativa* smoke to the same extent as those caused by smoking tobacco (Gauvin et al., 2018). Cadmium is a naturally occurring element and is found in trace amounts in the environment, including soil. Cadmium may be introduced to agricultural soils via contaminated manure and biosolids, phosphate fertilizers, mine waste, atmospheric deposition from smelter emissions, and industrial waste (Chaney, 2010; Sebastian et al., 2019).

Studies have documented the ability of hemp to survive on soils with relatively high levels of heavy metals, including Cd, copper (Cu), nickel (Ni), lead (Pb), zinc (Zn), mercury (Hg), and chromium (Cr) (Ahmad et al., 2015; Angelova et al., 2004; Citterio et al., 2003; Galic et al., 2019; Husain et al., 2019; Linger et al., 2002, 2005; Shi & Cai, 2009, 2010; Shi et al., 2012). Furthermore, hemp has been suggested to have the potential to remove Cd from contaminated soils for phytoremediation purposes. Hemp shares some traits with species of plants that can establish themselves in otherwise phytotoxic environments, such as an extensive and deep root system, efficient translocation of contaminants to the shoots, rapid life cycle, and high biomass production (Citterio et al., 2003; Nesler & Furini, 2012).

Numerous plant species have developed mechanisms to cope with exposure to high levels of heavy metals (Eutropio et al., 2016). The first step in Cd accumulation in plants is the uptake of Cd ions by the roots. The rhizosphere is critical in this process as it can provide an appropriate environment for the existence of bioavailable forms of metals, in terms of pH, redox potential, and presence of microorganisms (Chaney, 2010; Ismael et al., 2019; Lux et al., 2011; Sebastian et al., 2019). Subsequently, metal ions may enter roots through protein transporters and travel *via* apoplastic or symplastic pathways. Once in root cells, metal cations may be chelated with

phytochelatins, metallothioneins, organic acids, and amino acids and stored in the vacuoles in a chelated form, while also being loaded into the xylem where they are translocated to the shoots and leaves. Cadmium accumulation in hemp has been reported to be greater in roots when compared to other tissues, though translocation of Cd to aboveground portions of plants is still significant (Ahmad et al., 2015; Citterio et al., 2003; Galic et al., 2019; Linger et al., 2005; Shi & Cai, 2009; Shi et al., 2012).

Metal transporters mediate Cd movement within the plant. These proteins primarily transport divalent cations such as Cu, Zn, calcium (Ca), magnesium (Mg), and iron (Fe), which are essential nutrients for plants. When Cd is present in the soil solution, it competes with these essential nutrients for transport, and may be colloquially referred to as an "opportunistic hitchhiker" (Shah et al., 2019). Several transport protein families are involved in the uptake and movement of heavy metals in plants (Ismael et al., 2019; Lux et al., 2011; Sebastian et al., 2019). Transporting ATPases such as heavy metal ATPase 2 (*AtHMA2*) and heavy metal ATPase 4 (*AtHMA4*) have been reported to play an important role on Cd uptake and translocation in *Arabidopsis thaliana* (L.) Heynh (Wong & Cobbett, 2009), while *AtHMA3* (Ismael et al., 2019; Morel et al., 2009) and its orthologues *OsHMA3* in *Oriza sativa* L. are involved in vacuolar loading of Cd (Ismael et al., 2019; Miyadate et al., 2011).

Prior research has largely focused on the survival and accumulation of Cd in fiber hemp varieties grown for phytoremediation purposes. However, much of the hemp intended for human consumption is specifically grown for floral production, which is utilized for cannabinoid extraction or directly for smoking. Therefore, there is interest in determining the potential for Cd contamination in flower tissue. There is a lack of research-based information on heavy metal accumulation in hemp grown for the medicinal market and the relationship between Cd stress and

hemp growth and development (Ahmad et al., 2015; Yin et al., 2022). In this study we evaluated the potential for Cd accumulation and its impact on growth and physiological responses related to photochemical efficiency and chlorophyll content, and transcript abundance of metal transporter genes in an industrial hemp variety grown specifically for flower (cannabinoid) production.

Materials & Methods

Experimental Setting

Two independent greenhouse experiments utilizing a deep-water culture hydroponic system were conducted from Dec. 2020 through Mar. 2021 and Feb. 2021 through May 2021. Industrial hemp 'Purple Tiger', developed for flower production (The Hemp Mine, Fair Play, SC, USA), was propagated via rooted cuttings. This cultivar was chosen due to its long photoperiod requirements for vegetative growth (≥ 17 h) and ability to accumulate CBD in the flower tissue. Cuttings were taken from female plants in the active vegetative growth phase containing three nodes each. Cuttings were dipped in a commercial rooting gel (0.31% indole butyric acid; CLONEX, Growth Technology Ltd., Somerset, UK) and placed into engineered foam cubes (3.33 cm L x 2.54 cm W x 3.81 cm D; Oasis Grower Solutions, Kent, OH, USA) for rooting. Foam cubes were placed in plastic trays located on a heat mat set at 24 $^{\circ}$C under a mist system, which applied water approximately four-times daily for 3 min each. Cuttings were maintained for approximately 3 weeks, after which they were transferred to 37.9 L plastic containers (Rubbermaid Inc. Wooster, OH, USA) filled with 28 L of well water. Water was analyzed for nutrient concentrations prior to the experiment (Supplemental Table 3.1). A nutrient solution was added to the plastic containers using a complete hydroponic fertilizer (5N-4.8P-21.6K, Peters Professional Hydroponic Special; ICL, St. Louis, MO, USA) and calcium nitrate (14N-0P-0K,17Ca; Calcium + Micros, General Hydroponics, Santa Rosa, CA, USA) dissolved in the well water (Supplemental Table 3.1).

Four rooted cuttings were placed into plastic netted containers (4.7 cm W x 5.1 cm D) spaced equidistant (24.3 cm apart) through the lid of the plastic tub for a given treatment. There were four replicates for every given treatment. Welded wire mesh frames were attached to each lid to support plants. Plants were grown for 4 weeks in the base nutrient solution (Supplemental Table 3.1). A 15.2 cm aquarium air stone attached to an air pump (Active Aqua; Hydrofarm, Petaluma, CA, USA) was added to each plastic container to aerate the solution throughout the experiment. Container volume was maintained by adding well water every 2 d. Four weeks after transplant, nutrient solutions were replaced completely and Cd treatments added using $3CdSO_4 \cdot 8H_2O$, to achieve 0, 2.5, 10 and 25 $mg \cdot L^{-1}$ Cd. Cadmium concentrations were chosen based on the results of Huang et al. (2019), who evaluated hemp exposure to Cd in a hydroponic system. Nutrient solutions were maintained to a constant volume by adding water every 2 d and were completely replaced every 2 weeks for the next 10 weeks, except for the 25 $mg \cdot L^{-1}$ Cd tubs, which were harvested after 6 weeks of Cd exposure due to plant senescence caused by Cd toxicity. Electrical conductivity (EC) and pH of the solutions were measured at the start and mid-point of each 2-week period. Solution pH was adjusted to 5.5 when necessary (pH down; General Hydroponics, Santa Rosa, CA, USA). Supplemental light (104 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was used to provide 18/6 light/dark hours for 6 weeks of vegetative growth after planting. Supplemental lights were turned off to allow for flower induction for the remaining 8 weeks of production. Nutrient solutions were sampled at the beginning and end of each 2-week cycle using 20 mL scintillation vials (HDPE; Thermo-Fisher Scientific, Waltham, MS, USA), and stored at -4 $^{\circ}C$ until analysis.

Temperature and relative humidity (RH) of the greenhouse were monitored at canopy height hourly (VP4; Meter Group Inc., Pullman WA, USA) and averaged 19.4 ± 3.6 $^{\circ}C$ and 66% ± 14.0 RH, and 22.1 ± 4.9 $^{\circ}C$ and 63.8 ± 17.8 % RH for experiments 1 and 2, respectively.

Photosynthetic active radiation was also monitored hourly throughout the experiment (QSO-S; Meter Group Inc.) and the average daily light integral (DLI) was 30.9 ± 18.4 $mol \cdot m^{-2} \cdot d^{-1}$ in the first experiment and 47.8 ± 19.7 $mol \cdot m^{-2} \cdot d^{-1}$in the second experiment.

Mineral Analysis

Samples of the hydroponic solutions were filtered using a 0.45 μM PTFE membrane and acidified using 2% high purity nitric acid (HNO_3) prior to analysis. At harvest [68 d after Cd treatments, (DAT)], root, stem, leaf, and flower material were collected for metal analysis. Samples were taken prior to air drying whole plants for plant biomass quantification. For each replicate, sub-samples were taken from 3 plants per replicate and combined into a composite sample. In total, four replicates per treatment were used for determination of mineral composition. For roots, approximately 50 g of fresh material was collected from each replicate and triple washed with deionized water prior to drying. For leaves, ten of the youngest fully expanded leaves were collected from the top one-third of each plant (main stem and lateral branches) and rinsed with deionized water. Stem samples of approximately 50 g were collected from the bottom two-thirds of the main stem from each replicate. Approximately 20 g of fresh flower material was sampled from each replicate from the main stem and top one-third of plants.

Plant tissue samples were placed in a forced air oven to set at 55 $^{\circ}$C for 72 h until a constant weight was achieved. Dried plant material was then ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) and passed through a 20-mesh screen. The samples were digested following EPA Method 3052 (USEPA, 1995) as follows: 0.5 g samples were weighed and placed in fluorocarbon polymer microwave vessels. Then 10 mL of concentrated nitric acid was added to each vessel and sealed, placed in a microwave digester (Mars 6 Microwave; CEM Corp., Matthews, NC, USA), and heated to 200 $^{\circ}$C for 30 minutes. Digests (solutions) were then

transferred quantitatively into volumetric flasks and brought to 100 mL volume with deionized water prior to analysis.

Nutrient solutions and plant tissue digestions were analyzed for multiple elements (P, K, S, Ca, Mg, Fe, Mn, Al, B, Cu, Zn, Ni, and Cd) following EPA Method 200.8 (Creed et al., 1994) by Inductively Coupled Plasma - Optical Emission Spectroscopy (Spectro Arcos FHS16; Spectro Amertek USA, Wilmington, MA, USA). The instrument detection limit for Cd was 0.005 $mg \cdot L^{-1}$. Calibration standards utilized in this analysis were from a certified source (Inorganic Ventures, Christiansburg, VA, lot number: N2-MEB667614). Independent laboratory performance checks were also run with acceptable deviations for recoveries set at 100 ± 5.0%.

Plant Biomass and Photosynthetic Measurements

Plant height and leaf, flower, stem, and root biomass were determined at harvest. Whole plant samples (three per replicate) were air dried at ambient temperatures inside the greenhouse for 2 weeks and then separated into roots, stems, and combined flower and leaf biomass. To adjust for any remaining moisture content, subsamples were taken from the air-dried materials and further dried in a forced air oven set at 55 °C for 48 hours until a constant weight was achieved. The dry weights of the whole plant samples were then normalized based on subsample moisture content.

The maximum quantum yield of photosystem II (F_v/F_m) was measured during darkness with a fluorometer (Mini-Pam; Walz Company, Effeltrich, Germany) and chlorophyll content index (CCI) was measured at mid-morning with a handheld meter (MC-100; Apogee Instruments, Logan, UT, USA) at 14 and 45 DAT. Measurements were taken on three plants per replicate, on the youngest fully mature leaf on each of the plants.

Cannabinoid Analysis

Approximately 25 g of fresh flower tissue obtained from inflorescences located on the top one-third of the plants were sampled from each of the four replicates during week 8 of flowering (68 DAT) and dried separately from other samples for cannabinoid analysis. Four replicates per treatment were used for determination of cannabinoids. Flower material was spread evenly on a perforated baking sheet and dried to approximately 15% moisture content in a walk-in cooler with a temperature set point of 13 ˚C and 55% relative humidity for 14 d. Relative humidity was maintained using a dehumidifier. After 14 d, flower material was hand trimmed to remove leaves and sealed in a metalized resealable food bag (Uline, Braselton, GA) and stored at -4 ˚C for cannabinoid analysis.

The acidic and neutral (decarboxylated) forms of the cannabinoids, THC and CBD, were determined in dried flower material according to the method of Storm et al. (2020) by a commercial laboratory using high performance liquid chromatography and a diode array detector set to 230 nm (SJ Labs & Analytics, Macon, GA). In brief, a 200 mg sample of homogenized dried flower material was extracted with 20 mL of methanol in a 50 mL centrifuge tube. Tubes were vortexed for 10 min, centrifuged at 5000 rpm for 5 min and a 50 µL aliquot of supernatant diluted with 950 µL of methanol and filtered through a 0.45 µm regenerated cellulose syringe filter (4mm Captiva; Agilent, Santa Clara, CA, USA). Analysis was done using high performance liquid chromatography (1220 Infinity II LC; Agilent) with a variable wavelength diode array detector (Agilent). Ten µL of the methanol extract was injected into a 3.0 x 50 mm, 2.7 µm column liquid chromatography column (Infinity Lab Poroshell 120 EC-C18; Agilent). The flow rate was 1.0 $mL \cdot min^{-1}$ for the run. Eluents were A) 0.1% aqueous formic acid B) 0.1% formic acid in methanol. A gradient run was programmed as follows: 40% A and 60% B for 1 min, 40% to 23% A and 60%

to 77% B for the next 7 min, then 5% A and 95% B for 2 min. Total cannabinoid concentrations were calculated by the following formula: total cannabinoid = neutral + (acidic form x 0.877). Percentage dry matter for all samples was recorded and results reported on a dry weight basis.

Identification of Heavy Metal Transporter and Cannabinoid Biosynthesis Genes

In order to identify heavy metal transporters in *C. sativa*, orthologue sequences from Arabidopsis P1B-type ATPase gene family containing eight genes were used (Supplemental Table 3.2). Arabidopsis HMA protein sequences from National Center for Biotechnology Information (NCBI) were used to perform blast analysis using blastp function in NCBI against *C. sativa*. Reciprocal blast from *C. sativa* were performed to confirm the identity of the protein. Seven transporter genes were retrieved in *C. sativa*. All the accession numbers for protein sequences have been provided (Fig. 3.1). Two cannabinoid synthase genes sequences, *TETRAHYDROCANNABINOLIC ACID SYNTHASE* (XM_030625046.1) and *CANNABIDIOLIC ACID SYNTHASE* (XM_030624886.1), were retrieved from NCBI from accession numbers reported previously (Husain et al., 2019). Further, two *C. sativa* reference genes sequences, *ELONGATION FACTOR 1 ALPHA/HBS1-LIKE PROTEIN* (XM_030654944.1) and *UBIQUITIN-PROTEIN LIGASE/UBIQUITIN DOMAIN-CONTAINING PROTEIN DSK2B* (XM_030630092.1), were used to normalize the data and ensure validity of results. These reference genes were found to be stable for quantitative RT-PCR in *Cannabis sativa* L. (Guo et al., 2018). The accession numbers provided in this study were used to retrieve more updated sequences from the *C. sativa* Updated Annotation Release 100 with the accession numbers provided above.

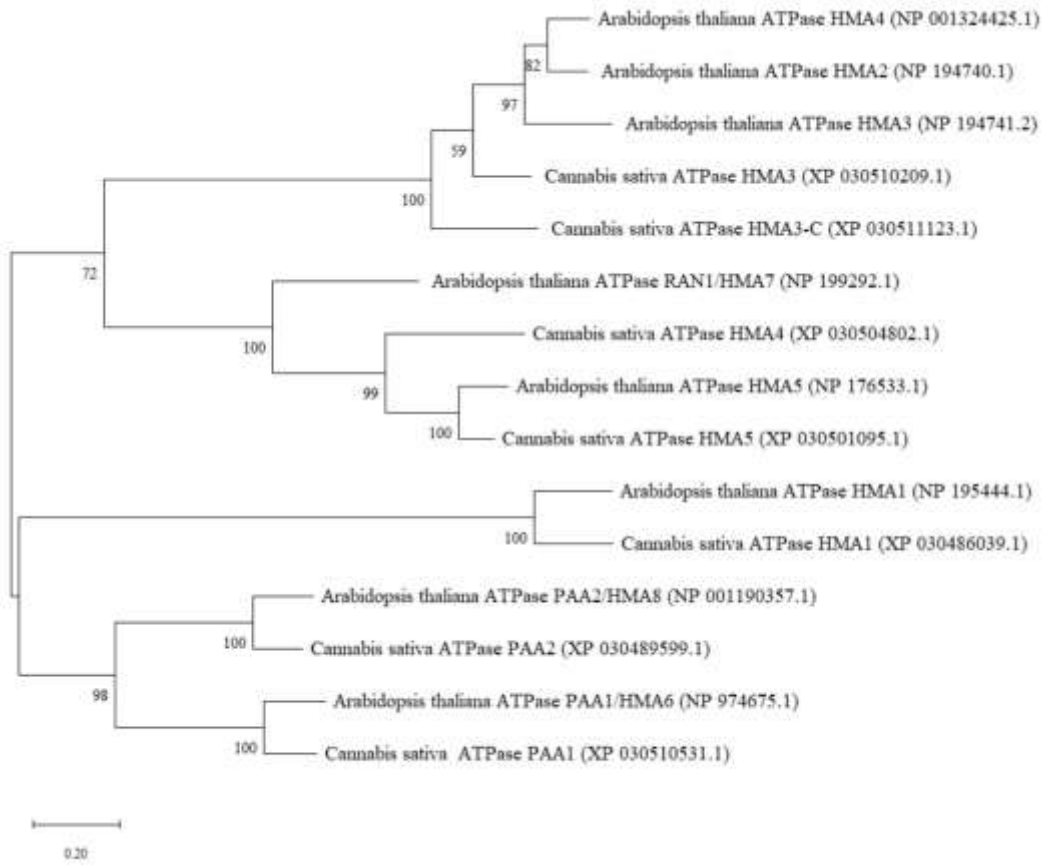


Figure 3.1. Maximum Likelihood (ML) phylogenetic tree of hemp (*Cannabis sativa* L.) heavy metal ATPases and their closest orthologues in *A. thaliana*.

Gene Expression Analysis

The transcript expression of seven heavy metal transporter genes and two cannabinoid biosynthetic genes identified from *C. sativa* were assessed in root, leaf, and flower tissues at different physiological time points: 2 DAT (vegetative stage), 45 DAT (4 weeks of flowering stage), and 68 DAT (8 weeks of flowering stage). Due to similar trends in plant responses between the two experiments, plant material from experiment 1 was analyzed for gene expression. Approximately 15 g of root, leaf, and flower tissue were collected at 2, 45, and 68 DAT at mid-morning, flash frozen in liquid nitrogen, and stored at –80 °C until RNA extraction. Since the plant was at the juvenile stage at 2 DAT, one whole plant per replicate was harvested. For the remaining

time points, sub-samples of roots, flowers, and young fully expanded leaves (from the top one-third of each plant) were collected from three plants per replicate and combined. Four replicates per treatment were used for gene expression analysis. For extraction, frozen plant tissues were ground into a fine powder by hand using a mortar and pestle and liquid nitrogen. Total RNA was extracted from 100 mg samples following TRIzol® methodology for leaf tissue and utilizing an E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA) for root and flower tissues, following the manufacturers' recommendation. RNA concentration, 260/280 absorbance ratios, and quality were assessed utilizing Nanodrop (Nanodrop 8000 Spectrophotometer, Thermo Fisher Scientific) and agarose gel electrophoresis.

Following RNA extraction, DNAse treatment was performed at 37 ˚C for 34 minutes in a thermocycler (Mastercycler® X50s Eppendorf SE, Hamburg, Germany). Subsequently, cDNA was synthesized using ImPromII Reverse Transcriptase (Mayorga-Gómez and Nambeesan, 2020). Quantitative RT-PCR reactions were performed in a 96-well plate using PowerUP SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), diluted cDNA, and 0.2 μM concentration of forward (Fw) and reverse (Rv) primers using AriaMx Real-time PCR System (Agilent, Santa Clara, CA, USA). Reaction conditions were as follows: 2 min at 50 ˚C, 5 min at 95 ˚C, 40 amplification cycles of 30 s at 95 ˚C followed by 1 min at 60 ˚C, and one final cycle for dissociation curve analysis of 1 min at 95 ˚C followed by 30 s at 55 ˚C followed by 30 s at 95 ˚C. Each reaction was performed at least in triplicates and PCR reaction efficiency was determined by LinRegPCR (v. 11.0). Primers were designed manually by importing sequences into MEGA software (v. 11.0, Tamura et al., 2021) and primer quality was verified using the OligoAnalyzer tool (Integrated DNA Technologies, Coralville, IA, USA). Primer sequences and accession numbers for the genes analyzed can be found in Supplemental Table 3.2.

Phylogenetic Analyses

Protein sequences of all the HMA genes in *A. thaliana* and *C. sativa* were used to construct a phylogenetic tree using MEGA software (v. 11.0, Tamura et al., 2021). The evolutionary history was inferred by using the Maximum Likelihood method and JTT matrix-based model. The tree with the highest log likelihood (-11628.46) was used for the construction of the tree. Multiple-sequence alignment was performed with MUSCLE. Phylogeny clusters reliability was tested with 1000 replicates (bootstrap analysis). Closest orthologue sequences were selected by blastp (query cover > 80% and identity > 60%) in the NCBI database.

Statistical and Correlational Analysis

Experiments followed a complete randomized block design. Statistical analysis was conducted using JMP© Pro 15 (SAS, Cary NC, USA). Data were subjected to a one-way ANOVA procedure with Tukey's honest significant difference (HSD) test (P<0.05) conducted for mean separation when appropriate. Tissue Cd concentrations were log-transformed to ensure equal variance prior to statistical analysis. Non-transformed data are presented. Correlations between Cd concentrations and gene transcript abundance in roots were determined using Spearman correlations using JMP© Pro 15 and p-values were adjusted using the false discovery rate (FDR) method. Heatmaps were created using RStudio (v. 2022.07.0) after transforming the data using Log2 (fold-change + 0.001), where 0.001 was added to account for the genes that showed no expression.

Results & Discussion

Plant Growth and Yield

Plants exposed to 0 and 2.5 mg·L^{-1} Cd grew similarly and displayed no visual symptoms of Cd toxicity throughout the experiment (Fig. 3.2A, E). Plants exposed to 10 mg·L^{-1} Cd exhibited

visual symptoms of Cd toxicity within 2 d of exposure (2 DAT), such as leaf curling and mild chlorosis, however symptoms decreased as plants continued to grow (Fig. 3.2B). Plants exposed to the highest level of Cd (25 $mg \cdot L^{-1}$) exhibited Cd toxicity symptoms within 2 d of exposure (Fig. 3.2C). However, unlike plants exposed to 10 $mg \cdot L^{-1}$ Cd, symptoms in plants exposed to 25 $mg \cdot L^{-1}$ Cd continued to worsen over time, with leaves and roots eventually becoming necrotic (Fig. 3.2C–E). Plants exposed to 0, 2.5, and 10 $mg \cdot L^{-1}$ Cd were harvested at week 8 of flowering (68 DAT); however, plants exposed to 25 $mg \cdot L^{-1}$ Cd senesced prematurely and were harvested prior to flowering (45 DAT).

(A)

(B)

(C)

(D)

(E)

Figure 3.2. Hemp (*Cannabis sativa* L.) 'Purple Tiger' grown hydroponically. From left to right: plants under 25, 10, 0, and 2.5 mg·L^{-1} Cd exposed to treatments for 11 d (A). Plants exposed to 10 and 25 mg·L^{-1} Cd for 2 d [(B, C), respectively]. Plants exposed to 25 mg·L^{-1} Cd for 11 d (D). From left to right: plants under 25, 10, 0, and 2.5 mg·L^{-1} Cd exposed to treatments for 30 d (E).

