

Accumulation and Histochemical Localization of Cadmium in Hemp (*Cannabis sativa* L.) Leaf and Root Tissue

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Abstract. As anthropogenic activities have facilitated the spread of heavy metals into the environment, plants have been identified that may have the ability to tolerate exposure to high levels of these metals. Industrial hemp (*Cannabis sativa* L.) has been suggested as potentially having the ability to accumulate elevated concentrations of some contaminants from soils, including cadmium (Cd), a heavy metal that has been linked to serious health risks to humans. Therefore, there is an interest to document the ability of hemp to tolerate exposure to Cd to determine if there is a risk when products for medicinal consumption are made from the hemp plant. The objectives of this study were to perform histochemical localization of Cd at the cellular level and document potential changes in plant tissues in response to hemp exposure. Plants were grown in hydroponic solutions for 4 weeks and then exposed to either 0 or 10 mg·L⁻¹ Cd for 17 days and harvested. There were no differences in dry weights (dw) of leaves, stems, and roots among treated and control 17 days after treatment (DAT). However, plants exposed to 10 mg·L⁻¹ Cd had Cd concentrations of 1448.0 mg·kg⁻¹ dw in roots and 23.2 mg·kg⁻¹ dw in leaves at 17 DAT, whereas Cd was not detected in roots or leaves of control plants. On staining with dithizone, Cd was primarily localized in leaf epidermis, mesophyll, and trichomes, and in root rhizodermis, cortex, and pericycle in hemp plants exposed to 10 mg·L⁻¹ Cd. Image analysis was able to further quantify these results based on area stained. Our results suggest that Cd is primarily accumulated throughout hemp roots, with lesser amounts translocated to the leaves, where it may be localized in the epidermis and trichomes.

Heavy metals refer to naturally occurring elements that exhibit the properties of metals, have relatively high atomic weights, and densities greater than 5 g·cm⁻³ (Dalcorsio 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); although all can be toxic to plants when present at elevated concentrations. Other heavy metals or metalloids are not beneficial for plant growth and can be toxic at low concentrations, including arsenic [(As) metalloid], cadmium (Cd), chromium, mercury, and lead (Pb) (Dalcorsio 2012; Mitra et al. 2014).

Anthropogenic actions have facilitated the spread of heavy metals into the environment through activities such as mining and smelting, use of contaminated bio-solids, and improper

disposal of industrial waste (Chaney 2010). Cd, a heavy metal that is mined extensively for industrial uses, may also be a contaminant in Zn and phosphorus (P) minerals that are used in 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 potentially harmful elements. Cadmium has been documented to pose serious health risks to humans, negatively affecting the function of 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 (Akeson and Chaney 2019; Chaney 2015). Because hemp flower may also be smoked in a manner similar to tobacco, Cd accumulation is of particular interest to hemp producers.

Cd 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 and Cai 2009; Shi et al. 2012). Because heavy metals such as Cd are not essential plant nutrients, they typically enter plants via nonspecific protein transporters and associated channels that are intended for the transport of other essential minerals such as Zn, Fe, or Cu (Paulose et al. 2008). When these nonessential metals enter plants, they may trigger different responses, such as being bound to the cell wall, chelation, compartmentalization within cells, and even translocation (Shah et al. 2019). Further, Cd accumulation in plant tissues may hinder chlorophyll and carotenoid biosynthesis, photosynthetic efficiency, and assimilation and transport of essential nutrients, while also triggering reactive oxygen species (Parmer et al. 2013; Shah et al. 2019; Singh et al. 2016).

Hyperaccumulator plants are those species that can tolerate exposure to otherwise toxic levels of heavy metals by accumulating and sequestering them in aboveground tissues (Greger 1999). These plants can avoid damage caused by some heavy metals through mechanisms that include sequestration in vacuoles, excretion, and exclusion (Nikalje and Suprasanna 2018). Because true hyperaccumulators are often small plants with limited biomass accumulation, they may not be routinely used for phytoremediation purposes. Instead, plants that can both tolerate adverse levels of heavy metals and produce significant biomass are often used for phytoremediation (Marques et al. 2009; Yan et al. 2020). Although not typically considered a hyperaccumulator, industrial hemp (*Cannabis sativa* L.) has been suggested as potentially suitable for phytoremediation purposes due to its rapid growth rate and large biomass accumulation (Ahmad et al. 2015; Angelova et al. 2004; Citterio et al. 2003; Flajsman et al. 2023; Galic et al. 2019; Husain et al. 2019; Nikalje and Suprasanna 2018; Shi and Cai 2009; Shi et al. 2012; Visković et al. 2023).

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In the United States, industrial hemp is legally defined as *C. sativa* that has a total delta-9 tetrahydrocannabinol (THC) concentration of less than 0.3% $\pm$ a measurement of uncertainty (Visković et al. 2023). Although there are differences in phenotype between varieties bred for fiber production and those intended for the medicinal market, THC concentrations are legally used to delineate industrial hemp from *C. sativa* grown for marijuana. With increased hemp production in the United States, there has been greater interest in the ability of hemp to tolerate exposure to Cd and how the potential for Cd accumulation could affect hemp grown for human consumption (Milan et al. 2024).

Several of the molecular and biochemical mechanisms underlying Cd tolerance in *C. sativa* have been documented (Huang et al. 2019; Luyckx et al. 2021a; Marabesi et al. 2023a; 2023b; Milan et al. 2024; Singh et al. 2016). Nevertheless, there is a lack of information regarding Cd cellular deposition and the structural changes in plant tissue associated with Cd exposure in hemp. Previously, accelerated endodermis formation and thickening of cell walls have been reported in roots of multiple plant species exposed to heavy metals (Shah et al. 2019). The objectives of this study were to assess Cd-induced alterations, document the ability of hemp to translocate Cd from the root system to leaves, and perform histochemical localization of Cd in roots and leaves.

