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Additional Sulfur Does Not Alleviate Cadmium Toxicity In Soybean

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Abstract

Cadmium (Cd) is non-essential and toxic. Sulfur (S) addition to contaminated soils reduces Cd toxicity in rice and corn. I aimed to determine the underlying mechanisms by which S reduces Cd toxicity in hydroponically- and soil-grown soybean. In the presence of Cd, plant biomass was reduced by ~20%, Cd accumulated up to 45 µg/g in roots and 15 µg/g shoots, and concentrations of Cd chelators increased by more than 10-fold. Addition of S to Cd-treated plants had no effect on plant biomass, concentrations of Cd in roots and shoots, or vacuolar Cd sequestration in the root cortex. While additional S visibly altered Cd localization in the roots, it had no effect on altering Cd concentration in root plaque. Additional S in the presence of Cd resulted in a 0- to 1.5-fold increase in Cd chelator concentrations; however, addition of S to alleviate Cd toxicity has no benefit in soybean.

Keywords

Soybean, Root plaque, Gamma-glutamyl-cysteine, Homoglutathione, Homophytochelatin.

Summary for Lay Audience

Cadmium (Cd) concentrations are increasing globally in agricultural soils due to anthropogenic contamination. Although it is a non-essential element, Cd can inhibit plant growth and pose a health risk to consumers. Sulfur (S) addition to Cd-contaminated soils can reduce Cd toxicity in rice and corn. Additional S might enhance the formation of an insoluble plaque (layers of oxidized compounds) on the root surface. Binding of Cd ions to the plaque would reduce Cd uptake. Increased availability of S may also increase the production of S-containing molecules called chelators that bind to and keep Cd within plant cells.

I designed this study to determine the underlying mechanisms behind reduction of Cd toxicity due to S addition in soybean. My specific objectives were to determine if S addition will reduce Cd toxicity by causing increased binding of Cd to the root plaque, decreased Cd uptake, increased chelator production, and increased accumulation of Cd in root cells. Hydroponic and soil experiments were performed.

As expected, biomass of soybean plants was reduced in response to Cd and the concentrations of chelators in roots and leaves increased at least 10-fold. In contrast to results of similar experiments on rice and corn, additional S did not reduce Cd toxicity in soybean, nor did it reduce the concentration of Cd in roots or shoots or cause accumulation of Cd on or in root cells. Additional S did visibly alter Cd localization in the roots but did not alter the concentration of Cd bound to the root plaque. Additional S also resulted in a 0 to 1.5-fold increase in some chelators but the pattern was not consistent with S dose.

Based on the findings of this study it can be concluded that addition of S in the presence of Cd does not reduce toxicity of Cd in soybean as it does in rice and corn. These results would best be applied by agronomists to prevent misconceptions about the benefits of additional S on reducing Cd uptake and toxicity in crop species such as soybean.

Co-Authorship Statement

The manuscript arising from this thesis will be co-authored by Dr. Sheila M. Macfie and myself. The experimental design and conduction of experiments was performed by me with guidance from Dr. Macfie. I conducted all statistical analysis and data collection with input from Dr. Macfie. Dr. Macfie helped me with the interpretation of the data. The manuscript will be written by me with editing done by Dr. Macfie.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

ANOVA……Analysis of Variance

ATP……Adenosine Triphosphate

βAla……Beta-Alanine

CAT……Catalase

DCB……Dithionite-Citrate-Bicarbonate

DNA……Deoxyribonucleic Acid

DTT……Dithiothreitol

DW……Dry Weight

γ-Glu-Cys……Gamma-Glutamyl-Cysteine

GR……Glutathione Reductase

GSH……Glutathione

Gly……Glycine

hGSH……Homoglutathione

HPLC……High Performance Liquid Chromatography

hPC……Homophytochelatin

ICP-MS……Inductively Coupled Plasma Mass Spectrometry

LC-MS……Liquid Chromatography - Mass Spectrometry

MT……..Metallothionein

NEM……..N-ethylmaleimide

NRAMP……..Natural Resistance-Associated Macrophage Proteins

POD.....Peroxidase

PC……..Phytochelatin

RO…….Reverse Osmosis

ROS……Reactive Oxygen Species

SEM-EDX.....Scanning Electron Microscopy-Energy Dispersive X-ray Spectroscopy

SEM-WDS.....Scanning Electron Microscopy-Wavelength Dispersive Spectroscopy

SOD……..Superoxide Dismutase

SRM……..Standard Reference Material

-SH…….Thiol Group

UV……Ultraviolet

ZIP……..Zn-Regulated Transporter, Iron-Regulated Transporter-Like Protein

Chapter 1

1 Literature review

Cadmium (Cd) is a widely studied metal contaminant that is toxic to both plants and animals. An increase in Cd accumulation in agricultural soils can lead to reduced crop yield and health risks to consumers. Sulfur (S) has been shown to reduce Cd toxicity in rice and corn by increasing defense mechanisms and Cd tolerance. The interaction and potential beneficial effects of S addition on mitigating Cd toxicity in soybean has not been studied.

1.1 Cadmium as an agricultural contaminant

1.1.1 Sources of cadmium in the environment

With ever-increasing global urbanization, there are increasing levels of environmental contamination (Chen, 2007). Main contaminants are toxic non-essential metals (Peralta-Videa et al., 2009), which can cause serious health problems for both plants and animals and propagate up the food chain (Canadian Council of Ministers of the Environment, 1999; Järup, 2003; Mahmood and Malik, 2014). One of the more concerning metal contaminants in Canadian agricultural soils is Cd (Grant et al., 1997). The main source for Cd contamination in agricultural soils is the use of Cd-contaminated phosphate fertilizers (Dharma-Wardana, 2018; François et al., 2009; Grant, 2011; Sabiha-Javied et al., 2009; Sheppard et al., 2009). The Canadian soil quality guideline for agricultural soil is 1.4 mg/kg (Canadian Council of Ministers of the Environment, 1999). Grant et al. (2000) found that the Cd concentration of southern Manitoba ranged from 0.1 to 0.3 mg/kg Cd in soil. The concentration of Cd ranged from 0.56 to 1.1 mg/kg Cd across Canadian soils in 1987 (Bewers et al., 1987). The mean concentration of Cd in Canadian water bodies ranged from 0.1 to 0.6 µg/L for those lakes and rivers not located near cadmium sources (Environment Canada, 1994). Those lakes and rivers in close proximity to metal smelters contained 5 to 286 µg/L of Cd (Environment Canada, 1994). The distribution of Cd in Europe is mostly around large cities and urban areas with a soil Cd concentration of 0.1-2 mg/kg (Birke et al., 2017; Tóth et al., 2016).

Most Cd is obtained as a by-product of zinc refining as Cd impurities are commonly found in zinc ores (Canadian Council of Ministers of the Environment, 1999; MacLatchy, 1992). In the 1990s it was estimated that the global production of refined Cd was around 22,000 tons (Hoskin, 1991). In 2018 it was estimated at 25,000 tons, which dropped from 26,000 tons two years prior (Callaghan, 2021). This level of global Cd production in the past 20 years has likely led to increased Cd pollution in the environment and agricultural soils. Canada is the fourth highest producer of refined Cd producing about 1600 tons annually in the 1990s, decreasing for 20 years, and increasing again back to 1600 tons by 2018 (Callaghan, 2021; Canadian Council of Ministers of the Environment, 1999). The main uses of Cd are for nickel-cadmium batteries (accounting for about 50% of total Cd usage), coatings (accounting for about 20% of total Cd usage), and pigments (accounting for about 20% of total Cd usage) (Callaghan, 2021; Canadian Council of Ministers of the Environment, 1999). Both nickel-cadmium batteries and coatings were primarily used in aircraft and aerospace purposes (Callaghan, 2021). The batteries were used for their power supply and reliability; however, these are being phased-out in favour of lithium-based batteries (Callaghan, 2021). The Cd-based coatings were used as anticorrosive protection on metal components to reduce surface corrosion (usually galvanic corrosion) (Callaghan, 2021). The Cd-based pigments were used for paints and plastics as a yellow or red colouring agent (Callaghan, 2021).

Cadmium is non-essential for both plant and animal life (Peralta-Videa et al., 2009) and is one of the most toxic metals due to it causing toxicity at very low concentrations (Canadian Council of Ministers of the Environment, 1999) and by competing with essential metal cation transporters (Benavides et al., 2005) . In soil, 2.5 to 4 mg Cd/kg reduces yield of a variety of crop species (Canadian Council of Ministers of the Environment, 1999). In humans, doses as low as 0.25 to 0.50 mg Cd/kg bodyweight can cause gastrointestinal issues and 5 mg Cd/kg bodyweight can cause serious, negative whole-body health effects (Canadian Council of Ministers of the Environment, 1999). Cadmium can impair protein form and function and interact with DNA (see section 1.1.2), causing liver, kidney, reproductive organ damage, as well as carcinogenesis (Canadian Council of Ministers of the Environment, 1999; Environment Canada, 1994).

In addition to the application of phosphate fertilizers, environmental contamination arises from other anthropogenic activities such as mining and smelting, industrial waste, fossil fuel burning, and sewage sludge application. (Canadian Council of Ministers of the Environment, 1999; Chary et al., 2008; Mahmood and Malik, 2014; Nawaz et al., 2021; Xing et al., 2020). Cadmium can also be deposited from air pollution, which can carry Cd hundreds of kilometers away from its original production site (Grant, 2011; Li et al., 2003; Yan et al., 2021). Most non-essential metals can accumulate in soil where they are deposited (Aprile and De Bellis, 2020; Environment Canada, 1994). This causes a build-up of toxic metal concentrations, which can reduce plant growth and lead to plant death (Aprile and de Bellis and, 2020)

Plants do not have a way to remove toxic metals once they are taken up, this leads to bioaccumulation and biomagnification (Aprile De Bellis, 2020). Bioaccumulation is a particular problem in contaminated agricultural soils as this can lead to health risks in humans and livestock after consumption of harvested crops (Chary et al., 2008; Mahmood and Malik, 2014). Bioavailability plays a large role in Cd accumulation in plants, as plants can readily take up water-soluble Cd from the soil solution (Sheoran et al., 2010). Cadmium becomes increasingly more bioavailable at a lower pH where it exists more in its ionic form Cd^{2+} (Environment Canada, 1994; Krishnamurti et al., 1997). At a higher pH Cd tends to be bound to organic matter, clay particles, and complexes with other compounds (Krishnamurti et al., 1997).

1.1.2 Cadmium-induced DNA damage and ROS production

When Cd enters a cell it can interfere with cellular processes, cause DNA damage, and disrupt protein formation (Nazar et al., 2012; Singh et al., 2016). The structural integrity of DNA can become compromised by Cd interference causing breakages and unfolding (Nazar et al., 2012; Singh et al., 2016). Cadmium can also directly bind to DNA bases (such as guanine and thiamine), which damages, destroys, and interferes with DNA structure and formation (Nazar et al., 2012). By binding to the S group of protein structures that are typically used for tertiary protein folding, Cd causes protein degradation, denaturation, and misfolding (Nagajyoti et al., 2010; Seregin and Ivanov, 2001). Nitrate reductase activity is decreased in the presence of Cd which

interferes with nitrogen assimilation within a plant (Seregin and Ivanov, 2001). Reduced cell growth and function are results of Cd interference with the synthesis of enzymes and proteins (Nagajyoti et al., 2010; Seregin and Ivanov, 2001). Cadmium stress alters gene expression and protein synthesis resulting in the production of reactive oxygen species (ROS) and chelator molecules, which help reduce Cd toxicity (Nazar et al., 2012; Seregin and Ivanov, 2001).

Intracellular Cd can also increase ROS formation (Amir et al., 2021; Hassan et al., 2020; Jung et al., 2020; Nazar et al., 2012). ROS are naturally produced in plants from UV exposure to side-products of electron transport chain reactions and CO_2 fixation (Benavides et al., 2005). ROS production increases when plants are exposed to stresses of different kinds. The main ROS species produced in response to Cd toxicity are superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2) (Amir et al., 2021; Hassan et al., 2020). While constitutive concentrations of ROS can be used as signal molecules in plants to help balance stress responses, a large build-up of ROS can lead to cell and whole organism death (Keyster et al., 2020; Seregin and Ivanov, 2001). ROS can oxidize most cellular components, such as DNA, proteins, lipids, and nucleic acids which alter cell morphology and cause intracellular damage (Hassan et al., 2020; Nagajyoti et al., 2010; Singh et al., 2016). Both ROS and Cd can interfere with the lipid structure of cell membranes causing fluidity issues and damaged cell structure (Nagajyoti et al., 2010; Nazar et al., 2012; Singh et al., 2016). Cadmium does not directly produce ROS, but instead causes ROS production by interfering with electron chain transfers and CO_2 fixation (Hassan et al., 2020; Seregin and Ivanov, 2001).

Since ROS are produced naturally, there are many antioxidant enzymes to reduce ROS concentrations in cells (Keyster et al., 2020). The main antioxidant enzymes plants produce are superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione reductase (GR) (Ferreira et al., 2002; Keyster et al., 2020). These antioxidant enzymes all target specific ROS with SOD targeting $O_2^{\cdot-}$, CAT targeting H_2O_2, POD targeting peroxide radicals, and GR which reduces glutathione (GSH) so it more favourably interacts with ROS (Keyster et al., 2020; Singh et al., 2016). Plants also produce non-protein antioxidant molecules such as ascorbic acid and GSH (Hassan et al.,

2020; Jung et al., 2020). Because GSH is used in the production of phytochelatin in response to Cd stress, this source of antioxidant defense can quickly become depleted at high Cd concentrations. Chelators and antioxidant enzymes are used to oxidize ROS, which reduces cell stress and possible cell death (Ferreira et al., 2002; Jung et al., 2020). Cadmium also suppresses antioxidant enzyme activity within cells, which leads to an accumulation of ROS since there is reduced antioxidant activity (Hassan et al., 2020).

1.1.3 Physiological and morphological changes

Cadmium toxicity can occur at very low concentrations and symptoms of Cd toxicity are easily noticed. Due to the high water solubility of Cd, it can readily move with the bulk flow of water and be more bioavailable to plants (Environment Canada, 1994). There is no dedicated Cd transporter on the root surface or plasma membranes for plants, as Cd is non-essential for growth, so it enters through passive or active transporters of other cations (Benavides et al., 2005; Cataldo et al., 1983; Song et al., 2017; Zhao et al., 2002). Due to the ionic properties of Cd^{2+} it can compete with essential metal cations, such as Zn^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, Ca^{2+}, K^{+}, and Mn^{2+}, at their transporters (Benavides et al., 2005; Shuo Zhang et al., 2020). This competition can result in reduced uptake of essential elements, with a higher impact on similarly charged cations.

There are also several micronutrient transporters in the ZIP (Zn-Regulated Transporter, Iron-Regulated Transporter-Like Protein) and NRAMP (Natural Resistance-Associated Macrophage Proteins) transporter families that allow for the uptake of Cd^{2+} (Benavides et al., 2005; Nazar et al., 2012; Song et al., 2017). The ZIP transporters are used to transport Zn^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, and Mg^{2+} within a plant and into the xylem (Benavides et al., 2005; Song et al., 2017). This is typically how Cd^{2+} gets transported from the roots to the shoots, along with some ATP-mediated transporters (Song et al., 2017). Because these ions are transported against their electrochemical gradient ATP must be hydrolyzed to facilitate their movement, which would be more costly if Cd^{2+} outcompeted one of the essential metals (Benavides et al., 2005).

Once Cd^{2+} is transported to the shoots there are visible signs of Cd stress. Leaf curling, chlorosis, and necrosis are all common signs of Cd toxicity in the shoots of plants (Benavides et al., 2005; Seregin and Ivanov, 2001). Reduced top-growth and leaf area are also signs of Cd toxicity (Hassan et al., 2020; G. Li et al., 2021). Cadmium has been shown to reduce stomatal opening in leaves by disrupting guard cell function (G. Li et al., 2021; Seregin and Ivanov, 2001). With reduced stomatal opening, leaf area, and increased leaf curling there is increased water stress placed on the plant and less CO_2 exchange occurring at the leaf surface (G. Li et al., 2021; Seregin and Ivanov, 2001). There is an indirect reduction in photosynthesis and increased chlorosis, which is caused by chloroplast degradation, reduced chlorophyll and carotenoid synthesis, and inhibition of enzymes in the Calvin cycle (Baker and Walker, 1989; Dobrikova et al., 2021; Singh et al., 2016). Cadmium also inhibits the electron transport chains in the chloroplast and mitochondria (Seregin and Ivanov, 2001; Singh et al., 2016). In the chloroplast, Cd inhibits photosynthesis by disrupting the electron transport flow by stealing the electrons in transport to photosystem II (Dobrikova et al., 2021). In the mitochondria, Cd disrupts the oxidative phosphorylation electron transport flow (Seregin and Ivanov, 2001; Singh et al., 2016). The Krebs cycle is also sensitive to Cd due to the reduction in production and activity of enzymes used (Seregin and Ivanov, 2001; Singh et al., 2016). In both cases, there is reduced ATP production and therefore reduced energy for growth and cellular processes. Cadmium impairs Rubisco synthesis and function by interfering with enzyme function resulting in reduced CO_2 fixation (Nagajyoti et al., 2010; Singh et al., 2016).

Both root and shoot growth are inhibited by Cd (Hassan et al., 2020; Jung et al., 2020). Root and shoot morphology are altered with fewer lateral roots produced and less branching of the stem (Baker and Walker, 1989; Peco et al., 2020). Cadmium can also cause reduced seed germination and interfere with cell division (Singh et al., 2016). Cadmium causes reduced growth through altered cell structure, apoptosis, and reduced nutrients and energy production (Dobrikova et al., 2021).

1.1.4 Mechanisms of cadmium tolerance in plants

Plants have three primary mechanisms to reduce metal toxicity to roots (Figure 1-1). The first is the formation of a plaque (layers of oxidized compounds on the root

surface), which form complexes with Cd^{2+} that precipitate Cd^{2+} out of solution, thereby, preventing it from entering the plant (Cao et al., 2018; Chen et al., 2014; Liu et al., 2021; Tai et al., 2018). The main oxidized compounds that form the plaque are Fe-hydroxides and Fe-oxides, which precipitate on the surface of roots (Cao et al., 2018; Yang et al., 2016). While most plants can produce a plaque on their roots, it is typically seen in aquatic plants or plants grown in hydroponic solution.