Plant Biomass

Shoot height and dry weight of stems, roots, and flower + leaves (classified as 'biomass' in industry standards) responded similarly to Cd exposure. Therefore, data from the two experiments were pooled. Shoot height was not affected by Cd for plants exposed to 0, 2.5, and 10 $mg \cdot L^{-1}$ Cd; however, shoot height was reduced in plants exposed to 25 $mg \cdot L^{-1}$ Cd (Fig. 3.3A). Dry weight of stems, roots, and flowers + leaves were also greater in the 0, 2.5, and 10 $mg \cdot L^{-1}$ Cd treatments compared to 25 $mg \cdot L^{-1}$ Cd (Fig. 3.3B). Compared to the 2.5 $mg \cdot L^{-1}$ Cd treatment, the 10 $mg \cdot L^{-1}$ Cd treatment displayed numerically lower stem biomass, however it was not significantly different from 0 $mg \cdot L^{-1}$ Cd treatment. Root dry weight was lower in the 25 $mg \cdot L^{-1}$ Cd treatment compared to all other treatments. There were no differences in flower and leaf biomass between the 0, 2.5, and 10 $mg \cdot L^{-1}$ Cd treatments, while plants grown with 25 $mg \cdot L^{-1}$ Cd began to senesce prior to full flower development resulting in low production of flower + leaf biomass. Stems, root, and flower + leaf biomass were reduced in the 25 $mg \cdot L^{-1}$ Cd treatment to a greater extent than shoot height, which is congruent with prior results suggesting that plant biomass is a more appropriate reference for analyzing Cd toxicity than shoot growth (Shi & Cai, 2009; Shi et al. 2012). Current results agree with prior studies, suggesting that diminished biomass is a common indicator of Cd toxicity in hemp plants and that Cd toxicity symptoms include leaf chlorosis, leaf curling, and growth inhibition (Linger et al., 2005; Luyckx et al., 2021; Shi & Cai, 2009; Shi et al. 2012). Although plants exposed to 10 $mg \cdot L^{-1}$ Cd visually displayed less branching and thinner stems compared to 0 and 2.5 $mg \cdot L^{-1}$ Cd treatments (personal observation), this was not reflected in the overall root, and flower + leaf biomass. These data also suggest that hemp 'Purple Tiger' can tolerate exposure to 10 $mg \cdot L^{-1}$ Cd without alterations in plant biomass.

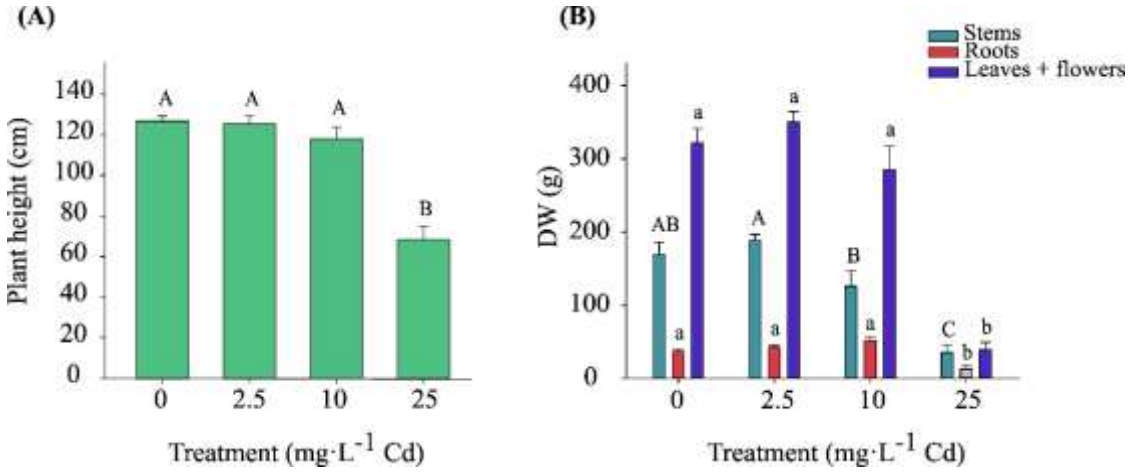


Figure 3.3. Shoot height ± SE at harvest (A). Dry weight of stems, roots, and flowers + leaves at harvest ± SE (B). Bars associated with the same letter(s) are not significantly different at $p \leq 0.05$ according to Tukey's HSD all pairwise comparison test.

Cadmium Concentration in Plant Tissues

Analysis of Cd in nutrient solutions at the beginning and end of each 14-d cycle indicates that Cd treatments were applied at intended levels and that Cd was not completely depleted during the growing cycle (Table 3.1). With increasing concentrations of Cd in the nutrient solution, plant uptake of Cd increased, particularly during the first 28 d of Cd exposure. However, after 28 d of exposure, Cd uptake declined in the 25 mg·L^{-1} Cd treatment. This was concurrent with a decline in plant health. In the 10 mg·L^{-1} Cd treatment, Cd uptake rate increased between 28 and 56 d of exposure but declined after 56 d of exposure. These data suggest a continued uptake of Cd during plant growth and development phase including flowering. However, considering that plants exhibited growth during this entire period, increased uptake may be due to increase in plant biomass.

Table 3.1. Mean concentrations of cadmium (Cd) in nutrient solutions measured at the beginning and end of each 14-d cycle and daily uptake. Values are averages ± SE of two experiments each with four replications per treatment.

		mg·L^{-1} Cd		µmol Cd·d^{-1}
Days after treatment	Treatment	Cd initial	Cd final	Average uptake

0-14	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 C[i]
	2.5	2.48 ± 0.03	1.96 ± 0.009	9.22 ± 0.31 C
	10	10.00 ± 0.13	7.77 ± 0.22	39.69 ± 3.85 B
	25	28.33 ± 0.37	21.27 ± 0.36	125.58 ± 20.41 A
14-28	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 B
	2.5	2.49 ± 0.02	1.23 ± 0.21	22.44 ± 1.97 B
	10	9.89 ± 0.18	7.17 ± 0.48	48.37 ± 6.22 AB
	25	28.05 ± 0.33	22.52 ± 1.26	98.32 ± 81.69 A
28-42	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 B
	2.5	2.33 ± 0.03	1.20 ± 0.19	20.04 ± 3.18 B
	10	10.96 ± 0.32	4.99 ± 0.57	106.15 ± 45.53 A
	25	25.32 ± 0.78	23.72 ± 0.56	29.02 ± 21.55 B
42-56	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 B
	2.5	2.59 ± 0.03	2.06 ± 0.08	7.87 ± 11.13 B
	10	11.41 ± 0.14	6.41 ± 0.34	88.85 ± 4.50 A
56-68	0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00 B
	2.5	2.62 ± 0.13	2.07 ± 0.07	9.86 ± 3.83 B
	10	10.03 ± 0.33	7.14 ± 0.07	51.33 ± 43.90 A

[i]Values followed by the same uppercase letter(s) within a column are not significantly different for each treatment cycle according to Tukey's Honest Significant Difference test ($p \leq 0.05$).

There was a significant Cd by experiment interaction for Cd concentrations in plant tissue; therefore, Cd accumulation data are presented separately for each experiment. Although Cd concentrations in the different tissues were generally higher in experiment 1, overall Cd accumulation trends were similar in both experiments (Table 3.2). Cadmium concentrations were greatest in root tissue, ranging from 2.5 to 12,663 $mg \cdot kg^{-1}$ in the 0 and 25 $mg \cdot L^{-1}$ Cd treatments, respectively. With increasing Cd concentrations in the solution, there was an increase in Cd concentration in root tissue in all Cd treatments compared to the control treatment. Cadmium concentration in the roots was approximately 150-times greater than in the leaves for the 2.5 $mg \cdot L^{-1}$ Cd treatment, indicating that hemp preferentially sequesters Cd in roots, as reported in previous studies (Angelova et al., 2004; Citterio et al., 2003; Linger et al., 2005; Luyckx et al., 2021; Shi & Cai, 2009; Shi et al. 2012).

Table 3.2. Average cadmium (Cd) concentration in hemp (*Cannabis sativa* L.) 'Purple Tiger' plant tissue on a dry weight (dw) basis.

Treatment ($mg \cdot L^{-1}$ Cd)	Flowers	Roots	Leaves	Stems
		Cd concentration ($mg \cdot kg^{-1}$)		
		Experiment 1		
0	ND C[i] b[ii]	2.5 C a	0.2 D b	ND D b
2.5	8.7 B d	1627.9 B a	11.2 C c	15.3 C b
10	66.2 A c	6146.4 A a	176.3 B b	147.1 B bc
25	NA	12663.5 A a	589.4 A c	1344.4 A b
		Experiment 2		
0	ND C b	4.7 C a	ND D b	ND D b
2.5	7.6 B c	1982.6 B a	13.2 C b	5.1 C c
10	25.5 A bc	4781.7 AB a	38.9 B b	20.7 B c
25	NA	7436.3 A a	381.9 A b	353.3 A b

[i]Values followed by the same uppercase letter(s) within a column are not significantly different according to Tukey's Honest Significant Difference test ($p \leq 0.05$).
[ii]Values followed by the same lowercase letter within Cd treatment indicate no significant differences among plant tissues according to Tukey's Honest Significant Difference test ($p \leq 0.05$).
ND = not detected.
NA = not available for sampling due to inability to form floral tissue.

The 10 $mg \cdot L^{-1}$ Cd treatment had higher Cd concentrations in leaf, flower, and stem tissues compared to 0 and 2.5 $mg \cdot L^{-1}$ Cd treatments. These results suggest that hemp plants subjected to 10 $mg \cdot L^{-1}$ Cd treatment tolerated increased Cd concentrations in multiple tissues without significant alterations in plant biomass. Plants in the 25 $mg \cdot L^{-1}$ Cd treatment exhibited the highest

Cd concentrations in leaf and stem tissues in both experiments. Plants exposed to 25 $mg \cdot L^{-1}$ Cd senesced before full flower development was completed; therefore, Cd concentration was not measured in floral tissue in this treatment.

Previously Cd concentrations in stems were reported to be greater than leaves in hemp fiber cultivars (Angelova et al., 2004; Luyckx et al., 2021). In the present study, Cd distribution within flower, leaf, and stem tissues was inconsistent between the two experiments, suggesting that environmental conditions may impact metal concentrations in different plant tissues. Average air temperatures were increased slightly in experiment 2 (22.1 ^{o}C) compared to experiment 1 (19.4 ^{o}C). However, the average DLI increased from 30.9 $mol \cdot m^{-2} \cdot d^{-1}$ in experiment 1 to 47.8 $mol \cdot m^{-2} \cdot d^{-1}$ in experiment 2 due to increasing day length and light intensities during the second experiment. While the role of light intensities on Cd accumulation has not been thoroughly evaluated, there is evidence that light spectrum can influence transcript abundance of the metal transporter gene *HMA3*, affecting Cd tolerance in cucumber (Guo et al., 2022). Further, *Cannabis* growth and morphology has been documented to respond positively to increasing DLI, which may have resulted in differences in Cd concentrations in the two experiments (Moher et al., 2022; Rodriguez-Morrison et al., 2021)

The cultivar used in current study was selected for cannabinoid production, suggesting that genetics may also affect metal accumulation in plant tissues. Although most previous work has focused on root and stem tissues, a study conducted in metal-contaminated soil reported significant high concentrations of Cd in hemp flowers compared to other plant tissue Angelova et al. (2004).

Our results also suggest that when exposed to significant levels of plant available Cd, such as is the case in a hydroponic production system, Cd can accumulate in floral material in plants with a typical morphology (Fig. 3.2). Current limits for Cd in hemp flower range from 0.2 to 0.82

μg/g (California Code of Regulations, 2018; The Maryland Medical Cannabis Commission, 2020; Washington State Liquor and Cannabis Board, 2021). Our data indicate that concentration of Cd in plants exposed to 2.5 mg·L^{-1} Cd was approximately 40 times higher than the legal limit for Cd in hemp flower in several states in the U.S.

Photochemical Efficiency

Because there was no significant interaction between Cd treatments and experiments, F_v/F_m data were pooled. There was no impact of Cd on F_v/F_m in plants growing under 0, 2.5, and 10 mg·L^{-1} Cd treatments at 14 or 45 DAT. At 14 and 45 DAT, Cd levels of 25 mg·L^{-1} Cd reduced F_v/F_m to 0.78 and 0.62 respectively, indicating that photosystem II of these plants was damaged in response to the high Cd concentrations (Fig. 3.4). Reductions in F_v/F_m indicate photoinhibition damage or other damage to photosystem II in response to environmental stresses (Maxwell and Johnson, 2000).

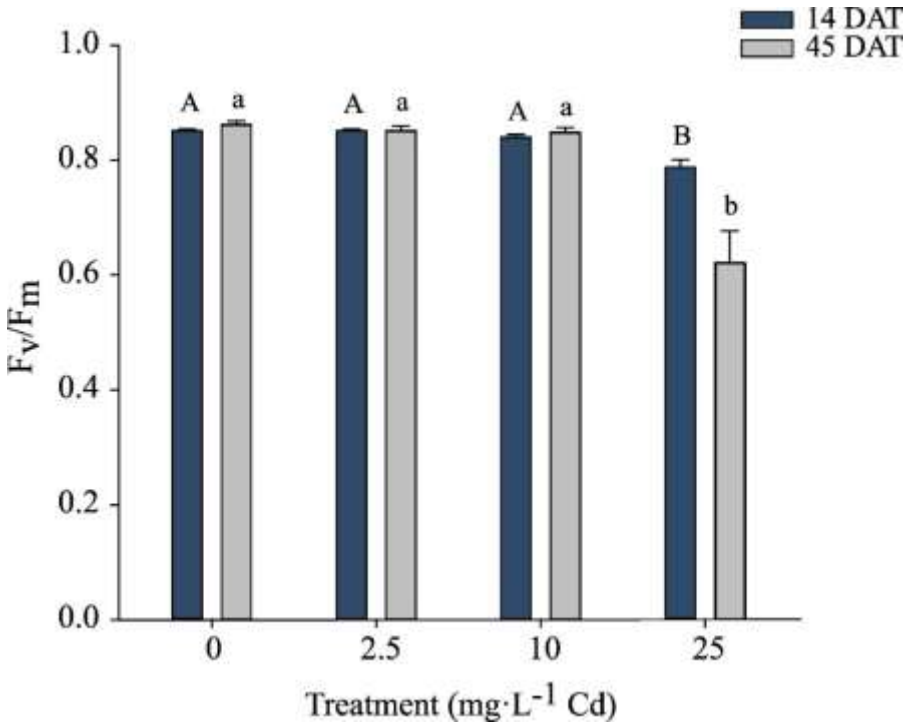


Figure 3.4. Maximum photochemical yield of Photosystem II (F_v/F_m) ± SE in hemp (*Cannabis sativa* L.) 'Purple Tiger' 14 and 45 d after treatment (DAT). Bars associated with the same

letter(s) are not significantly different at $p \leq 0.05$ according to Tukey's HSD all pairwise comparison test.

The CCI was measured at 14 and 45 DAT. Despite similar trends, results for the two experiments are shown separately, as there was a significant interaction between experiment and Cd treatment. In experiment 1, plants exposed to 10 and 25 mg·L^{-1} Cd had a lower CCI at 14 DAT when compared to lower Cd treatments (0 and 2.5 mg·L^{-1} Cd) (Fig. 3.5A). At 45 DAT, plants exposed to 10 mg·L^{-1} Cd had a similar CCI to the 0 and 2.5 mg·L^{-1} Cd treatments (Fig. 3.5C), suggesting that the CCI in the 10 mg·L^{-1} Cd had recovered during the subsequent growth period. However, in experiment 2, the CCI in plants exposed to 10 mg·L^{-1} Cd remained lower than the 0 mg·L^{-1} Cd treatment at 45 DAT. Overall this decrease in CCI at 10 mg·L^{-1} Cd did not affect PSII efficiency (Fig. 3.4). This may explain why biomass at 10 mg·L^{-1} Cd was not reduced compared to lower Cd treatments. The CCI in plants exposed to 25 mg·L^{-1} Cd were significantly lower than all the other treatments, throughout both studies, suggesting that the Cd concentrations in leaf tissue in the 25 mg·L^{-1} Cd treatment limited CCI and photosynthetic efficiency. Consistently, leaf chlorosis was observed at this treatment level. The reductions in F_v/F_m at 25 mg·L^{-1} Cd in this study may suggest photoinhibition damage, which has been observed in response to numerous environmental stresses (Maxwell and Johnson, 2000).

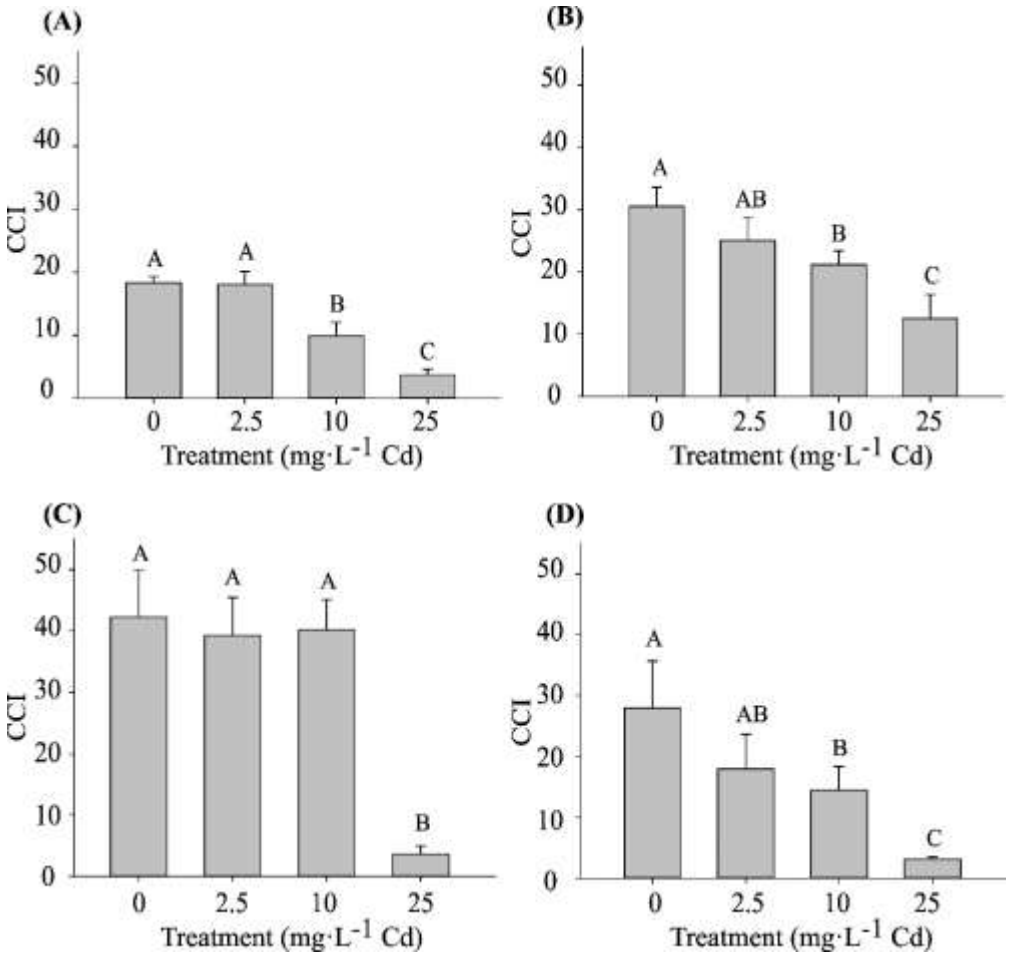


Figure 3.5. Chlorophyll Content Index (CCI) ± SE in in hemp (*Cannabis sativa* L.) 'Purple Tiger' experiment 1 at 14 d after treatment (DAT) (A) and 45 DAT (C) and CCI in experiment 2 at 14 DAT (B) and 45 DAT (D). Bars associated with the same letter(s) are not significantly different at $p \leq 0.05$ according to Tukey's HSD all pairwise comparison test.

Cadmium stress can also trigger oxidative stresses, leading to the accumulation of reactive oxygen species (ROS) (Eutropio et al., 2016; Gillet et al., 2006; Singh et al., 2016; Taiz et al., 2015). Further, metals such as Cd have been reported to replace essential nutrients, disrupting essential reactions in plants (Taiz et al., 2015). These processes can trigger a Cd-induced stress response that can inhibit chlorophyll and carotenoid biosynthesis, disturb chloroplast structure, and ultimately hamper photosynthetic efficiency (Gillet et al., 2006; Küpper et al., 2002; Mallick and Mohn 2003; Parmar et al., 2013).

Total THC and CBD concentrations

There was an interaction between experiments and Cd treatments for total CBD concentrations, therefore, results from each experiment are presented separately. Total CBD concentrations in the flower material were not impacted by Cd treatments in experiment 1, with average concentrations of 4.94%. However, in experiment 2, CBD concentrations decreased from 9.52% to 7.47 % (1.27-fold reduction) in the 0 $mg \cdot L^{-1}$ and 10 $mg \cdot L^{-1}$ Cd treatments, respectively (Table 3.3). Similar to CBD, total THC concentrations were not different among treatments and averaged 0.14 % in experiment 1. In experiment 2, there was a reduction from 0.39 to 0.31% (1.25-fold reduction) in the 0 and 10 $mg \cdot L^{-1}$ Cd treatments, respectively.

Table 3.3. Average total CBD and THC in hemp (*Cannabis sativa* L.) Purple Tiger.

	Concentration in flowers (% dw)			
	Experiment 1		Experiment 2	
Treatment ($mg \cdot L^{-1}$ Cd)	Total CBD	Total THC	Total CBD	Total THC
0	5.22 A[i]	0.15 A	9.52 A	0.39 A
2.5	4.86 A	0.14 A	8.34 AB	0.35 AB
10	4.73 A	0.14 A	7.47 B	0.31 B
25	NA[ii]	NA	NA	NA

[i]Values followed by the same uppercase letter(s) within a column are not significantly different according to Tukey's Honest Significant Difference test ($P < 0.05$).
NA = not available for sampling due to inability to form floral tissue.

The legal threshold concentrations of THC imposed by the U.S. for industrial hemp is 0.3%. In experiment 2, these threshold levels were exceeded even under the 0 $mg \cdot L^{-1}$ Cd treatment, suggesting that changes in environmental conditions between experiment 1 and 2 may have influenced total THC content. It is not uncommon for high CBD hemp cultivars, such as Purple Tiger, to have THC concentrations exceeding the 0.3% threshold (Coolong et al., 2023; Stack et al. 2019; Yang et al. 2020). Although variation in cannabinoid concentrations is largely impacted

by genetics, it is also understood that there are genetic and environmental interactions that may impact cannabinoid concentrations (Trancoso et al., 2022). Rodriguez-Morrison et al. (2021) and Potter and Duncombe (2011) reported no significant impact of light intensity on cannabinoid concentration in floral tissue, though due to overall increases in yield, total cannabinoid production per plant increased with increasing light intensity. Plants in both experiments were harvested at the same number of days after planting; however, the slightly increased air temperature and increased DLI in experiment 2 may have hastened flower development, which could have led to increased concentrations of cannabinoids in experiment 2 (Yang et al., 2020)

Our results contrast with Husain et al. (2019), who reported total CBD concentrations were greater in the hemp cultivar Fedora 17, grown in mine-land soil containing 0.34 $mg \cdot L^{-1}$ Cd, when compared to plants grown in a commercial substrate containing < 0.25 $mg \cdot L^{-1}$ Cd. However, in agreement with our results, total THC concentrations were higher in plants grown in the commercial substrate compared to Cd-contaminated soil (Husain et al. 2019). Our results do not clearly suggest a link between Cd and concentrations of THC and CBD, particularly since differences were noted only in experiment 2. Further, Cd levels in flower tissue were 2.6-fold lower in experiment 2 than in experiment 1 suggesting a potential interaction between Cd concentration in flowers and environmental factors, leading to a decrease in resources allocated for cannabinoid production (Trancoso et al. 2022).