Materials and Methods

Hydroponic experiment. Cuttings from female hemp plants ‘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 (3.3 cm L $\times$ 2.5 cm W $\times$ 3.8 cm D; Oasis Grower Solutions, Kent, OH, USA) for rooting. Vegetative propagation was chosen to reduce genetic heterogeneity among plants in the study. Further, the cultivar was chosen because of prior research demonstrating a tolerance to Cd exposure in a hydroponic system (Marabesi et al. 2023a). The cubes containing the cuttings were placed on a heat mat set at 24 °C. The cuttings received water four times daily for 3 min each from an overhead mist system. At 3 weeks, cuttings and foam cubes were placed into mesh plastic containers (4.7 cm W $\times$ 5.1 cm D) spaced equidistantly (24.3 cm apart) in the lid of 37.9 L polyethylene tubs (Rubbermaid Inc. Wooster, OH, USA). Tubs were then filled with 28.0 L of nutrient solution containing a commercial hydroponic fertilizer (5.0N–4.8P–21.6K, Peters Professional Hydroponic Special; ICL, St. Louis, MO, USA) and calcium nitrate (14.0N–0P–0K, 17.0 Ca; Calcium + Micros, General Hydroponics, Santa Rosa, CA, USA). Both fertilizers were solubilized in well water (Table 1). Each container supported five plants. During the study, a 15.2-cm aquarium aerator attached to a pump (Active Aqua; Hydrofarm, Petaluma, CA, USA) was added to each tub to provide additional oxygen to the solution. Solution volumes were

Table 1. Mineral nutrient concentrations in well water and the nutrient solution before adding cadmium. Values are the average of four replicates of each solution.

	N	P	K	Ca	Mg	B (mg·L ⁻¹)	Cu	Mo	Fe	Mn	Zn
Well water	<LOD	<LOD	2.6	10.8	2.1	<0.01	<0.05	<0.01	0.3	0.09	<0.05
Nutrient solution	150.0	56.6	240.4	132.8	58.2	0.8	0.4	0.1	4.6	1.1	0.4
Total concentration	150.0	56.6	243.0	143.6	60.3	0.8	0.4	0.1	4.9	1.1	0.4

LOD = limit of detection; nutrient solution composed of the following compounds: KNO₃, K₂HPO₄, MgSO₄, H₃BO₃, Cu-EDTA, Fe-EDTA, Mn-EDTA, Na₂ MoO₄, Zn-EDTA, Ca(NO₃)₂·NH₄NO₃·10H₂O, (NH₄)₂MoO₄, Ca-EDTA, and Na₂B₄O₇·10H₂O.

maintained by adding well water every 2 d and nutrient solutions were completely replaced every 2 weeks. Electrical conductivity and pH of the solutions were monitored twice per week and solution pH was adjusted to 5.5 (pH down; General Hydroponics). A deep-water culture system was chosen to more accurately and evenly distribute Cd to plants as well as avoid any effects of pH and organic matter

on metal availability as could occur if plants were grown in a soil-based media (Prasad and Freitas 2003).

Supplemental light ($\sim 100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was used to provide 18/6 light/dark hours for the duration of the experiment. Plants were grown in the nutrient solution for 4 weeks for acclimation. After 4 weeks of growth, plants were exposed to either 0 (control) or 10 mg·L⁻¹

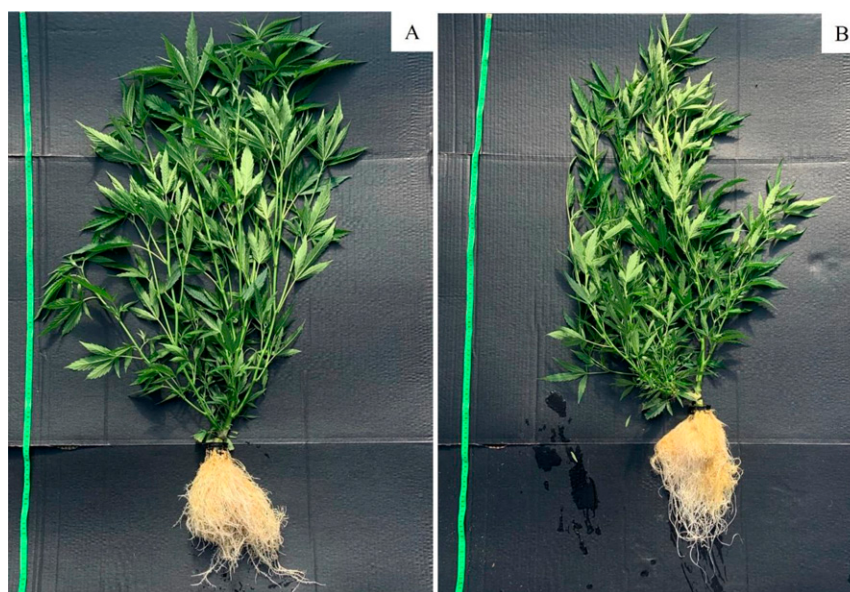


Fig. 1. Entire hemp plants at harvest in the 0 mg·L⁻¹ Cd (A) and 10 mg·L⁻¹ Cd (B).