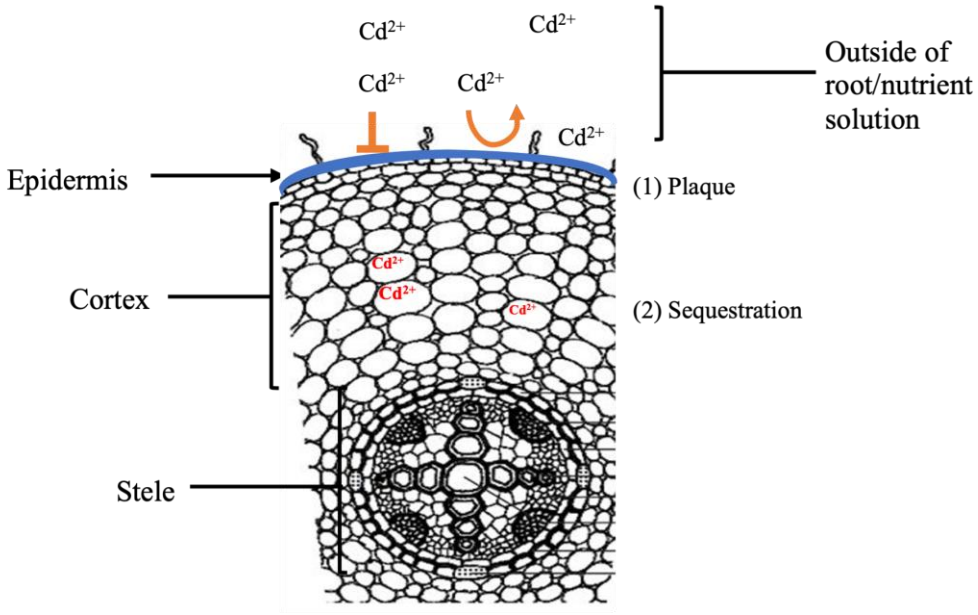

Figure 1-1. Mechanisms of metal tolerance in the root of a plant. In addition to binding of Cd^{2+} to cell walls, cadmium toxicity can be reduced by (1) the formation of a plaque on the surface of the root and (2) the sequestration of metals in the vacuoles of cortex cells. The orange "T" bar indicates adsorption and precipitation of Cd ions to Fe-hydroxides on the root plaque; curved arrow represents Cd ions returning to the hydroponic solution. The black band around the endodermal cells which surround the stele is the Casparian band. Image modified from Tutors Globe©.

The second mechanism is adsorption of metal ions to cell walls, especially those of the root epidermis (Fan et al., 2011; Nishizono et al., 1989). This traps Cd in the extracellular matrix, reducing its toxicity. Plants can alter their cell wall composition through the deposit of lignin, suberin, pectin, cellulose and hemicellulose (Fan et al., 2011; Jia et al., 2020; Wan and Zhang, 2012; Wu et al., 2020). This helps to reduce the amount of Cd that can enter a cell by increasing the amount of Cd that is adsorbed to the cell wall (Fan et al., 2011; Jia et al., 2020; Nishizono et al., 1989). Located between root endodermal cells that surround the stele, the Casparian band is a physical barrier to apoplastic transportation within a root (Tester and Leigh, 2001; Tylová et al., 2017). This

band contains large amounts of suberin and can be altered in response to abiotic stress (Tylová et al., 2017). When corn was grown in the presence of Cd, suberin increased in concentration within cell walls and the Casparian band in roots widened (Tylová et al., 2017). By increasing the width of the Casparian band and the amount of structural components of cell walls, there is reduced transportation and diffusion of Cd within a plant (Fan et al., 2011; Tylová et al., 2017; Wu et al., 2020), thereby reducing Cd toxicity. If a plant can sequester Cd within its roots, then that would result in less toxicity and less health risk for consumers of the aboveground portions of the plant.

The third mechanism is the production of chelators, including thiol-containing phytochelatins, which form complexes with metal ions (especially Cd^{2+}) that are transported into vacuoles where the metal ions remain sequestered, thereby reducing Cd translocation from the roots to the shoots (Akhter et al., 2012; Li et al., 2017; Seregin and Ivanov, 2001). Within a cell, free Cd^{2+} or chelator-bound Cd can be transported into the vacuole either by a H^+/metal cation antiport transporter or through an ATP-dependent chelator transporter (P. Li et al., 2021; Park et al., 2012; Raskin et al., 1998). Once inside the vacuole the chelator-bound Cd is removed from the chelator and binds to organic acids which have a higher affinity for Cd^{2+} than the chelators (Seregin and Ivanov, 2001). The chelator is then removed from the vacuole and reused to bind more Cd^{2+} (Seregin and Ivanov, 2001).

Other chelating compounds produced by plants to reduce Cd and metal toxicity are metallothioneins (MTs) and organic acids (Clemens, 2001; Cobbett, 2000; Songlin Zhang et al., 2020). While phytochelatins are produced mainly in response to Cd and other toxic metals, MTs are constitutively produced in plants (Cobbett, 2000). These MTs are used to regulate essential metals such as Zn within a cell but can also be used to bind to Cd to reduce its toxicity (Clemens, 2001; Clemens and Simm, 2003; Lee et al., 2004). Organic acids are naturally produced in plants and are used to reduce metal toxicity via metal chelation (Songlin Zhang et al., 2020). Organic acid chelators are found both within a plant and are also exuded from roots to bind to metals in the surrounding environment (Clemens, 2001; Kazemi Movahed, 2020; Songlin Zhang et al., 2020).

These organic acids have a high affinity for metal ions as they can contain thionyl- or carbonyl- groups (Clemens, 2001; Songlin Zhang et al., 2020).

1.2 Role of sulfur in plants

1.2.1 Sources of sulfur in the environment

Main sources of S in the environment formerly included acid rain due to high levels of atmospheric pollution (Chinoim et al., 1997). However, because of environmental reforms and reduced pollution emissions, the amount and frequency of acidic rain has decreased (Chinoim et al., 1997). Reduced deposition of atmospheric S is most prevalent in agricultural fields, which has caused diminishing concentrations of S in soils and the need for some farmers to supplement their fields with S (Chinoim et al., 1997). This supplementation comes in the form of fertilizers that also contain nitrogen and phosphorus. Another form of S addition to the environment is through S-reducing bacteria, which reduce elemental S and release bioavailable sulphate (SO_4^{2-}) (Chinoim et al., 1997; Komarnisky et al., 2003). The application of compost and manure could also introduce S into fields much the same as sewage sludge application. The main form of S that plants take up from the environment is SO_4^{2-} (Chinoim et al., 1997; Komarnisky et al., 2003). Other sources of S that can be used by plants include sulfite, sulfide, and hydrogen sulfide (Chinoim et al., 1997; Komarnisky et al., 2003).

1.2.2 Uses of sulfur in plants

Sulfur is essential for plant growth and development as it is a key component of the amino acids methionine and cysteine (Cys), which are incorporated into some enzymes and most proteins (Giovanelli, 1971). The S in Cys is used in tertiary protein structure formation through the linking of disulfide bridges (Giovanelli, 1971; Komarnisky et al., 2003). Thus, plants rely heavily on the presence of S in their environment for continued growth.

Sulfur is an essential component of chelators that contain thiol (-SH) groups such as phytochelatin (PC), Gamma-Glutamyl-Cysteine (γ-Glu-Cys), and glutathione (GSH); many of these molecules are important for reducing oxidative stress and promoting metal

tolerance (Cao et al., 2015, 2018; Li et al., 2017; Seregin and Ivanov, 2001; Xiao et al., 2020). However, in soybean, and most bean species, homophytochelatin (hPC) and homoglutathione (hGSH) are more abundant than PC and GSH, respectively (Saboori-Robat et al., 2019). The difference between GSH and hGSH is that GSH contains a terminal glycine (Gly) amino acid (γ-Glu-Cys-Gly) (Moran et al., 2000), while hGSH contains a terminal beta-alanine (βAla) amino acid (γ-Glu-Cys-βAla) (Carnegie, 1963). The difference between PC and hPC is that PC contains a terminal glycine (Gly) amino acid ($(\gamma\text{-Glu-Cys})_n$-Gly) where n = 2-11, while hPC contains a terminal beta-alanine (βAla) amino acid ($(\gamma\text{-Glu-Cys})_n$-βAla) where n = 2-11 (Grill et al., 1986).

The significance of these SH-containing chelators (also called non-protein thiols) is that they all contain cysteine residues and can be used in the synthesis of larger chelator molecules (Giovanelli, 1971). Cysteine is a precursor to larger chelator molecules such as γ-Glu-Cys (Moran et al., 2000). While γ-Glu-Cys can chelate metals, it is not found in abundance when soybean experiences metal stress. Instead, γ-Glu-Cys is used to synthesize hGSH using hGSH synthetase (Moran et al., 2000). Homoglutathione molecules are combined to form hPC (mainly hPC2) using hPC synthetase (Grill et al., 1986; Saboori-Robat et al., 2019). These two compounds, hGSH and hPC2, are used by soybean to reduce metal and ROS toxicity when metal concentrations build-up past a threshold (Grill et al., 1986; Moran et al., 2000). Due to the large size of hPCs they can chelate multiple metal ions at one time, depending on how long the chain is (Clemens and Simm, 2003). Both γ-Glu-Cys and hPC2 are produced in low concentrations when a plant is not under stress, as the γ-Glu-Cys is more of a precursor molecule and hPC2 is more specialized for metal and ROS targeting (Grill et al., 1986; Moran et al., 2000). Soybean constitutively produce hGSH as it is used as a ROS regulator. Most of the chelators a plant produces are in the roots rather than in the shoots (Moran et al., 2000). This is likely because the roots are where the uptake of nutrients and potentially toxic compounds occurs.

1.2.3 Interactions between cadmium and sulfur in plants

In addition to being an essential element for plant growth and development, S reduces Cd (and other metal) toxicity in many plants including rice (*Oryza sativa L.*)

(Cao et al., 2018), corn (*Zea mays L.*) (Li et al., 2017) wheat (*Triticum aestivum L.*) (Matraszek-Gawron and Hawrylak-Nowak, 2019), barley (*Hordeum vulgare L.*) (Astolfi et al., 2012), foxtail millet (*Setaria italica L.*) (Han et al., 2018), and *Arabidopsis thaliana* (Yamaguchi et al., 2016). In solution, Cd and S can bind to each other and form $CdSO_4$; however, this is unlikely in plants, as Cd readily binds to other ions and molecules.

The availability of S can influence the interactions between Cd and S in plants. If S supply is low, then there would be less expression of Cd tolerance mechanisms. If there is an increase in S supply then there would be an increased production of chelators, especially in the presence of a stressor such as Cd (Cao et al., 2018). Binding of Cd to cell walls would be the next important mechanism affected by S supply. The importance of S is that S can increase Cd adsorbed to cell walls and reduce Cd transportation across the cell wall through increased Cd-S complex formation and SH-containing cell wall components (Jia et al., 2020). Formation of a root plaque is the least important mechanism for Cd tolerance of the three primary mechanisms plants use to increase Cd tolerance. The addition of S increases plaque formation on the surface of roots, which helps to reduce Cd uptake into the roots (Cao et al., 2018). This is done by increasing the concentration of Fe^{2+} on the surface of the root resulting from the reduction of S^{2-} (Cao et al., 2018). The downside to this plaque formation is reduced water and nutrient uptake at the site of plaque formation.

In rice, Cao et al. (2018) reported that the addition of 5.28 mM S (double the concentration used in their control nutrient solution) enhanced both the formation of root plaques and subsequent external complexation of Cd, as well as the internal production of phytochelatins and subsequent chelation and sequestration of Cd in vacuoles. It is not yet known if S will have the same stimulatory effects on Cd exclusion, vacuolar sequestration, and reduced toxicity in soybean. The addition of exogenous S should increase the formation of plaques on the root surface, the production of chelators in the plant, and sequestration of Cd in cell vacuoles, thereby reducing Cd uptake, translocation, and toxicity (Cao et al., 2018; Jia et al., 2020; Li et al., 2017; Xiao et al., 2020).

1.3 Soybean importance and study aim

Soybean (*Glycine max L.*) is an important economic crop plant and very important as a food source for humans and livestock (Grassini et al., 2020; Zhi et al., 2020). Soybean is in the top five cultivated crop species in Canada and results in millions of dollars of revenue a year worldwide (Ciampitti and Salvagiotti, 2018; Hungria et al., 2005; Keyser and Li, 1992). Soybean is also the fourth most important crop globally (Ciampitti and Salvagiotti, 2018; Grassini et al., 2020). Because soybeans can contribute to fixing nitrogen from the atmosphere, they add nitrogen to the soil in which they are grown (Hungria et al., 2005). This added nitrogen can be used by other crop species that are planted in the same field as the soybeans were grown in (Hungria et al., 2005).

Soybean is used in many foods including tofu, oils, protein powders, and edamame (Grassini et al., 2020; Keyser and Li, 1992; Naresh et al., 2019). Soybean is very rich in proteins and amino acids and is an excellent source of nutrition compared to other vegetables (Grassini et al., 2020; Naresh et al., 2019). Because soybeans contain all essential amino acids for human growth and development they are also commonly used in meat-substitutes and meat-alternatives (Grassini et al., 2020; Naresh et al., 2019). With the use of soybean in various food products increasing, there is now more of a need to reduce Cd and other toxic metals from entering and accumulating in soybean. Besides being used as a food source, soybean products are used in candle wax, cosmetic products, biofuels, oils, and adhesives (Keyser and Li, 1992). Soy products have a wide variety of other uses and further development of non-consumption and consumption uses are ongoing (Naresh et al., 2019).

Soybeans show the same symptoms of Cd toxicity as other plants such as chlorosis, leaf curling, reduced growth, and reduced yield (Shamsi et al., 2010). Soybeans also experience reduced biomass and increased stress in the presence of Cd (Shamsi et al., 2010). Based on my prediction that S would reduce Cd toxicity in soybean as it has been shown to do in other crop species (see section 1.2.3), my aim was to determine the physiological mechanisms by which S reduces the uptake and translocation of Cd in soybean. This information will inform agronomic practices to minimize accumulation of Cd in soybean plants and to increase plant growth in Cd-contaminated soil. The

mechanisms were determined by quantifying dry biomass, Cd localization and distribution in roots using histochemical analysis, Cd concentration in roots, shoots, and root plaque using inductively coupled plasma mass spectrometry (ICP-MS), and thiol-containing compounds in roots and shoots using high performance liquid chromatography (HPLC). This thesis follows the integrated article format. Chapter 2 contains the article that will be submitted for publication and Chapter 3 contains a more general discussion that includes the limitations of the work as well future research directions arising from my experiments.

1.4 References

Akhter, F., McGarvey, B., Macfie, S.M., 2012. Reduced translocation of cadmium from roots is associated with increased production of phytochelatins and their precursors. Journal of Plant Physiology 169, 1821–1829. https://doi.org/10.1016/j.jplph.2012.07.011

Amir, S.B., Rasheed, R., Ashraf, M.A., Hussain, I., Iqbal, M., 2021. Hydrogen sulfide mediates defense response in safflower by regulating secondary metabolism, oxidative defense, and elemental uptake under drought. Physiologia Plantarum 172, 795–808. https://doi.org/10.1111/ppl.13267

Aprile, A., De Bellis, L., 2020. Heavy metals accumulation, toxicity and detoxification in plants. International Journal of Molecular Sciences 21, 1–5.

Astolfi, S., Zuchi, S., Neumann, G., Cesco, S., di Toppi, L.S., Pinton, R., 2012. Response of barley plants to Fe deficiency and Cd contamination as affected by S starvation. Journal of Experimental Botany 63, 1241–1250. https://doi.org/10.1093/jxb/err344

Baker, A.J.M., Walker, P.L., 1989. Physiological responses of plants to heavy metals and the quantification of tolerance and toxicity. Chemical Speciation and Bioavailability 1, 7–17. https://doi.org/10.1080/09542299.1989.11083102

Benavides, M.P., Gallego, S.M., Tomaro, M.L., 2005. Cadmium toxicity in plants. Brazilian Journal of Plant Physiology 17, 21–34.

Bewers, J., Barry, P., MacGregor, D., 1987. Distribution and cycling of cadmium in the environment. Advances in Environmental Science and Technology 1–18.

Birke, M., Reimann, C., Rauch, U., Ladenberger, A., Demetriades, A., Jähne-Klingberg, F., Oorts, K., Gosar, M., Dinelli, E., Halamić, J., 2017. GEMAS: Cadmium distribution and its sources in agricultural and grazing land soil of Europe — original data versus clr-transformed data. Journal of Geochemical Exploration 173, 13–30. https://doi.org/10.1016/j.gexplo.2016.11.007

Callaghan, R.M., 2021. Cadmium. U.S. Geological Survey Minerals Yearbook 15.1–15.9.

Canadian Council of Ministers of the Environment, 1999. Canadian soil quality guidelines for the protection of environmental and human health. Canadian Environmental Quality Guidelines.

Cao, F., Cai, Y., Liu, L., Zhang, M., He, X., Zhang, G., Wu, F., 2015. Differences in photosynthesis, yield and grain cadmium accumulation as affected by exogenous cadmium and glutathione in the two rice genotypes. Plant Growth Regulation 75, 715–723. https://doi.org/10.1007/s10725-014-9973-1

Cao, Z.Z., Qin, M.L., Lin, X.Y., Zhu, Z.W., Chen, M.X., 2018. Sulfur supply reduces cadmium uptake and translocation in rice grains (*Oryza sativa* L.) by enhancing iron plaque formation, cadmium chelation and vacuolar sequestration. Environmental Pollution 238, 76–84. https://doi.org/10.1016/j.envpol.2018.02.083

Carnegie, P.R., 1963. Structure and properties of a homologue of glutathione. The Biochemical Journal 89, 471–478.

Cataldo, D.A., Garland, T.R., Wildung, R.E., 1983. Cd uptake kinetics in intact soybean plants. Plant Physiology 73, 844–848.

Chary, N.S., Kamala, C.T., Raj, D.S.S., 2008. Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. Ecotoxicology and Environmental Safety 69, 513–524. https://doi.org/10.1016/j.ecoenv.2007.04.013

Chen, J., 2007. Rapid urbanization in China: A real challenge to soil protection and food security. Catena 69, 1–15. https://doi.org/10.1016/j.catena.2006.04.019

Chen, M., Cao, L., Song, X., Wang, X., Qian, Q., Liu, W., 2014. Effect of Fe plaque and Se on Cd uptake and translocation in rice seedlings (*Oryza sativa*) grown in solution. International Journal of Agriculture & Biology 16, 1159–1164.

Chinoim, N., Lefroy, R.D.B., Blair, G.J., 1997. Effect of crop duration and soil type on the ability of soil sulfur tests to predict plant response to sulfur. Soil Research 35, 1131–1142.