Transcript Abundance of Metal Transporter and Cannabinoid Biosynthetic Genes

Spatial and temporal expression patterns of genes coding for metal transporters, *CsHMA1*, *CsHMA3, CsHMA3-C, CsHMA4, CsHMA5, CsPAA1*, and *CsPAA2,* were analyzed in roots, leaves, and flowers of plants exposed to Cd treatments at three time points: 2, 45, and 68 (harvest) DAT. In addition, transcript expression of cannabinoid synthase genes, *CsTHCAS* and *CsCBDAS,* were

analyzed in leaf and flower tissues. In flowers, none of the transporter and cannabinoid genes analyzed displayed quantifiable expression except for *CsHMA5*. Further, transcript abundance of *CsHMA5* in flowers was also low and not significantly affected by treatments at harvest (Supplemental Fig. 3.1A). The transcript abundance of the reference genes in flower tissue was comparable to that in other tissues suggesting optimal RNA integrity. Thus, it is very likely that genes analyzed in this study were not expressed or expressed at very low levels (below the detection limit) in the flower tissue.

In general, all metal transporters tested had higher transcript abundance in roots when compared to leaves at 2 DAT (Fig. 3.6A), and at other time points (data not shown). Transcript abundance of *CsHMA5* in leaves was upregulated by 3-fold in response to the 25 $mg \cdot L^{-1}$ Cd treatment when compared to the control (0 $mg \cdot L^{-1}$ Cd) at 45 DAT (Supplemental Fig.3.1B). However due to Cd toxicity and leaf yellowing, only two replications were analyzed at this stage. Transcript abundance of all seven metal transporters in root tissues was normalized to that of *CsHMA3* exposed to 0 mg $Cd \cdot L^{-1}$ at 2 DAT to identify the most abundantly expressed genes. Among the seven metal transporter genes evaluated, *CsHMA5* and *CsPAA2* were generally highly expressed in roots with *CsHMA1*, *CsHMA3*, and *CsPAA1* showing intermediate expression, and *CsHMA3-C* and *CsHMA4* being least abundant (Fig. 3.6B).

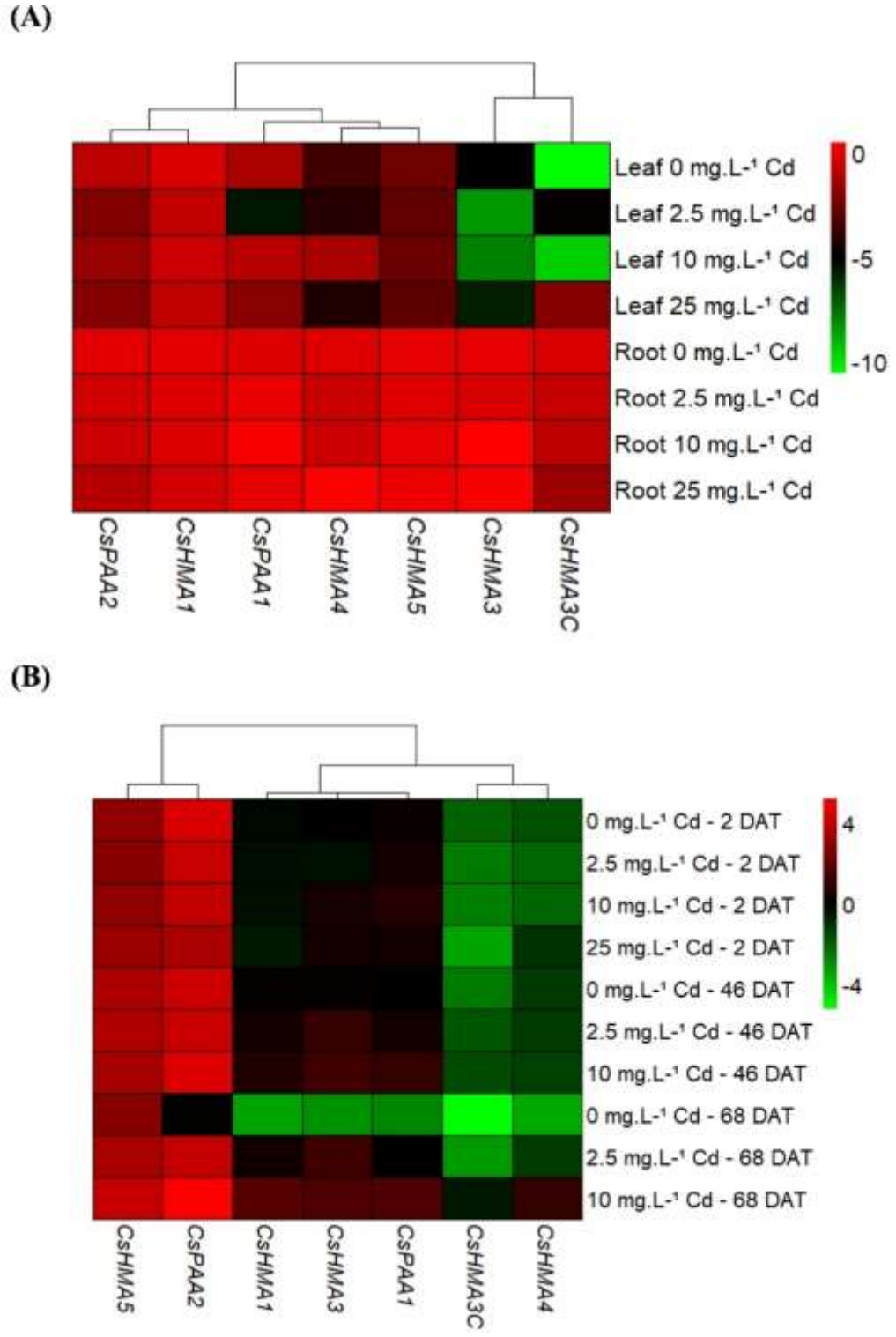


Figure 3.6. Spatial expression of metal transporter genes at 2 d after treatment (DAT). Transcript abundance of each gene in both tissues was normalized to its expression in roots at 2 DAT (A). Temporal expression of metal transporter genes in root tissues. Transcript abundance of each gene in both tissues was normalized to *CsHMA3* expression at 2 DAT (B).

CsHMA3 was upregulated by 2.7- and 3-fold in plants exposed to 10 mg·L^{-1} Cd at 45 and 68 DAT, respectively (Fig. 3.7B). *CsHMA1*, *CsHMA4*, and *CsHMA5* were upregulated by 4-, 6-, and 2-fold, respectively, in plants exposed to 10 mg·L^{-1} Cd at 68 DAT (Fig. 3.7A, D, E). The three remaining transporter genes, *CsHMA3-C*, *CsPAA1*, and *CsPAA2* showed a trend of increasing abundance under 10 mg·L^{-1} Cd treatment at 68 DAT, but were not significantly different compared to the control treatment (Fig. 3.7C, F, G). Transcript abundance of two transporter genes at harvest, *CsHMA1* and *CsHMA5* were positively correlated to Cd concentrations in roots (Table 3.4).

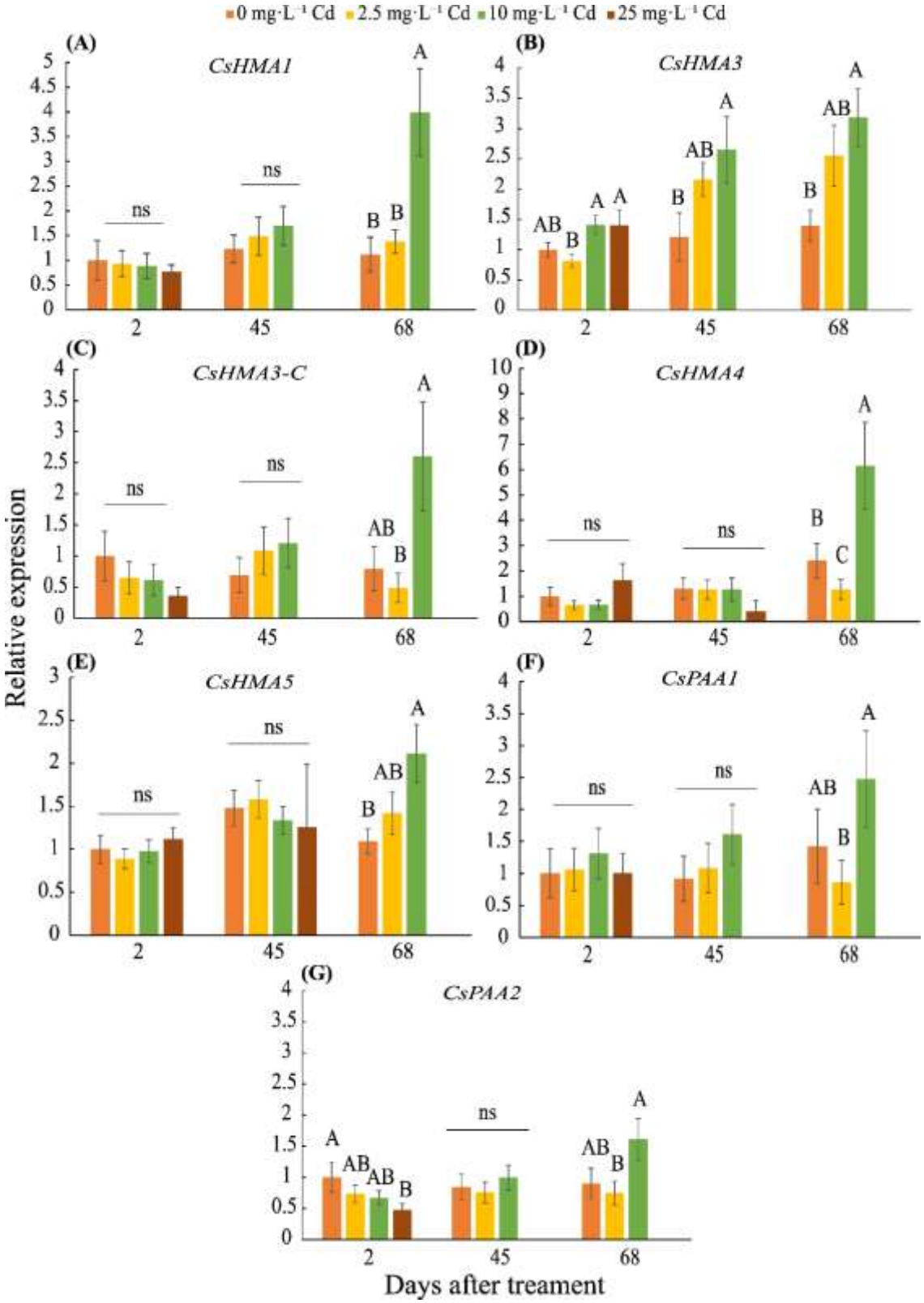


Figure 3.7. Relative expression ± SE of *CsHMA1* (A), *CsHMA3* (B), *CsHMA3-C* (C), *CsHMA4* (D), *CsHMA5* (E), *CsPAA1* (F), and *CsPAA2* (G) in roots of plants exposed to 0, 2.5, 10, and 25

mg.L^{-1} Cd at 2, 45, and 68 d after Cd treatment (DAT). Bars associated with the same letter(s) are not significantly different at $p \leq 0.05$ according to Tukey's HSD all pairwise comparison test. ns, not significant.

Table 3.4. Correlation between transcript expression of Cs metal transporter genes and Cd concentration in roots at 68 DAT.

Cs metal transporter gene	Correlation to Cd concentration	*p* value
CsHMA1	**0.82**	0.02
CsHMA3	0.52	ns
CsHMA3-C	0.70	ns
CsHMA4	0.52	ns
CsHMA5	**0.81**	0.02
CsPAA1	0.56	ns
CsPAA2	0.68	ns

Significant positive correlations are in bold and ns indicates not significant.

Metal transporters from the heavy metal ATPase (HMA) family have been categorized into two subgroups based on their specificity of metal substrates. These are the Zn-ATPases (Zn^{2+} /Cd^{2+}/Co^{2+} /Pb^{2+} specificity) and Cu-ATPases (Cu^{2+}/Ag^{2+} specificity) which may be regulated within certain tissues or subcellular compartments (Takahashi et al., 2012; Wang et al., 2018; Zhang et al., 2018). *A. thaliana* has eight HMA genes of which *AtHMA1-4* belong to the former group whereas *AtHMA5-8* are closely related to the Cu^{2+}/Ag^{2+} subclass. Based on the phylogenetic analysis, most similarities were observed between *CsHMA1* and *AtHMA1*, *CsHMA3*/*CsHMA3-C* and *AtHMA2-4*, *CsHMA4*/*CsHMA5* and *AtHMA7* and *AtHMA5*, *CsPAA1* and *AtHMA6*, and *AtHMA8* and *CsPAA2* (Fig. 3.1).

AtHMA1 has been shown to play role in detoxification of excess Zn and is localized to the chloroplast envelope (Kim et al., 2009). Transcript abundance of *CsHMA1*, most closely related to *AtHMA1*, was upregulated in response to 10 mg·L^{-1} Cd at 68 DAT, suggesting that this gene is

transcriptionally regulated during advanced growth stages, perhaps leading to greater plant Cd uptake and accumulation. Previous findings reported on the role of *AtHMA3* and its orthologues on Cd sequestration into root vacuoles (Ismael et al., 2019; Miyadate et al., 2011; Morel et al., 2009). Further, *AtHMA2* and *AtHMA4* were reported to be involved in Cd uptake and translocation, being expressed in tissues adjacent to the root vascular bundle for loading Cd into the xylem (Ismael et al., 2019; Wong & Cobbett, 2009). *CsHMA3* was the only transporter induced during mid and late growth stages at 45 and 68 DAT, and similar to *AtHMA2-4* may be involved in Cd uptake, sequestration into the root vacuoles, and possibly xylem loading. Migocka et al. (2015) reported that in cucumber (*Cucumis sativus*) *HMA3* was upregulated in roots in response to Cd exposure and conferred tolerance to heavy metal detoxification through sequestration in the vacuole. Further, *HMA3* expression was greater in response to Cd exposure compared to *HMA4* in cucumber, though activity of *HMA4* was higher when exposed to elevated levels of Zn, suggesting that *HMA4* may have been more efficient as a Zn^{2+}-ATPase compared to a Cd^{2+}-ATPase (Migocka et al. 2015).

As noted earlier, *AtHMA5-8* belong to the Cu^{2+}/Ag^{2+} ATPase subgroup and functions in Cu transport. *AtHMA5* was predicted to be mainly expressed in roots and involved in Cu^{2+} transport, compartmentalization, and detoxification (Andrés-Colás et al., 2006; Axelsen & Palmgren, 2001). Although *CsHMA4* and *CsHMA5* fall within this subclass, our data indicate the upregulation of these transporter genes under long-term Cd stress. Similarly, *Populus tomentosa HMA5* shared similarity with *AtHMA5*, and overexpression of *PtoHMA5* in tobacco plants increased Cd translocation from roots to leaves, suggesting its role in Cd transport (Wang et al., 2018). Similarly, upregulation of a putative Cu-transporting ATPase, *HMA5* was found to be induced by Cd exposure in peanut (Chen et al., 2019) and durum wheat (Aprile et al., 2018).

Overall, the seven transporter genes were expressed in root tissue and potentially involved in cation uptake, including Cd. When Cd levels increased during the course of plant growth under the 10 $mg \cdot L^{-1}$ Cd treatment, *CsHMA1*, *CsHMA3*, *CsHMA4* and *CsHMA5* transcript abundance were upregulated in the roots possibly to allow for increased uptake, sequestration, and translocation of Cd. These transporters may play a role in leaf Cd uptake as well; however, leaf Cd concentrations were substantially lower compared to that in roots and may not elicit an increase in their transcript abundance.

Of the two cannabinoid biosynthetic genes, *CsCBDAS* and *CsTHCAS*, analyzed in leaf tissue, only the expression of *CsTHCAS* was downregulated at harvest in plants exposed to 10 $mg \cdot L^{-1}$ Cd (Supplemental Fig. 3.2B). Cannabinoid levels in flower tissue did not change in experiment 1 and transcript abundance of cannabinoid biosynthetic genes in leaf tissues with cannabinoid levels in flower tissue may not be well correlated. Although previous studies have indicated upregulation of *CsCBDAS* in flowers with higher total CBD concentrations in Cd contaminated soil (Husain et al., 2019), other studies have proposed that transcript levels of both *CsCBAS* and *CsTHCAS* are not well correlated with CBD and THC synthesis (Apicella et al., 2022).

Conclusions

Our findings indicate that *C. sativa* may tolerate Cd exposure of 2.5 and 10 $mg \cdot L^{-1}$ Cd in a hydroponic solution. Concentrations of 25 $mg \cdot L^{-1}$ Cd severely restricted growth, reduced F_v/F_m, CCI, and caused premature death. As expected, Cd concentrations were highest in roots, but there was significant accumulation of Cd in leaves, stems, and flowers, with increasing Cd in the nutrient solution. Plant biomass and total CBD and THC in flowers were not significantly different between plants in the control treatment and plants exposed to up to 10 $mg \cdot L^{-1}$ Cd. Transcript abundance

of *CsHMA1*, *CsHMA3*, *CsHMA4*, and *CsHMA5* in the roots of *C. sativa* were upregulated in 10 $mg \cdot L^{-1}$ Cd treatments, suggesting that they may be associated with Cd uptake, sequestration in roots and xylem loading. While Cd concentrations that were available to plants used in the present study are greater than what would be encountered on agricultural soils, our results indicate that hemp plants have the potential to accumulate Cd in floral tissue. Therefore, heavy metal testing in *C. sativa* consumer products may be of importance.

Acknowledgements

We thank Dr. Allison Justice at The Hemp Mine for providing plant material for this research.

Supplementary Materials

Supplemental Table 3.1. Mineral nutrient concentrations ($mg \cdot L^{-1}$) used in this study.

	N	P	K	Ca	Mg	B	Cu	Mo	Fe	Mn	Zn
Well Water	ND	0.1	2.9	10.5	1.9	1.7	<0.05	<0.01	0.3	0.2	<0.05
Nutrient Solution[i]	150	50	200	125	65	3	0.3	0.1	4	1	0.3
Total Concentration	150	50.1	202.9	135.5	66.9	4.7	0.3	0.1	4.3	1.2	0.3

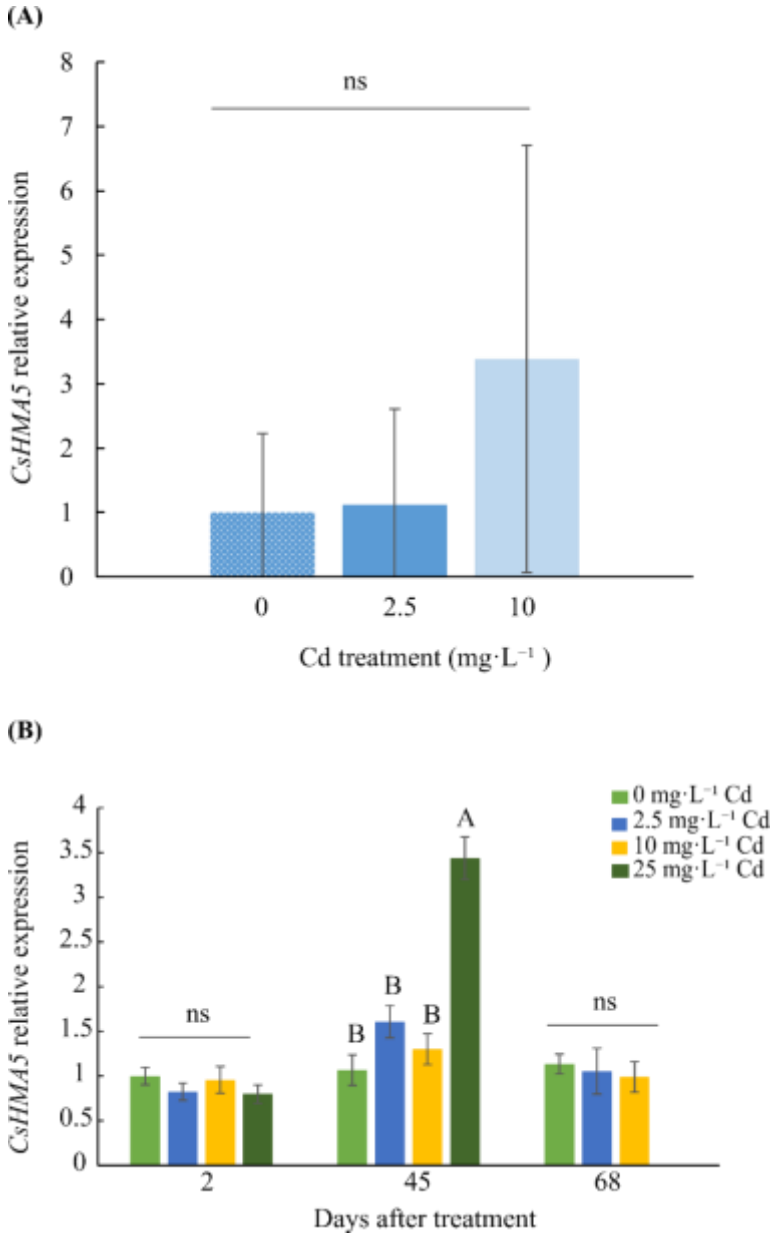
ND = not detected.

[i]Nutrient solution comprised from the following compounds: KNO_3, K_2HPO_4, $MgSO_4$, H_3BO_3, Cu-EDTA, Fe-EDTA, Mn- EDTA, Na_2MoO_4, Zn-EDTA, $Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$, $(NH_4)_2MoO_4$, Ca-EDTA, and $Na_2B_4O_7 \cdot 10H_2O$.

Supplemental Table 3.2. Primer sequences and accession numbers of metal transporter, cannabinoid biosynthetic, and reference genes.

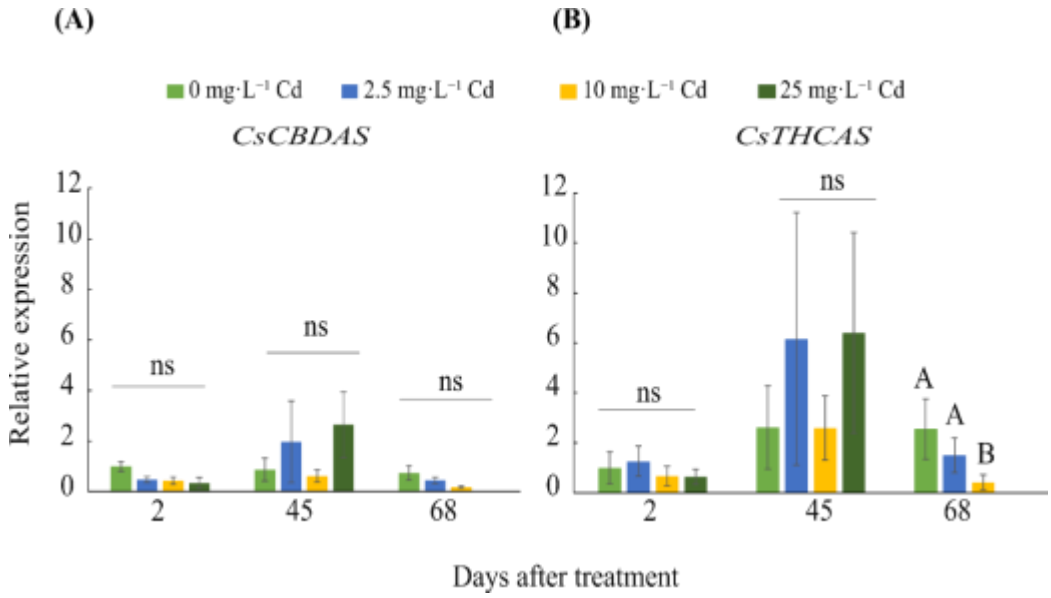
Gene name	NCBI reference	Fw sequence	Rv sequence
Cs probable cadmium/zinc-transporting ATPase HMA1	XM_030630179.1	GACGGAAGCTCAGAAAGCG	ACCTGTAGAACCGCAACAC
Cs putative inactive cadmium/zinc-transporting ATPase HMA3	XM_030654349.1	AGGGAAGTGCTGTAAACCT	CCGAGTTCCCGCTCTTCT
Cs putative inactive cadmium/zinc-transporting ATPase HMA3 Complement	XM_030655263.1	GGTCATGGCCTGTGGATTATTAC	GTGAGGTTCCAAATGGAGGT
Cs copper-transporting ATPase HMA4	XM_030648942.1	CTTCAAGGTCAGGCTGTGA	CGGGAAACTCCTCAGCTGT
Cs probable copper-transporting ATPase HMA5	XM_030645235.1	CAATAGCTGCAGGAGCACTC	GAG CAG CAA ACA ACA CTG AC
Cs copper-transporting ATPase PAA1	XM_030654671.1	GTGGAAGAAGCCCAAAGTAGG	AAGGTAGCAGTAGAGAGAGCC
Cs copper-transporting ATPase PAA2	XM_030633739.1	TGGTCGTGAAGGAGAAGGC	TCCCCGGATAAGAGCAAAGT
Cs inactive tetrahydrocannabinolic acid synthase	XM_030625046.1	CCTTCAAATGTCTCCCATATCCAGG	TGTAGGACAAACCCTCAGC
Cs cannabidiolic acid synthase-like	XM_030624886.1	GTCACTCCTTCACATGTCTCTC	GGGACTTGAGATATGTAGGACATG

Cs Elongation factor 1 alpha/HBS1-like protein (reference gene)	XM_030654944.1	TTGCACGGATCAGTTTGCG	AATGCCGACCGCTACAGTT
Cs ubiquitin-protein ligase/ubiquitin domain-containing protein DSK2b) (reference gene)	XM_030630092.1	TACGAGGAGCAGCTGTAGAT	ATAGAGTGAGGGTGGGAGAAGA



Supplemental Figure 3.1. Relative expression ± SE of ATPase *CsHMA5* in flower tissue of plants exposed to 0, 2.5, and 10 mg·L^{-1} Cd at 68 d after Cd treatment (DAT) (A). Relative expression ± SE of ATPase *CsHMA5* in leaf tissue at 2, 45, and 68 DAT (B). Plants were

exposed to 0, 2.5, 10, and 25 mg·L^{-1} Cd. Bars associated with the same letter(s) are not significantly different at $p \leq 0.05$ according to Tukey's HSD all pairwise comparison test.