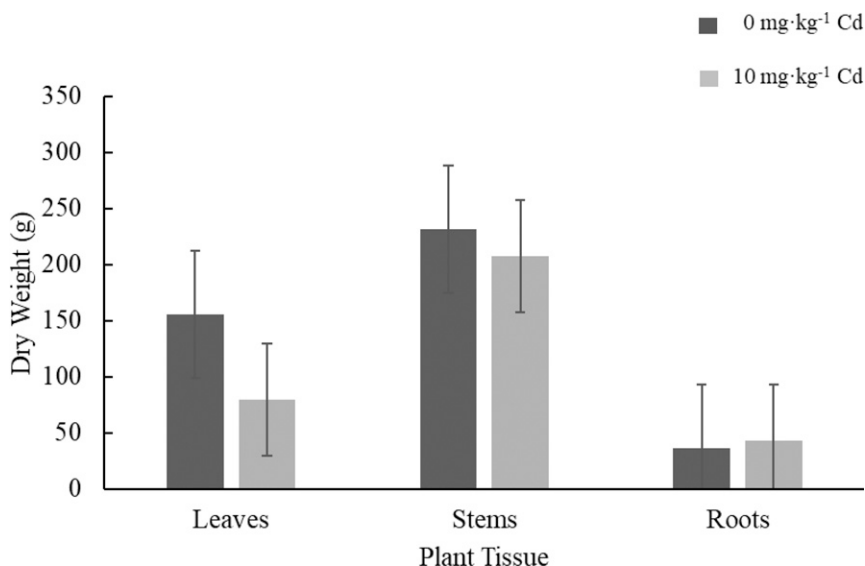


Fig. 2. Hemp, dry weight biomass for leaves, stems, and roots at 17 d after exposure to 0 and 10 mg·L⁻¹ Cd.

Cd by adding $3\text{CdSO}_4 \cdot 8\text{H}_2\text{O}$ 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 $10\text{ mg}\cdot\text{L}^{-1}$ Cd in a deep-water culture hydroponic system (Marabesi et al. 2023a). Plants were exposed to the Cd-containing solutions for 17 d. The experiment followed a randomized complete block design containing two treatments with four replications per treatment and five plants per replication.

Nutrient solutions were sampled at the start and termination of each 2-week period (20-mL scintillation vials; HDPE; Thermo-Fisher Scientific, Waltham, MS, USA), and stored at 4°C until analysis. The air temperature and relative humidity (RH) of the greenhouse were measured hourly at the canopy level (VP4; Meter Group Inc., Pullman WA, USA). Average air temperature and RH during the experiment were $17.3 \pm 5.6^\circ\text{C}$ and $78.7\% \pm 14.6\%$, respectively. Photosynthetic active radiation was recorded every hour during the study (QSO-S; Meter Group Inc.) and the average daily light integral was $19.6 \pm 7.5\text{ mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$.

Root and leaf samples were collected at 5 DAT (visual symptoms started) and 17 DAT (plants recovered and had no visual symptoms) and kept on moist filter paper inside sealed plastic bags (1.0 Gallon Ziploc Freezer Bag; SC Johnson, Racine, WI, USA) to maintain turgor until further processing for scanning electron microscopy (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 terminus of the apically dominant branch at harvest. To determine biomass yield, five plants each from the four replicates were air dried at ambient temperatures inside the greenhouse for 2 weeks and then separated into roots, stems, and leaf biomass. 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.

Inductively coupled plasma optical emission spectroscopy analysis. Root and leaf material from 5 DAT and 17 DAT were subjected to inductively coupled plasma optical emission spectroscopy (ICP OES) analysis. Subsamples for elemental analysis were taken from intact plants before biomass sampling and drying (Marabesi et al. 2023b). Five plants from each of the four replicates were used for a composite sample. Root samples were $\sim 50\text{ g}$ (fresh weight) and were triple rinsed with deionized water and then oven dried. Foliar samples were obtained by taking 10 of the youngest fully expanded leaves from the terminal third of each plant to ensure that leaves were exposed to Cd throughout the entirety of their development. Foliar samples were rinsed with deionized water and then

Table 2. Average cadmium (Cd) $\pm$ SE accumulation in hemp (*Cannabis sativa* L.) ‘Purple Tiger’ plant tissue on a dry weight (dw) basis at 5 and 17 d after treatment (DAT) using inductively coupled plasma optical emission spectroscopy analysis. Values are the average of four replicates.

Treatment (mg·L ⁻¹ Cd)	Roots		Leaves	
	(Cd mg·kg ⁻¹ dw)			
	5 DAT	17 DAT	5 DAT	17 DAT
0	<LOD	<LOD	<LOD	<LOD
10	628.6 ± 54.1 B	1448.0 ± 148.5 A	24.8 ± 6.0 A	23.2 ± 2.1 A

LOD = Limit of detection; 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 ($P \leq 0.05$).

dried in a forced air oven to set to 55°C for $\sim 48\text{ h}$ or until a constant weight was achieved. Dry plant material was ground in a stainless-steel Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) and put through a 20-mesh stainless-steel screen. Samples were digested using 0.5 g of dry plant tissue placed in a fluorocarbon polymer microwave vessel. Concentrated nitric acid (10 mL) was added to each vessel, placed in a microwave digester (Mars 6 Microwave; CEM Corp., Matthews, NC, USA), and heated to 200°C for 30 min. The microwaved digests were then placed into volumetric flasks and brought to 100 mL with deionized water before analysis. To ensure no cross contamination of heavy metals during the grinding and digestion processes, an internal quality control sample

(pecan leaves, *Carya illinoensis*) with established concentration ranges was analyzed with each batch. Samples of the hydroponic solutions were filtered using a $0.45\text{-}\mu\text{M}$ polytetrafluoroethylene (PTFE) membrane and acidified using 2% high-purity nitric acid (HNO_3) before analysis. The ICP OES detection limit for Cd was $0.001\text{ mg}\cdot\text{L}^{-1}$.