Ciampitti, I.A., Salvagiotti, F., 2018. New insights into soybean biological nitrogen fixation. Agronomy Journal 110, 1185–1196. https://doi.org/10.2134/agronj2017.06.0348

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475–486. https://doi.org/10.1007/s004250000458

Clemens, S., Simm, C., 2003. *Schizosaccharomyces pombe* as a model for metal homeostasis in plant cells: The phytochelatin-dependent pathway is the main cadmium detoxification mechanism. New Phytologist 159, 323–330. https://doi.org/10.1046/j.1469-8137.2003.00811.x

Cobbett, C.S., 2000. Phytochelatins and their roles in heavy metal detoxification plants make two types of peptide metal-binding ligands: MTs and PCs. Plant Physiology 123, 825–832.

Dharma-Wardana, M.W.C., 2018. Fertilizer usage and cadmium in soils, crops and food. Environmental Geochemistry and Health 40, 2739–2759.

Dobrikova, A.G., Apostolova, E.L., Hanć, A., Yotsova, E., Borisova, P., Sperdouli, I., Adamakis, I.D.S., Moustakas, M., 2021. Cadmium toxicity in *Salvia sclarea* L.: An integrative response of element uptake, oxidative stress markers, leaf structure and photosynthesis. Ecotoxicology and Environmental Safety 209, 1–13. https://doi.org/10.1016/j.ecoenv.2020.111851

Environment Canada, 1994. Cadmium and its compounds. Environment Canada.

Fan, J.-L., Wei, X.-Z., Wan, L.-C., Zhang, L.-Y., Zhao, X.-Q., Liu, W.-Z., Hao, H.-Q., Zhang, H.-Y., 2011. Disarrangement of actin filaments and Ca^{2+} gradient by $CdCl_2$

alters cell wall construction in *Arabidopsis thaliana* root hairs by inhibiting vesicular trafficking. Journal of Plant Physiology 168, 1157–1167. https://doi.org/10.1016/j.jplph.2011.01.031

Ferreira, R.R., Fornazier, R.F., Vitória, A.P., Lea, P.J., Azevedo, R.A., 2002. Changes in antioxidant enzyme activities in soybean under cadmium stress. Journal of Plant Nutrition 25, 327–342.

François, M., Grant, C., Lambert, R., Sauvé, S., 2009. Prediction of cadmium and zinc concentration in wheat grain from soils affected by the application of phosphate fertilizers varying in Cd concentration. Nutrient Cycling in Agroecosystems 83, 125–133. https://doi.org/10.1007/s10705-008-9204-0

Giovanelli, J., 1971. Sulfur amino acids of plants: an overview. Methods in Enzymology 143, 419–426.

Grant, C.A., 2011. Phosphate effects on Cd accumulation in crops and soil. Pedologist 54, 143–155.

Grant, C.A., Buckley, W.T., Bailey, L.D., Selles, F., 1997. Cadmium accumulation in crops. Canadian Journal of Plant Science. Revue Canadienne de Phytotechnie 78, 1–17

Grant, C.A., Dribnenki, J.P., Bailey, L.D., 2000. Cadmium and zinc concentrations and ratios in seed and tissue of solin (cv Linola^{TM} 947) and flax (cvs McGregor and Vimy) as affected by nitrogen and phosphorus fertiliser and Provide (*Penicillium bilaji*). Journal of the Science of Food and Agriculture 80, 1735–1743.

Grassini, P., Cafaro La Menza, N., Rattalino Edreira, J.I., Monzón, J.P., Tenorio, F.A., Specht, J.E., 2020. Soybean, in: Crop physiology case histories for major crops. Elsevier, 282–319. https://doi.org/10.1016/B978-0-12-819194-1.00008-6

Grill, E., Gekeler, W., Winnacker, E.-L., Zenk, H.H., 1986. Homo-phytochelatins are heavy metal-binding peptides of homo-glutathione containing Fabales. FEBS Letters 205, 47–50.

Han, Y., Wu, M., Hao, L., Yi, H., 2018. Sulfur dioxide derivatives alleviate cadmium toxicity by enhancing antioxidant defense and reducing Cd^{2+} uptake and translocation in foxtail millet seedlings. Ecotoxicology and Environmental Safety 157, 207–215. https://doi.org/10.1016/j.ecoenv.2018.03.084

Hassan, M.J., Raza, M.A., Rehman, S.U., Ansar, M., Gitari, H., Khan, I., Wajid, M., Ahmed, M., Shah, G.A., Peng, Y., Li, Z., 2020. Effect of cadmium toxicity on growth, oxidative damage, antioxidant defense system and cadmium accumulation in two sorghum cultivars. Plants 9, 1–14. https://doi.org/10.3390/plants9111575

Hoskin, W.M.A., 1991. Cadmium in Canadian minerals yearbook 1990. Energy, Mines, and Resources Canada. 11–15.

Hungria, M., Franchini, J.C., Campo, R.J., Graham, P.H., 2005. The importance of nitrogen fixation to soybean cropping in South America, in: Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment 4, 25–39.

Järup, L., 2003. Hazards of heavy metal contamination. British Medical Bulletin 68, 167–182. https://doi.org/10.1093/bmb/ldg032

Jia, H., Wang, X., Shi, C., Guo, J., Ma, P., Ren, X., Wei, T., Liu, H., Li, J., 2020. Hydrogen sulfide decreases Cd translocation from root to shoot through increasing Cd accumulation in cell wall and decreasing Cd^{2+} influx in *Isatis indigotica*. Plant

Physiology and Biochemistry 155, 605–612. https://doi.org/10.1016/j.plaphy.2020.08.033

Jung, H. il, Lee, B.R., Chae, M.J., Lee, E.J., Lee, T.G., Jung, G.B., Kim, M.S., Lee, J., 2020. Ascorbate-mediated modulation of cadmium stress responses: reactive oxygen species and redox status in *Brassica napus*. Frontiers in Plant Science 11, 1–15. https://doi.org/10.3389/fpls.2020.586547

Kazemi Movahed, L., 2020. Soybean root exudates increase the physiological diversity of bacteria in cadmium-treated soil. Electronic Thesis and Dissertation Repository 6908. https://ir.lib.uwo.ca/etd/6908

Keyser, H.H., Li, F., 1992. Potential for increasing biological nitrogen fixation in soybean. Plant and Soil 141, 119–135

Keyster, M., Niekerk, L.A., Basson, G., Carelse, M., Bakare, O., Ludidi, N., Klein, A., Mekuto, L., Gokul, A., 2020. Decoding heavy metal stress signaling in plants: towards improved food security and safety. Plants 9, 1–26. https://doi.org/10.3390/plants9121781

Komarnisky, L.A., Christopherson, R.J., Basu, T.K., 2003. Sulfur: its clinical and toxicologic aspects. Nutrition 19, 54–61.

Krishnamurti, G.S.R., Huang, P.M., Kozak, L.M., Rostad, H.P.W., Rees, K.C.J. van, Rees, V., 1997. Distribution of cadmium in selected soil profiles of Saskatchewan, Canada: speciation and availability. Canadian Journal of Soil Science 77, 613–619.

Lee, J., Shim, D., Song, W.-Y., Hwang, I., Lee, Y., 2004. Arabidopsis metallothioneins 2a and 3 enhance resistance to cadmium when expressed in *Vicia faba* guard cells. Plant Molecular Biology 54, 805–815.

Li, C., Cornett, J., Ungar, K., 2003. Long-term decrease of cadmium concentrations in the Canadian Arctic air. Geophysical Research Letters 30, 60-1–60-4. https://doi.org/10.1029/2002gl016723

Li, G., Shah, A.A., Khan, W.U., Yasin, N.A., Ahmad, A., Abbas, M., Ali, A., Safdar, N., 2021. Hydrogen sulfide mitigates cadmium induced toxicity in *Brassica rapa* by modulating physiochemical attributes, osmolyte metabolism and antioxidative machinery. Chemosphere 263, 1–12. https://doi.org/10.1016/j.chemosphere.2020.127999

Li, M., Hao, P., Cao, F., 2017. Glutathione-induced alleviation of cadmium toxicity in *Zea mays*. Plant Physiology and Biochemistry 119, 240–249. https://doi.org/10.1016/j.plaphy.2017.09.005

Li, P., Luo, T., Pu, X., Zhou, Y., Yu, J., Liu, L., 2021. Plant transporters: roles in stress responses and effects on growth and development. Plant Growth Regulation 93, 253–266. https://doi.org/10.1007/s10725-020-00684-3

Liu, N., Lou, X., Li, X., Shuai, Z., Liu, H., Jiang, Z., Wei, S., 2021. Rhizosphere dissolved organic matter and iron plaque modified by organic amendments and its relations to cadmium bioavailability and accumulation in rice. Science of the Total Environment 792, 1–16. https://doi.org/10.1016/j.scitotenv.2021.148216

MacLatchy, J., 1992. Metals data from base metal smelters and refineries. Environment Canada, Industrial Programs Branch, Ottawa.

Mahmood, A., Malik, R.N., 2014. Human health risk assessment of heavy metals via consumption of contaminated vegetables collected from different irrigation sources

in Lahore, Pakistan. Arabian Journal of Chemistry 7, 91–99. https://doi.org/10.1016/j.arabjc.2013.07.002

Matraszek-Gawron, R., Hawrylak-Nowak, B., 2019. Sulfur nutrition level modifies the growth, micronutrient status, and cadmium distribution in cadmium-exposed spring wheat. Physiology and Molecular Biology of Plants 25, 421–432. https://doi.org/10.1007/s12298-018-00635-3

Moran, J.F., Aki Iturbe-Ormaetxe, I., Matamoros, M.A., Rubio, M.C., Clemente, M.R., Brewin, N.J., Becana, M., 2000. Glutathione and homoglutathione synthetases of legume nodules. Cloning, expression, and subcellular localization. Plant Physiology 124, 1381–1392.

Nagajyoti, P.C., Lee, K.D., Sreekanth, T.V.M., 2010. Heavy metals, occurrence and toxicity for plants: A review. Environmental Chemistry Letters 8, 199–216. https://doi.org/10.1007/s10311-010-0297-8

Naresh, S., Ong, M.K., Thiagarajah, K., Muttiah, N.B.S.J., Kunasundari, B., Lye, H.S., 2019. Engineered soybean-based beverages and their impact on human health. Non-Alcoholic Beverages 6, 329–361. https://doi.org/10.1016/B978-0-12-815270-6.00011-6

Nawaz, H., Anwar-ul-Haq, M., Akhtar, J., Arfan, M., 2021. Cadmium, chromium, nickel and nitrate accumulation in wheat (*Triticum aestivum* L.) using wastewater irrigation and health risks assessment. Ecotoxicology and Environmental Safety 208, 1–8. https://doi.org/10.1016/j.ecoenv.2020.111685

Nazar, R., Iqbal, N., Masood, A., Khan, M.I.R., Syeed, S., Khan, N.A., 2012. Cadmium toxicity in plants and role of mineral nutrients in its alleviation. American Journal of Plant Sciences 03, 1476–1489. https://doi.org/10.4236/ajps.2012.310178

Nishizono, H., Kubota, K., Suzuki, S., Ishii, F., 1989. Accumulation of heavy metals in cell walls of *Polygonum cuspidatum* roots from metalliferous habitats. Plant and Cell Physiology 30, 595–598.

Park, J., Song, W.Y., Ko, D., Eom, Y., Hansen, T.H., Schiller, M., Lee, T.G., Martinoia, E., Lee, Y., 2012. The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. Plant Journal 69, 278–288. https://doi.org/10.1111/j.1365-313X.2011.04789.x

Peco, J.D., Campos, J.A., Romero-Puertas, M.C., Olmedilla, A., Higueras, P., Sandalio, L.M., 2020. Characterization of mechanisms involved in tolerance and accumulation of Cd in *Biscutella auriculata* L. Ecotoxicology and Environmental Safety 201, 1–13. https://doi.org/10.1016/j.ecoenv.2020.110784

Peralta-Videa, J.R., Lopez, M.L., Narayan, M., Saupe, G., Gardea-Torresdey, J., 2009. The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. International Journal of Biochemistry and Cell Biology 41, 1665–1677. https://doi.org/10.1016/j.biocel.2009.03.005

Raskin, I., Salt, D.E., Smith, R.D., 1998. Phytoremediation. Plant Molecular Biology 49, 643–668.

Sabiha-Javied, Mehmood, T., Chaudhry, M.M., Tufail, M., Irfan, N., 2009. Heavy metal pollution from phosphate rock used for the production of fertilizer in Pakistan. Microchemical Journal 91, 94–99. https://doi.org/10.1016/j.microc.2008.08.009

Saboori-Robat, E., Joshi, J., Pajak, A., Renaud, J., Marsolais, F., Saboori-Robat, E., Sol, M., Maccelli, C., Joshi, J., Marsolais, F., Joshi, J., Mohsenpour, M., 2019. Common

bean (*Phaseolus vulgaris* L.) accumulates most s-methylcysteine as its γ-glutamyl dipeptide. Plants 8, 1–15. https://doi.org/10.3390/plants8050126

Seregin, I. v., Ivanov, V.B., 2001. Physiological aspects of cadmium and lead toxic effects on higher plants. Russian Journal of Plant Physiology 48, 523–544.

Shamsi, I.H., Jiang, L., Wei, K., Jilani, G., Hua, S., Zhang, G.P., 2010. Alleviation of cadmium toxicity in soybean by potassium supplementation. Journal of Plant Nutrition 33, 1926–1938. https://doi.org/10.1080/01904167.2010.512052

Sheoran, V., Sheoran, A.S., Poonia, P., 2010. Soil reclamation of abandoned mine land by revegetation: a review. International Journal of Soil, Sediment and Water 3, 1–20.

Sheppard, S.C., Grant, C.A., Sheppard, M.I., de Jong, R., Long, J., 2009. Risk indicator for agricultural inputs of trace elements to Canadian soils. Journal of Environmental Quality 38, 919–932. https://doi.org/10.2134/jeq2008.0195

Singh, S., Singh, A., Bashri, G., Prasad, S.M., 2016. Impact of Cd stress on cellular functioning and its amelioration by phytohormones: An overview on regulatory network. Plant Growth Regulation 80, 253–263. https://doi.org/10.1007/s10725-016-0170-2

Song, Y., Jin, L., Wang, X., 2017. Cadmium absorption and transportation pathways in plants. International Journal of Phytoremediation 19, 133–141. https://doi.org/10.1080/15226514.2016.1207598

Tai, Y., Tam, N.F.Y., Wang, R., Yang, Yang, Lin, J., Wang, J., Yang, Yufen, Li, L., Sun, Y., 2018. Iron plaque formation on wetland-plant roots accelerates removal of water-borne antibiotics. Plant and Soil 433, 323–338. https://doi.org/10.1007/s11104-018-3843-y

Tester, M., Leigh, R.A., 2001. Partitioning of nutrient transport processes in roots. Journal of Experimental Botany 52, 445–457.

Tóth, G., Hermann, T., da Silva, M.R., Montanarella, L., 2016. Heavy metals in agricultural soils of the European Union with implications for food safety. Environment International 88, 299–309. https://doi.org/10.1016/j.envint.2015.12.017

Tylová, E., Pecková, E., Blascheová, Z., Soukup, A., 2017. Casparian bands and suberin lamellae in exodermis of lateral roots: An important trait of roots system response to abiotic stress factors. Annals of Botany 120, 71–85. https://doi.org/10.1093/aob/mcx047

Wan, L., Zhang, H., 2012. Cadmium toxicity. Plant Signaling & Behaviour 7, 345–348. https://doi.org/10.4161/psb.18992

Wu, X., Song, H., Guan, C., Zhang, Z., 2020. Boron mitigates cadmium toxicity to rapeseed (*Brassica napus*) shoots by relieving oxidative stress and enhancing cadmium chelation onto cell walls. Environmental Pollution 263, 1–9. https://doi.org/10.1016/j.envpol.2020.114546

Xiao, Q., Wang, Y., Lü, Q., Wen, H., Han, B., Chen, S., Zheng, X., Lin, R., 2020. Responses of glutathione and phytochelatins biosynthesis in a cadmium accumulator of *Perilla frutescens* (L.) Britt. under cadmium contaminated conditions. Ecotoxicology and Environmental Safety 201, 1–10. https://doi.org/10.1016/j.ecoenv.2020.110805

Xing, W., Liu, H., Banet, T., Wang, H., Ippolito, J.A., Li, L., 2020. Cadmium, copper, lead and zinc accumulation in wild plant species near a lead smelter. Ecotoxicology and Environmental Safety 198, 1–9. https://doi.org/10.1016/j.ecoenv.2020.110683

Yamaguchi, C., Takimoto, Y., Ohkama-Ohtsu, N., Hokura, A., Shinano, T., Nakamura, T., Suyama, A., Maruyama-Nakashita, A., 2016. Effects of cadmium treatment on the uptake and translocation of sulfate in *Arabidopsis thaliana*. Plant and Cell Physiology 57, 2353–2366. https://doi.org/10.1093/pcp/pcw156

Yan, Y., Sun, Q., Yang, J., Zhang, X., Guo, B., 2021. Source attributions of cadmium contamination in rice grains by cadmium isotope composition analysis: a field study. Ecotoxicology and Environmental Safety 210, 1–7. https://doi.org/10.1016/j.ecoenv.2020.111865

Yang, Junxing, Liu, Z., Wan, X., Zheng, G., Yang, Jun, Zhang, H., Guo, L., Wang, X., Zhou, X., Guo, Q., Xu, R., Zhou, G., Peters, M., Zhu, G., Wei, R., Tian, L., Han, X., 2016. Interaction between sulfur and lead in toxicity, iron plaque formation and lead accumulation in rice plant. Ecotoxicology and Environmental Safety 128, 206–212. https://doi.org/10.1016/j.ecoenv.2016.02.021

Zhang, Songlin, Chen, H., He, D., He, X., Yan, Y., Wu, K., Wei, H., 2020. Effects of exogenous organic acids on Cd tolerance mechanism of *Salix variegata* Franch. under Cd stress. Frontiers in Plant Science 11, 1–13. https://doi.org/10.3389/fpls.2020.594352

Zhang, Shuo, Li, Q., Nazir, M.M., Ali, S., Ouyang, Y., Ye, S., Zeng, F., 2020. Calcium plays a double-edged role in modulating cadmium uptake and translocation in rice. International Journal of Molecular Sciences 21, 1–18. https://doi.org/10.3390/ijms21218058

Zhao, F.-J., Hamon, R.E., Lombi, E., Mclaughlin, M.J., Mcgrath, S.P., 2002. Characteristics of cadmium uptake in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. Journal of Experimental Botany 53, 535–543.