Supplemental Figure 3.2. Relative expression ± SE of *CsCBDAS* (A) and *CsTHCAS* (B) in leaf tissue at 2, 45, and 68 days after Cd treatment (DAT). Plants were exposed to 0, 2.5, 10, and 25 mg·L^{-1} Cd. Bars associated with the same letter(s) are not significantly different at $p \leq 0.05$ according to Tukey's HSD all pairwise comparison test.

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CHAPTER 4

CADMIUM BIOCONCENTRATION AND TRANSLOCATION POTENTIAL IN DAY NEUTRAL AND PHOTOPERIOD SENSITIVE HEMP GROWN HYDROPONICALLY FOR THE MEDICINAL MARKET[2]

[2] Marabesi, A. O., Lessl, J. T., & Coolong, T. W. (2023). *Water, 15*(12), 2176; https://doi.org/10.3390/w15122176. Reprinted here with permission of the publisher.

Abstract

Heavy metal contamination of agricultural soils is potentially concerning when growing crops for human consumption. Industrial hemp (*Cannabis sativa* L.) has been reported to tolerate the presence of heavy metals such as cadmium (Cd) in the soil. Therefore, the objectives of this study were to evaluate Cd uptake and translocation in two day-length sensitive (DLS) and two day-neutral (DN) hemp varieties grown for the medicinal market, and to determine the impact of Cd exposure on cannabinoid concentrations in flowers. A hydroponic experiment was conducted exposing plants to 0 $mg \cdot L^{-1}$ Cd and 2.5 $mg \cdot L^{-1}$ Cd in the nutrient solution. Cadmium concentrations ranged from 16.1 to 2274.2 $mg \cdot kg^{-1}$ in roots, though all four varieties accumulated significant concentrations of Cd in aboveground tissues, with translocation factors ranging from 6.5 to 193. Whole-plant bioconcentration factors ranged from 20 to 1051 $mg \cdot kg^{-1}$. Cannabinoid concentrations were negatively impacted by Cd exposure in DN varieties but were unaffected in DLS varieties. Biomass was reduced by Cd exposure demonstrating that these varieties might not be suitable for growth on contaminated soil or for phytoremediation. There is potential for Cd accumulation in flowers, showing the need for heavy metal testing of *C. sativa* consumer products.

Introduction

For centuries, humans have cultivated hemp (*Cannabis sativa* L.) for its fiber, seed, therapeutic, and psychoactive properties. During the domestication process, wild *Cannabis* populations have been subject to selection, giving rise to the multiple varieties that exist today [1]. The term industrial hemp is commonly used to refer to *C. sativa* plants [2] with total tetrahydrocannabinol (THC) concentrations below 0.3%. Plants with total THC concentrations above 0.3% are classified as marijuana and subject to federal prohibition in the United States (U.S.) [3]. Current industrial hemp breeding efforts target fiber, seed, or cannabinoid production, based

on the end user. Hemp varieties with high cannabidiol (CBD) concentrations are often selected for medicinal and therapeutic uses. There is also a focus on developing varieties that are day-neutral (DN) or minimally sensitive to photoperiod in order to expand production opportunities [4].

Hemp is generally considered a qualitative short-day plant that flowers in response to decreasing photoperiods. Hemp selections that flower in response to photoperiod are known as day length sensitive (DLS). After emergence, hemp undergoes a photoperiod-dependent vegetative phase maintained by exposure to approximately 14-18 h or more of light daily [5,6]. When hemp is planted during periods of short days (< 13 h light), it may flower prematurely. Premature flowering, prior to complete vegetative development, can result in yield reductions [7,8]. In contrast, some hemp varieties exhibit DN flowering tendencies known colloquially as "auto-flower" hemp. These DN varieties are relatively insensitive to photoperiod for flower induction. The DN trait is speculated to arise from *Cannabis ruderalis* (*C. sativa* ssp. *ruderalis*) and may have originated from hemp located in high latitudes where photoperiods can be long and growing seasons are typically short or regions with relatively short and constant photoperiods [5,9,10]. Advantages of DN hemp varieties include the ability to flower in regions that have little variation in photoperiod throughout the year (tropics) or during times of the year when photoperiods may be inadequate to grow DLS varieties. However, many DN types of hemp have been reported to be particularly sensitive to environmental stressors such as high temperatures and may have lower yields than comparable DLS varieties [11].

In addition to uses for fiber, seed, and medicinal purposes, hemp has also been proposed as a candidate for use in phytoremediation, which utilizes plants to remove contaminants, such as heavy metals or other chemicals from soils [12,13]. Accumulator plant species can uptake heavy metals from soils, even at low external concentrations, and concentrate them in plant tissues [14].

By growing accumulator plants in contaminated soil, it is possible to realize *in situ* decontamination, an economically viable approach that preserves physicochemical soil characteristics, while removing contaminants [15]. The morphophysiological characteristics of hemp, such as high biomass production, deep roots, and short life cycle make it a potential candidate for phytoremediation [16-20].

Heavy metal contamination of agricultural soils is a concern when growing crops for food or medicinal purposes, due to potential harm to human and animal health [21-23]. Cadmium (Cd) contamination in the environment has been linked to anthropogenic activities, such as mining and smelting. Further, Cd can be introduced to soils via contaminated manure, sewage sludge, and phosphate fertilizers [24]. Cadmium is known to cause health issues when ingested in amounts greater than the provisional tolerable monthly intake (PTMI) of 25 $\mu g \cdot kg^{-1}$ of body weight [25]. In previous studies utilizing naturally and artificially contaminated soil and substrate containing from 0 to up to 200 $mg \cdot kg^{-1}$ Cd, hemp varieties grown for fiber production accumulated Cd in aboveground tissues at levels that could be harmful to human health [26-29]. For instance, the hemp fiber variety Silistrinski grown in naturally contaminated soil containing 12.2 $mg \cdot kg^{-1}$ Cd accumulated 1.22 $mg \cdot kg^{-1}$ Cd in its flowers [27].

There are multiple indicators that can be used to determine the accumulation potential of a plant species. Bioconcentration factor (BCF) is the ratio between the metal concentration in plant tissues and the initial metal concentration in the soil or growing solution [20,30-33]. This indicator has also been used interchangeably with terms such as accumulation factor (AF) [28], biological absorption coefficient (BAC) or index of bioaccumulation (IBA) [23]. A separate indicator of accumulation potential is the translocation factor (TF), which is the ratio between the metal concentration in the above ground biomass and the metal concentration in the roots [31,32].

Additionally, plant growth parameters can be assessed to determine the tolerance index (TI), calculated as the ratio between growth in contaminated and non-contaminated soils [20,30]. There is significant variability in BCF among plant species and chemical elements. It has been proposed that plants with BCF >100 $mg \cdot kg^{-1}$ Cd on a dry weight (DW) basis in its leaves could be referred to as hyperaccumulators [14]. Conversely, [12] suggested that true hyperaccumulators are able to accumulate higher concentrations of metals in leaves than in roots (TF > 1).

Few studies have evaluated heavy metal accumulation in hemp flowers, with most research utilizing fiber hemp varieties to determine heavy metal uptake for phytoremediation purposes [16,17,19,34]. Due to the harmful effects of Cd and other heavy metals to human health, the U.S. hemp industry has attempted to implement standards regarding maximum allowable levels of metals in *C. sativa* consumer products, which vary by state [35]. As hemp flowers are increasingly grown for the medicinal market, determining Cd distribution among plant organs, as well as bioconcentration and root-to-shoot translocation factors, are of importance. We hypothesize that there are distinctions in Cd accumulation and distribution among plant tissues in hemp varieties with different growth and flowering habits. Therefore, the objectives of this study were to evaluate nutrient partitioning and Cd uptake, translocation, and accumulation in DLS and DN hemp varieties, and to determine the impact of Cd exposure to cannabinoids in plant flowers.

Materials & Methods

Experimental Setting

The experiment was conducted in a greenhouse in Watkinsville, GA, USA (lat. 33° 5’N, long. 83° 3’W) from Jan. 2022 to Apr. 2022. Feminized seed from two DLS hemp varieties, T1 and Von (Sunbelt Hemp Source, Moultrie, GA, USA), and two DN varieties Apricot Auto (Blue Forest Farms, New York, NY, USA) and Auto CBD Alpha Explorer (Alpha Explorer) (Phylos

Bioscience, Portland, OR, USA) were sown into engineered foam cubes (3.33 cm L × 2.54 cm W × 3.81 cm D; Oasis Grower Solutions, Kent, OH, USA) for germination. Foam cubes were placed in plastic trays over a germination mat set at 24 °C exposed to a mist irrigation system, which applied water twice daily for 1 min each. Supplemental lighting (approximately 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was used during germination. Seedlings were maintained under these conditions for 4 weeks, after which they were placed into plastic netted containers (4.7 cm W ×5.1 cm D) and transferred to 37.9 L plastic containers (Rubbermaid Inc. Wooster, OH, USA) filled with 28 L of well water. The well water was analyzed for nutrient concentrations periodically throughout the experiment (Table 4.1). Four seedlings per replicate were placed equidistantly (24.3 cm apart) in holes drilled in the container lid. Welded wire mesh frames were attached to each lid to support plants. A 15.2 cm aquarium air stone attached to an air pump (Active Aqua; Hydrofarm, Petaluma, CA, USA) was placed inside the container to aerate the nutrient solution throughout the experiment. Container volume was maintained by adding well water every 2–3 days. At transplant, a nutrient solution was added to the plastic containers using a half-strength Hoagland's solution [36] (Table 4.1).

Table 4.1. Mineral nutrient concentrations in the nutrient solution used in this study.

	N	P	K	Ca	Mg	B	Cu	Mo	Fe	Mn	Zn
						($mg \cdot L^{-1}$)					
Well water	ND	<0.01	2.7	12.1	2.1	<0.01	<0.05	<0.01	<0.1	<0.1	<0.1
Nutrient solution[i]	105	15.5	117	100	24.3	0.3	0.01	0.005	0.5	0.25	0.025
Total concentration	105	15.5	119.7	112.1	26.4	0.3	<0.05	<0.01	0.5	0.25	<0.1

ND = not detected.

[i]Nutrient solution comprised from the following compounds: $Ca(NO_3)_2 \cdot 4H_2O$, KNO_3, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, H_3BO_3, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $H_2MoO_4 \cdot H_2O$, and Sequestrene 330.

Plants were grown for 3 weeks in the base nutrient solution after which nutrient solutions were replaced completely and Cd treatments added using $CdSO_4 \cdot 8H_2O$, to achieve 0 (control) and 2.5 $mg \cdot L^{-1}$ Cd. Cadmium concentrations were chosen based on the results of previous studies [34, 35], which evaluated hemp exposure to Cd in hydroponic systems. The experimental treatments were arranged in a randomized complete block design with four hemp varieties exposed to two levels of Cd with four replicates each. Nutrient solutions were maintained to a constant volume by adding water every 2–3 days and nutrients were replaced every 3 weeks for the remainder of the experiment. Electrical conductivity (EC) and pH of the solutions were measured weekly. Solution pH was adjusted to 5.5 when necessary (pH down; General Hydroponics, Santa Rosa, CA, USA). Supplemental light (approximately 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was used to provide 18/6 light/dark hours for 4 weeks of vegetative growth after transplanting seedlings into containers. Supplemental lights were turned off to allow for flower induction in the DLS varieties for the remaining 7 weeks of production (average day length 12 h 38min). The DN varieties (Apricot Auto and Auto CBD Alpha Explorer) exhibited visually detectable flower development 1 week prior to the induction of flowering in the DLS varieties (Von and T1). Therefore, the DN varieties were harvested 1 week prior to the DLS varieties to ensure that plants flowered for the same length of time.

Nutrient solutions were sampled at the beginning and end of each 3-week cycle using 20 mL scintillation vials (HDPE; Thermo-Fisher Scientific™, Waltham, MS, USA), and stored at - 4 $^{\circ}C$ until the analysis of mineral nutrient concentrations (Supplemental Table 4.1). Temperature and relative humidity (RH) of the greenhouse were monitored at canopy height hourly (VP4; Meter Group Inc., Pullman WA, USA) and averaged 19.1 ± 3.1 $^{\circ}C$ and 74 ± 0.1 % RH for the experiment. Photosynthetic active radiation was also monitored hourly throughout the experiment (QSO-S;

Meter Group Inc.) and plants were exposed to an average daily light integral (DLI) average of 21.6 ± 9.0 $mol \cdot m^{-2} \cdot d^{-1}$.

Mineral Analysis

Samples of fresh root, stem, leaf, and flower tissues were collected for Cd analysis at harvest. Composite samples (50 g fresh material) were taken from each of the four plants in a replicate (container). Roots were triple washed with deionized water after removal. Ten of the youngest fully expanded leaves were collected from the top one-third of each plant (main stem and lateral branches) and rinsed with deionized water. Stem samples were collected from the bottom two-thirds of the main stem. Flower material was sampled from the top of the main stem and top one-third of plants. Samples were placed in a forced air oven set at 55 °C for 72 h until a constant weight was achieved. Dried plant material was then ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) and passed through a 20-mesh screen. Samples were digested using EPA Method 3052 [37]. In brief, 0.5 g samples were placed in fluorocarbon polymer microwave vessels, adding 10 mL of concentrated nitric acid to each vessel which was then sealed. The microwave digester (Mars 6 Microwave; CEM Corp., Matthews, NC, USA) was heated to 200 °C for 30 min and digested (solutions) were then transferred quantitatively into volumetric flasks and brought to 100 mL volume with deionized water prior to analysis.

Samples of the hydroponic solutions were filtered using a 0.45 µM PTFE membrane (Thermo-Fisher Scientific™ Choice™ Polypropylene Syringe Filters) and acidified using 2% (V/V) high purity nitric acid (HNO_3) (Certified ACS Plus, Fisher Scientific, Pittsburgh, PA, USA) prior to analysis. Hydroponic solutions and plant tissues were analyzed for multiple elements (P, K, S, Ca, Mg, Fe, Mn, Al, B, Cu, Zn, Ni, and Cd) following EPA Method 200.8 [38] by Inductively Coupled Plasma — Optical Emission Spectroscopy (Spectro Arcos FHS16; Spectro Ametek USA,

Wilmington, MA, USA). The instrument parameter settings and wavelengths used are displayed in the supplementary materials (Supplemental Tables 4.3, 4.4, and Supplemental Fig. 4.1). The instrument reporting limit for Cd was <0.005 $mg \cdot L^{-1}$. Results were expressed as $mg \cdot L^{-1}$. Calibration standards utilized in this analysis were from a certified source (Inorganic Ventures, Christiansburg, VA, lot number: N2-MEB667614). Independent laboratory performance checks were also run with acceptable deviations for recoveries set at 100 ± 5.0%.

The BCF was calculated by dividing the Cd concentration in plant tissues by the initial Cd concentration in the nutrient solution [20,30-33]. The TF (%) was calculated by dividing the sum of Cd concentration in leaves, flowers, and stems by the Cd concentration in roots, and multiplying by 100 [31,32]. Cadmium uptake rates ($\mu mol \cdot plant^{-1} \cdot d^{-1}$) were calculated by the following equation [adapted from 30]:

(1) $((([Cd_{initial} - Cd_{final}]) / \text{number of plants}) / \text{treatment days}) / \text{root biomass}$

Plant Growth and Biomass Yield

Plant height was determined by measuring the distance from the base of the stem to tip of the apically dominant flower at harvest [11]. To quantify leaf, flower, stem, and root biomass, four whole plants per replicate were air dried at ambient temperatures inside the greenhouse for 2 weeks and then separated into roots, stems, and leaf and flower biomass. Dry leaf and flower materials were manually pulled from plants following industry standards used for hemp biomass intended in cannabinoid extraction. Subsamples were taken from the air-dried materials and further dried in a forced air oven set at 55 ˚C for 48 h until a constant weight was achieved. The dry weights of the whole plant samples were then normalized based on subsample moisture content [11].

Cannabinoid Analysis

Approximately 25 g of fresh flower tissue sampled from inflorescences located on the top one-third of the plants were sampled during weeks 6 (DN varieties) and 7 (DLS varieties) of flowering (49 and 56 days after treatment (DAT), respectively) and dried separately from other samples as follows. Flower material was placed on a perforated aluminum baking sheet and dried to approximately 15% moisture content in a walk-in cooler with a temperature set point of 13 °C and 55% relative humidity for 14 days. The appropriate relative humidity was maintained using a dehumidifier. The dried material was hand trimmed to remove leaves, sealed in a metalized resealable food bag (Uline, Braselton, GA, USA) and stored at -4 °C for cannabinoid analysis. The acidic and neutral forms of the cannabinoids, THC and CBD, were determined in dried flower material by a commercial laboratory using high performance liquid chromatography and a diode array detector set to 230 nm (SJ Labs & Analytics, Macon, GA, USA). The limit of detection for THC and CBD was 0.02%. Total cannabinoid concentrations were calculated by the following formula: total cannabinoid = neutral + (acidic form × 0.877). Percentage dry matter for all samples was recorded and results reported on a dry weight basis.

Statistical Analysis

Statistical analysis was conducted using JMP© Pro 15 (SAS, Cary NC, USA). Data were subjected to a one-way ANOVA procedure with Student's t test ($p < 0.05$) or Tukey's Honest Significant Difference test ($p < 0.05$) conducted for mean separation when appropriate. Tissue Cd and cannabinoid concentrations were log-transformed to ensure equal variance prior to statistical analysis. Non-transformed data are presented.

Results & Discussion

Plant Height and Biomass Yield

Plant heights were significantly reduced by Cd exposure in DN varieties, Apricot Auto and Alpha Explorer. Plants exposed to 2.5 mg·L^{-1} Cd were shorter than plants in the control (0 mg·L^{-1} Cd) treatment (Fig. 4.1). Plant heights of the two DLS varieties, Von and T1, were not significantly affected by Cd treatment. Nevertheless, the average decline in plant height of plants exposed to Cd was 20.4% in the DLS varieties and 35.6% in the DN varieties. Exposure to Cd also significantly reduced flower and leaf, stem, and root biomass in the DN varieties, Apricot Auto and Alpha Explorer (Table 4.2). In the DLS variety Von, dry weight of flower and leaf tissues significantly decreased in the 2.5 mg·L^{-1} Cd treatment compared to the control, while dry weights of stem and root tissues were unaffected by Cd. Dry weight of flower and leaf, and root tissue were significantly reduced by exposure to Cd in the DLS variety T1. Whole plant biomass in all four varieties were significantly reduced by Cd treatments, with an average reduction of 74.4% in the DN varieties and 50.2% in the DLS varieties.

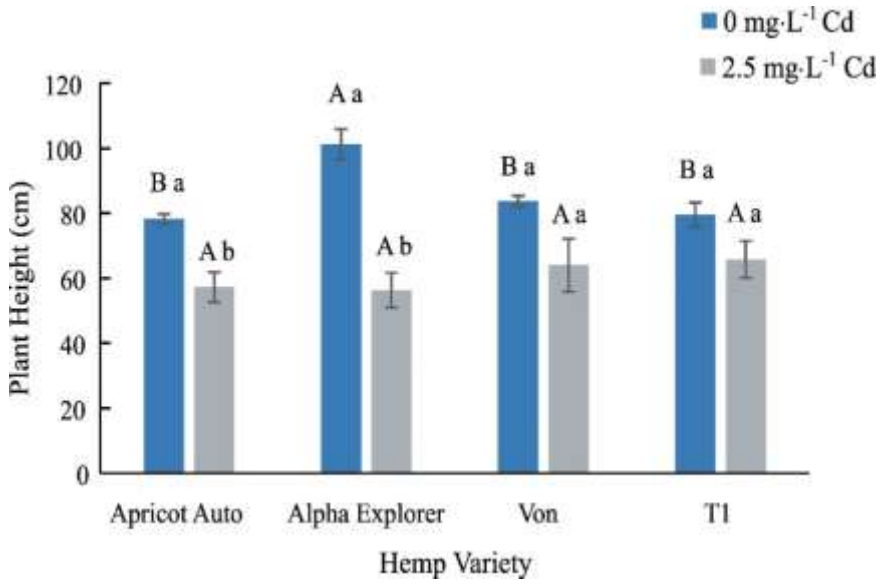


Figure 4.1. Average plant height at harvest ± SE of four hemp (*Cannabis sativa* L.) varieties. Values correspond to averages of four replicates with four plants each. Bars associated with the

same uppercase letter(s) indicate no significant differences among hemp varieties at $p \leq 0.05$ according to Tukey's HSD test. Bars associated with the same lowercase letter indicate no significant differences between control and treated plants for a given hemp variety at $p \leq 0.05$ according to Student's t-test.

The DLS varieties, Von and T1, generated significantly greater root biomass relative to the DN varieties when not exposed to Cd. In Cd treated plants, root biomass was significantly greater in Von compared to the two DN varieties. Root biomass in T1 was not significantly different from any other variety when exposed to Cd. Flower and leaf tissue yields were highest in DLS variety T1 in both control and Cd exposed treatments. Exposure to Cd resulted in a significant decrease in flower and leaf biomass of 75% and 78%, in Apricot Auto and Alpha Explorer DN varieties, respectively. Exposure to Cd significantly reduced flower and leaf biomass in the DLS varieties Von and T1 by 52% and 41%, respectively. Whole-plant, stem, and root biomass was also significantly reduced to a greater extent in DN varieties compared to the DLS varieties. This suggests that the DLS varieties used in the present study may be more tolerant to Cd exposure at 2.5 $mg \cdot L^{-1}$ than the DN varieties evaluated. These results agree with previous studies that have reported a decrease in shoot biomass of hemp plants exposed to Cd [26,34,35,39].

Table 4.2. Dry weight of flowers + leaves, stems, roots, and whole plant at harvest ± SE of four hemp (*Cannabis sativa* L.) varieties exposed to 0 (control) and 2.5 (treated) $mg \cdot L^{-1}$ Cd in a nutrient solution.