Plant tissue digestions and nutrient hydroponic solutions were analyzed according to EPA Method 200.8 (Creed et al. 1994) by synchronous vertical dual view ICP OES (Agilent 5110; Agilent Technologies, Inc., Santa Clara, CA, USA). Elements analyzed were Al, B, Ca, Cu, Cd, Fe, K, Mg, Mn, Ni, P, Pb, S, and Zn. Basic ICP OES conditions were as follows: read time 6 s, radiofrequency power 1.2 kW , stabilization time 15 s, viewing

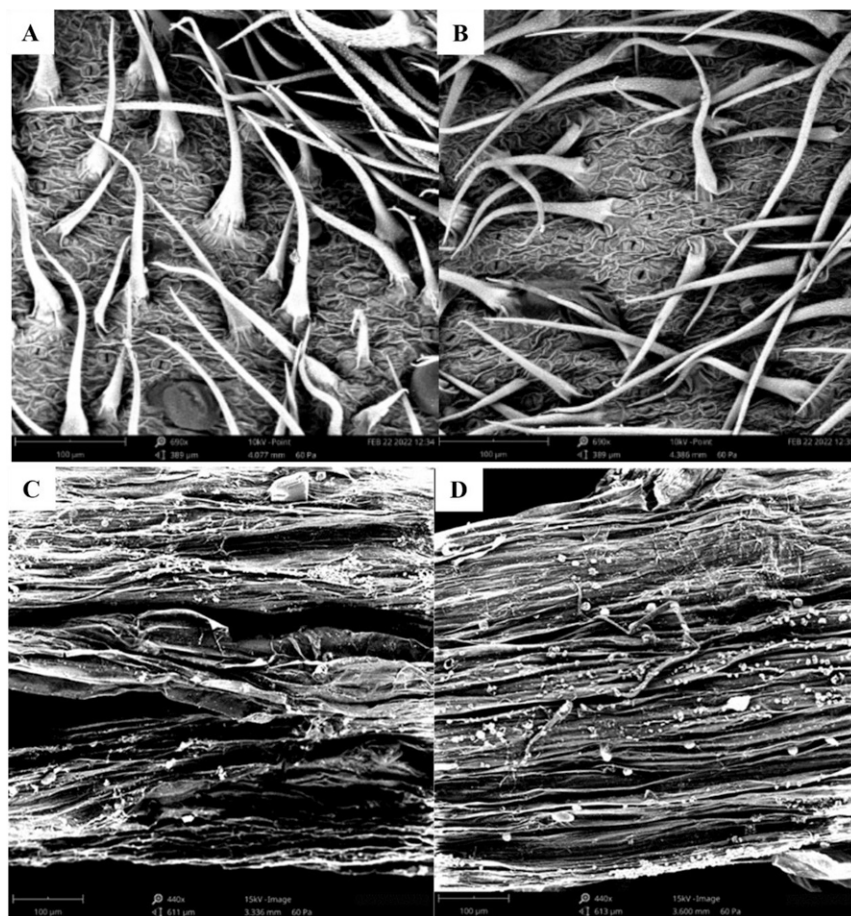


Fig. 3. Abaxial surface of hemp leaves exposed to $0\text{ mg}\cdot\text{L}^{-1}$ Cd (A) and $10\text{ mg}\cdot\text{L}^{-1}$ Cd (B) at 17 d after exposure. Surface of hemp root exposed to $0\text{ mg}\cdot\text{L}^{-1}$ Cd (C) and $10\text{ mg}\cdot\text{L}^{-1}$ Cd (D) at 17 DAT. Scale bar is $100\text{ }\mu\text{m}$ in all images.

mode axial, viewing height 8 mm, nebulizer flow 0.7 L·min⁻¹, plasma flow 12.0 L·min⁻¹, and auxiliary flow 1.0 L·min⁻¹. For internal quality plant standards, The University of Georgia Agricultural & Environmental Services laboratory collects and composites pecan leaves from a local commercial grove to use as an internal quality control standard. Quality control samples are dried, ground in the Wiley mill, and digested alongside each group of experimental samples analyzed. Initial ranges of the composited leaf tissue are established by grinding and digesting 20 samples.

For each batch of samples, the laboratory runs an internal sample (pecan leaf) along with independent calibration verification (ICV), continuing calibration verification (CCV), and control calibration blanks (CCB). The CCV (Lot no. S220830028; SCP Science, Champlain, NY, USA) is a certified standard and the ICV (Lot No. N2-MEB6667614; Inorganic Ventures, Christiansburg, VA, USA) is a separate certified standard from a different source than the CCV. During the ICP OES analysis the ICV, CCV, and CCB samples are run at the beginning, every 20 samples, and again at the end of the run. Recoveries of the ICV and CCV must be 100% ± 10% and the CCB must remain below detection for any analysis to continue.

SEM imaging. All SEM imaging and histochemical analyses were carried out at the Fort Valley State University Center for Ultrastructure Research in Fort Valley, GA, USA. An SEM (Phenom XL; Thermo-Fisher Scientific) equipped with an energy dispersive spectroscopy (EDS) system (Silicon Drift Detector; Mn Kα ≤ 132 eV, Thermo-Fisher Scientific) for elemental analysis to provide a semiquantitative analysis of Cd present in roots and leaves of hemp. For sample preparation, roots and leaves from 0 and 10 mg·L⁻¹ Cd treatments were dried in the Incushaker™ 10L (Benchmark, Tempe AZ, USA) overnight at 35 °C, and cut into pieces to fit aluminum stubs (Ted Pella Inc., Redding, CA, USA). Samples (four replicates of each treatment) were mounted with a double-sided adhesive tape on aluminum stubs and dusted with compressed air before placing in the SEM. No coatings were applied to samples before SEM placement.