Zhi, Y., Sun, T., Zhou, Q., Leng, X., 2020. Screening of safe soybean cultivars for cadmium contaminated fields. Scientific Reports 10, 12965. https://doi.org/10.1038/s41598-020-69803-4

Chapter 2

2 Additional sulfur does not alleviate cadmium toxicity in soybean

With the increase in urbanization globally, there are increasing levels of environmental contamination (Chen, 2007). These environmental contaminants range from abiotic to biotic contaminants and can have disastrous effects on local ecosystems and agricultural production. One of the most dangerous contaminants are toxic non-essential metals (Peralta-Videa et al., 2009). These are hazardous because they are not used by plants and animals for cellular functions, can interfere with essential metals within cells, and can cause DNA damage and cell death. Many of these metals, such as Cd, are toxic at very low concentrations, which makes them an important area of research (Benavides et al., 2005). A plant can take up and bioaccumulate Cd, but because plants cannot excrete the Cd once it has been taken up the Cd remains in the plant (Chary et al., 2008). If that plant is eaten, then the metal is transferred to the other organism and may result in toxic metal propagation up the food chain (Järup, 2003).

2.1 Sources of cadmium and its effect on plants

For Canadian agricultural soils, Cd is one of the more dangerous and monitored toxic metals (Grant et al., 1997). Cadmium contamination of soils arises from anthropogenic activities. These activities include impure phosphate fertilizer application, mining and smelting runoff, industrial waste and pollution, and sewage sludge application to crop fields (Canadian Council of Ministers of the Environment, 1999; Nawaz et al., 2021; Sabiha-Javied et al., 2009; Xing et al., 2020). The dangers of Cd come from its severe toxicity and low dosage required to cause toxicity (Canadian Council of Ministers of the Environment, 1999; Environment Canada, 1994). Bioavailability of Cd plays a large role in regulating accumulation in plants, as plants can only take up Cd that is available to them in the soil solution (Environment Canada, 1994; Sheoran et al., 2010). The bioavailability of Cd is influenced by soil properties (Environment Canada, 1994; Sheoran et al., 2010). A more acidic soil will contain more ionic Cd^{2+}, which can easily move through soil solution and interact with plants, as it is

very water soluble (Environment Canada, 1994; Krishnamurti et al., 1997). Soil texture and physical properties also impact Cd availability by increasing or decreasing Cd bound to soil particles or organic compounds in soil (Krishnamurti et al., 1997; Sheoran et al., 2010).

There are many mechanisms for Cd tolerance in plants that also work for other metals. The three main mechanisms used by plants to reduce toxicity of Cd are: (1) reduced uptake of Cd ions into the roots, (2) reduced movement of Cd into root cells and into the stele, and (3) sequestration and compartmentalization of Cd in cell vacuoles. The first mechanism involves increased root plaque formation to physically block and chemically bind Cd ions on the surface of roots (Cao et al., 2018; Tai et al., 2018). This plaque consists of layers of oxidized Fe-hydroxides and Fe-oxides on the surface of the root (Chen et al., 2014; Liu et al., 2004; Tai et al., 2018). The Cd^{2+} ions are bound to the plaque and Cd-oxides precipitate out of soil solution, which reduces entry into the roots (Cao et al., 2018; Chen et al., 2014; Liu et al., 2021; Tai et al., 2018). The second mechanism involves increased structural component deposition in and on cell walls, which involves increased synthesis and deposition of lignin, cellulose, hemicellulose, and pectin into and onto the cell wall structure (Fan et al., 2011; Jia et al., 2020; Nishizono et al., 1989; Wu et al., 2020). Increased binding of Cd to cell walls reduces Cd translocation and movement from roots to shoot (Jia et al., 2020; Wan and Zhang, 2012). Along with cell wall adsorption, the Casparian band helps regulate extracellular movement from the cortex of the root to the stele providing a physical barrier to apoplastic movement, thereby regulating translocation from roots to shoots (Tester and Leigh, 2001; Tylová et al., 2017). The Casparian band widens in response to Cd stress to reduce the amount of Cd that can enter the xylem and be transported to the shoots (Tylová et al., 2017). The third and final mechanism involves the increased production of chelators within a plant. These chelators include SH-containing molecules that bind to Cd and transport it to the vacuoles of cells (Cao et al., 2018; Seregin and Ivanov, 2001). Within the vacuole the Cd is sequestered and made insoluble by binding with organic acids, which reduces its toxicity, transportation, and translocation (Cao et al., 2018; Li et al., 2017; Seregin and Ivanov, 2001). The main chelator that plants use is PC (or hPC in beans), which has a high affinity for metal ions (Cobbett, 2000).

The above mechanisms to reduce Cd uptake and tolerance cannot completely prevent Cd stress if the amount of Cd oversaturates these mechanisms. When the mechanisms are saturated, intracellular Cd inhibits a wide range of physiological processes including enzyme activity, water balance, and photosynthesis (Benavides et al., 2005; G. Li et al., 2021; Seregin and Ivanov, 2001). Cadmium-induced leaf curling, reduced leaf area and inhibition of critical enzymes contribute to poor water balance and impaired photosynthesis (G. Li et al., 2021; Seregin and Ivanov, 2001). The primary impacts of Cd toxicity in plants are oxidative stress, caused by an increase of reactive oxygen species (ROS), and water stress due to reduced leaf area and increased stomatal closure (Benavides et al., 2005; G. Li et al., 2021; Nazar et al., 2012; Seregin and Ivanov, 2001). Increased ROS production results in DNA, protein, and lipid damage (Benavides et al., 2005; Hassan et al., 2020; Jung et al., 2020; Seregin and Ivanov, 2001), decreased membrane fluidity and altered cell morphology (Benavides et al., 2005; Nazar et al., 2012) eventually causing cell death. Symptoms of Cd toxicity in a plant include chlorosis of the leaves, necrosis of tissues, mild to severe growth reduction, and reduced plant yield (Benavides et al., 2005; Nazar et al., 2012; Seregin and Ivanov, 2001).The relatively low concentration of Cd required to cause stress and be toxic to a plant means these symptoms become apparent very quickly and plant growth is easily impaired. By alleviating these symptoms and reducing toxicity, agricultural yield can be increased in mildly contaminated soils.

2.2 Sources of sulfur and its role in plants

The main form of environmental S is SO_4^{2-} with sulfite and sulfide occurring less frequently (Komarnisky et al., 2003; Yi et al., 2010). Because of reductions in S pollution to the atmosphere there are less instances of acid rain and less S addition to the environment (Chinoim et al., 1997). As a result, there are now very low concentrations of S in crop soil and fields (Chinoim et al., 1997). To correct for this, S fertilization practices are now more common and excess S is applied to crop fields to promote increased growth and yield (Chinoim et al., 1997).

Plants will take up SO_4^{2-} from the soil as their main S supply (Komarnisky et al., 2003; Yi et al., 2010). Internally, SO_4^{2-} is reduced to sulfide, which is used in the

synthesis of cysteine (Giovanelli, 1971; Yi et al., 2010). Many proteins and chelators contain cysteine (Giovanelli, 1971). The importance of cysteine in tertiary protein structure is to form disulfide bridges for protein stability (Komarnisky et al., 2003). Chelators take advantage of the S in cysteine as it is the thiol group that binds to metals during chelation (Moran et al., 2000; Yi et al., 2010). The main chelators in plants are GSH and PC, with hGSH and hPC being the most common forms in beans (Saboori-Robat et al., 2019). The only difference between the homo and non-homo forms of these molecules is that the homo forms contain a terminal βAla while the non-homo versions contain a terminal Gly (Carnegie, 1963). While chelators are typically produced only under stress, some are produced at baseline levels to prevent accumulation of side-products of cellular processes such as ROS production (Amir et al., 2021). Chelators can also be used to regulate intracellular concentrations of essential metals by binding to them and allowing the non-reactive chelate-metal molecule to be transported inter- and extracellularly (Clemens, 2001; Seregin and Ivanov, 2001).

2.3 Interactions between cadmium and sulfur in plants

Additional S supply has been shown to reduce Cd toxicity in rice (Cao et al., 2018) and wheat (Chen et al., 2007). Sulfur and Cd can directly interact with each other by binding together to form complexes (usually $CdSO_4$). This can occur in solution but is unlikely to occur in soil because Cd can more readily bind to other ions, molecules, and soil particles (Krishnamurti et al., 1997). Since S supply is essential for the production and formation of Cd tolerance mechanisms, additional S is expected to increase the efficacy of these mechanisms (Cao et al., 2018; Han et al., 2018). First, the addition of S increases root plaque formation (Cao et al., 2018; Li et al., 2018). Oxides in plaques form complexes with local metal ions, preventing them from entering the plant (Chen et al., 2014; Liu et al., 2004; Syu et al., 2013; Tai et al., 2018; Yang et al., 2016). Reduced Cd uptake should arise if S treatment results in the formation of plaques on the surface of roots (Cao et al., 2018; Yang et al., 2016). However, plaque formation can also reduce nutrient and water uptake as there is a physical barrier formed on the root surface. Second, increased S has been shown to increase Cd bound to cell walls, which reduces Cd transportation across the cell wall through increased pectin, cellulose, and

hemicellulose deposition (Fan et al., 2011; Jia et al., 2020). As a result of increased Cd adsorption to cell walls less Cd would be able to enter the cell resulting in less Cd toxicity (Jia et al., 2020; Nishizono et al., 1989). Third, because chelator concentrations are affected by supply of S and the presence of stressors within a plant, increased availability of S should enhance the synthesis of thiol-containing chelators (Cao et al., 2018; Li et al., 2017). This should result in increased chelation of Cd^{2+} ions and decreased Cd toxicity in roots. Reduced Cd translocation to shoots could also occur if S treatment results in increased sequestration in root cell vacuoles (Cao et al., 2018; Li et al., 2017; Seregin and Ivanov, 2001).

2.4 Rationale and hypotheses

Being one of the top five cultivated crops in Canada and in the top four cultivated crops worldwide, soybean is an important crop plant (Grassini et al., 2020; Zhi et al., 2020). Soy production is a multimillion-dollar industry ranging from plant growth to food production (Grassini et al., 2020). Soybean components are used in a variety of non-consumption purposes such as oil and candle production, as well as in the production of plant-based plastics. Another important feature of soybean is the fixation of nitrogen from the atmosphere and the subsequent release of nitrogen to the surrounding soil (Ciampitti and Salvagiotti, 2018; Hungria et al., 2005; Keyser and Li, 1992). This feature can be used to benefit crop rotation and reduce the need for nitrogen fertilizers (Hungria et al., 2005; Keyser and Li, 1992). With increasing contamination of agricultural soils with Cd, it is important to understand how agronomic practices such as S fertilization could reduce Cd toxicity in soybean.

Knowing the mechanism(s) of S-induced alteration of Cd uptake and translocation in soybean is important to the agricultural industry. If the primary mechanisms for S-related reduction of Cd toxicity involve reduced oxidative stress and/or vacuolar Cd sequestration, then one would expect S-treated plants to take up as much Cd as non-S-treated plants. On the other hand, if S treatment results in reduced uptake and translocation of Cd (Cao et al., 2018; Chen et al., 2007), then S-supplementation could be used in mildly contaminated soil to grow edible soybeans.

Specific research objectives were to determine if S: (i) increased soybean biomass in the presence of Cd, (ii) reduces Cd uptake into the roots by increased root plaque formation, (iii) increases the production of chelators in the roots and shoots, (iv) influences the distribution of Cd between the roots and shoots, and (v) alters localization of Cd in roots.

- Hypothesis 1: S will alter Cd uptake into the roots of the soybean due to external precipitation of Cd^{2+} in root plaques.

- Hypothesis 2: S will impact Cd translocation from root to shoot by the production of chelators that bind to and sequester Cd^{2+} in the roots.

2.5 Materials and methods

To study the effects of additional S on reducing Cd toxicity and the mechanisms involved in soybean, hydroponic and soil experiments were performed. The hydroponic experiments allowed for harvesting of full, intact roots for histochemical and root desorption experiments. However, they are not a realistic simulation of agricultural conditions and Cd-S interactions within soil. Soil experiments allowed for a more realistic effect of S addition in the presence of Cd in the growth medium but prohibited the use of the roots for histochemical or root desorption experiments.

2.5.1 Plant materials, growth conditions, and experimental treatments

Soybean (*Glycine max* (L.) Merr.) cv NK Syngenta Roundup Ready 2 XTend (variety: S09-R8X) seeds treated with optimized MD ST inoculant (containing *Bradyrhizobium japonicum* and lipo-chitooligosaccharide, groups 4/7/12 (phenylamide, carboxamide, and phenylpyrroles, respectively) fungicide, and group 28 (ryanodine receptor modulators) insecticide were provided by Caroline Rasenberg at the Environmental Sciences Western field station. Seeds were germinated on filter paper wetted with reverse osmosis (RO) water in Petri dishes in the dark for 72 h. Seedlings were grown in hydroponic culture (section 2.5.2) or potted topsoil (section 2.5.3) in a

controlled environment chamber set to 23 °C with a 16 h:8 h light:dark cycle, a light intensity of 187 ± 1.5 μmol•m^{-2}•sec^{-1} and 60% relative humidity.

2.5.2 Hydroponic experimental treatments

Growth conditions were as described by Akhter and Macfie (2012) with slight modifications. After 72 h, germinating seedlings were transferred to pots (15 cm diameter) filled with sand that was saturated with quarter-strength Hoagland's nutrient solution (Table 2-1; (Hoagland and Arnon, 1950)).

Table 2-1. Chemical composition of Hoagland's nutrient solution

	Hoagland's Solution
Macronutrients	Concentration (mM)
$Ca(NO_3)_2$•$4H_2O$	1.0
K_2HPO_4	1.0
KNO_3	0.4
$Mg(NO_3)_2$•$6H_2O$	0.3
NH_4NO_3	0.3
K_2SO_4	0.1
$FeCl_3$•$6H_2O$	0.01
Na_2EDTA	0.01
Micronutrients	Concentration (μM)
H_3BO_3	6.0
$MnCl_2$•$4H_2O$	2.0
$ZnSO_4$•$7H_2O$	0.5
$CuSO_4$•$5H_2O$	0.15
Na_2MoO_4	0.1

After four days, the plants were transferred to 1 L glass jars filled with half-strength Hoagland's nutrient solution set to pH 6.0 and containing the treatment conditions – Cd (0 or 0.05 μM $CdCl_2$) and S (0, 1, 5, or 10 mM Na_2SO_4) – in a full factorial design. The Cd concentration of 0.05 μM was chosen from a dose response curve that was generated in a preliminary experiment (Appendix A, Figure A1). This Cd concentration resulted in 20-30% reduction in dry biomass relative to control, which indicates a mild Cd stress on the plants (Baker and Walker, 1989). Having mild Cd stress on the plants allowed for observation of any effects the S addition might have on the Cd

stress because too much Cd stress could overshadow the beneficial effects of S addition. Sulfur concentrations were chosen based on Cao et al. (2018), who used concentrations of 0, 2.64, and 5.28 mM S, which represented no S, baseline S, and double S concentration based on Yoshida's nutrient solution. I used the same strategy by adding zero to 10 mM S, representing no, mild, moderate, and excess S in solution.

Trace amounts of S-containing compounds in the basal nutrient solution (i.e., K_2SO_4, $ZnSO_4 \cdot 7H_2O$, and $CuSO_4 \cdot 5H_2O$, ~ 0.1003 mM total) had negligible effects on the S treatment conditions. Visual Minteq, a chemical equilibrium model that estimates the chemical speciation of inorganic ions in aqueous solutions, was used to verify that the S treatments did not alter Cd availability to plants. As seen in Figure 2-1A, when control solution (no additional Na_2SO_4) was combined with 1 μM $CdCl_2$ at pH below 6 the model predicted virtually no Cd-S complex ($CdSO_4$) formation and most of the Cd was free Cd^{2+}. Among treatments with 1, 5, or 10 mM added Na_2SO_4 (Figure 2-1B-D), there was a predicted increase in $CdSO_4$ and a decrease in Cd^{2+} availability with increasing Na_2SO_4 concentrations. However, because $CdSO_4$ is soluble, bioavailable Cd was 100% across all pH settings.

In each jar, one seedling was suspended in a soft foam support that was placed in an opening of the lid of the jar. Solutions were aerated and the plants were provided with fresh nutrient solution (including the corresponding Cd and S treatments) every week. Plants were harvested approximately 14 days after being placed in the experimental treatments.

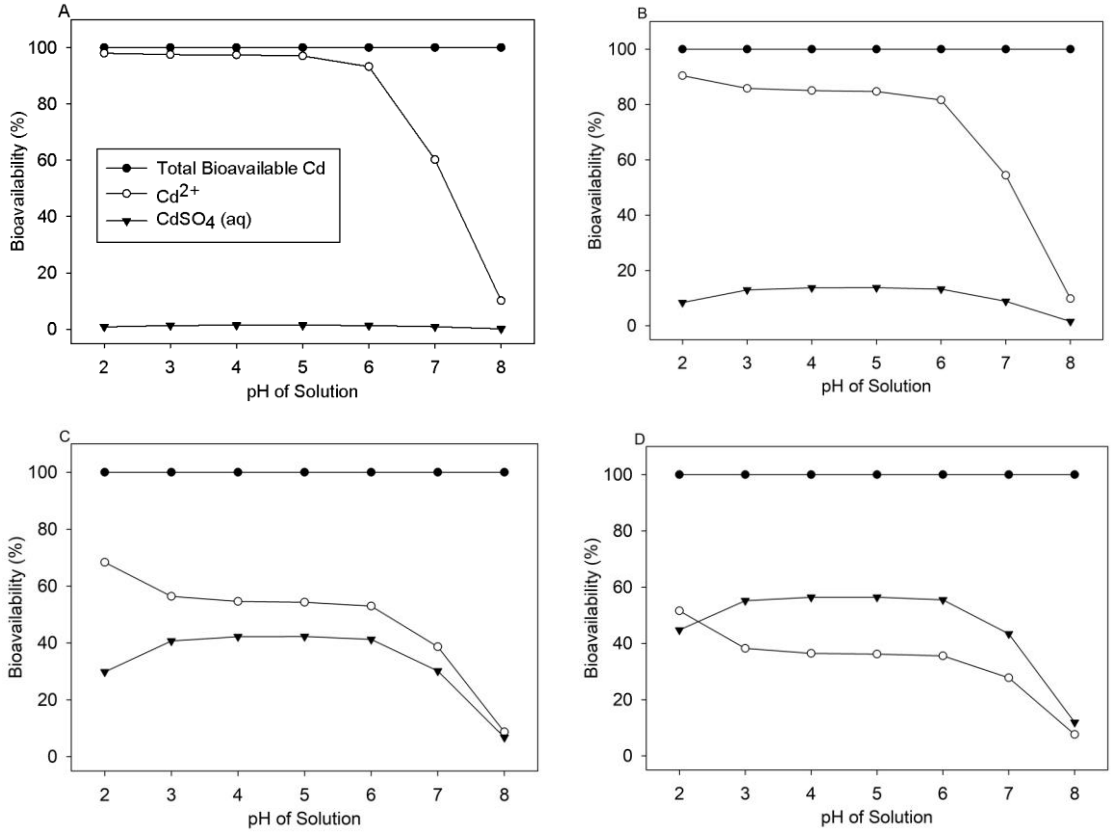

Figure 2-1. Bioavailability of cadmium in hydroponic solution. Bioavailable cadmium (Cd) chemical compounds were estimated using Visual Minteq for solutions with (A) no additional Na_2SO_4 (S), (B) 1 mM added S, (C) 5 mM added S, and (D) 10 mM added S. Closed circles indicate total bioavailable Cd in solution. Open circles indicate available Cd^{2+} in solution. Closed, inverted triangles indicate available $CdSO_4$ in solution.