	Flower + Leaf		Stems		Roots		Whole Plant	
	Biomass ($g \cdot plant^{-1}$)[i]							
Variety	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Apricot Auto	40.0 ± 2.3 AB a	9.9 ± 2.0 B b	17.7 ± 1.2 A a	6.5 ± 1.0 A b	8.3 ± 0.3 B a	3.2 ± 0.4 B b	66.0 ± 3.2 B a	19.5 ± 2.7 AB b
Alpha Explorer	37.8 ± 2.2 B a	8.2 ± 1.4 B b	23.6 ± 1.5 A a	4.5 ± 0.8 A b	7.1 ± 0.7 B a	2.1 ± 0.4 B b	68.4 ± 3.3 B a	14.8 ± 2.4 B b
Von	46.2 ± 1.3 AB a	22.2 ± 7.2 AB b	29.3 ± 1.7 A a	15.2 ± 5.6 A a	20.1 ± 1.0 A a	13.3 ± 2.9 A a	95.6 ± 1.5 A a	50.7 ± 15.3 A b
T1	48.6 ± 2.4 A a	28.5 ± 5.6 A b	27.7 ± 6.5 A a	13.9 ± 2.7 A a	22.9 ± 2.0 A a	9.7 ± 2.7 AB b	99.2 ± 6.4 A a	52.2 ± 9.6 A b

[i]Values followed by the same uppercase letter(s) indicate no significant differences among hemp varieties for each plant tissue according to Tukey's Honest Significant Difference test ($p < 0.05$).Values followed by the same lowercase letter within each individual hemp variety row indicate no significant differences between control and Cd treated plants for each plant tissue according to Student's t test ($p < 0.05$).

Cd Concentration in Hemp Tissues

Cd concentrations in hemp tissues were affected by plant variety and Cd treatment (Table 4.3). In DN varieties, Apricot Auto and Alpha Explorer, and the DLS variety Von, Cd concentrations were highest in roots. In contrast, the DLS variety, T1 had similar Cd concentrations in roots, leaf, and stem tissue upon exposure to Cd. These results are consistent with previous literature, which reported that roots were the preferred tissue for Cd accumulation in hemp [27,34,35,40,41] and that Cd accumulation increased with increasing Cd concentrations in the growing media [39]. For instance, Cd concentrations in the roots of *C. sativa* fiber variety Santhica 27 exposed to 20 μM Cd (2.25 $mg \cdot L^{-1}$ Cd) for one week averaged 2687 $mg \cdot kg^{-1}$ dw, while Cd concentrations in stems and leaves averaged 1243 $mg \cdot kg^{-1}$ dw and 717 $mg \cdot kg^{-1}$ dw, respectively [34]. Additionally, the *C. sativa* medicinal variety Purple Tiger exposed to 2.5 $mg \cdot L^{-1}$ Cd for 68 days had average Cd concentrations of 1982.6 $mg \cdot kg^{-1}$ dw in roots, 13.2 $mg \cdot kg^{-1}$ dw in leaves, 5.1 $mg \cdot kg^{-1}$ dw in stems, and 7.6 $mg \cdot kg^{-1}$ dw in flowers [35].

All varieties had increased concentrations of Cd in all plant tissues exposed to Cd in the nutrient solution, except in the floral tissues of T1. Cadmium treated DN varieties, Apricot Auto and Alpha Explorer, had increased Cd concentration in flower and stem tissues, and Alpha Explorer had significantly greater Cd concentrations in leaf tissue compared to the DLS varieties. These results suggest that Cd concentration in plant tissues is negatively correlated to biomass accumulation, as the DN varieties had a higher reduction in flower and leaf biomass when exposed to 2.5 $mg \cdot L^{-1}$ Cd, compared to the DLS varieties. It should be noted that detectable Cd levels were observed in the control tissues. We expected all the control tissues to be below detection and can only speculate at the source of the Cd. It could have been slight cross contamination due to the aeration system that connected all the tanks. Another possibility is the Cd was already in the plant

tissues before the treatments were applied. We analyzed the propagation foam cubes and found some Cd in the material (0.25 $mg \cdot L^{-1}$), which potentially could have slightly contaminated the tanks.

Whole-plant BCF ranged from 1051.8 in Alpha Explorer to 20.9 in T1 (Table 4.4 and Supplemental Fig. 4.2). In Alpha Explorer, whole-plant, root, and leaf BCF were significantly greater than the other three varieties evaluated. The BCF in stems and flowers were significantly higher in the DN varieties, Alpha Explorer and Apricot Auto, when compared to the DLS varieties, Von and T1. In Alpha Explorer, Apricot Auto, and Von varieties, BCF values were the highest in roots, while in T1 plants BCF was the highest in stems. For all varieties, the BCF in floral tissue was significantly less than that of roots, except for T1, which accumulated most Cd in stem tissues instead of roots. Previous studies reported that BCF values were consistently higher in roots when compared to above-ground biomass [39,41]. Furthermore, in a review of the capacity of different varieties of *C. sativa* to accumulate heavy metals, root BCF values in plants exposed to different Cd treatments ranged from 0.08 to 30.99 [17]. Most prior studies utilized contaminated soil or a soil-like substrate, which makes it challenging to determine the exact concentration of Cd that was available to plants. Nevertheless, our results supports previous literature demonstrating that plant genetics play a role on Cd tolerance and accumulation potential of hemp plants.

Table 4.3. Average cadmium (Cd) concentration at harvest in plant tissues of four hemp (*Cannabis sativa* L.) varieties exposed to 0 (control) and 2.5 (treated) $mg \cdot L^{-1}$ Cd in a nutrient solution on a dry weight (dw) basis.

	Flower		Root		Leaf		Stem	
	Cd concentration ($mg \cdot kg^{-1}$ dw)[i]							
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
Apricot Auto	1.2 A ab	38.1 A c	3.8 A a	1056.8 A a	1.0 A b	44.9 AB c	0.5 A c	116.3 A b
Alpha Explorer	0.6 A ab	51.0 A d	3.4 A a	2274.2 A a	3.0 A a	92.5 A c	0.3 A b	176.4 A b
Von	0.4 A a	11.9 B b	1.1 A a	512.4 A a	2.1 A a	18.2 BC b	ND A b	1.8 C c
T1	ND B a	0.2 C b	ND B a	16.1 B a	0.8 A a	8.22 C a	0.6 A a	26.8 B a

[i]Values followed by the same uppercase letter(s) indicate no significant differences among hemp varieties for each plant tissue according to Tukey's Honest Significant Difference test ($p < 0.05$).
Values followed by the same lowercase letter(s) within a hemp variety row indicate no significant differences among plant tissues for each treatment (control and treated) according to Tukey's Honest Significant Difference test ($p < 0.05$).
ND = not detected.

Table 4.4. Translocation factor (TF) and bioconcentration factor (BCF) in whole plants, roots, leaves, stems, and flowers of the hemp (*Cannabis sativa* L.) varieties, Apricot Auto, Alpha Explorer, Von, and T1 exposed to 2.5 mg·L^{-1} Cd in a nutrient solution.

	BCF[i]					TF (%)
	Whole Plant	Root	Leaf	Stem	Flower	Whole Plant
Apricot Auto	527.5 B	446.1 B a	18.3 B b	47.6 AB b	15.5 A b	28.5 B
Alpha Explorer	1051.8 A	920.1 A a	37.9 A b	73.2 A b	20.6 A b	14.3 B
Von	213.8 BC	201.4 BC a	7.0 BC b	0.7 C b	4.8 B b	6.5 B
T1	20.9 C	6.2 C ab	3.4 C b	11.2 BC a	0.1 B b	193.0 A

[i]Values followed by the same uppercase letter(s) within a column indicate no significant differences among hemp varieties for a plant tissue according to Tukey's Honest Significant Difference test ($p < 0.05$).
Values followed by the same lowercase letter within each individual hemp variety row indicate no significant differences among plant tissues (root, leaf, stem, and flower) according to Tukey's Honest Significant Difference test ($p < 0.05$).

The TF was calculated as the ratio between Cd concentration in aboveground tissue (stems, leaves, and flowers) and Cd concentration in roots. The variety, T1, had the highest TF compared to the other three varieties (Table 4.4). Our results suggest T1 had the highest translocation factor due to increased Cd accumulation in stems, and relatively low accumulation in roots compared to other varieties. Therefore, this DLS variety may favor Cd sequestration in aboveground parts of the plant while the other three varieties accumulated more Cd in the roots. Tolerance index, which was calculated as the ratio between biomass accumulation (whole plant) in the 2.5 mg·L^{-1} treatment and biomass accumulation in the control treatment, was not significantly different among hemp varieties (data not shown).

Cadmium uptake rate was significantly greater in Alpha Explorer compared to the other three hemp varieties (Fig. 4.2). Both BCF and Cd uptake rates were the highest in Alpha Explorer, suggesting that this variety was the most efficient in taking up Cd from the solution, accumulating

it primarily in the roots but also translocating it to the shoots. However, Alpha Explorer had significantly lower whole-plant biomass than the DLS varieties, Von and T1, and biomass is considered a more accurate indicator of Cd toxicity than plant height [39]. In a typical field production scenario, DN varieties would generally have a shorter life cycle than the DLS varieties and significant differences in growing degree-day requirements for maturity in DN and DLS hemp varieties have been reported [11]. Although all plants in the present study were harvested at the same time, the increased maturation rate of the DN varieties likely led to increased rates of Cd uptake. Reduced Cd uptake rates in Von and T1 varieties could potentially allow these plants to better cope with Cd exposure.

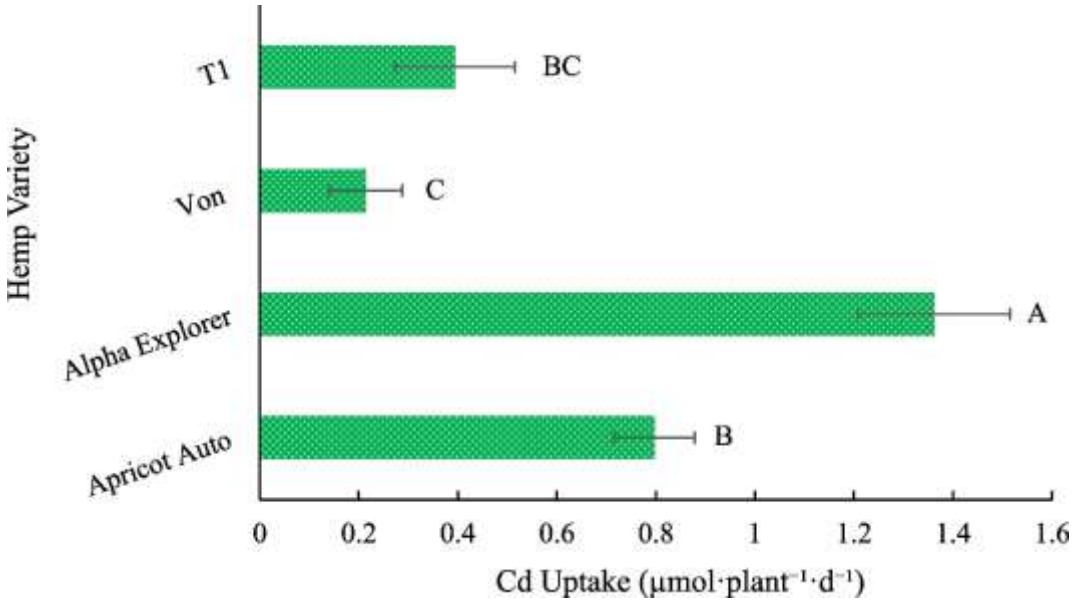


Figure 4.2. Average Cd uptake rates ± SE in four hemp (*Cannabis sativa* L.) varieties exposed to 2.5 mg·L^{-1} Cd. Bars associated with the same letter(s) indicate no significant differences among hemp varieties at $p \leq 0.05$ according to Tukey's HSD test.

Although the TF was the highest in T1, the other three hemp varieties had significant TF values within or above the range of approximately 2.5 to 12% that has been reported in previous studies [39,41], suggesting that hemp may tolerate Cd stress (Table 4.4). Furthermore, the TF of

all hemp varieties were above 1% and, therefore, these varieties could be classified as high-efficiency plants for metal translocation from roots to above ground organs [42]. Although BCF was above 100 $mg \cdot kg^{-1}$ DW in leaves, which is the minimal value for hyperaccumulator plants [14], whole plant biomass was reduced by Cd exposure. Therefore, these hemp varieties might not be candidates to be considered Cd hyperaccumulators as previously indicated [34]. Nevertheless, our data suggests that hemp characteristics related to metal uptake and distribution within plant tissues can fluctuate by variety.

Nutrient Partitioning

The concentrations of the macronutrients N, P, K, and Ca were affected by both plant variety and Cd treatment (Supplemental Table 4.2). However, no clear trends for any macronutrients were observed in response to Cd or among varieties. Previous data on nutrient distribution under metal stress is contradictory. For instance, the high-THC *C. sativa* variety "NB100" growing in uncontaminated substrate supplied with a commercial fertilizer (65, 17, 90 ppm N, P, and K, respectively) had higher N, P, and K concentrations in flower tissue, when compared to inflorescence leaves, fan leaves, and stems (roots were not analyzed), while Ca concentration was higher in fan leaves [43]. Conversely, there was a significant reduction in N, P, K, and Ca content in edible parts of tomato and lettuce grown in spiked soil containing 2.5 $mg \cdot kg^{-1}$ Cd, when compared to plants grown in non-contaminated soil [44]. Furthermore, chickpea plants (*Cicer arietinum* L.) exposed to Cd in nutrient solution (approximately 68 $mg \cdot L^{-1}$ Cd) showed a significant decrease in root and shoot Ca concentrations when compared to plants growing in uncontaminated nutrient solution [45]. Nitrogen, P, and K concentrations in the shoots of *Pfaffia glomerata* were reported to increase with increasing Cd concentrations in the growing media [46]. These macronutrients are involved in the synthesis of Cd-detoxifying chelator molecules, such as

glutathione and phytochelatins, and in the increase in activity of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidade (APX) [46,47].

Total THC and CBD in Hemp Flower

Total CBD concentrations were significantly lower in Apricot Auto and Alpha Explorer plants exposed to 2.5 $mg \cdot L^{-1}$ Cd compared to the 0 $mg \cdot L^{-1}$ Cd treatment, while total CBD concentrations in T1 and Von plants were not significantly affected by Cd exposure (Table 4.5). Similarly, total THC was significantly lower (below the detection limit) in Apricot Auto and Alpha Explorer plants exposed to 2.5 $mg \cdot L^{-1}$ Cd, compared to the control. Interestingly, THC concentrations in the DLS varieties, Von and T1, were not significantly affected by Cd exposure. Both DN varieties exhibited a significant reduction in total THC and CBD when exposed to Cd, while the DLS varieties did not. In addition, DN varieties had significantly greater Cd uptake rates (Fig. 4.2) and BCF (Supplemental Fig. 4.2) than DLS plants. Further, both DN varieties had significantly greater Cd accumulation in the floral tissue compared to the DLS varieties (Table 4.3). This suggests that the increased Cd uptake and accumulation in the floral tissue in Apricot Auto and Alpha Explorer may have led to a decrease in cannabinoid synthesis. A previous study [28] reported no differences in THC content in leaves of the DLS fiber hemp variety "Fibranova" grown in substrate contaminated with 25 and 100 $\mu g \cdot g^{-1}$ Cd. Previous results suggest that plant genetics might play a role in cannabinoid synthesis under metal stress [28,48].

Table 4.5. Average total CBD and THC ± SE in the hemp (*Cannabis sativa* L.) varieties Apricot Auto, Alpha Explorer, Von, and T1 exposed to 0 (control) and 2.5 (treated) $mg \cdot L^{-1}$ Cd in a nutrient solution.

	Concentration in flowers (% dw)[i]			
	Total THC		Total CBD	
	Control	Treated	Control	Treated
Apricot Auto	0.53 ± 0.05 A a	ND B b	9.18 ± 0.69 A a	1.82 ± 0.86 B b
Alpha Explorer	0.36 ± 0.01 A a	ND B b	8.37 ± 0.30 A a	1.18 ± 0.84 B b

Von	0.53 ± 0.07 A a	0.43 ± 0.04 A a	11.38 ± 1.29 A a	9.44 ± 1.31 A a
T1	0.43 ± 0.04 A a	0.36 ± 0.05 A a	10.19 ± 0.90 A a	7.64 ± 1.04 A a

[i]Values followed by the same uppercase letter(s) within a column indicate no significant differences among hemp varieties according to Tukey's Honest Significant Difference test ($p < 0.05$).
Values followed by the same lowercase letter within a hemp variety row indicate no significant differences between control and treated plants according to Student's t test ($p < 0.05$).
ND = not detected.

While total THC concentrations in all hemp varieties in the control treatment reached concentrations above the legal threshold (0.3%), this is not uncommon in both DLS and DN high CBD hemp varieties [11,49] as age, genetics, and environmental factors may impact cannabinoid synthesis [50]. These results indicate that the impact of Cd stress on CBD and THC synthesis is variety dependent, and exposure to 2.5 $mg \cdot L^{-1}$ Cd may affect cannabinoid synthesis in some varieties of *C. sativa*.

Conclusions

The impact of Cd on plant growth as well as BCF, uptake rate, and TF were affected by variety. Whole plant biomass yield in all four varieties were significantly reduced by the Cd treatment, suggesting that the hemp studied here may not be classified as hyperaccumulator as they may accumulate Cd with other ions in a nutrient solution until it becomes toxic. While Cd concentration was significantly higher in roots, all four varieties were efficient in translocating Cd from roots to shoots, with Cd concentrations in flowers ranging from 0.2 to 51 $mg \cdot kg^{-1}$ Cd DW in T1 and Alpha Explorer varieties, respectively. Flower and leaf biomass were significantly reduced in all four varieties in response to Cd. Further, the DN varieties, Alpha Explorer, and Apricot Auto, had a significant decrease in total THC and CBD concentrations in plants exposed to Cd when compared to plants in the control treatment, while the DLS varieties did not. Additional studies are warranted to determine if there are different Cd tolerance mechanisms in DN compared to DLS

hemp varieties. All four hemp varieties analyzed on this study are suitable for the medicinal market, given that heavy metal testing is conducted throughout production and on finished consumer products.

Supplementary Materials

Supplemental Table 4.1. Mean concentrations of cadmium (Cd) in nutrient solutions measured at the beginning and end of each cycle. Values are averages ± SE of four replications per treatment.

			$mg \cdot L^{-1}$ Cd	
Days after treatment	Variety	Treatment	Cd initial	Cd final
0-21	Apricot	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	2.46 ± 0.13	0.66 ± 0.12
	Alpha	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	2.50 ± 0.19	0.79 ± 0.14
	Von	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	2.51 ± 0.20	0.38 ± 0.18
	T1	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	2.48 ± 0.24	0.40 ± 0.16
21-42	Apricot	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.66 ± 0.12	0.51 ± 0.12
	Alpha	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.77 ± 0.13	0.50 ± 0.15
	Von	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.39 ± 0.20	0.39 ± 0.22
	T1	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.38 ± 0.15	0.29 ± 0.15
42-49	Apricot	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.51 ± 0.12	0.59 ± 0.16
	Alpha	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.50 ± 0.15	0.44 ± 0.18
42-56	Von	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.40 ± 0.22	0.44 ± 0.25
	T1	0	0.00 ± 0.00	0.00 ± 0.00
		2.5	0.29 ± 0.16	0.29 ± 0.16

Supplemental Table 4.2. Average concentrations of nitrogen (N), phosphorus (P), potassium (K), and calcium (Ca) among different tissues in the hemp (*Cannabis sativa* L.) varieties, Apricot Auto, Alpha Explorer, Von, and T1 on a dry weight (dw) basis exposed to 0 (control) and 2.5 (treated) $mg \cdot L^{-1}$ Cd in a nutrient solution.

		N		P		K		Ca	
Hemp Variety		($mg \cdot g^{-1}$ dw)							
		Control	Treated	Control	Treated	Control	Treated	Control	Treated
Apricot Auto	Flower	32.3 A[i] b[ii]	58.9 A a	8.4 A b	13.6 AB a	19.4 B b	32.6 A a	22.0 B a	21.8 B a
	Root	22.7 AB b	38.4 B a	2.7 C a	4.4 C a	23.9 A a	24.6 B a	2.9 C b	4.1 C a
	Leaf	18.4 B a	22.3 B a	5.1 B b	10.3 BC a	16.0 B b	21.5 B a	42.8 A b	62.6 A a
	Stem	7.9 C b	19.4 B a	1.4 D b	20.8 A a	9.27 C a	2.4 C b	3.0 C a	4.9 C a
Alpha Explorer	Flower	34.8 A[i] b[ii]	56.3 A a	21.9 A a	34.0 A a	8.0 A b	11.6 A a	18.7 B b	25.7 B a
	Root	24.9 B b	40.4 B a	22.4 A b	27.9 AB a	3.4 B b	5.4 B a	3.7 C b	5.0 C a
	Leaf	15.2 C b	23.5 C a	20.5 A a	20.5 B a	6.2 A a	6.7 B a	49.6 A b	81.9 A a
	Stem	6.7 D b	14.7 D a	13.8 B b	25.4 AB a	1.0 B b	2.3 C a	3.8 C b	5.6 C a
Von	Flower	30.5 A[i] a[ii]	45.0 A a	5.1 A a	8.7 A a	14.8 A a	27.5 A a	11.2 B a	14.8 B a
	Root	14.1 B a	23.6 AB a	2.5 B a	3.2 AB a	12.8 AB a	16.7 A a	2.5 C a	3.3 B a
	Leaf	7.2 BC a	19.8 B a	2.2 B b	8.0 A a	8.4 B b	17.6 A a	24.1 A b	47.7 A a
	Stem	5.0 C a	14.0 B a	0.02 C a	0.05 B a	0.1 C a	0.3 B a	0.05 C a	0.1 B a
T1	Flower	36.2 A[i] a[ii]	40.0 A a	0.1 C a	0.1 B a	0.4 B a	0.5 B a	0.2 B a	0.3 B a
	Root	17.5 B b	29.1 A a	0.04 C b	0.07 B a	0.3 B a	0.3 B a	0.04 B b	0.1 B a
	Leaf	8.3 BC b	17.7 A a	3.1 A a	6.9 A a	8.8 A a	14.4 A a	43.2 A a	47.6 A a
	Stem	5.2 C a	18.1 A a	1.4 B b	2.1 B a	8.6 A b	16.8 A a	3.0 B a	4.4 B a

[i]Values followed by the same uppercase letter(s) within a column for each hemp variety indicate no significant differences among plant tissues according to Tukey's Honest Significant Difference test ($p < 0.05$).

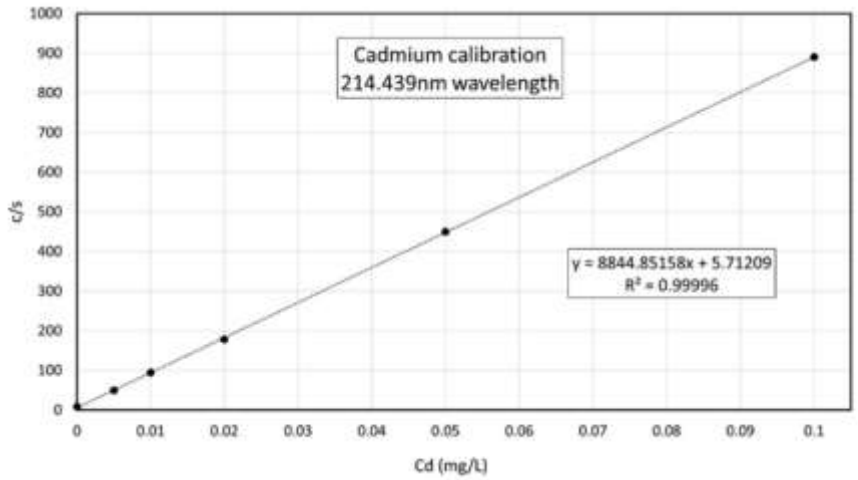
[ii]Values followed by the same lowercase letter within a Cd treatment row indicate no significant differences between control and treated plants (separate for each nutrient) according to Student's t test ($p < 0.05$).

Supplemental Table 4.3. ICP-OES parameters.