Histochemical analysis. Fresh leaf and root samples from a minimum of four replicates of each Cd treatment 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 cryoembedding compound (Cryomatrix; Thermo-Fisher Scientific) and immediately frozen in a Peltier freezing unit in the cryostat. Sections were cut to a thickness of 12 μm and adhered to positively charged microscope slides. These slides were left to dry for 20 min and then stained using diphenylthiocarbazone (dithizone) (Sigma-Aldrich, St. Louis, MO, USA) solution following the protocol developed by Seregin and Ivanov (1997).

Fresh samples were fixed in HistoChoice Clearing Agent (Sigma-Aldrich) for microtomy

and stored at 4.0 °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-Fisher Scientific) set to 65 °C. Sectioning was performed at three thickness (12, 15, and 20 μm) with a microtome, and left to dry overnight on a slide warmer at 37 °C. Next, slides were deparaffinized in xylene for 5 min to remove the paraffin before staining. For staining, we used a diphenylthiocarbazone (dithizone) (Sigma-Aldrich) solution following methodology proposed by Seregin and Ivanov (1997). When dithizone is in contact with Cd or Pb, it reacts and precipitates red or dark blue/black insoluble salts (Pandey et al. 2020; Seregin and Ivanov 1997). For staining, the dithizone solution is prepared fresh. In brief, 30.0 mg diphenylthiocarbazone is dissolved in 60.0 mL of 100% acetone and then 20.0 mL deionized water is added (3:1 mixture of acetone and deionized water). One to two drops of glacial acetic acid (99.9%, Sigma-Aldrich) were added to

6.0 mL of the solution. According to Seregin and Ivanov (1997), adding glacial acetic acid to the solution enhances the reaction with Cd as it is more specific in a slightly acidic medium. Slides were stained for 20 min with dithizone solution, washed with deionized water, and observed with a light microscope with ×10 and ×40 objective lenses and a ×10 ocular lens. Image analysis was conducted using Fiji v. 2.11.0 (Schindelin et al. 2012) following the US National Institutes of Health (NIH) methodology for stained sections (NIH 2018). The scale bar was not deleted from the images, and it was accounted as part of the stained area for all images due to the color threshold methodology that was used. Dithizone reacts with Pb and Cd and other ions (Zn, Cr, Cu, Fe) that have been noted to interfere with staining in some instances (Seregin and Ivanov 1997; Seregin and Kozhevnikova 2011); therefore, it was expected that some residual red staining would be present in control samples.

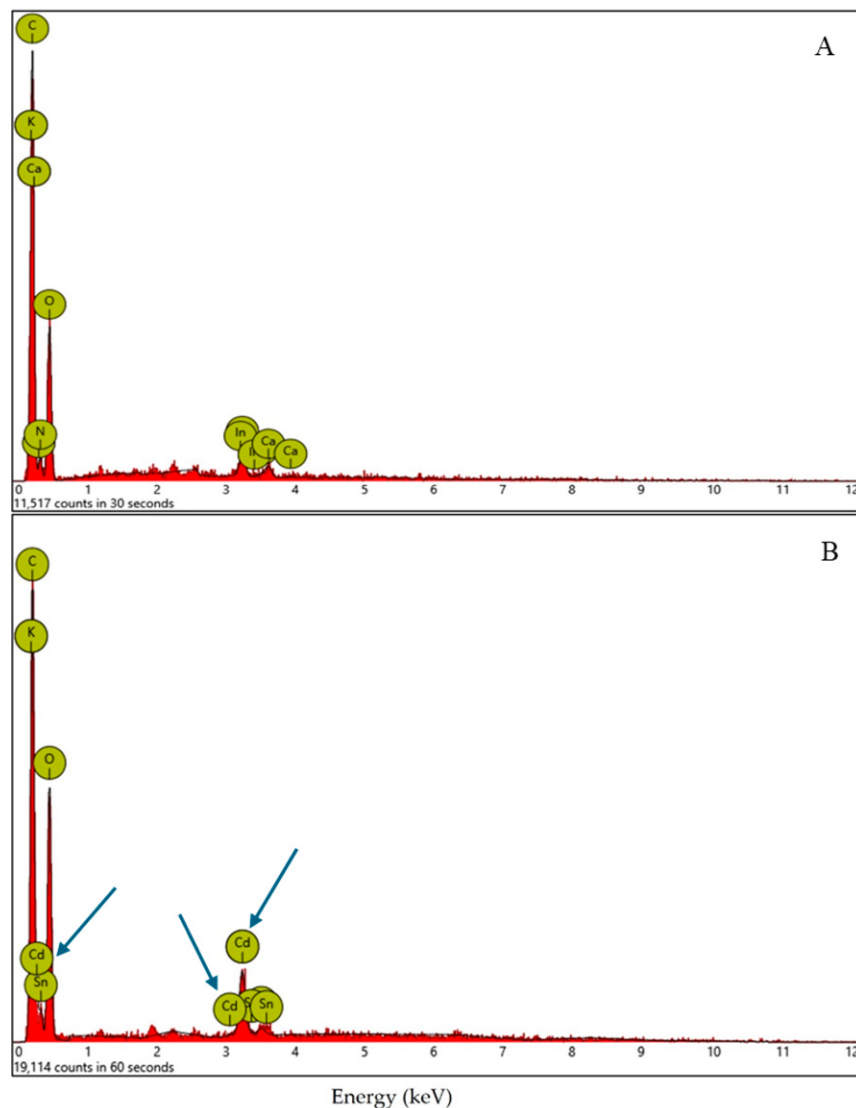


Fig. 4. Scanning electron microscope energy dispersive spectroscopy spectrum for roots exposed to 0 mg·L⁻¹ Cd (A) and 10 mg·L⁻¹ Cd (B). Cadmium was not observed in the control sample (A).