Three sets of plants were grown in hydroponic solutions (Appendix B, Figure B1). One was used to measure dry biomass (section 2.5.4.1), Cd and S concentrations (2.5.7), and localization of Cd in root cross sections (section 2.5.5) with n=8. The second set was used to measure amounts of Cd adsorbed to the root surface (section 2.5.6) with n=4. The third set was used to measure concentrations of non-protein thiols (section 2.5.8) with n=3.

2.5.3 Soil experimental treatments

Topsoil was purchased from North London Landscape Supplies and three subsamples were sent to A&L Laboratories (London, ON) for the following physicochemical analyses. The soil texture was loam with about 50% sand, 30% silt, and

20% clay. Relative to typical agricultural soils in Ontario, the soil contained very low concentrations of sulfur (7 mg/kg), phosphorus (12 mg/kg) and sodium (11 mg/kg); low concentrations of magnesium (160 mg/kg); medium concentrations of potassium (110 mg/kg) and zinc (3.5 mg/kg); and very high concentrations of calcium (4100 mg/kg). The soil pH was around 8 and was left unadjusted for the experiment. The basal soil Cd concentration was 0.68 mg/kg before treatments were applied. The soil used was unfertilized and unaltered from purchase aside from adding the treatment conditions and sifting the soil.

Soil was sifted, first through a 1 cm sieve then through a 2 mm sieve to achieve uniform particle size and remove large debris, insects, worms, vegetation, and clay chunks. Perlite was added at 10% v:v to reduce soil clumping and maintain aeration. Soil was stored at 4°C prior to use to inhibit bacterial, fungal, and plant growth. Any seedlings that grew in the soil were removed to prevent nutrient loss both before and after the soil was added to pots for treatment.

Cadmium concentrations of 300 and 400 mg/kg were chosen from a dose response curve that was generated in a preliminary experiment. These concentrations induced a mild Cd stress of 20-30% reduction of dry biomass compared to control (Appendix A, Figure A2). Cd treatments (0, 300, or 400 mg/kg $CdCl_2$) were added to 7-inch pots filled with 750 g of soil two months prior to seedling placement to ensure equilibration of Cd in soil solution. Pots were initially watered to 70% field capacity, which is 70% of the volume of water that the soil can hold against gravity, then watered as needed (approximately every four days) to maintain this moisture level. The S treatments (0, 1, 5, or 10 mM Na_2SO_4) were applied three days prior to seedling placement to mimic fertilization of an agricultural field prior to sowing. The pots were covered with clear acrylic sheets during this time to reduce evaporative water loss.

After 72 h, germinating seedlings were transferred directly to pots that contained the treatment conditions: Cd (0, 300, or 400 mg/kg $CdCl_2$) and S (0, 1, 5, or 10 mM Na_2SO_4), in a full factorial design with three replicates per treatment. In the middle of each pot, one seedling was placed and partially covered with soil to reduce desiccation.

Plants were watered as needed (approximately every three days for the first week and then every two days thereafter) to prevent them from wilting. To maintain high humidity, pots were covered with clear acrylic sheets until the seedlings touched the sheets. Plants were harvested three weeks after being placed in soil.

One set of plants were grown for soil experiments (n=3) (Appendix B, Figure B2). For roots and shoots, biomass (section 2.5.4.2), concentrations of Cd and S (section 2.5.7) and concentrations of non-protein thiols (Section 2.5.8) were measured.

2.5.4 Biomass

2.5.4.1 Hydroponically-grown plants

Harvest procedures were performed as described by Akhter and Macfie (2012) with slight modifications. At harvest, roots and shoots were separated, rinsed with RO water, and blotted dry. The effects of S on Cd toxicity were determined by measuring plant biomass. Plants were individually placed into small paper bags and immediately placed in an oven and dried to constant weight at 60 ℃, after which dry mass was recorded. Dried tissues were used for the quantification of Cd and S concentrations in roots and shoots (section 2.5.7).

2.5.4.2 Soil-grown plants

Plants were removed from pots and the loose soil was carefully separated from the roots by hand to avoid root breakage. Roots were then rinsed in RO water baths to gently remove remaining soil and perlite. Once roots were sufficiently rinsed, they were patted dry, separated from the stem, and immediately placed in liquid nitrogen for determination of non-protein thiol concentration (section 2.5.8). Shoots were then placed into small paper bags and immediately placed in an oven and dried to constant weight at 60 ℃, after which dry mass was recorded. Dried shoot tissues were used for the quantification of Cd concentrations and for determination of S concentrations in shoots (section 2.5.7).

2.5.5 Histochemical localization of cadmium in the roots

Subcellular localization of Cd in the roots was determined using the methods of Akhter et al. (2014). Fresh samples (approx. 3.0 g) of each root were rinsed with RO water, fixed in 2% (v/v) glutaraldehyde, and kept at room temperature overnight. Tissue subsamples were removed from the glutaraldehyde solution and 80-100 micron-thick cross sections were taken, by hand, 1 cm above the root tip. This allowed for sampling of mature cells. Sections were placed on a glass slide with 2-3 drops of dithizone, which produces an insoluble red salt in the presence of Cd, and covered with a cover slip. Samples were immediately viewed using compound microscopy, and digital images were captured. The general patterns of Cd localization (e.g., in the outer cell layers, cortex, endodermis, and stele) were observed.

2.5.6 Desorption of root plaque

To estimate the amount of Cd in the root plaque, roots were processed. Desorption of Cd from the root surface (i.e., in the plaque) was done according to Wang et al. (2009) with slight modification. Plants were removed from hydroponic solution and dipped into a beaker of RO water to remove any nutrient solution on the roots. The plants were placed on paper towel and blotted dry. The entire root system was separated from the stem and placed in a 40 mL solution of dithionite-citrate-bicarbonate (DCB) for 60 mins at room temperature (~23 °C). The DCB solution consisted of 0.03 M sodium citrate, 0.125 M sodium bicarbonate, and 0.11 M sodium dithionite. The DCB solution was used to remove the plaque from the surface of the roots. The roots were incubated on an orbital mixer at 100 rpm to allow for gentle agitation in solution. After incubation in the DCB solution, the solution was filtered through VWR® Grade 413 filter paper into 50 mL Falcon® tubes. Roots were rinsed with RO water, which was added to the DCB extract, blotted dry, and placed into small paper bags and immediately placed in an oven and dried to constant weight at 60 ℃, after which dry mass was recorded. Dried tissues were used for the quantification of Cd inside the roots. Beakers were rinsed with RO water once and added to the DCB extract to maximize solution recovery. To each tube containing DCB solution, 1 mL of pure (OmniTrace®, Sigma Aldrich) nitric acid was added, and the final volume was brought to 50 mL. Roots as well as the DCB extracts

were analyzed using ICP-MS to determine the concentrations of Cd in roots and on the surface of the roots (section 2.5.7).

2.5.7 Cadmium and sulfur concentrations

Cadmium and S concentrations in shoot and roots were measured following the method of Akhter and Macfie (2012). Dried tissue was hand-chopped and ground in a mortar using a pestle. A 0.1 g sample (or all the sample if mass did not reach 0.1 g) was placed into a 10 mL test tube, covered with a glass marble, and placed in a rack. One reagent blank (a test tube containing only nitric acid) and one standard reference material sample (NIST spinach SRM 1570a; approximately 0.1 g) were processed alongside the plant samples in each rack. The reagent blank was used to detect possible contamination of the RO water with Cd. The standard reference material was used to quantify the efficiency of acid digestion. To all test tubes, 1 mL of pure (OmniTrace®, Sigma Aldrich) nitric acid was added. The test tubes were left overnight to digest at room temperature. The next day, the samples were heated to 90-100 °C until vapors appeared transparent. The samples were cooled to room temperature and filtered through VWR® Grade 413 filter paper into 50 mL disposable centrifuge tubes. RO water was used to rinse the tubes and to bring the final sample volume to 50 mL. The samples were sent to the Water Quality Centre at Trent University, Peterborough ON, for Cd and S analysis by inductivity-coupled plasma mass spectrometry (ICP-MS).

2.5.8 Concentrations of non-protein thiol compounds

Root and leaf samples were placed in 15 mL Falcon® tubes, immediately frozen in liquid nitrogen and then lyophilized to prevent degradation of non-protein thiols. Dried samples were ground in their tubes to a rough powder, which was then transferred to microcentrifuge tubes. Two stainless steel ball bearings were added to each microcentrifuge tube and the tubes were placed into a Tissuelyser II (QIAGEN©) to homogenize the samples. The Tissuelyser II (QIAGEN©) was set to 300 revolutions per sec for 2 mins. To perform the non-protein thiol extraction, a subsample of 20 mg of powdered tissue was added to a separate microcentrifuge tube. To each microcentrifuge tube, 1 mL of extraction buffer (20 mL of 250 mM ammonium bicarbonate solution pH

6.5, adjusted with formic acid, and 80 mL of methanol) was added. Samples were then vortexed for 15 sec and sonicated at room temperature for 10 mins. Once sonicated, 40 µL of 0.5 M dithiothreitol (DTT) solution was added to each sample and vortexed for 15 sec. Samples were then incubated on an orbital mixer at 1000 rpm and 45 °C for 30 mins. Samples were allowed to cool to room temperature. Once cooled, 60 µL of 1 M N-ethylmaleimide (NEM) solution was added to each sample and then vortexed for 15 sec. The use of NEM was done to enhance the peak area of each analyzed chelator to produce a stronger signal through HPLC analysis (Appendix C, Figure C1). Samples were placed on an orbital mixer, covered with aluminum foil, and incubated at room temperature (23 °C) at 400 rpm for 20 mins. Samples were vortexed for 15 sec and centrifuged at 10,000 g at 4 °C for 10 mins. The supernatants were filtered into 2 mL amber glass HPLC vials using 0.2 µm PTFE syringe filters. The samples were then analyzed using liquid chromatography coupled with mass spectrometry (LC-MS) at the London Research Development Centre of Agriculture and Agri-Food Canada. The standard for hPC2 was purchased from AnaSpec (Fremont, CA, USA). The standard for hGSH was purchased from Toronto Research Chemicals (North York, ON, CAN). The standards for γ-Glu-Cys, GSH and PC were purchased from Sigma-Aldrich (Oakville, ON, CAN).

The extracts were screened using a Q-Exactive Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific), coupled to an Agilent 1290 high-performance liquid chromatography (HPLC) system with a Zorbax Eclipse Plus RRHD C18 column maintained at 35 °C (2.1 × 50 mm, 1.8 µm; Agilent). Mobile phase A (0.1% formic acid in LC-MS grade H_2O, Thermo Fisher Scientific) began at 100% and was held for 1.25 mins. Mobile phase B (0.1% formic acid in LC-MS grade acetonitrile, Thermo Fisher Scientific) was then increased to 50% over 1.75 mins, and 100% over 0.5 mins. Mobile phase B was maintained at 100% for 1.5 min and returned to 0% over 0.5 mins. The following heated electrospray ionization parameters were used in positive ionization mode: spray voltage, 3.9 kV; capillary temperature, 250°C; probe heater temperature, 450 °C; sheath gas, 30 arbitrary units; auxiliary gas, 8 arbitrary units; and S-Lens RF level, 60%. High resolution, full MS was used to detect any possible hPC2, hGSH, and γ-Glu-Cys, by accurate mass, while concurrent MS/MS scans monitored hPC2 (m/z 554 →

193.0648) all at normalized collision energies of 24. The automatic gain control target and maximum injection time were 3 × 106 and 256 ms, respectively. Data were analyzed and all theoretical masses were calculated with XcaliburTM software.

2.6 Statistical analysis

Normality and homogeneity of variance were verified for each data set. Two-way ANOVA was used to determine the main treatment effects and interactions between Cd and S. If ANOVA detected significant main effects, a Holm-Sidak post-hoc test was used to determine significant differences among treatment means ($P < 0.05$). A regression analysis was performed using Microsoft Excel to confirm the main effects of Cd and S treatments for all data sets (Appendix D, Table D). Statistical analyses and graphing were performed using SigmaPlot v11.

2.7 Results and discussion

2.7.1 Biomass

For hydroponically-grown plants, roots were more affected by Cd than shoots (Figure 2-2). The Cd-treated shoots had about a 20% reduction in biomass compared to control (Figure 2-2A). The roots had roughly a 30% decrease in biomass compared to control (Figure 2-2B). Soil-grown plants showed no effect of S addition nor Cd treatment (Figure 2-3). Additional S without Cd (white bars) did not alter shoot or root dry weight in hydroponically- or soil-grown plants (Figure 2-2 and Figure 2-3, respectively). In hydroponically-grown plants the shoots decreased in dry weight at 1 and 10 mM additional S in the presence of Cd (black bars) (Figure 2-2A). The roots of hydroponically-grown plants also decreased in dry weight with the addition of Cd and at all S concentrations. The soil-grown plants had no effects from S or Cd on altering dry weight in shoots or roots (Figure 2-3). There was no interaction between S and Cd in the shoots or roots of hydroponically- or soil-grown plants.

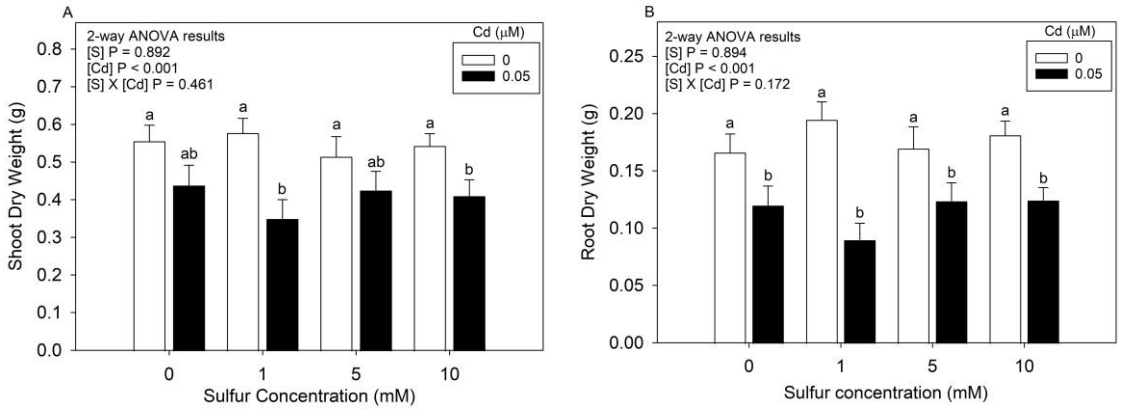

Figure 2-2. Dry biomass for hydroponically-grown plants. Dry biomasses were measured for A) shoots and B) roots of hydroponically-grown plants treated with either 0 or 0.05 μM of $CdCl_2$ and either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in dry weight, with n = 8. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top left of each graph.

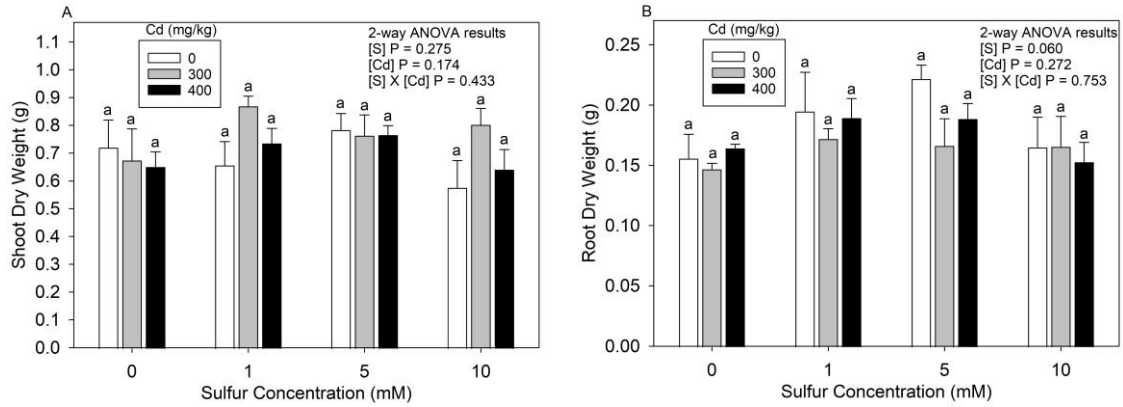

Figure 2-3. Dry Biomass for soil-grown plants. Dry biomasses were measured for A) shoots and B) roots of soil-grown plants treated with either 0, 300, or 400 mg of $CdCl_2$ per kg of soil and either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in dry weight, with n = 3. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top right of each graph.

Not surprisingly, in the presence of Cd, shoot and root biomass decreased in accordance with the dose response curves generated in preliminary experiments (Appendix A) in hydroponically-grown plants (Figure 2-2). I expected these results based on previous research with soybean and rice where Cd addition reduced growth and biomass of plants. Zhou et al. (2022) found that the addition of 20 μM Cd resulted in a 40% decrease in shoot and 51% decrease in root fresh weight of soybean. Shamsi et al. (2010) found that root and shoot dry weights decreased by 10% in the presence of 1 μM Cd in soybean. Hussain et al. (2020) reported that rice root biomass decreased by 40% at

96 μM and 144 μM Cd while shoot biomass decreased from 40-60% at 48, 96, and 144 μM Cd. While these other researchers also found a decrease in soybean biomass in the presence of Cd, the concentrations of Cd they used were 2 or more orders of magnitude higher than what I used for hydroponic experiments. This difference was due in part to the cultivar used in my experiment being highly sensitive to Cd stress. Also, it is possible that the chemistry of the other researchers' solutions resulted in less bioavailable Cd, hence less Cd uptake than the modified Hoagland's solution used here.

I predicted that plants given additional S would have higher biomass in the presence of Cd for both hydroponic and soil experiments, but this did not happen. While no other researchers have conducted S addition experiments to alleviate Cd toxicity in soybean, there have been many studies in other crop species. Cao et al. (2018) found that root and shoot dry weight increased by about 40% with the addition of 50 μM Cd and 5.28 mM S compared to no added S in rice. Han et al. (2018) found that shoot fresh weight increased by 30% with the addition of 250 μM Cd and 1 mM sulfur dioxide compared to no sulfur dioxide added in foxtail millet. The results of S addition to alleviate Cd toxicity in soybean could differ from the results reported in other plant species due to soybean being a legume. Soybean could utilize the added S in a different way than rice and foxtail millet or fail to use it properly for Cd mediation. There could be more S being used for protein formation rather than chelator production which would have no effect on reducing Cd toxicity and increasing biomass as the Cd is causing too much damage to the cells.