	ICP Settings	
Replicates	3	readings
Uptake delay	15	seconds
Read time	15	seconds
Pump speed	12	rpm
RF power (kW)	1.2	kW
Stabilization time	15	seconds
Viewing height	8	mm
Nebulizer flow	0.7	L/min
Plasma Flow	12	L/min
Auxiliary Flow	1.0	L/min

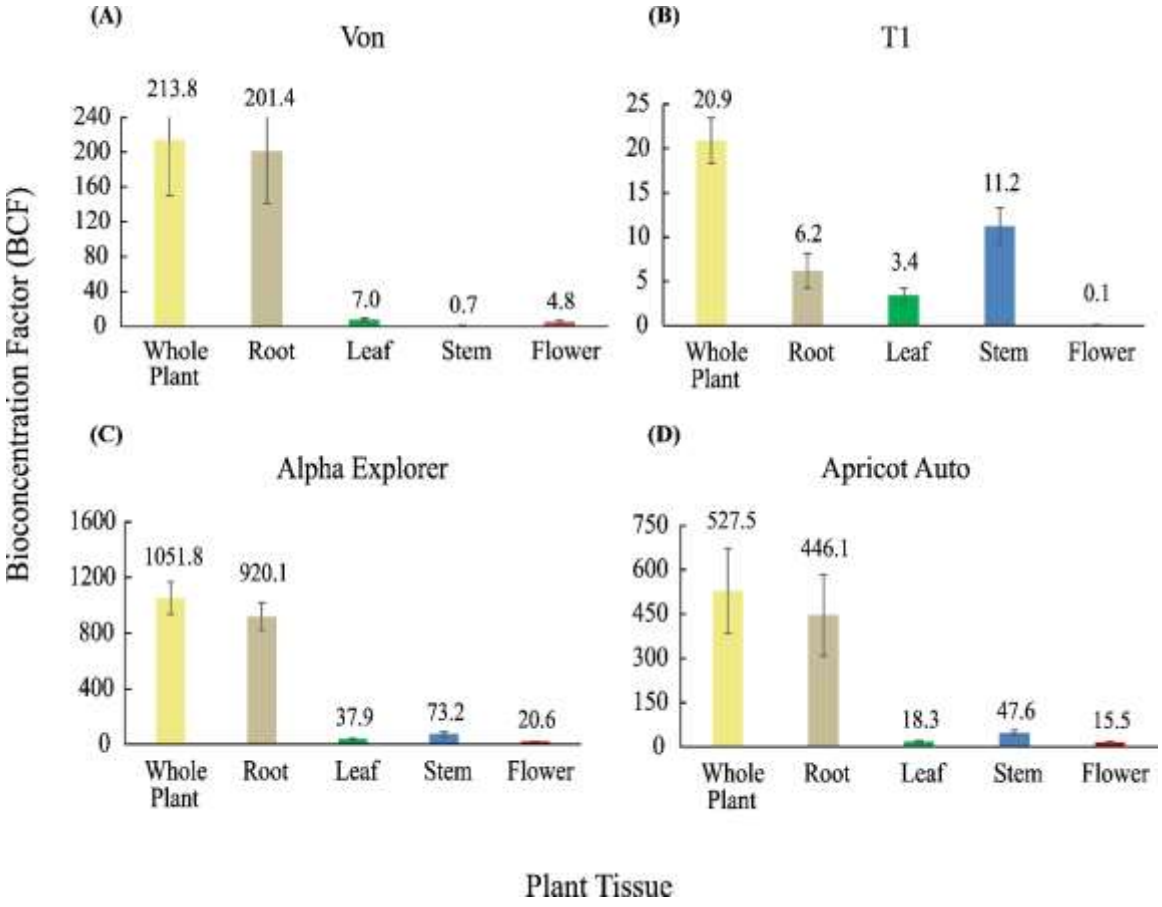
Supplemental Table 4.4. ICP-OES wavelengths used.

Element	Wavelength (nm)
Al	394.401
As	188.980
B	249.772
Ca	315.887
Cd	214.439
Cr	267.716
Cu	324.754
Fe	259.940
K	766.491
Mg	279.553
Mn	257.610
Mo	202.032
Na	589.592
Ni	231.604
P	178.222
Pb	220.353
S	180.669
Si	251.611
Zn	213.857



Cd (mg/L)	Cd 214.439 c/s
0	8.1647
0.005	49.6544
0.010	94.5087
0.020	178.5735
0.050	449.4156
0.100	890.2532

Supplemental Figure 4.1. Calibration curve of cadmium on ICP-OES.



Supplemental Figure 4.2. Bioconcentration factor (BCF) for Cd ± SE in plant tissues of four hemp (*Cannabis sativa* L.) varieties exposed to 2.5 mg·L^{-1} Cd.

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CHAPTER 5

EVALUATION OF HEMP (*CANNABIS SATIVA* L.) EXPOSED TO CADMIUM: STRUCTURAL CHANGES AND METAL ACCUMULATION AND LOCALIZATION[3]

[3] Marabesi, A. O., Sherman, S., Joshee, N., Coolong, T. W., & Lessl, J. T. To be submitted to *HortScience*.

Abstract

As anthropogenic activities have facilitated the spread of heavy metals into the environment, plants have been used for phytoremediation. Industrial hemp (*Cannabis sativa* L.) has been suggested as potentially suitable for removing contaminants from soils. One of them is cadmium (Cd), a heavy metal linked to serious health risks to humans. In addition to industrial uses, hemp has been consumed medicinally. Therefore, there is an increased interest in the ability of hemp to tolerate exposure to Cd from phytoremediation and human consumption perspectives. The objectives of this study were to assess Cd-induced alterations at the cellular level in hemp roots, assess the ability of hemp to translocate Cd from the root system to the shoot, and perform histochemical localization of Cd at the cellular level in roots and leaves. Upon staining with dithizone, Cd was primarily localized in the leaf epidermis, spongy mesophyll, and trichomes, and root rhizodermis, cortex, and pericycle in hemp plants exposed to 10 $mg \cdot L^{-1}$ Cd in a hydroponic experiment. Our results suggest that Cd is taken up by roots and translocated to the leaves, although the root is the primarily affected tissue.

Introduction

Heavy metals refer to naturally occurring elements that exhibit the properties of metals, and have relatively high atomic weights with densities greater than 5 $g \cdot cm^3$ (DalCorso, 2012; Mitra et al., 2014). Some are considered essential or beneficial for plant growth and development, including iron (Fe), cobalt (Co), copper (Cu), molybdenum (Mo), manganese (Mn), nickel (Ni), and zinc (Zn); though all are toxic to plants when present at high concentrations. Other heavy metals are not beneficial for plant growth and development and can be toxic at low concentrations, for example, arsenic (As), cadmium (Cd), chromium (Cr) mercury (Hg), and lead (Pb) (DalCorso, 2012; Mitra et al., 2014).

Anthropogenic activities have facilitated the spread of heavy metals into the environment through mining and smelting, using contaminated bio-solids, and improper disposing of industrial waste (Chaney et al., 2010). Cadmium, a heavy metal mined for extensively industrial uses, may also be a contaminant in Zn and phosphorus (P) based agricultural fertilizers (Kubier et al., 2019). When plants grown for human consumption accumulate Cd or other heavy metals, there is a potential for increased exposure to these harmful elements. Cadmium has been documented to pose serious health risks to humans, negatively affecting the function of the lungs, liver, and kidneys (Gauvin et al., 2018; Ismael et al., 2019). Multiple Cd-related and life-threatening conditions have been correlated with consuming rice (*Oryza sativa* L.) and tobacco (*Nicotiana tabacum* L.) grown in Cd-contaminated soils (Akesson & Chaney, 2019; Chaney, 2015).

Cadmium may be toxic to plants at low concentrations and visual symptoms of Cd toxicity in hemp plants have generally been described as leaf chlorosis, leaf curling, and stunted growth (Linger et al., 2005; Luyckx et al., 2021a; Marabesi et al., 2023a; Shah et al., 2019; Shi & Cai, 2009; Shi et al. 2012). Previous studies reported that Cd^{2+} cations enter root cells via associated transporters and channels, triggering different response mechanisms in plants, such as exclusion, binding of metal to the cell wall, chelation, compartmentalization, and translocation (Shah et al., 2019). Further, Cd accumulation in plant tissues may affect or hinder chlorophyll and carotenoid biosynthesis, photosynthetic efficiency, and assimilation and transport of essential nutrients while also triggering reactive oxygen species (Parmar et al., 2013; Shah et al., 2019; Singh et al., 2016; Taiz et al., 2015).

Hyperaccumulator plants can tolerate exposure to otherwise toxic levels of heavy metals by accumulating and sequestering them in roots and aboveground tissues (Greger, 1999). These plants can avoid damage caused by some heavy metals through accumulation and sequestration in

vacuoles, excretion, and exclusion (Nikalje & Suprasanna, 2018). Industrial hemp (*Cannabis sativa* L.) has been suggested as potentially suitable for phytoremediation purposes (Ahmad et al., 2015; Angelova et al., 2004; Citterio et al., 2003; Galic et al., 2019; Husain et al., 2019; Linger et al., 2002; Shi & Cai, 2009; Shi et al., 2012. In the United States (U.S.), widespread industrial hemp production was prohibited until the passage of the 2018 Agriculture Improvement Act, which allowed for widespread legal cultivation. Industrial hemp is defined as *C. sativa* with a total delta-9 tetrahydrocannabinol concentration of less than 0.3% [U.S. Department of Agriculture (USDA), 2019]. With the removal of the prohibition of hemp production in the U.S., there has been an increased interest in the ability of hemp to tolerate exposure to Cd from phytoremediation and human consumption perspectives.

The molecular and biochemical mechanisms underlying Cd tolerance in *C. sativa* have been documented (Huang et al., 2019; Luyckx et al., 2021b; Marabesi et al., 2023a; Singh et al., 2016). Nevertheless, there is a lack of information regarding Cd cellular deposition and the structural changes in plant tissues associated with Cd exposure in hemp. Previously, accelerated endodermis formation and thickening of cell walls have been reported in the roots of multiple plant species exposed to heavy metals (Shah et al., 2019). To help elucidate Cd tolerance mechanisms in hemp, the objectives of this study were to assess Cd-induced alterations at the cellular level in hemp roots, assess the ability of hemp to translocate Cd from the root system to the shoot, and perform histochemical localization of Cd at the cellular level in roots and leaves.

Materials & Methods

Hydroponic Experiment

The experiment was conducted in a greenhouse in Watkinsville, GA (33.884670, -83.419440). Cuttings from documented female plants of *C. sativa* L. ‘Purple Tiger’ (Hemp Mine,

Fair Play, SC, USA) containing at least three nodes were dipped in a commercial rooting gel (0.31% indole butyric acid; CLONEX, Growth Technology LLC., Somerset, UK) and placed into engineered foam cubes (3.33 cm L × 2.54 cm W × 3.81 cm D; Oasis Grower Solutions, Kent, OH, USA) for rooting. This cultivar was chosen due to prior research demonstrating tolerance to Cd exposure in a hydroponic system (Marabesi et al. 2023a). Foam cubes were placed in plastic trays on a heat mat set at 24 $^{\circ}$C under a mist system, which applied water approximately four-times daily for 3 min each. After three weeks, rooted cuttings were placed into plastic netted pots (4.7 cm W × 5.1 cm D) spaced equidistantly (24.3 cm apart) through the lid of 37.9 L plastic containers (Rubbermaid Inc. Wooster, OH, USA) filled with 28 L of nutrient solution containing a complete hydroponic fertilizer (5N-4.8P-21.6K, Peters Professional Hydroponic Special; ICL, St. Louis, MO, USA) and calcium nitrate (14N-0P-0K,17Ca; Calcium + Micros, General Hydroponics, Santa Rosa, CA, USA) dissolved in well water (Table 5.1). Each container supported five plants. A 15.2 cm long aquarium air stone attached to an air pump (Active Aqua; Hydrofarm, Petaluma, CA, USA) was added to each plastic container to aerate the solution throughout the experiment. Container volume was maintained by adding well water every two days, nutrient solutions were drained, and new solutions were added after two weeks. The electrical conductivity (EC) and pH of the solutions were measured twice per week. Solution pH was adjusted to 5.5 when necessary (pH down; General Hydroponics, Santa Rosa, CA, USA). Supplemental light (approximately 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was used to provide 18/6 light/dark hours for the duration of the experiment. Plants were kept in the nutrient solution for four weeks for acclimation. After four weeks of growth, plants were exposed to either 0 (control) or 10 $mg \cdot L^{-1}$ Cd by adding $3CdSO_4 \cdot 8H_2O$ to the nutrient solutions. This concentration of Cd was chosen based on prior research that indicated that the same cultivar of hemp demonstrated tolerance to 10 $mg \cdot L^{-1}$ Cd in a deep-water culture hydroponic

system (Marabesi et al, 2023a). Plants were exposed to the Cd-containing solutions for 17 days. The experiment followed a randomized complete block design containing two treatments with four replications per treatment and five plants per replication.

Table 5.1. Mineral nutrient concentrations (mg $\cdot L^{-1}$) in well water and nutrient solution.

	N	P	K	Ca	Mg	B	Cu	Mo	Fe	Mn	Zn
Well Water	ND	<0.02	2.6	10.8	2.1	<0.01	<0.05	<0.01	0.3	0.09	<0.05
Nutrient Solution[ii]	150	56.6	240.4	132.8	58.2	0.8	0.4	0.1	4.6	1.1	0.4
Total Concentration	150	56.6	243.0	143.6	60.3	0.8	0.4	0.1	4.9	1.1	0.4

ND = not detected.

[ii]Nutrient solution comprised from the following compounds: KNO_3, K_2HPO_4, $MgSO_4$, H_3BO_3, Cu-EDTA, Fe-EDTA, Mn- EDTA, Na_2MoO_4, Zn-EDTA, $Ca(NO_3)_2 \cdot NH_4NO_3 \cdot 10H_2O$, $(NH_4)_2MoO_4$, Ca-EDTA, and $Na_2B_4O_7 \cdot 10H_2O$.

Nutrient solutions were sampled at the beginning and end of the experiment using 20 mL scintillation vials (HDPE; Thermo-Fisher Scientific®, Waltham, MS, USA), and stored at 4 °C until analysis. The greenhouses temperature and relative humidity (RH) of were monitored at canopy height hourly (VP4; Meter Group Inc., Pullman WA, USA), and the averages during the experiment were 17.3 ± 5.6 °C and 78.7 ± 14.6 % RH. Photosynthetic active radiation was monitored hourly throughout the experiment (QSO-S; Meter Group Inc.), and the average daily light integral (DLI) was 19.6 ± 7.5 $mol \cdot m^{-2} \cdot d^{-1}$.

Plants started showing visual symptoms of Cd exposure at approximately 5 d after Cd treatment (DAT) was introduced in the solutions. Symptoms were similar to leaf epinasty, where leaves bend downwards, having a claw-like appearance. At 17 d after Cd treatments (DAT) were initiated, plants had visually recovered and were showing no symptoms. Root and leaf samples were collected at 5 DAT (visual symptoms initiated) and 17 DAT (plants recovered and had no

visual symptoms) and kept on moist filter papers inside sealed plastic bags to maintain turgor until further processing for scanning electron microscope (SEM) and histochemical analyses or immediately processed for mineral analysis.

Plant Growth and Biomass Yield

Plant height and leaf, stem, and root biomass were determined at harvest (17 DAT). Plant height was determined by measuring the distance from the base of the stem to the end of the apically dominant branch at harvest. To determine biomass yield, five plants per replicate were air dried at ambient temperatures inside the greenhouse for 2 weeks and then separated into roots, stems, and leaf biomass. Subsamples were further dried in a forced air oven set at 55 ˚C for 48 hours until a constant weight was achieved. The dry weights of the whole plant samples were then normalized based on subsample moisture content.

Mineral Analysis

Root and leaf material were collected at 5 DAT and 17 DAT for elemental analysis. Samples at harvest were taken prior to air drying whole plants for plant biomass quantification. Each replicate consisted of five plants combined into a composite sample and four replicates per treatment were used for elemental analysis. For roots, approximately 50 g of fresh material was collected from each replicate and triple washed with deionized water prior to drying. For leaves, ten of the youngest fully expanded leaves were collected from the top one-third of each plant (main stem and lateral branches) and rinsed with deionized water prior to drying. Plant tissue samples were placed in a forced air oven set to 55 ˚C for approximately 48 h until a constant weight was achieved. Dried plant material was ground in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) and passed through a 20-mesh screen. The samples were digested following EPA Method 3052 (USEPA, 1995): 0.5 g samples were weighed and placed in fluorocarbon polymer microwave

vessels. Then 10 mL of concentrated nitric acid was added to each vessel, sealed, placed in a microwave digester (Mars 6 Microwave; CEM Corp., Matthews, NC, USA), and heated to 200 ˚C for 30 minutes. Digests were then transferred quantitatively into volumetric flasks and brought to 100 mL volume with deionized water before analysis.

Plant tissue digestions and nutrient hydroponic solutions were analyzed for multiple elements (P, K, S, Ca, Mg, Fe, Mn, Al, B, Cu, Zn, Ni, and Cd) following EPA Method 200.8 (Creed et al., 1994) by Inductively Coupled Plasma - Optical Emission Spectroscopy (Spectro Arcos FHS16; Spectro Amertek USA, Wilmington, MA, USA). Samples of the hydroponic solutions were filtered using a 0.45 µM PTFE membrane and acidified using 2% high-purity nitric acid (HNO_3) prior to analysis. The instrument detection limit for Cd was 0.005 $mg \cdot L^{-1}$. Calibration standards utilized in this analysis were from a certified source (Inorganic Ventures, Christiansburg, VA, lot number: N2-MEB667614). Independent laboratory performance checks were also run with acceptable deviation for recovery set at 100 ± 5.0%.

SEM Analysis

The SEM and histochemical analyses were conducted at Fort Valley State University (Fort Valley, GA). An SEM (Phenom XL, Thermo-Fisher Scientific®) equipped with energy dispersive spectroscopy (EDS) at 15kV provided a quantitative analysis of Cd and other elements present in the roots and leaves of hemp. For sample preparation, roots and leaves from both treatments were dried in the Incushaker™ 10L (Benchmark, Tempe AZ, USA) overnight at 35˚ C, broken into pieces to fit the aluminum specimen mounting stubs (Ted Pella Inc., Redding CA, USA), mounted with a double-sided adhesive tape, and dusted with compressed air before being placed in the SEM. Observations made with the SEM also allowed to assess Cd-induced alterations in hemp roots and leaves at the cellular level.

Histochemical Analysis

Fresh leaf and root samples were fixed in 70% methanol for use in the cryostat (HM525, NX cryostat, Thermo-Fisher Scientific®) and stored at 4 ˚C. For sectioning with the cryostat, fixed samples were embedded in cryomatix cryoembedding compound (Thermo-Fisher Scientific®) and immediately frozen using the peltzer freezing unit in the cryostat. Sections were cut at a thickness of 12 µm and adhered to positively charged microscope slides. These slides were left to dry for 30 minutes and then stained for 20 minutes using diphenylthiocarbazone (dithizone) (Sigma Aldrich®, St. Louis, MO, USA) solution following the protocol developed by Seregin and Ivanov (1997).

Fresh leaf and root samples were fixed in HistoChoice Clearing Agent (Sigma Aldrich®) for microtomy and stored at 4°C. In the paraffin method, samples were submitted to an alcohol series with isopropanol alcohol for dehydration of samples, followed by embedding with paraffin wax Type 9 (Thermo-Fisher Scientific®) in a vacuum oven (VT6025; Thermo Scientific®) set to 65˚C. Samples were sectioned at three thickness (12, 15, and 20 µm) performed with a microtome, and left to dry overnight on a slide warmer at 37 ˚C. Next, slides were deparrafinized in xylene for 5 min to remove the paraffin prior to staining. For staining, we utilized a diphenylthiocarbazone (dithizone) (Sigma Aldrich®) solution following the methodology of Seregin and Ivanov (1997). In brief, 30 mg diphenylthiocarbazone was dissolved in 60 mL of 100% acetone and then 20 mL deionized water was added (3:1 mixture of acetone and deionized water). To improve the sensitivity of reaction 1-2 drops of acetic acid was added to 6 mL of the solution. Presence of Cd or lead (Pb) is indicated by the formation of red insoluble precipitate when stained with dithizone (Seregin and Ivanov, 1997). Sections on the slides were stained for 20 min with dithizone solution, washed with deionized water, and observed with a light microscope (Olympus BX 43, Olympus, USA). Image analysis was conducted using Fiji v. 2.11.0 (Schindelin et al., 2012) following the

National Institutes of Health (Bethesda, MD, USA) methodology for stained sections. The scale bar was not deleted from the images and was accounted as part of the stained area for all images due to the color threshold methodology employed.

Statistical Analysis

Experiments followed a complete randomized block design. Statistical analysis was conducted using JMP© Pro 15 (SAS, Cary NC, USA). Data were subjected to Student's t-test ($p \leq 0.05$) to determine the level of significance between means.

Results & Discussion

Plant Growth and Biomass Yield

At 17 DAT, plants exposed to 10 $mg \cdot L^{-1}$ Cd did not show visual toxicity symptoms and were not visually distinguishable from plants grown in the 0 $mg \cdot L^{-1}$ Cd control treatment. There were no differences in plant height and dry weight of leaves, stems, and roots between treatments, emphasizing the short-term tolerance of *C. sativa* 'Purple Tiger' to up to 10 $mg \cdot L^{-1}$ Cd in solution (Marabesi et al., 2023a). Nevertheless, these results are in contrast with previous studies that have reported a decrease in the shoot biomass of hemp plants exposed to Cd (Angelova et al., 2004; Luyckx et al., 2021a, Marabesi et al., 2023b; Shi and Cai, 2009).

Mineral and SEM Analysis

Mineral analysis did not detect Cd in the roots or leaves of plants in the control treatment at either sampling time (Table 5.2). Cadmium concentrations were greater in the roots at 5 DAT (628.6 $mg \cdot L^{-1}$) and 17 DAT (1448.0 $mg \cdot L^{-1}$) compared to leaves at 5 DAT (24.8 $mg \cdot L^{-1}$) and 17 DAT (23.2 $mg \cdot L^{-1}$). The Cd concentration was approximately 25 times greater in roots at 5 DAT, and about 62 times higher at 17 DAT compared to leaves. Hemp has previously been reported to preferentially accumulate Cd in roots (Angelova et al. 2004; Cacic et al., 2019; Marabesi et al.,

2023a; Marabesi et al., 2023b Shi et al., 2012). Cadmium concentrations in leaves were not different between the two time points, despite continued plant growth during Cd exposure. This suggests that plants could still uptake and translocate Cd to the leaves at a relatively constant rate.

Table 5.2. Average cadmium (Cd) ± SE accumulation in hemp (*Cannabis sativa* L.) 'Purple Tiger' plant tissue on a dry weight (dw) basis.

	Cd in Roots ($mg \cdot kg^{-1}$ dw)		Cd in Leaves ($mg \cdot kg^{-1}$ dw)	
Treatment	5 DAT	17 DAT	5 DAT	17 DAT
0 $mg \cdot L^{-1}$ Cd	ND	ND	ND	ND
10 $mg \cdot L^{-1}$ Cd	628.6 ± 54.1 B[i]	1448.0 ± 148.5 A	24.8 ± 6.0 A	23.2 ± 2.1 A

[i]Values followed by the same uppercase letter(s) indicate no significant differences between 5 DAT and 17 DAT time points for each plant tissue according to Student's t-test ($p > 0.05$).

ND = not detected

Despite an absence of visual toxicity symptoms, analysis of hemp roots at 5 DAT using SEM, indicated damage and deformity to the root surface (Fig. 5.1A) upon exposure to 10 $mg \cdot L^{-1}$ Cd. In contrast, the root surface of untreated plants remained visually intact (Fig. 5.1B). Analysis using SEM at 17 DAT suggests that while damage to the root surface was visible, the differences between roots in the Cd exposed plants (Fig. 5.1C) and those in the control (Fig. 5.1D) were less than those observed at 5 DAT. In prior research, hemp grown with 10 $mg \cdot L^{-1}$ Cd, showed mild toxicity symptoms for several days, but recovered within a few weeks after exposure (Marabesi et al. 2023a; Marabesi et al., 2023b), suggesting that hemp may be able to recover from some concentrations of Cd exposure. While hemp in the present study showed no visual symptoms of Cd toxicity, analysis using SEM indicates damage at the cellular level at 5 DAT that seemed to be

reduced at 17 DAT. No visual differences were observed on the surface of hemp leaves under control and 10 mg·L^{-1} Cd treatment (images not shown). The elemental analysis detected Cd in the treated root tissue (1.13% weight concentration), showing a clear peak for Cd, which was non-existent in the control samples (data not shown).