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

Plant growth and biomass yield. Plants started showing visual symptoms of Cd exposure at ~5 DAT. Symptoms were similar to leaf epinasty, with leaves bent downward, having the aspect of a claw. No visual toxicity symptoms were observed in plant roots at any time after Cd exposure. At 17 DAT, plants exposed to $10 \text{ mg}\cdot\text{L}^{-1}$ Cd did not show visual toxicity symptoms and were not visually distinguishable from plants grown in the $0 \text{ mg}\cdot\text{L}^{-1}$ Cd control treatment (Fig. 1A and B). There were no differences in plant height and dry weight of leaves, stems, and roots between treatments (Fig. 2).

ICP OES analysis and SEM imaging. Mineral analysis did not detect Cd in roots or leaves of plants in the control treatment at either sampling time (Table 2). Complete elemental analysis is provided in Supplemental Table 1. Cadmium concentrations were greater in the roots at 5 DAT ($628.6 \text{ mg}\cdot\text{kg}^{-1} \text{ dw}$) and 17 DAT ($1448.0 \text{ mg}\cdot\text{kg}^{-1} \text{ dw}$) compared with leaves at 5 DAT ($24.8 \text{ mg}\cdot\text{kg}^{-1} \text{ dw}$) and 17 DAT ($23.2 \text{ mg}\cdot\text{kg}^{-1} \text{ dw}$). The Cd concentration in the roots was ~25 times greater than in leaves at 5 DAT, and ~62 times higher at 17 DAT.

Using SEM imaging, no visual differences were observed on the abaxial surface of hemp leaves between control (Fig. 3A) and treated plants (Fig. 3B) measured at 17 DAT. Analysis of hemp roots at 17 DAT suggests that there were minimal differences between control (Fig. 3C) and Cd-treated plants (Fig. 3D) as well. Additional elemental analysis using SEM EDS detected Cd in the treated root tissue, showing clear peaks for Cd, which were not present in the control samples (Fig. 4A and B).

Cadmium localization in hemp leaves and roots. Reactions with dithizone in leaves and roots were similar at 5 and 17 DAT, therefore images from staining conducted at the end of the experiment (17 DAT) are presented. In control leaf samples, reactions with dithizone were largely negative (Fig. 5A and B); however, cross sections from the midrib of leaves grown in the $10 \text{ mg}\cdot\text{L}^{-1}$ Cd solution at 17 DAT showed a clear positive reaction with dithizone (Fig. 5C and D). The red color in the leaf epidermis, spongy mesophyll, and trichomes suggests that the reaction was more intense in those regions, indicating Cd localization here. Although large areas of leaf tissue were stained red, there were black precipitates in vacuoles in epidermal cells of Cd-exposed plants (Fig. 5D). These were likely precipitates of dithizone reacting with Cd (He et al. 2022; Pandey et al. 2020). Roots of plants exposed to $0 \text{ mg}\cdot\text{L}^{-1}$ Cd had a minimal reaction to dithizone staining (Fig. 5E). In roots of plants exposed to $10 \text{ mg}\cdot\text{L}^{-1}$ Cd at 17 DAT,