2.7.2 Histochemical localization of cadmium in the roots

The addition of S to Cd-treated plants did not visibly increase Cd localization in root cortical cells or epidermal cells (Figure 2-4). For both plants given additional S and those with no additional S added to solution, there appeared to be strong localization of Cd in the stele of the roots.

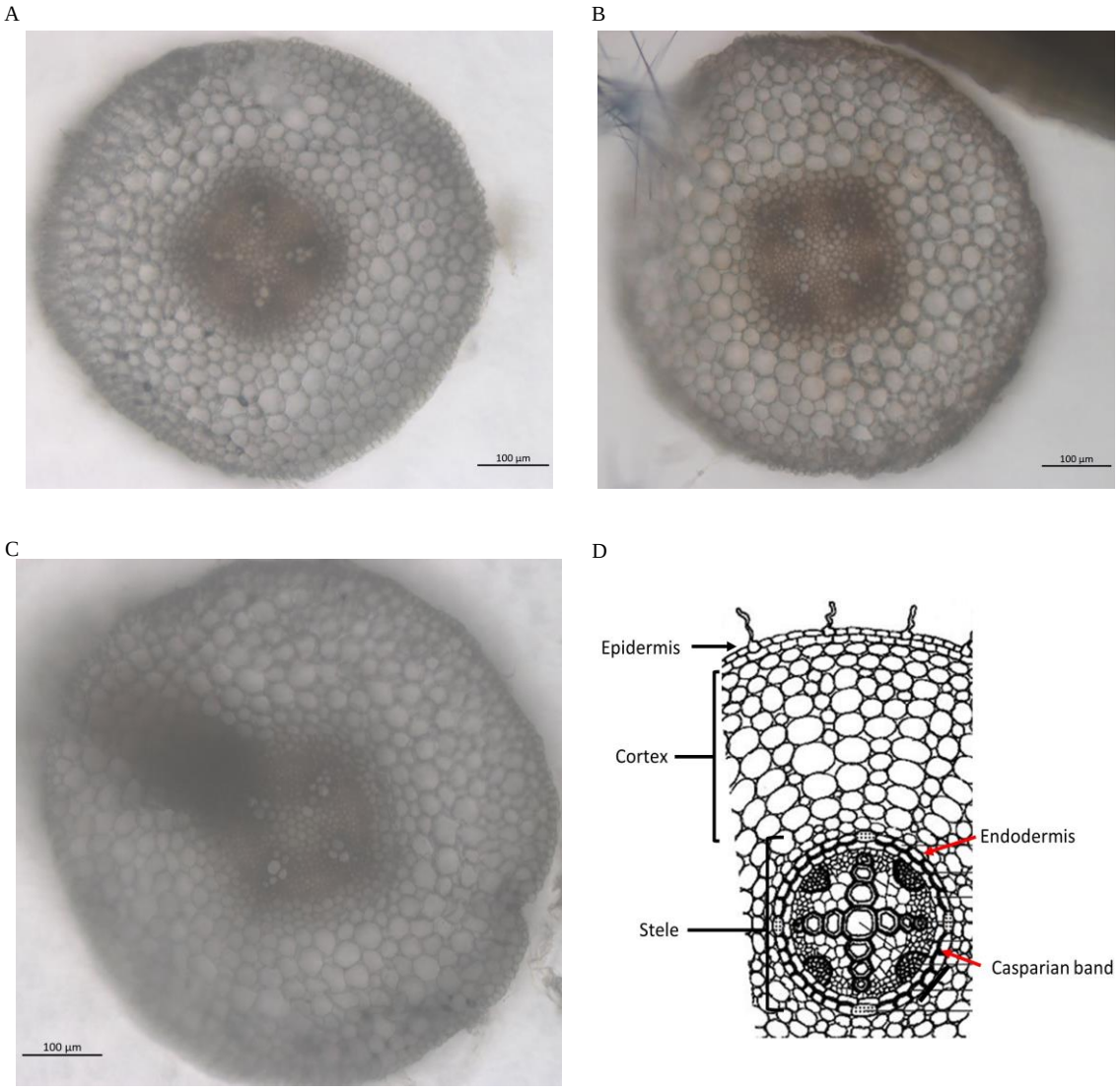

Figure 2-4. Cadmium localization in root cross-sections stained with dithizone. Roots of plants treated with either A) 10 mM Na_2SO_4 and 0.05 µM of $CdCl_2$, B) 0 mM Na_2SO_4 and 0.05 µM of $CdCl_2$, or C) 0 mM Na_2SO_4 and 0 µM of $CdCl_2$. A diagram D) of a root cross-section with anatomical locations labelled. Both Cd treated cross-sections have darkened steles relative to the 0 Cd control; however, in root in (B) has darker and thicker plaque formation on the epidermis and more Cd localization within cortical cells. Images were taken with a Zeiss microscope using brightfield optical microscopy. Image D was modified from Tutors Globe©.

Contrary to what was predicted, additional S resulted in little to no Cd localization in root cortical cells and epidermal cells (Figure 2-4A). I expected that the addition of S would have resulted in more Cd being localized in root cortical cells, epidermal cells, and cell walls for both previously mentioned cell types as a response to the presence of Cd (Seregin and Ivanov, 1997). Increased localization in root cortical cells should have resulted from increased Cd sequestration in cortical cell vacuoles due to increased

chelator production (Seregin and Ivanov, 1997, 2001). While there was hardly any Cd located in root cortical cells, there also appeared to be no Cd bound to cortical cell walls with the addition of S (Figure 2-4). Increased epidermal Cd could have been a result of increased Cd sequestration in epidermal cell vacuoles, increased Cd binding to epidermal cell walls, and/or increased Cd binding to the root surface or root plaque (Fang et al., 2020; Seregin and Ivanov, 1997). What is shown in Figure 2-4B was expected for plants grown in the presence of Cd with no additional S added. However, the addition of S was expected to greatly increase this distribution resulting in stronger, more intense staining in the cortex and epidermal regions of the root (Fang et al., 2020; Seregin and Ivanov, 1997).

2.7.3 Desorption of root plaque

In the Cd-control extraction solutions (white bars) at 10 mM added S there was a 3-fold increase in Cd concentration compared to the extraction solution from plants given no added S (Figure 2-5A). However, the magnitude of this increase (less than 0.1 μg/g Cd) is unlikely to illicit a biological response such as a measurable increase in plaque formation or Cd adsorption. Indeed, concentrations of Cd remaining in the roots did not vary with S treatment (Figure 2-5B). While there was a significant effect of Cd treatment on increasing Cd concentrations in the extraction solution and in the desorbed roots, there were no significant interactions between S and Cd (Figure 2-5).

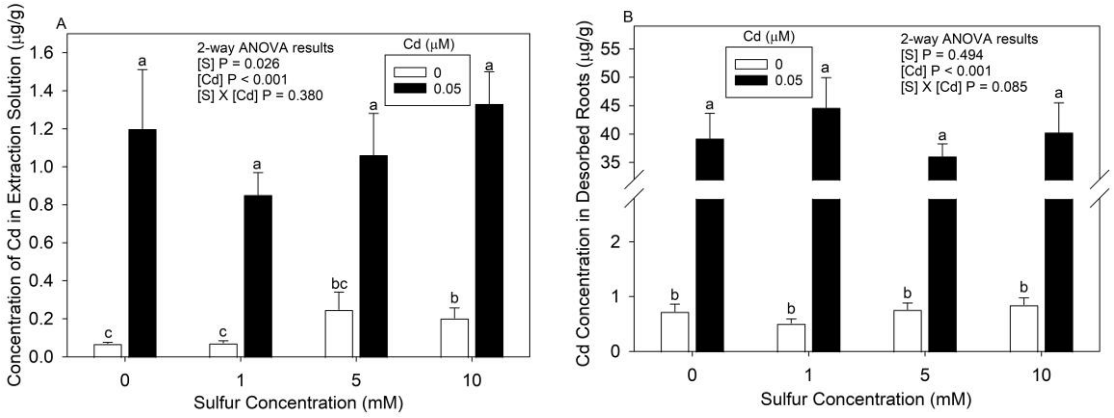

Figure 2-5. Concentration of cadmium in desorbed roots and root plaque. Concentrations were measured for A) desorption solution and B) desorbed roots of hydroponically-grown plants treated with either 0 or 0.05 μM of $CdCl_2$ and either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in Cd concentration, with n = 4. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top of each graph.

I expected that additional S in solution would have resulted in an increase of Cd bound to the root plaque and to the root surface. Yang et al. (2016) found that S addition resulted in increased Fe plaque formation in rice. Cao et al. (2018) also found that S addition increased Fe plaque formation in rice, but also in the presence of Cd; however, this was not the case here. The addition of S did not increase Cd bound to the root plaque nor to the root surface (Figure 2-5A). This could be because there was not enough Fe in solution to form a substantive plaque on the root surface. Since extracellular Cd was expected to be bound to Fe-hydroxides on the root surface that make up the root plaque, the root plaque was desorbed to target those potential Fe-hydroxides. If Fe was limited in solution, then there might not have been enough Fe to form a plaque with which to bind Cd.

2.7.4 Cadmium and sulfur concentrations

2.7.4.1 Cadmium concentrations

Not surprisingly, when the concentration of Cd in the growth medium increased, so too did the Cd concentration in the plant (Figure 2-6). Both the shoots and roots of hydroponically- and soil-grown plants had increased concentrations of Cd when Cd was added to solution (Figure 2-6). However, there was no interaction between S and Cd for both hydroponically- and soil-grown plants (Figure 2-6). It was interesting to see that the shoots of hydroponically-grown plants had a 2-fold higher Cd concentration than the soil-grown plants. This could be an indication that reduced translocation is occurring in soil-grown plants than hydroponically-grown plants, which would account for the higher root Cd concentration. For both hydroponically- and soil-grown plants, additional S did not affect the Cd concentrations in shoots or roots.

The proportions of Cd in shoots compared to overall Cd in the plant for hydroponically- and soil-grown plants are shown in Figure 2-7. In hydroponic culture, the proportion of Cd in control shoots (white bars) decreased by 40% with up to 10 mM additional S but the was no effect of S on the proportion of Cd in Cd-treated shoots and there were no Cd × S interactions (Figure 2-7A). There was no effect of additional S on the proportion of Cd in the shoots of soil-grown control or Cd-treated plants (Figure 2-

7B). Contrary to what I expected, the proportion of Cd in shoots of Cd-treated soil-grown plants did not change with the addition of S, and it was surprising to find that the proportion of Cd in shoots was consistently around 13% (Figure 2-7B). This was surprising because the effect found in hydroponically-grown plants was expected to be seen in soil-grown plants as well.

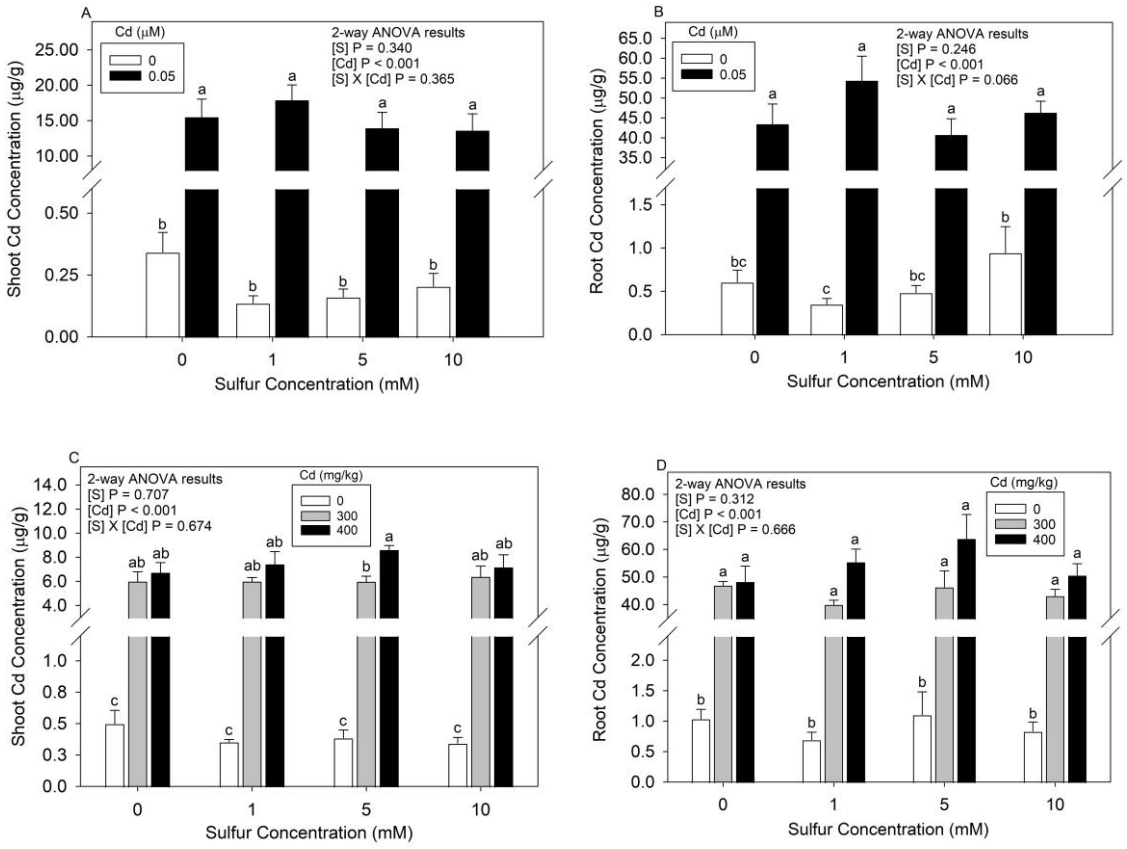

Figure 2-6. Concentrations of cadmium in plant tissues. Concentrations were measured for A) shoots and B) roots of hydroponically-grown plants treated with either 0 or 0.05 μM of $CdCl_2$ and C) shoots and D) roots of soil-grown plants treated with either 0, 300, or 400 mg of $CdCl_2$ per kg of soil. Both hydroponically- and soil-grown plants were treated with either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in Cd concentration, with n = 8 for hydroponically-grown plants and n = 3 for soil-grown plants. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top of each graph.

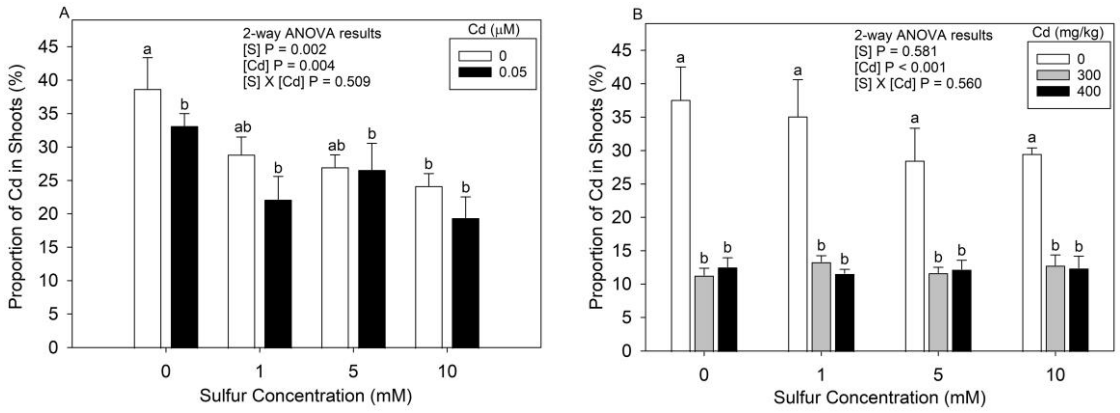

Figure 2-7. Proportion of cadmium in shoot tissue. Proportions were determined for A) hydroponically-grown plants treated with either 0 or 0.05 µM of $CdCl_2$ and B) soil-grown plants treated with either 0, 300, or 400 mg of $CdCl_2$ per kg of soil. Both hydroponically- and soil-grown plants were treated with either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in proportion of Cd, with n = 8 for hydroponically-grown plants and n = 3 for soil-grown plants. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top of each graph.

I expected that increased S would have resulted in reduced concentrations of Cd in shoots and roots of Cd-treated plants. With increased S I expected there to be increased root plaque formation and Cd sequestration on cell walls and in vacuoles of root cells. I expected these to increase which would have resulted in reduced Cd concentrations in the soybean plants overall. My results could be an indication that soybean does not use the same defense mechanisms as previously studied plants as additional S does not reduce Cd uptake, translocation, and toxicity.

2.7.4.2 Sulfur concentrations

As I expected, with additional S in solution or soil there was an increase in the concentration of S in the shoots and roots (Figure 2-8). The concentration of S in shoots of hydroponically-grown plants increased by about 1.3-fold when 10 mM additional S was added in the presence of Cd and by 1.4-fold without Cd (Figure 2-8A). In Cd-control roots of hydroponically-grown plants (white bars), S concentrations increased by 2-fold at 10 mM added S compared to no added S and by 2.2-fold with added Cd (Figure 2-8B). The S concentrations in shoots of soil-grown plants were mostly unaffected by additional S (Figure 2-8C). The roots of soil-grown plants were affected by additional S only when no Cd was added (white bars); at 10 mM additional S there was 1.25-fold more S compared to no S added (Figure 2-8D). The only significant interaction between S and

Cd was found in the roots of hydroponically-grown plants (Figure 2-8B). This interaction might be explained by the greater difference in S concentrations between plants from the two Cd treatments when no additional S was provided relative to when 5 or 10 mM was provided. This shows that when S is added in the presence of Cd there is an increase in the concentration of S kept in the roots.