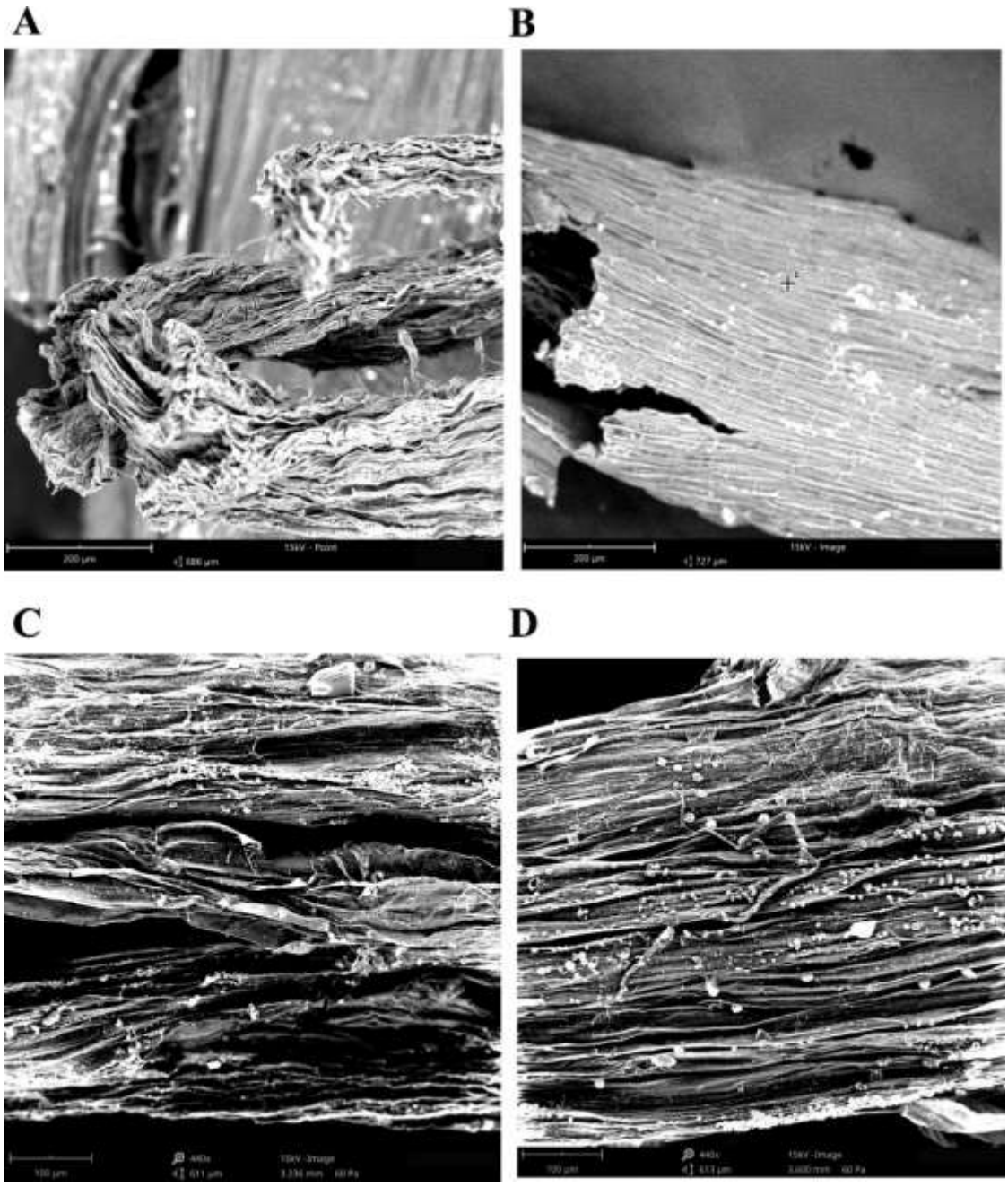


Figure 5.1. Surface of hemp root exposed to 10 mg·L^{-1} Cd (A) and 0 mg·L^{-1} Cd (B) at 5 DAT. Surface of hemp root exposed to 10 mg·L^{-1} Cd (C) and 0 mg·L^{-1} Cd (D) at 17 DAT.

Cadmium Localization in Hemp Leaves and Roots

In control leaf samples, reactions with dithizone were negative (Fig. 5.2A, B); however, leaf cross sections of plants exposed to 10 $mg \cdot L^{-1}$ Cd at 5 DAT showed a positive reaction with dithizone (Fig. 5.2C, D). The bright red color in the leaf epidermis, spongy mesophyll, and trichomes suggest that the reaction was more intense in those regions, indicating Cd localization. Although there was a positive reaction with dithizone on treated plants, there was no apparent cell damage or anatomical alterations in the leaf tissue compared to untreated plants. The lack of cell damage in leaf tissue was in agreement to a lack of visual differences between leaf surfaces of treated and non-treated plants when using SEM. As noted previously, concentrations of Cd in the leaf tissue were lower than those found in the roots (Table 5.2). The same reaction pattern was observed in leaf cross sections of plants exposed to 10 $mg \cdot L^{-1}$ Cd at 17 DAT (Fig. 5.3A–D).

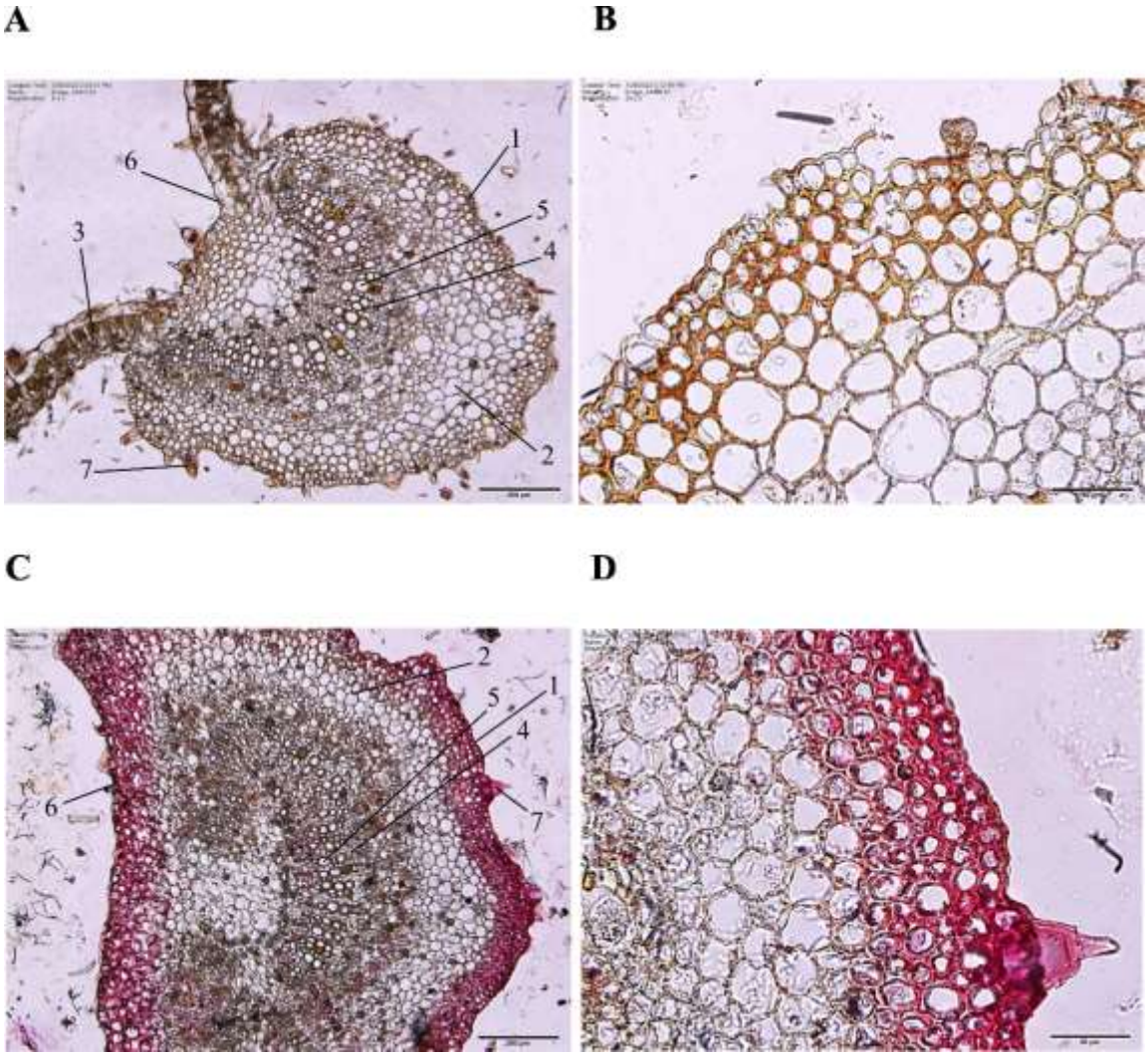


Figure 5.2. Cross sections of leaves exposed to 0 mg·L^{-1} Cd (A, B) and 10 mg·L^{-1} Cd (C, D) at 5 DAT. Images A and C were taken with a 10x objective while images B and D were taken with a 40x objective. (1) Lower epidermis; (2) Spongy mesophyll; (3) Palisade mesophyll; (4) Phloem; (5) Xylem; (6) Upper Epidermis; (7) Trichome.

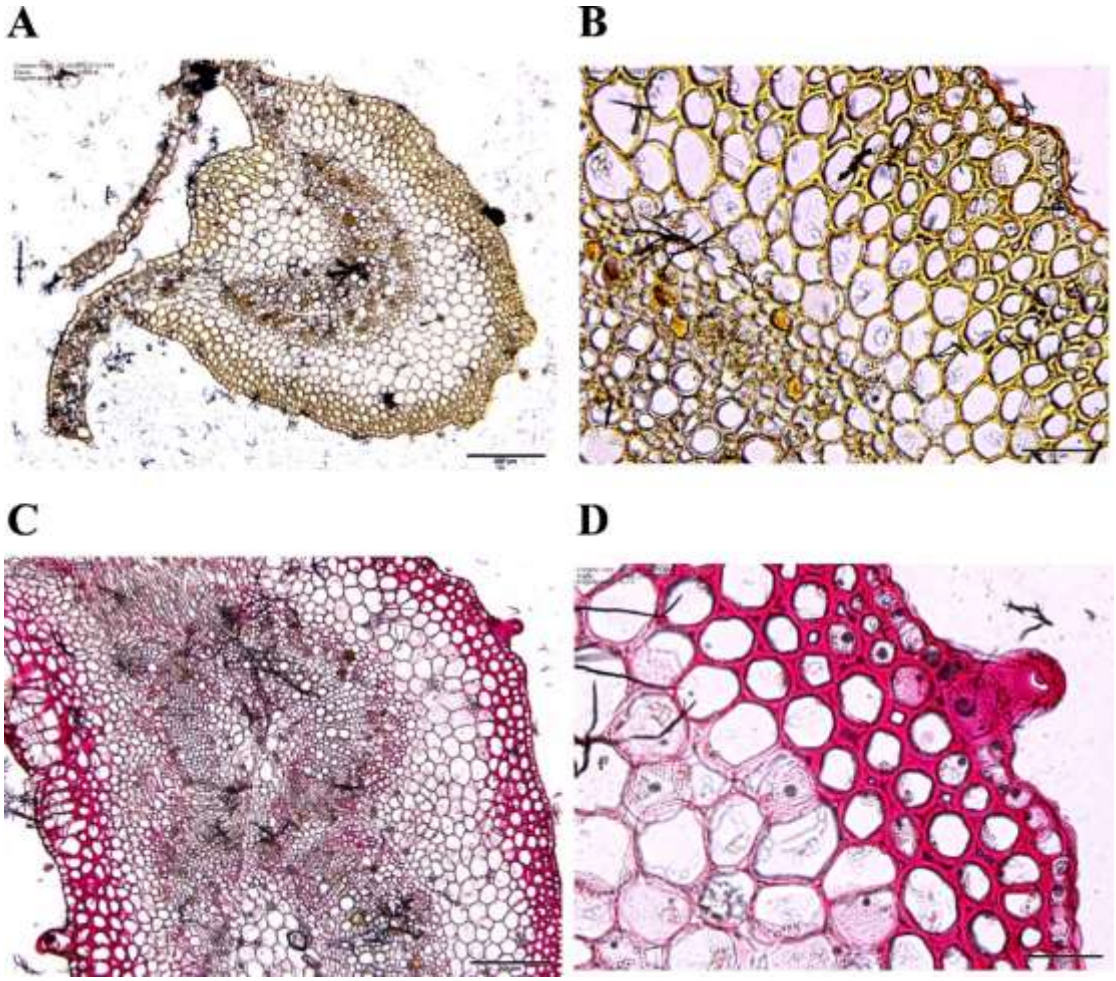


Figure 5.3. Cross sections of leaves exposed to 0 mg·L^{-1} Cd (A, B) and 10 mg·L^{-1} Cd (C, D) at 17 DAT. Images A and C were taken with a 10x objective while images B and D were taken with a 40x objective.

Image analysis of stained leaf sections showed an increase in the percent stained area in plants exposed to 10 mg·L^{-1} Cd. At 5 DAT, the percent areas stained on the 0 mg·L^{-1} Cd control samples were 0.33% and 0.38% compared to 4.31% and 4.0% for the Cd treated samples, at 100x and 400x magnifications, respectively (Supplemental Fig. 5.1A–D). In 17 DAT samples, the percent areas stained on the control samples were 0.99 and 2.52% compared to 10.51 and 17.96% on the Cd treated sample, at 100x and 400x magnifications, respectively (Supplemental Fig. 5.2A–D).

Similar to leaf tissue, roots of plants exposed to 0 $mg \cdot L^{-1}$ Cd showed minimal staining after exposure to dithizone (Fig. 5.4A, B). In roots of plants exposed to 10 $mg \cdot L^{-1}$ Cd at 5 DAT, the reaction with dithizone was notable throughout the cortex, endodermis, and pericycle (Fig. 5.4C, D). The damage to the cortex and rhizodermis of both control and treated plants (Fig. 5.4A, C), was possibly due to the sectioning and slide preparation processes. A similar reaction pattern to dithizone was also observed in leaf cross sections of plants exposed to 10 $mg \cdot L^{-1}$ Cd for 17 days (Fig. 5.5A–D). In several plant species, the root cell wall can immobilize heavy metal ions due to the presence of pectic sites, hystidyl groups, and extracellular callose and mucilage, therefore preventing the uptake of harmful metals into the cytosol (Jan and Parray, 2016). Although the techniques utilized in this study did not allow for the localization of Cd inside plant cells, it is possible to see Cd deposition in the intercellular spaces (Fig. 5.4C, D), suggesting that *C. sativa* cell wall can partially retain Cd in the intercellular spaces.

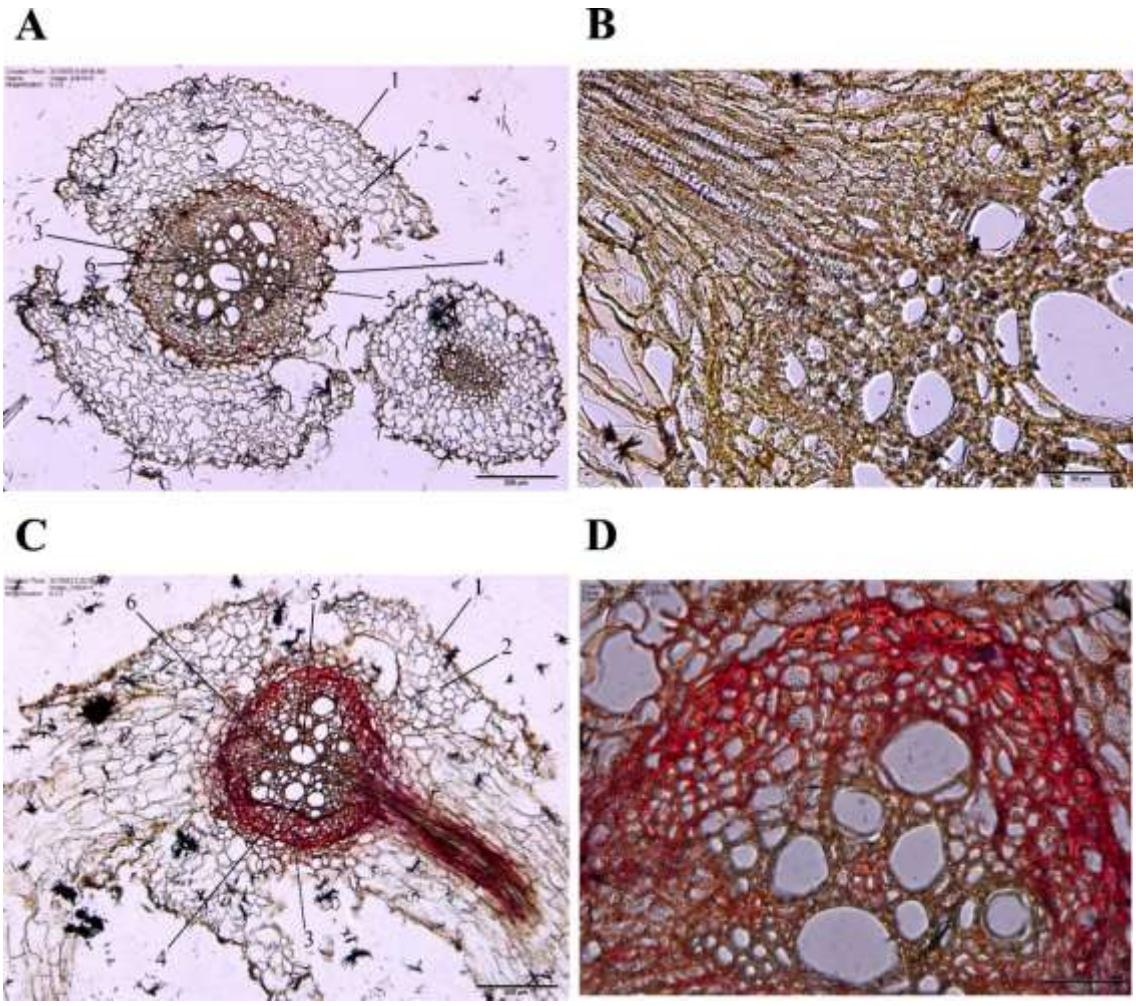


Figure 5.4. Cross sections of roots exposed to 0 mg·L^{-1} Cd (A, B) and 10 mg·L^{-1} Cd (C, D) at 5 DAT. Images A and C were taken with a 10x objective, while images B and D were taken with a 40x objective. (1) Rhizodermis; (2) Cortex; (3) Endodermis; (4) Pericycle; (5) Xylem; (6) Phloem.

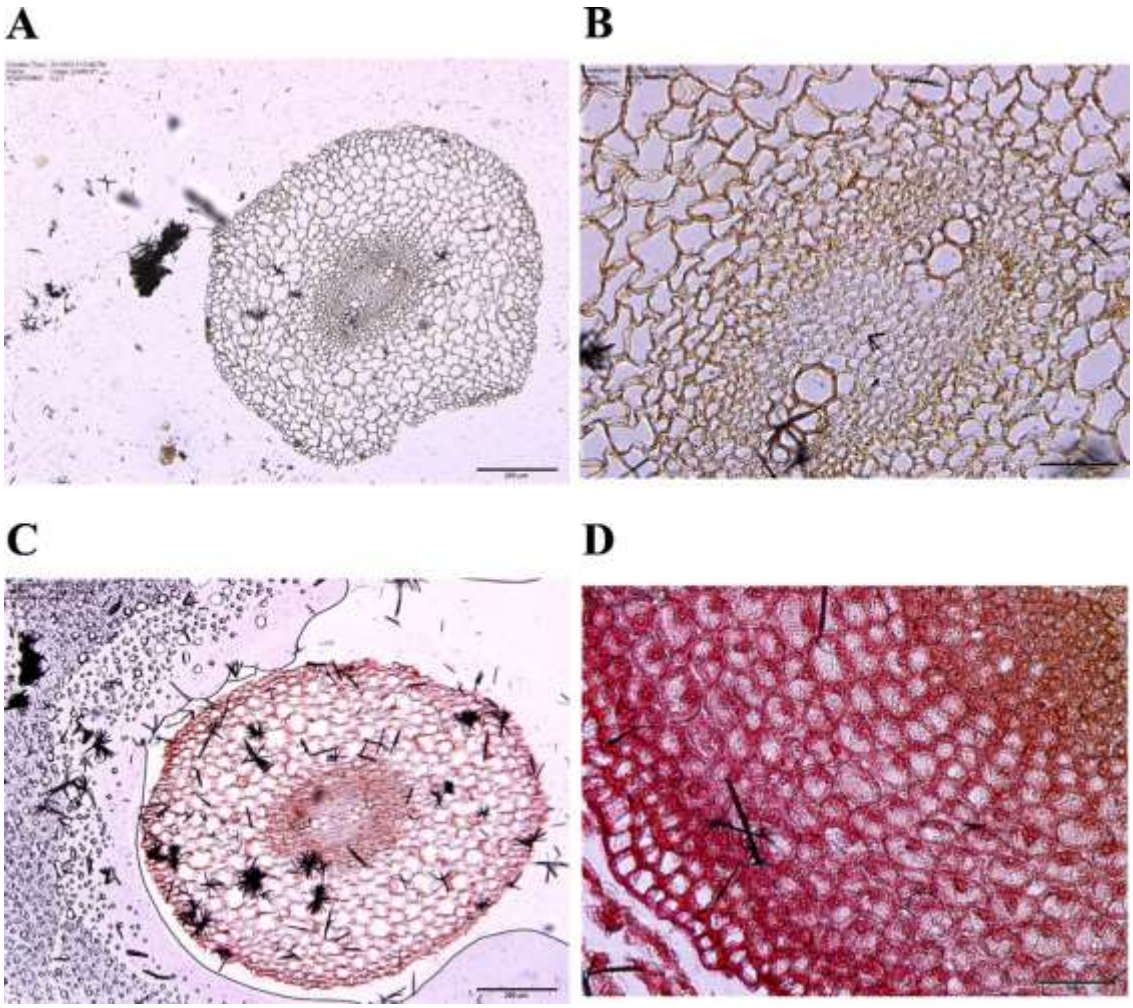


Figure 5.5. Cross sections of roots exposed to 0 mg·L^{-1} Cd (A, B) and 10 mg·L^{-1} Cd (C, D) at 17 DAT. Images A and C were taken with a 10x objective, while images B and D were taken with a 40x objective.

At 5 DAT, the percent area stained on the control samples was 1.32% and 1.19 % compared to 4.50% and 12.81 % on the Cd treated samples, at 100x and 400x magnifications, respectively (Supplemental Fig. 5.3A, B, C, D). In recovered samples, the percent area stained on the control samples was 1.74 and 0.58 % compared to 2.91 and 6.35 % on the Cd treated samples, at 100x and 400x magnifications, respectively (Supplemental Fig. 5.4A, B, C, D). Despite not accumulating any detectable levels of Cd in roots or leaf tissue, there was a small area of control samples that

displayed staining; this was due to certain portions of tissue where excess dye residues were unable to be completely washed from slides (Fig. 5.4A).