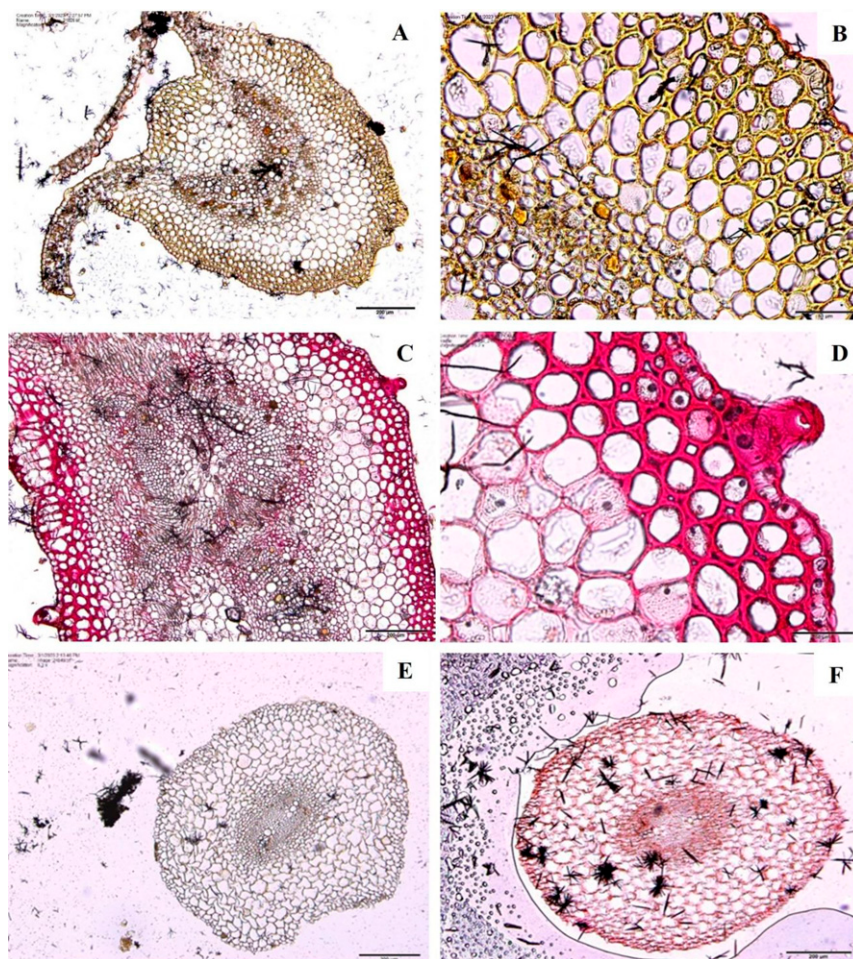


Fig. 5. Cross sections of dithizone-stained hemp leaves (A–D) and roots (E, F) that were exposed to $0 \text{ mg}\cdot\text{L}^{-1}$ Cd (A, B, E) and $10 \text{ mg}\cdot\text{L}^{-1}$ Cd (C, D, F) at 17 d after treatment. In panels A, C, E, and F the scale bar is 200 μm , and in panels B and D the scale bar is 50 μm .

staining with dithizone was observed throughout the root cross section (Fig. 5F). There were some dark precipitates to the exterior of the root cross section for plants grown in the $0 \text{ mg}\cdot\text{L}^{-1}$ Cd (Fig. 5E), which were likely excess dithizone that was unable to be rinsed from the section after staining. There were also several dark precipitates in observed root cross sections that were likely excess dithizone reacting with Cd and precipitating (Fig. 5F); however, there is a possibility that they could be related to staining of Cd-phytochelatin (Ahmad et al. 2019).

Although Cd levels in the roots and leaves of the control plants were below the level of detection, there were small areas of control samples that were stained. This was most likely due to excess dye residue that was unable to be completely washed from slides, contributing to overall stained area. It should be noted that dithizone can also be used to detect the presence of Pb in plant materials (Seregin and Ivanov 1997). Low concentrations of Pb were detected in all tissues and treatments, averaging 1.2 and $1.6 \text{ mg}\cdot\text{kg}^{-1} \text{ dw}$ in leaf and root tissues, respectively (Supplemental Table 1). Although present in all tissue, Pb concentrations were a small fraction of Cd concentrations in treated plants. The

presence of Pb in the tissue was likely an artifact of the nutrient salts used in the study or other Pb-containing items that came in contact with the nutrient solution.

Imaging analysis of stained sections. Imaging analysis was used to determine if the area of dithizone staining could be quantified on sectioned plant material. Imaging analysis of stained leaf sections showed an increase in stained area in plants grown in a $10 \text{ mg}\cdot\text{L}^{-1}$ Cd hydroponic solution. At 17 DAT 1.0% and 2.5% of the total area was stained in the $0 \text{ mg}\cdot\text{L}^{-1}$ Cd control samples compared with 10.5% and 18.0% in the $10 \text{ mg}\cdot\text{L}^{-1}$ Cd samples, at $\times 100$ and $\times 400$ magnifications, respectively (Fig. 6A–D).

In plant root cross sections, at 17 DAT, the percent area stained in the control samples was 0.8% compared with 2.9% on the Cd-treated samples, at $\times 100$ magnification (Fig. 6E and F). Although the percentage of viewable area that was stained was greater in treated samples compared with control samples, there were no significant correlations between area stained and Cd concentrations in leaf or root tissue and area stained using image analysis. Although image analysis using dithizone may be able to demonstrate relative locations of where Cd is sequestered in plant tissue, it would not

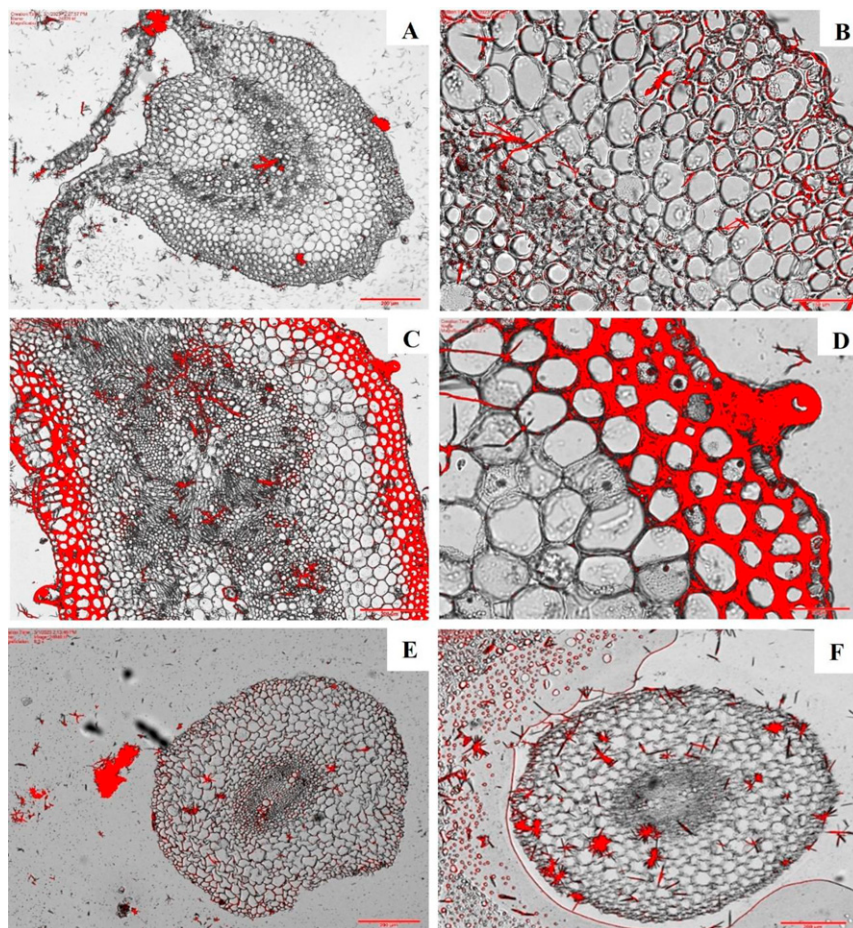


Fig. 6. Image analysis of dithizone-stained cross sections of hemp leaves (A–D) and roots (E, F) exposed to 0 mg·L⁻¹ Cd (A, B, E) and 10 mg·L⁻¹ Cd (C, D, F) at 17 d after treatment. In panels A, C, E, and F the scale bar is 200 µm, and in panels B and D the scale bar is 50 µm.

be able to determine relative concentrations of Cd in hemp.