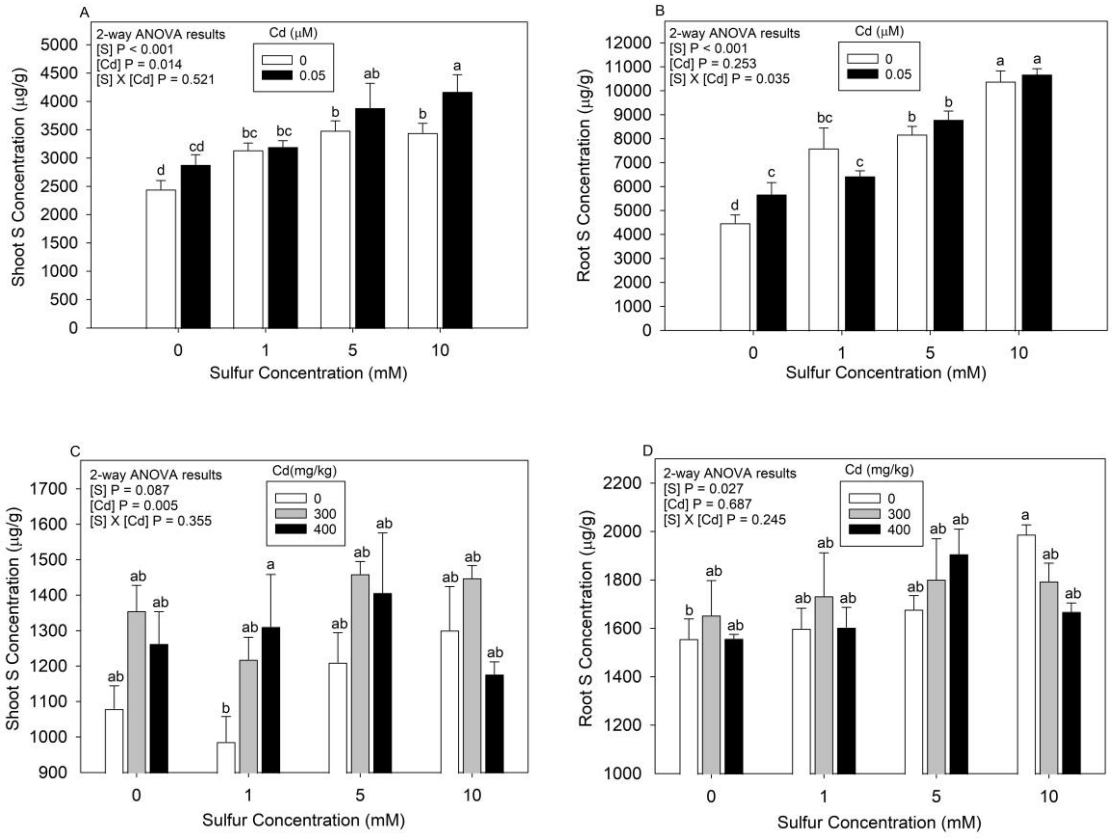

Figure 2-8. Concentrations of sulfur in plant tissue. Concentrations were measured for A) shoots and B) roots of hydroponically-grown plants treated with either 0 or 0.05 μM of $CdCl_2$ and C) shoots and D) roots of soil-grown plants treated with either 0, 300, or 400 mg of $CdCl_2$ per kg of soil. Both hydroponically- and soil-grown plants were treated with either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in S concentration, with n = 8 for hydroponically-grown plants and n = 3 for soil-grown plants. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top left of each graph.

As I expected, increasing the concentration of S added to solution resulted in an increase in S concentration in shoots and roots. This effect was more apparent in hydroponically-grown plants (Figure 2-8A&B) but was still present in soil-grown plants (Figure 2-8C&D). Sulfur concentrations were higher in roots than shoots for both

hydroponically- and soil-grown plants. This could be an indication that the increased S concentration in the roots is being used for increased chelator production. Since the roots are the site of S uptake into the plant it is expected that the roots would contain more S concentration than the shoots.

2.7.5 Concentrations of non-protein thiol compounds

2.7.5.1 Concentrations of gamma-glutamyl-cysteine

In hydroponically-grown plants, Cd-treatment resulted in higher concentrations of γ-Glu-Cys, with a few exceptions. With 0 or 1 mM S in hydroponic solution, concentrations of γ-Glu-Cys were at least 2-fold higher in Cd-treated shoots (black bars) compared to control (white bars, Figure 2-9A). In roots (Figure 2-9B) concentrations were 1.3- to 2.3-fold higher for all Cd-treated plants compared to control plants. Addition of S generated an interesting response. In hydroponically-grown plants, concentrations of γ-Glu-Cys increased as much as 2-fold in response to S for control leaves (white bars, Figure 2-9A) and increased by 25% in control roots (white bars, Figure 2-9B). However, in Cd-treated plants (black bars in Figure 2-9A&B), concentrations of γ-Glu-Cys did not vary with S treatment except for roots of plants given 10 mM S (Figure 2-9B). The consistent increases in γ-Glu-Cys in response to additional S for plants grown without Cd and the lack of this effect in Cd-treated plants explains the significant Cd × S interactions that were found.

In soil-grown plants, concentrations of γ-Glu-Cys in leaves did not vary with either Cd or S treatment in shoots (Figure 2-9C). In roots (Figure 2-9D) concentrations were up to 2.5-fold higher in Cd-treated plants compared to control plants and there was no Cd × S interaction.

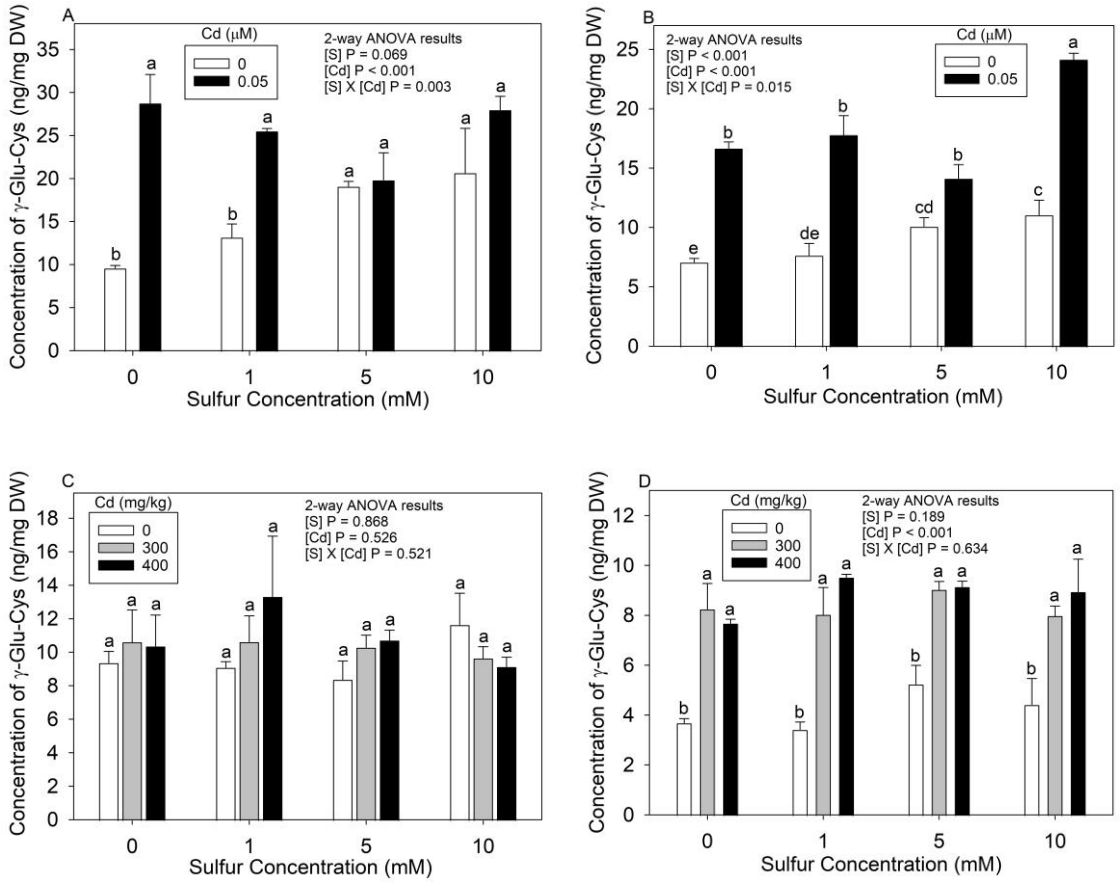

Figure 2-9. Concentrations of gamma-glutamyl-cysteine (γ-Glu-Cys) in plant tissue. Concentrations were measured for A) leaves and B) roots of hydroponically-grown plants treated with either 0 or 0.05 μM of $CdCl_2$ and C) leaves and D) roots of soil-grown plants treated with either 0, 300, or 400 mg of $CdCl_2$ per kg of soil. Both hydroponically- and soil-grown plants were treated with either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in γ-Glu-Cys concentration, with n = 3 for hydroponically- and soil-grown plants. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top of each graph.

Concentrations of γ-Glu-Cys were expected to increase in hydroponically- and soil-grown plants in response to both additional S and the presence of Cd. This increase in γ-Glu-Cys could come from increased S supply increasing chelator production. The increase in γ-Glu-Cys was also expected as a response to Cd. Although γ-Glu-Cys is a precursor molecule for larger chelators and would be used up during synthesis of these larger molecules, the addition of S was expected to increase replenishment of γ-Glu-Cys, resulting in higher concentrations with and without Cd. I expected that the roots of both hydroponically- and soil-grown plants had the most consistent increase in γ-Glu-Cys concentrations in the presence of Cd compared to the shoots of the same plants. Since the

roots are the site of Cd introduction and contain the highest Cd concentrations in plant, I expected that chelator concentrations would be higher there.

There seemed to be a trend of increasing γ-Glu-Cys concentrations as S concentration increased, which was more evident in hydroponically-grown plants. While no other studies were found in which γ-Glu-Cys concentrations were analyzed in response to Cd and S addition, the concentrations of γ-Glu-Cys were still expected to increase. These results show that γ-Glu-Cys concentrations increase in response to Cd presence, but also show that they increase in response to additional S addition in soybean. Akhter et al. (2012) did find that γ-Glu-Cys concentrations increased in the roots of barley in the presence of Cd.

2.7.5.2 Concentrations of homoglutathione

There was no consistent effect of additional S on hGSH concentrations in the leaves or roots of hydroponically- and soil-grown plants (Figure 2-10). The only differences were found in the leaves of hydroponically-grown plants. Concentrations of hGSH increased in response to Cd by more than 2-fold at 0 mM additional S and by about 30% at 1 mM added S in the leaves of hydroponically-grown plants (Figure 2-10A). In plants grown without Cd, concentrations of hGSH up to 40% higher in plants given additional S relative to plants not given additional S (white bars, Figure 2-10A). Concentrations of hGSH decreased by about 10-30% in the leaves of Cd-treated hydroponically-grown plants as added S concentration increased (black bars, Figure 2-10A). Concentrations of hGSH in roots of hydroponically-grown plants did not vary with Cd or S treatment (Figure 2-10B).

The concentrations of hGSH in leaves of soil-grown plants did not vary with the additions of Cd or S (Figure 2-10C). The hGSH in roots of soil-grown plants at 1 mM added S was 25% higher for plants given no added Cd relative to those given 300 mg/kg; however, this difference is would not illicit a biological response because the two values are statistically the same as most other values in the figure (Figure 2-10D).

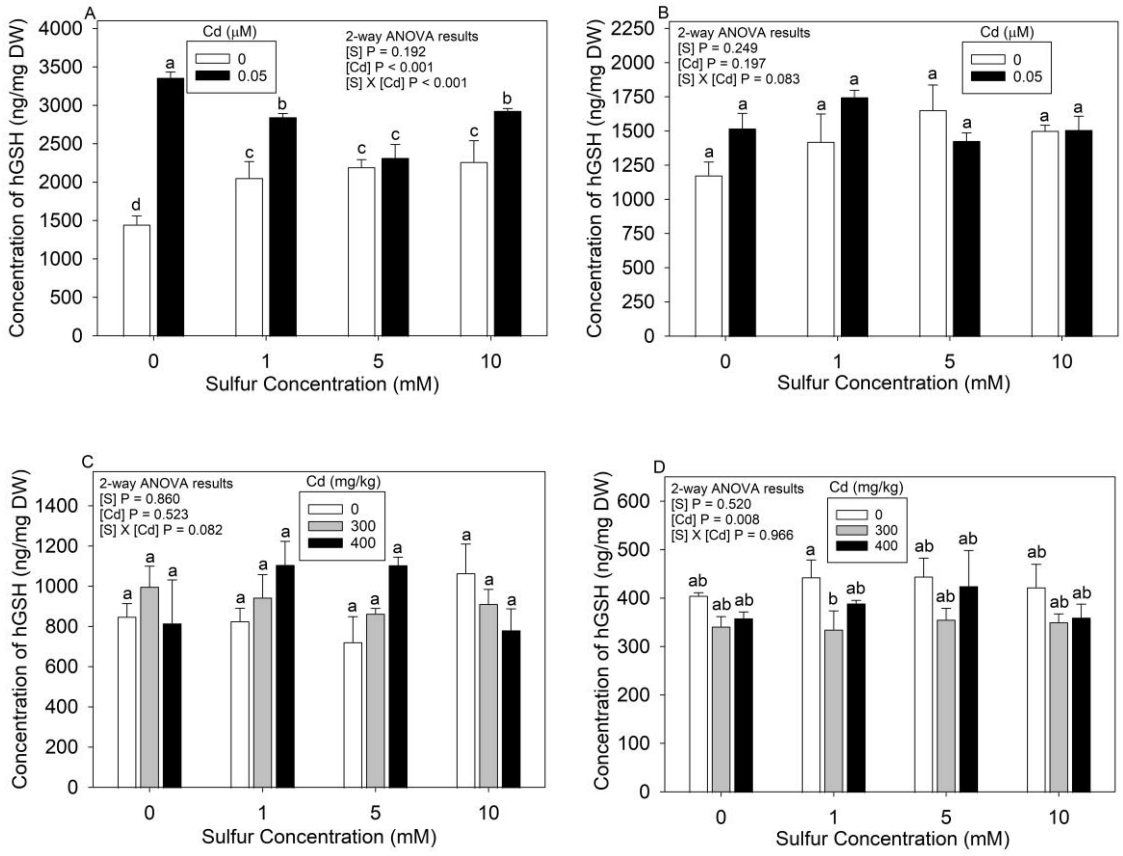

Figure 2-10. Concentrations of homoglutathione (hGSH) in plant tissue. Concentrations were measured for A) leaves and B) roots of hydroponically-grown plants treated with either 0 or 0.05 μM of $CdCl_2$ and C) leaves and D) roots of soil-grown plants treated with either 0, 300, or 400 mg of $CdCl_2$ per kg of soil. Both hydroponically- and soil-grown plants were treated with either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in hGSH concentration, with n = 3 for hydroponically- and soil-grown plants. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top of each graph.

Homoglutathione was expected to increase with the addition of S and in the presence of Cd. Like γ-Glu-Cys, hGSH is both a precursor molecule and a chelator. From this I expected that increased S and the addition of Cd would result in increased production of hGSH. Additional S appeared to increase hGSH production in shoots and roots of hydroponically- and soil-grown plants, but the increase was significant only in the shoots of hydroponically-grown plants. It was interesting to see no effect on hGSH concentrations in soil-grown plants in response to Cd or S because I expected the soil-grown plants to have some changes in hGSH concentration similar to the hydroponically-

grown plants. Perhaps the soil-grown plants better regulated hGSH production and hPC synthesis to maintain a consistent concentration of hGSH.

2.7.5.3 Concentrations of homophytochelatin-2

In Cd-treated plants, concentrations of hPC2 increased by at least 10-fold in hydroponically-grown plants (Figure 2-11A&B) and at least 3-fold in soil-grown plants (Figure 2-11C&D). This increase in hPC2 concentration is likely caused by increased hPC2 production as a result of Cd stress. Although not statistically significant, concentrations of hPC2 tended to increase in the roots of hydroponically- and soil-grown plants with increasing S concentrations (Figure 2-11B&D). There was a significant interaction between Cd and S in the hydroponically-grown plants (Figure 2-11A&B). The leaves of hydroponically-grown plants had a 25% decrease in hPC2 concentration at 5 mM added S compared to 0 added S in the presence of Cd (black bars) (Figure 2-11A). In the roots the addition of 10 mM S in the presence of Cd resulted in a 1.5-fold increase in hPC2 concentration compared to 0 added S and added Cd (Figure 2-11B).

The leaves of soil-grown plants grown with the addition of 400 mg/kg Cd seemed to have higher hPC2 concentrations when increasing concentrations of S were added (black bars); however, this trend was not statistically significant (Figure 2-11C). The same can be said for the roots of hydroponically-grown plants (Figure 2-11B), where hPC2 concentrations showed an increasing trend with added S concentration without Cd added (white bars), but this was also not statistically significant.

There were significantly lower hPC2 concentrations in the leaves of both hydroponically- and soil-grown plants (Figure 2-11A&C) compared to the roots (Figure 2-11B&D). This distribution of hPC2 concentration is the same with and without Cd, indicating that plants perhaps produce and store more hPC2 in the roots as a main site of oxidative and metal stress. Since the roots are the main source of nutrient and chemical uptake for a plant, it makes sense that more hPC2 would be stored in the roots compared to the leaves.

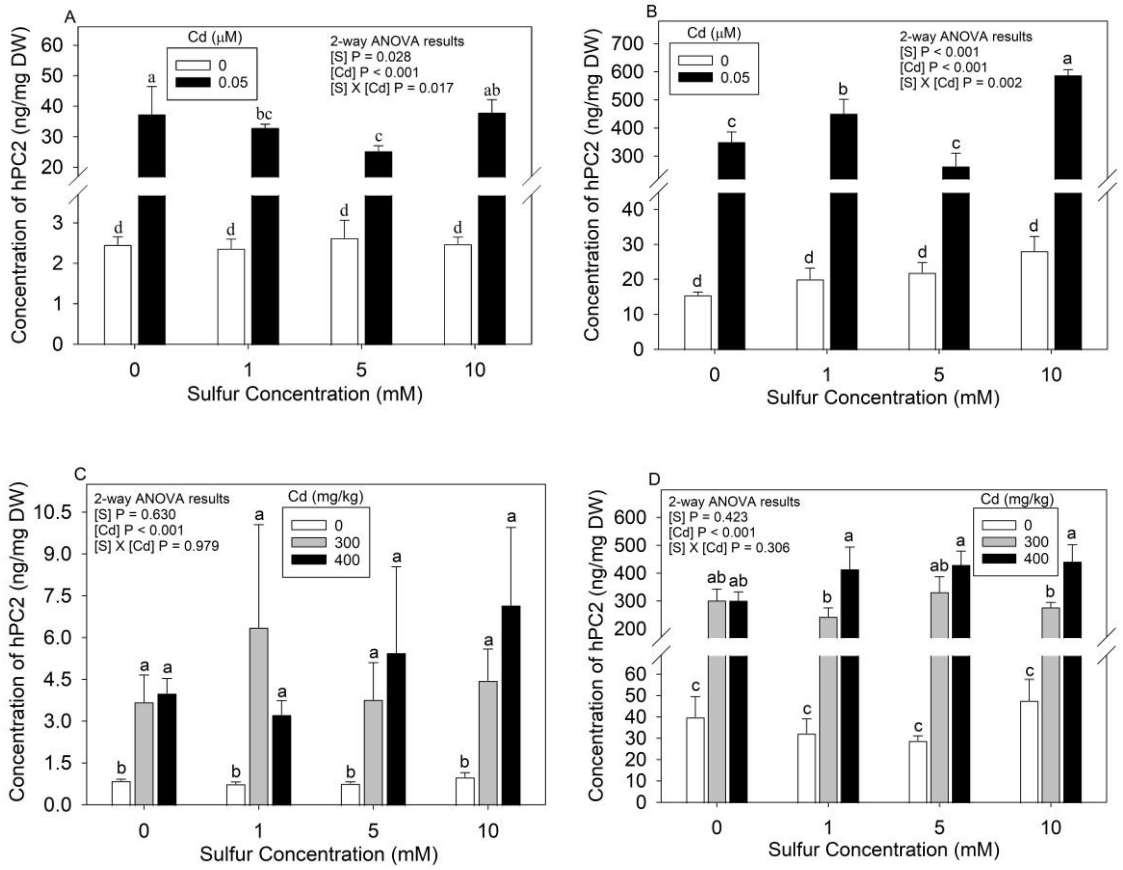

Figure 2-11. Concentrations of homophytochelatin-2 (hPC2) in plant tissue. Concentrations were measured for A) leaves and B) roots of hydroponically-grown plants treated with either 0 or 0.05 μM of $CdCl_2$ and C) leaves and D) roots of soil-grown plants treated with either 0, 300, or 400 mg of $CdCl_2$ per kg of soil. Both hydroponically- and soil-grown plants were treated with either 0, 1, 5, or 10 mM added Na_2SO_4. Different letters indicate a significant difference in hPC2 concentration, with n = 3 for hydroponically- and soil-grown plants. Values plotted are mean ± SE. The results of a two-way ANOVA test are shown at the top of each graph.