Plant roots take up Cd via metal transporters and primarily accumulates in the root system, with smaller amounts being translocated to the shoot (Husain et al., 2019; Ismael et al., 2019; Shi et al., 2012). Indeed, Shi et al. (2012) have described hemp as an excluder rather than a hyperaccumulator. In the present study, plants exposed to 10 $mg \cdot L^{-1}$ Cd in hydroponic solutions could uptake Cd from the solution and translocate it to the leaves, where it was deposited on the leaf epidermis, spongy mesophyll, and trichomes. In the present study, plants exposed to 10 $mg \cdot L^{-1}$ Cd in hydroponic solutions could uptake Cd from the solution and translocate it to the leaves, where it was deposited on the leaf epidermis, spongy mesophyll, and trichomes. In fact, transcript abundance of the heavy metal associated transporter genes *CsHMA1*, *CsHMA3*, *CsHMA4*, and *CsHMA5* were upregulated in the roots of the hemp variety Purple Tiger exposed to 10 $mg \cdot L^{-1}$ Cd in a hydroponic solution, suggesting their role in Cd uptake and sequestration in roots (Marabesi et al., 2023a).

Previously, Pielichowska and Wierzbicka (2004) called attention to the role of leaf hairs in Cd detoxification. The authors claimed that *Biscutella laeviagata* (a Cd hyperaccumulator species) had leaf hairs that could accumulate and eliminate Cd ions, although this mechanism was not described in detail. Similarly, Balestri et al. (2014) reported that trichomes of *Pteris vittata*, a perennial fern used on phytoremediation of sites contaminated with As, could be a site for Cd accumulation, therefore, protecting the plant against biotic stresses. Trichomes on hemp leaves may execute similar functions, however, further research is necessary to validate this hypothesis. Additionally, Balestri et al. (2014) reported that Cd had a negative effect on mitotic activity in the root apical meristem of *Pteris vittata*, as they observed a reduction in root size of plants exposed

to up to 11.2 mg·L^{-1} Cd (100 μM $CdCl_2$). The authors suggested this could be a defense mechanism to avoid Cd toxicity in the root meristem.

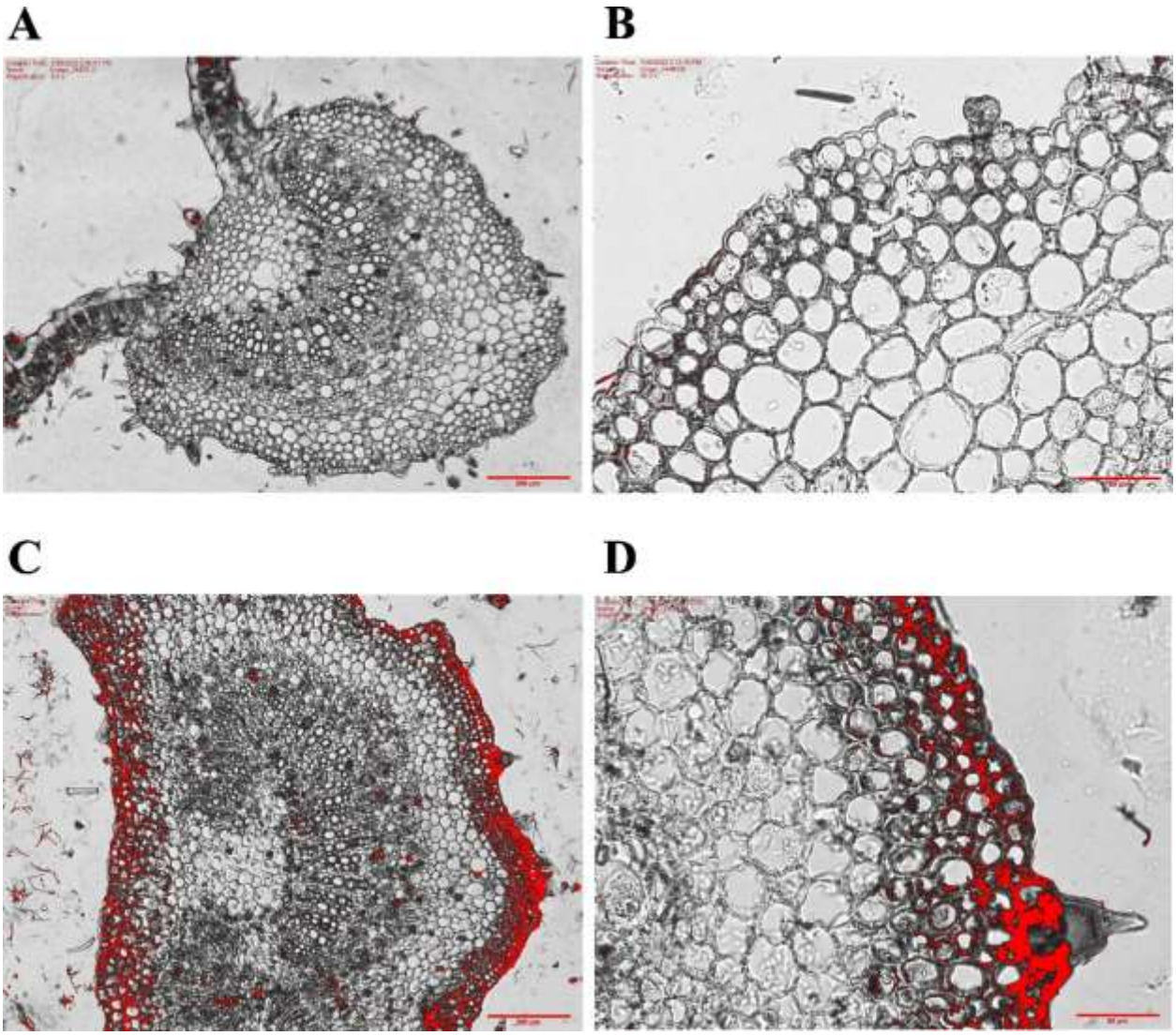
In *C. sativa*, there is a lack of research on Cd localization in plant tissues. Arru et al. (2004) conducted a study to localize Cu in hemp leaves. They suggested that Cu was mainly accumulated in the upper epidermis although also found in non-glandular hairs and abaxial trichomes, which is consistent with our results. We could also localize Cd in the roots of Cd treated plants, mainly throughout the cortex, endodermis, and pericycle. Extensive damage to the root surface was observed through SEM analysis. These results support data from Yang et al. (2015), who have found Cd to be localized mainly in the root cortex of *Salix matsudana* Koidz. seedlings exposed to 5.6 mg·L^{-1} Cd (50 μM $CdCl_2$) for 10 hours; and from Seregin and Ivanov (1997), who have observed an intense dithizone reaction on the rhizodermis, cortex, and endodermis in the roots of maize seedlings. Seregin and Ivanov (1997) reported damage to the rhizodermis and inner cortical cell at 120 hours of incubation with 100 μM of $Cd(NO_3)_2$. Interestingly, our study's Cd concentration of 10 mg·L^{-1} Cd was not lethal to well-developed hemp plants. Nevertheless, plant genetics were reported to influence Cd tolerance in hemp as different varieties had distinct responses to Cd exposure (Marabesi et al., 2023b).

Conclusions

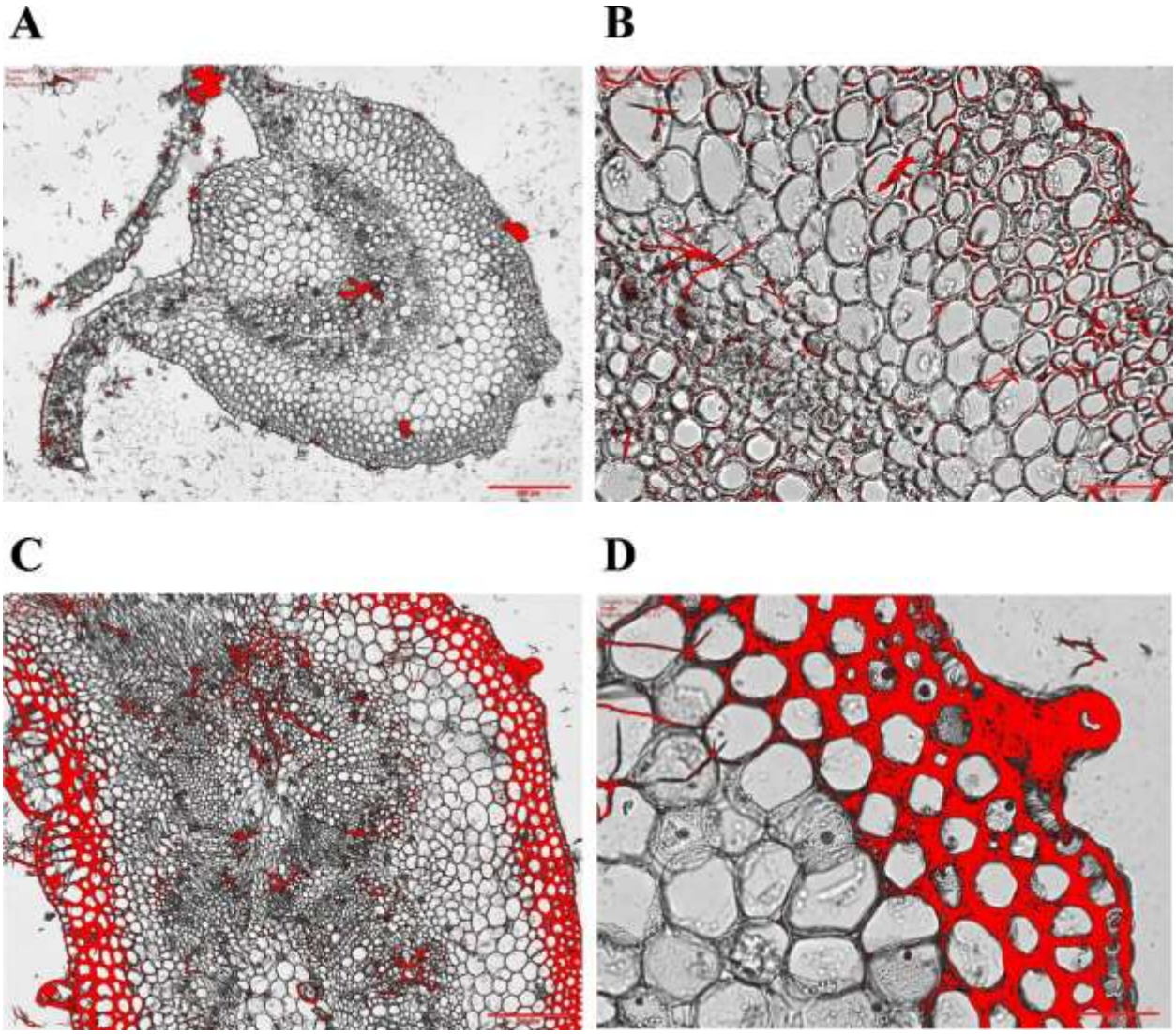
Cadmium was primarily localized in leaf epidermis, spongy mesophyll, and trichomes, and root rhizodermis, cortex, and pericycle in hemp plants exposed to 10 mg·L^{-1} Cd. Our results suggest that Cd is taken up by roots and translocated to aboveground tissues, although the root is the primarily affected tissue, with notable damage to the root surface at 5 DAT. Further, upon staining with dithizone, we could distinguish between Cd treated and untreated plants in both leaf and root tissue. Additionally, as visual toxicity symptoms lessened and Cd concentration in roots

increased over time, the accumulation mechanism employed by hemp might play a role in plant tolerance to Cd.

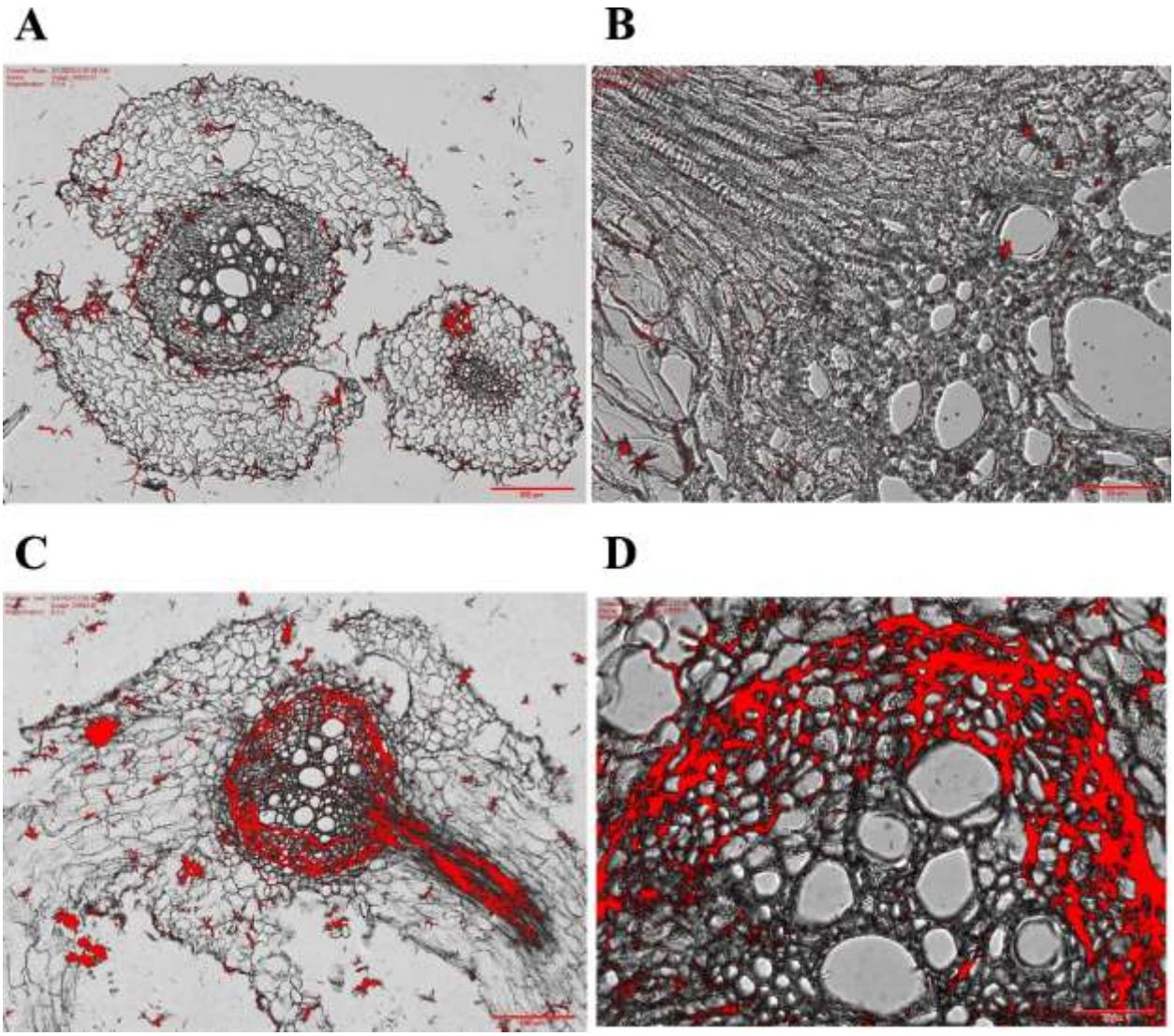
Supplementary Materials



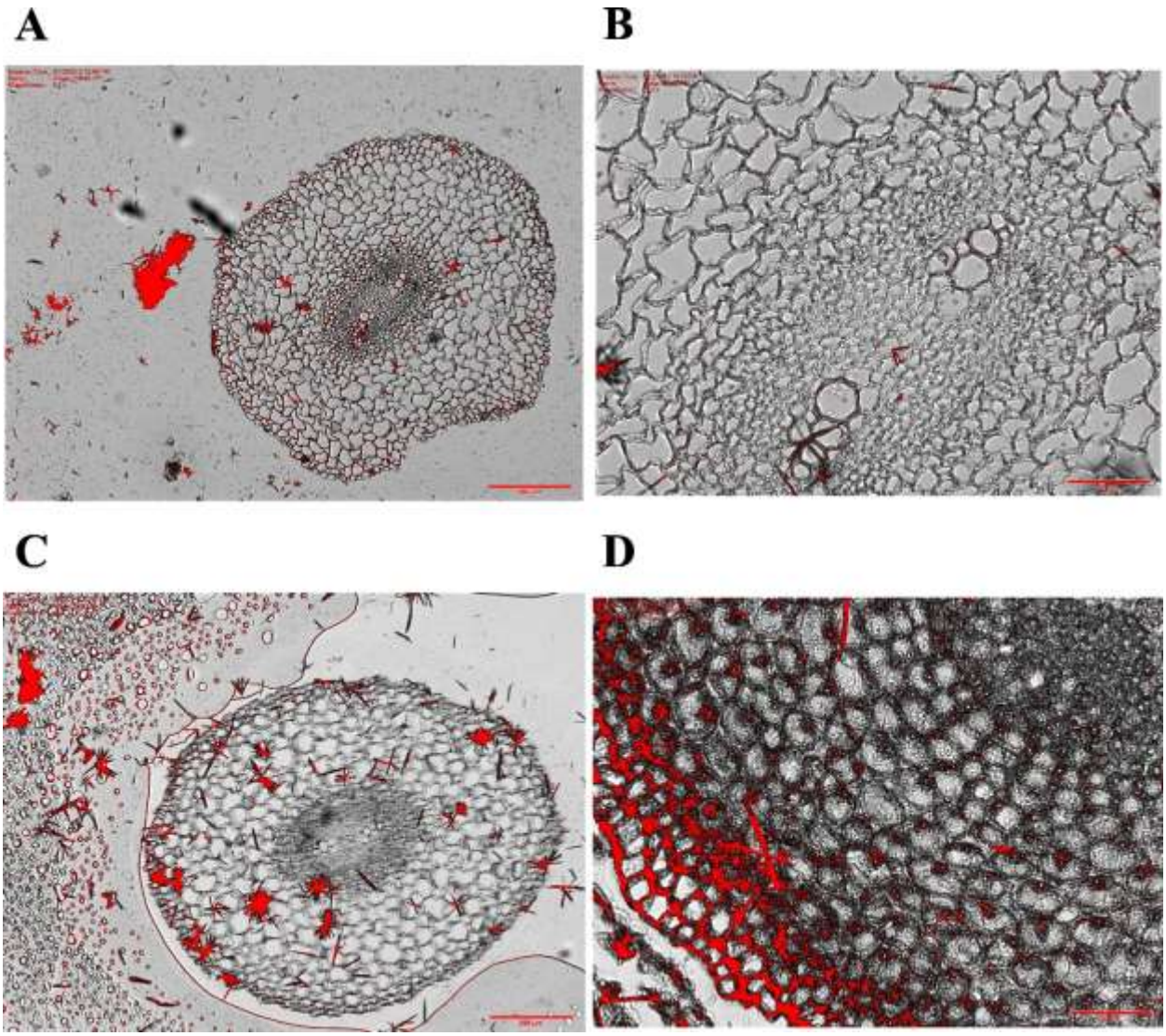
Supplemental Figure 5.1. Image analysis of leaf cross sections of plants exposed to 0 $mg \cdot L^{-1}$ Cd (A, B) and 10 $mg \cdot L^{-1}$ Cd (C, D) for 5 days. Images A and C were taken with a 10x objective while images B and D were taken with a 40x objective.



Supplemental Figure 5.2. Image analysis of leaf cross sections of plants exposed to 0 $mg \cdot L^{-1}$ Cd (A, B) and 10 $mg \cdot L^{-1}$ Cd (C, D) for 17 days. Images A and C were taken with a 10x objective while images B and D were taken with a 40x objective.



Supplemental Figure 5.3. Image analysis of root cross sections of plants exposed to 0 mg·L^{-1} Cd (A, B) and 10 mg·L^{-1} Cd (C, D) for 5 days. Images A and C were taken with a 10x objective while images B and D were taken with a 40x objective.



Supplemental Figure 5.4. Image analysis of root cross sections of plants exposed to 0 $mg \cdot L^{-1}$ Cd (A, B) and 10 $mg \cdot L^{-1}$ Cd (C, D) for 17 days. Images A and C were taken with a 10x objective while images B and D were taken with a 40x objective.

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CHAPTER 6

CONCLUSIONS

Hemp has agronomic traits that could facilitate the accumulation of heavy metals such as a large and deep root system, rapid life cycle, and high biomass production. As a result, fiber varieties have been reported as potential candidates for phytoremediation. Nevertheless, companies producing hemp for floral material and subsequent cannabinoid extraction have expressed an interest in determining typical levels of heavy metals in hemp flowers due to the potential harm of heavy metals to human health. There is a need for research on heavy metal accumulation in industrial hemp grown for the medicinal market. Therefore, in the present dissertation, we aimed to evaluate the potential for heavy metal accumulation for industrial hemp varieties grown for cannabinoid production.

Our first study evaluated the potential for cadmium uptake and its impact on growth, physiological responses, and transcript expression of metal transporter genes in a hemp variety grown for flower production. The cultivar 'Purple Tiger' was exposed to 0, 2.5, 10, and 25 $mg \cdot L^{-1}$ Cd in a greenhouse hydroponic study in two independent experiments. Plants exposed to 25 $mg \cdot L^{-1}$ Cd displayed stunted plant growth characteristics, reduced photochemical efficiency, and premature senescence suggesting Cd toxicity. At the two lower concentrations of Cd (2.5 and 10 $mg \cdot L^{-1}$ Cd), plant height, biomass, and photochemical efficiency were not affected, with CCI being slightly lower at 10 $mg \cdot L^{-1}$ Cd, compared to 2.5 $mg \cdot L^{-1}$ Cd. The two experiments had no consistent differences in total CDB and THC concentrations in flower tissues at 2.5 and 10 $mg \cdot L^{-1}$ Cd, compared to the control treatment. Root tissue accumulated the highest amount of Cd compared to other tissues for all the Cd treatments, suggesting preferential root sequestration of this heavy

metal in hemp. Transcript abundance analysis of HMA transporter genes suggested that all seven members of this gene family are expressed in hemp, albeit with higher expression in the roots than in the leaves. In roots, *CsHMA3* was up-regulated at 45 and 68 DAT, and *CsHMA1*, *CsHMA*4, and *CsHMA*5 were upregulated only under long-term Cd stress at 68 DAT, at 10 $mg \cdot L^{-1}$ Cd. Results suggest that expression of multiple HMA transporter genes in the root tissue may be upregulated in hemp exposed to 10 $mg \cdot L^{-1}$ Cd in a nutrient solution. These transporters could be involved in Cd uptake in the roots *via* regulating its transport and sequestration, and xylem loading for long-distance transport of Cd to shoot, leaf, and flower tissues.

The objectives of our second study were to evaluate Cd uptake and translocation in two DLS and two DN hemp varieties grown for the medicinal market, and to determine the impact of Cd exposure on cannabinoid concentrations in flowers. A hydroponic experiment was conducted by exposing plants to 0 $mg \cdot L^{-1}$ Cd and 2.5 $mg \cdot L^{-1}$ Cd in the nutrient solution. Cadmium concentrations ranged from 16.1 to 2274.2 $mg \cdot kg^{-1}$ in roots, though all four varieties accumulated significant concentrations of Cd in aboveground tissues, with TFs ranging from 6.5 to 193. Whole-plant BCFs ranged from 20 to 1051 $mg \cdot kg^{-1}$. Cannabinoid concentrations were negatively impacted by Cd exposure in DN varieties but were unaffected in DLS varieties. Biomass was reduced by Cd exposure, demonstrating that these varieties might not be suitable for growth on contaminated soil or for phytoremediation.

To further elucidate Cd tolerance mechanisms in hemp, in our third study we assessed Cd-induced alterations at the cellular level in hemp roots and the ability of hemp to translocate Cd from the root system to the shoot, and performed histochemical localization of Cd at the cellular level in roots and leaves. Upon staining with dithizone, Cd was primarily localized in the leaf epidermis, spongy mesophyll, and trichomes, and in root rhizodermis, cortex, and pericycle in

hemp plants exposed to 10 $mg \cdot L^{-1}$ Cd in a hydroponic experiment. Our results suggest that roots take up Cd and translocate it to the leaves, although the root is the primarily affected tissue.

In summary, our results indicated a potential for Cd accumulation in hemp flowers of the varieties studied in the present work, showing the need for heavy metal testing of *C. sativa* consumer products.