Discussion

In prior research, the cultivar Purple Tiger was grown with 10 mg·L⁻¹ Cd, and exhibited mild toxicity symptoms for several days, but recovered within a few weeks after exposure, emphasizing the potential short-term tolerance of this cultivar to up to 10 mg·L⁻¹ Cd in solution (Marabesi et al. 2023a). This suggests that some cultivars of hemp may be able to recover from limited Cd exposure. Our results were in contrast with previous studies using different cultivars of hemp that have reported a notable decrease in shoot biomass of hemp plants exposed to Cd (Angelova et al. 2004; Luyckx et al. 2021a; Marabesi et al. 2023b; Shi and Cai 2009). Although plants in the current study were harvested before flowering, Marabesi et al. (2023a) reported the impact of 10 mg·L⁻¹ Cd on flower cannabinoid concentrations using 'Purple Tiger' hemp.

Cadmium is taken up by plant roots via nonspecific 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). Transporting ATPases such as *AtHMA4* and *AtHMA2* may play a role in Cd uptake and

translocation in *Arabidopsis thaliana* for example (Wong and Cobbett 2009). Shi et al. (2012) described hemp as an excluder rather than a hyperaccumulator, and hemp has previously been reported to preferentially accumulate Cd in roots (Angelova et al. 2004; Ćaćić et al. 2019; Luyckx et al. 2021b; Marabesi et al. 2023a; Milan et al. 2024; Shi et al. 2012; Visković et al. 2023). In the present study, plants exposed to 10 mg·L⁻¹ Cd in hydroponic solutions were able to uptake Cd from the solution and translocate it to the leaves, where it was deposited in the leaf epidermis, spongy mesophyll, and trichomes. Cd concentrations in leaves were not different between the two sample times, despite continued plant growth during Cd exposure. This suggests that plants were still able to uptake and translocate Cd to the leaves at a constant rate to maintain a relatively consistent concentration in the dry plant material, despite continued growth. Interestingly, the Cd concentration of 10 mg·L⁻¹ Cd used in our study was not lethal to 7-week-old (age from day of seeding at Cd exposure) hemp plants. Balestri et al. (2014) reported that Cd has 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 (100.0 µM CdCl₂). The authors suggested that this could

be a defense mechanism to avoid Cd toxicity in the root meristem.

Previously Pielichowska and Wierzbicka (2014) discussed the role of trichomes in Cd detoxification. The authors reported that *Biscutella laevigata*, a Cd hyperaccumulator species had trichomes that were able to accumulate and sequester 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. In the present study, Cd staining was present in trichomes of hemp leaves, suggesting that they could possibly serve a similar purpose; however, further research is necessary to validate this hypothesis. Interestingly, trichomes located on the abaxial leaf surface exhibited no notable differences in appearance between control and Cd-treated plants (Fig. 3A and B).

Although information regarding the status of metal accumulation in hemp has increased recently (Milan et al. 2024), there is still limited research on Cd localization in hemp tissues. Arru et al. (2004) conducted a study to localize Cu in hemp leaves and suggested that Cu was mainly accumulated in the upper epidermis although also found in nonglandular and abaxial trichomes, which is consistent with our results. We were also able to stain Cd in the roots of Cd-treated plants, primarily throughout the cortex, endodermis, and pericycle. These results support data from Yang et al. (2015), who found Cd to be localized mainly in the root cortex of corkscrew willow, *Salix matsudana*, seedlings exposed to 5.6 mg·L⁻¹ Cd (50.0 µM CdCl₂) for 10 h; and from Seregin and Ivanov (1997), who observed a dithizone reaction in the rhizodermis, cortex, and endodermis in the roots of maize (*Zea mays* L.) seedlings. Seregin and Ivanov (1997) reported damage to the rhizodermis and inner cortical cell of roots at 5 d of incubation with 100 µM of Cd(NO₃)₂. It should be noted that by dehydrating (fixing) samples in alcohol before treatment with dithizone, some Cd likely was mobilized from the vacuoles to intercellular spaces. This movement of Cd would reduce the ability to localize Cd within cells of the plants. Nonetheless, our results do demonstrate general trends in Cd movement and deposition in hemp material. The Cd concentration of 10 mg·L⁻¹ Cd used in our study was not lethal to 7-week-old (age from day of seeding at Cd exposure) hemp plants.

Conclusions

The purpose of this study was to document potential changes in tissue morphology and Cd accumulation for industrial hemp plants exposed to Cd in a greenhouse setting. Our results suggest that although Cd was translocated to aboveground portions of the plant, Cd accumulation in roots accounted for most of the Cd in plant tissue. On staining with dithizone, we were able to distinguish

between Cd-treated and untreated plants in both leaf and root tissue. Although much of the Cd accumulation in plants occurred in roots, trichomes and epidermal cells were a notable location for Cd accumulation in leaves.

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