It is unsurprising that hPC2 concentrations increased in the presence of Cd as that is one of the primary chelators soybeans use to reduce Cd toxicity. The addition of S altered shoot and root hPC2 concentrations mostly in the presence of Cd. This could be because plants produce more hPC2 in the presence of Cd so additional S could allow for more hPC2 to be produced. Xiao et al. (2020) found that PC2 concentrations increased by more than 100-fold in the presence of 48 μM Cd compared to control in *Perilla frutescens* (L.) Britt. and increased by 30-60% on average at lower Cd concentrations.

2.8 Conclusions

Sulfur addition was expected to illicit the same responses in soybean as have been seen in rice and corn (Cao et al., 2018; Li et al., 2017), but this was not the case. The addition of S had no effect on reducing Cd toxicity in soybean. Sulfur addition did not result in increased biomass, altered Cd localization within roots, reduced Cd concentrations in roots and shoots, increased root plaque formation, or increased concentration of Cd in the root plaque. Sulfur addition did result in increased γ-Glu-Cys and hPC2 concentrations along with the presence of Cd. However, additional S did not result in changes in hGSH concentrations. While S addition did not result in reduced Cd toxicity and have beneficial effects on soybean in the presence of Cd, there were no harmful effects of S addition either. These results should be applied to agronomy and agricultural practices when adding S fertilizers to crop fields. If S fertilizer is being added to soybean fields for the purpose of reducing Cd uptake or toxicity, then there will be no effect. Applying S fertilizer to soybean fields could still be beneficial for increasing plant growth and yield to supplement the low S concentrations naturally found in soil.

2.9 References

Akhter, F., Macfie, S.M., 2012. Species-specific relationship between transpiration and cadmium translocation in lettuce, barley and radish. Journal of Plant Studies 1, 2–13. https://doi.org/10.5539/jps.v1n1p2

Akhter, F., McGarvey, B., Macfie, S.M., 2012. Reduced translocation of cadmium from roots is associated with increased production of phytochelatins and their precursors. Journal of Plant Physiology 169, 1821–1829. https://doi.org/10.1016/j.jplph.2012.07.011

Akhter, F., Omelon, C.R., Gordon, R.A., Moser, D., Macfie, S.M., 2014. Localization and chemical speciation of cadmium in the roots of barley and lettuce. Environmental and Experimental Botany 100, 10–19.

Amir, S.B., Rasheed, R., Ashraf, M.A., Hussain, I., Iqbal, M., 2021. Hydrogen sulfide mediates defense response in safflower by regulating secondary metabolism, oxidative defense, and elemental uptake under drought. Physiologia Plantarum 172, 795–808. https://doi.org/10.1111/ppl.13267

Baker, A.J.M., Walker, P.L., 1989. Physiological responses of plants to heavy metals and the quantification of tolerance and toxicity. Chemical Speciation and Bioavailability 1, 7–17. https://doi.org/10.1080/09542299.1989.11083102

Benavides, M.P., Gallego, S.M., Tomaro, M.L., 2005. Cadmium toxicity in plants. Brazilian Journal of Plant Physiology 17, 21–34.

Canadian Council of Ministers of the Environment, 1999. Canadian soil quality guidelines for the protection of environmental and human health. Canadian Environmental Quality Guidelines.

Cao, Z.Z., Qin, M.L., Lin, X.Y., Zhu, Z.W., Chen, M.X., 2018. Sulfur supply reduces cadmium uptake and translocation in rice grains (*Oryza sativa* L.) by enhancing iron plaque formation, cadmium chelation and vacuolar sequestration. Environmental Pollution 238, 76–84. https://doi.org/10.1016/j.envpol.2018.02.083

Carnegie, P.R., 1963. Structure and properties of a homologue of glutathione. The Biochemical Journal 89, 471–478.

Chary, N.S., Kamala, C.T., Raj, D.S.S., 2008. Assessing risk of heavy metals from consuming food grown on sewage irrigated soils and food chain transfer. Ecotoxicology and Environmental Safety 69, 513–524. https://doi.org/10.1016/j.ecoenv.2007.04.013

Chen, J., 2007. Rapid urbanization in China: A real challenge to soil protection and food security. Catena 69, 1–15. https://doi.org/10.1016/j.catena.2006.04.019

Chen, M., Cao, L., Song, X., Wang, X., Qian, Q., Liu, W., 2014. Effect of Fe plaque and Se on Cd uptake and translocation in rice seedlings (*Oryza sativa*) grown in solution. International Journal of Agriculture & Biology 16, 1159–1164.

Chen, S., Sun, L., Sun, T., Chao, L., Guo, G., 2007. Interaction between cadmium, lead and potassium fertilizer (K_2 SO_4) in a soil-plant system. Environmental Geochemistry and Health 29, 435–446. https://doi.org/10.1007/s10653-007-9088-y

Chinoim, N., Lefroy, R.D.B., Blair, G.J., 1997. Effect of crop duration and soil type on the ability of soil sulfur tests to predict plant response to sulfur. Soil Research 35, 1131–1142.

Ciampitti, I.A., Salvagiotti, F., 2018. New insights into soybean biological nitrogen fixation. Agronomy Journal 110, 1185–1196. https://doi.org/10.2134/agronj2017.06.0348

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475–486. https://doi.org/10.1007/s004250000458

Cobbett, C.S., 2000. Phytochelatins and their roles in heavy metal detoxification plants make two types of peptide metal-binding ligands: MTs and PCs. Plant Physiology 123, 825–832.

Environment Canada, 1994. Cadmium and its compounds. Environment Canada.

Fan, J.-L., Wei, X.-Z., Wan, L.-C., Zhang, L.-Y., Zhao, X.-Q., Liu, W.-Z., Hao, H.-Q., Zhang, H.-Y., 2011. Disarrangement of actin filaments and Ca^{2+} gradient by $CdCl_2$ alters cell wall construction in *Arabidopsis thaliana* root hairs by inhibiting vesicular trafficking. Journal of Plant Physiology 168, 1157–1167. https://doi.org/10.1016/j.jplph.2011.01.031

Fang, Z., Hu, Z., Yin, X., Song, G., Cai, Q., 2020. Exogenous glutathione alleviation of cd toxicity in Italian ryegrass (*Lolium multiflorum*) by modulation of the cd absorption, subcellular distribution, and chemical form. International Journal of Environmental Research and Public Health 17, 1–12. https://doi.org/10.3390/ijerph17218143

Giovanelli, J., 1971. Sulfur amino acids of plants: an overview. Methods in Enzymology 143, 419–426.

Grant, C.A., Buckley, W.T., Bailey, L.D., Selles, F., 1997. Cadmium accumulation in crops. Canadian Journal of Plant Science. Revue Canadienne de Phytotechnie 78, 1–17

Grassini, P., Cafaro La Menza, N., Rattalino Edreira, J.I., Monzón, J.P., Tenorio, F.A., Specht, J.E., 2020. Soybean, in: Crop physiology case histories for major crops. Elsevier, 282–319. https://doi.org/10.1016/B978-0-12-819194-1.00008-6

Han, Y., Wu, M., Hao, L., Yi, H., 2018. Sulfur dioxide derivatives alleviate cadmium toxicity by enhancing antioxidant defense and reducing Cd^{2+} uptake and translocation in foxtail millet seedlings. Ecotoxicology and Environmental Safety 157, 207–215. https://doi.org/10.1016/j.ecoenv.2018.03.084

Hassan, M.J., Raza, M.A., Rehman, S.U., Ansar, M., Gitari, H., Khan, I., Wajid, M., Ahmed, M., Shah, G.A., Peng, Y., Li, Z., 2020. Effect of cadmium toxicity on growth, oxidative damage, antioxidant defense system and cadmium accumulation in two sorghum cultivars. Plants 9, 1–14. https://doi.org/10.3390/plants9111575

Hoagland, D.R., Arnon, D.I., 1950. The water-culture method for growing plants without soil. California Agricultural Experiment Station 347, 23–32.

Hungria, M., Franchini, J.C., Campo, R.J., Graham, P.H., 2005. The importance of nitrogen fixation to soybean cropping in South America, in: Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment 4, 25–39.

Hussain, B., Li, J., Ma, Y., Tahir, N., Ullah, A., 2020. Effects of Fe and Mn cations on Cd uptake by rice plant in hydroponic culture experiment. PLoS ONE 15, 1–15. https://doi.org/10.1371/journal.pone.0243174

Järup, L., 2003. Hazards of heavy metal contamination. British Medical Bulletin 68, 167–182. https://doi.org/10.1093/bmb/ldg032

Jia, H., Wang, X., Shi, C., Guo, J., Ma, P., Ren, X., Wei, T., Liu, H., Li, J., 2020. Hydrogen sulfide decreases Cd translocation from root to shoot through increasing Cd accumulation in cell wall and decreasing Cd^{2+} influx in *Isatis indigotica*. Plant Physiology and Biochemistry 155, 605–612. https://doi.org/10.1016/j.plaphy.2020.08.033

Jung, H. il, Lee, B.R., Chae, M.J., Lee, E.J., Lee, T.G., Jung, G.B., Kim, M.S., Lee, J., 2020. Ascorbate-mediated modulation of cadmium stress responses: reactive oxygen species and redox status in *Brassica napus*. Frontiers in Plant Science 11, 1–15. https://doi.org/10.3389/fpls.2020.586547

Keyser, H.H., Li, F., 1992. Potential for increasing biological nitrogen fixation in soybean. Plant and Soil 141, 119–135

Komarnisky, L.A., Christopherson, R.J., Basu, T.K., 2003. Sulfur: its clinical and toxicologic aspects. Nutrition 19, 54–61.

Krishnamurti, G.S.R., Huang, P.M., Kozak, L.M., Rostad, H.P.W., Rees, K.C.J. van, Rees, V., 1997. Distribution of cadmium in selected soil profiles of Saskatchewan, Canada: speciation and availability. Canadian Journal of Soil Science 77, 613–619.

Li, G., Shah, A.A., Khan, W.U., Yasin, N.A., Ahmad, A., Abbas, M., Ali, A., Safdar, N., 2021. Hydrogen sulfide mitigates cadmium induced toxicity in

Brassica rapa by modulating physiochemical attributes, osmolyte metabolism and antioxidative machinery. Chemosphere 263, 1–12. https://doi.org/10.1016/j.chemosphere.2020.127999

Li, M., Hao, P., Cao, F., 2017. Glutathione-induced alleviation of cadmium toxicity in *Zea mays*. Plant Physiology and Biochemistry 119, 240–249. https://doi.org/10.1016/j.plaphy.2017.09.005

Li, Yunyun, Li, Hailong, Yu, Y., Zhao, J., Wang, Y., Hu, C., Li, Hong, Wang, G., Li, Yufeng, Gao, Y., 2018. Thiosulfate amendment reduces mercury accumulation in rice (*Oryza sativa* L.). Plant and Soil 430, 413–422. https://doi.org/10.1007/s11104-018-3726-2

Liu, N., Lou, X., Li, X., Shuai, Z., Liu, H., Jiang, Z., Wei, S., 2021. Rhizosphere dissolved organic matter and iron plaque modified by organic amendments and its relations to cadmium bioavailability and accumulation in rice. Science of the Total Environment 792, 1–16. https://doi.org/10.1016/j.scitotenv.2021.148216

Liu, W.J., Zhu, Y.G., Smith, F.A., Smith, S.E., 2004. Do iron plaque and genotypes affect arsenate uptake and translocation by rice seedlings (*Oryza sativa* L.) grown in solution culture? Journal of Experimental Botany 55, 1707–1713. https://doi.org/10.1093/jxb/erh205

Moran, J.F., Aki Iturbe-Ormaetxe, I., Matamoros, M.A., Rubio, M.C., Clemente, M.R., Brewin, N.J., Becana, M., 2000. Glutathione and homoglutathione synthetases of legume nodules. Cloning, expression, and subcellular localization. Plant Physiology 124, 1381–1392.

Nawaz, H., Anwar-ul-Haq, M., Akhtar, J., Arfan, M., 2021. Cadmium, chromium, nickel and nitrate accumulation in wheat (*Triticum aestivum* L.) using wastewater irrigation and health risks assessment. Ecotoxicology and Environmental Safety 208, 1–8. https://doi.org/10.1016/j.ecoenv.2020.111685

Nazar, R., Iqbal, N., Masood, A., Khan, M.I.R., Syeed, S., Khan, N.A., 2012. Cadmium toxicity in plants and role of mineral nutrients in its alleviation. American Journal of Plant Sciences 03, 1476–1489. https://doi.org/10.4236/ajps.2012.310178

Nishizono, H., Kubota, K., Suzuki, S., Ishii, F., 1989. Accumulation of heavy metals in cell walls of *Polygonum cuspidatum* roots from metalliferous habitats. Plant and Cell Physiology 30, 595–598.

Peralta-Videa, J.R., Lopez, M.L., Narayan, M., Saupe, G., Gardea-Torresdey, J., 2009. The biochemistry of environmental heavy metal uptake by plants: implications for the food chain. International Journal of Biochemistry and Cell Biology 41, 1665–1677. https://doi.org/10.1016/j.biocel.2009.03.005

Sabiha-Javied, Mehmood, T., Chaudhry, M.M., Tufail, M., Irfan, N., 2009. Heavy metal pollution from phosphate rock used for the production of fertilizer in Pakistan. Microchemical Journal 91, 94–99. https://doi.org/10.1016/j.microc.2008.08.009

Saboori-Robat, E., Joshi, J., Pajak, A., Renaud, J., Marsolais, F., Saboori-Robat, E., Sol, M., Maccelli, C., Joshi, J., Marsolais, F., Joshi, J., Mohsenpour, M., 2019. Common bean (*Phaseolus vulgaris* L.) accumulates most s-methylcysteine as its γ-glutamyl dipeptide. Plants 8, 1–15. https://doi.org/10.3390/plants8050126

Seregin, I. v., Ivanov, V.B., 1997. Histochemical investigation of cadmium and lead distribution in plants. Russian Journal of Plant Physiology 44, 791–796.

Seregin, I. v., Ivanov, V.B., 2001. Physiological aspects of cadmium and lead toxic effects on higher plants. Russian Journal of Plant Physiology 48, 523–544.

Shamsi, I.H., Jiang, L., Wei, K., Jilani, G., Hua, S., Zhang, G.P., 2010. Alleviation of cadmium toxicity in soybean by potassium supplementation. Journal of Plant Nutrition 33, 1926–1938. https://doi.org/10.1080/01904167.2010.512052

Sheoran, V., Sheoran, A.S., Poonia, P., 2010. Soil reclamation of abandoned mine land by revegetation: a review. International Journal of Soil, Sediment and Water 3, 1–20.

Syu, C.H., Jiang, P.Y., Huang, H.H., Chen, W.T., Lin, T.H., Lee, D.Y., 2013. Arsenic sequestration in iron plaque and its effect on As uptake by rice plants grown in paddy soils with high contents of As, iron oxides, and organic matter. Soil Science and Plant Nutrition 59, 463–471. https://doi.org/10.1080/00380768.2013.784950

Tai, Y., Tam, N.F.Y., Wang, R., Yang, Yang, Lin, J., Wang, J., Yang, Yufen, Li, L., Sun, Y., 2018. Iron plaque formation on wetland-plant roots accelerates removal of water-borne antibiotics. Plant and Soil 433, 323–338. https://doi.org/10.1007/s11104-018-3843-y

Tester, M., Leigh, R.A., 2001. Partitioning of nutrient transport processes in roots. Journal of Experimental Botany 52, 445–457.

Tylová, E., Pecková, E., Blascheová, Z., Soukup, A., 2017. Casparian bands and suberin lamellae in exodermis of lateral roots: an important trait of roots system response to abiotic stress factors. Annals of Botany 120, 71–85. https://doi.org/10.1093/aob/mcx047

Wan, L., Zhang, H., 2012. Cadmium toxicity. Plant Signaling & Behaviour 7, 345–348. https://doi.org/10.4161/psb.18992

Wang, H., Jia, Y., Wang, S., Zhu, H., Wu, X., 2009. Bioavailability of cadmium adsorbed on various oxides minerals to wetland plant species *Phragmites australis*. Journal of Hazardous Materials 167, 641–646. https://doi.org/10.1016/j.jhazmat.2009.01.012

Wu, X., Song, H., Guan, C., Zhang, Z., 2020. Boron mitigates cadmium toxicity to rapeseed (*Brassica napus*) shoots by relieving oxidative stress and enhancing cadmium chelation onto cell walls. Environmental Pollution 263, 1–9. https://doi.org/10.1016/j.envpol.2020.114546

Xiao, Q., Wang, Y., Lü, Q., Wen, H., Han, B., Chen, S., Zheng, X., Lin, R., 2020. Responses of glutathione and phytochelatins biosynthesis in a cadmium accumulator of *Perilla frutescens* (L.) Britt. under cadmium contaminated conditions. Ecotoxicology and Environmental Safety 201, 1–10. https://doi.org/10.1016/j.ecoenv.2020.110805

Xing, W., Liu, H., Banet, T., Wang, H., Ippolito, J.A., Li, L., 2020. Cadmium, copper, lead and zinc accumulation in wild plant species near a lead smelter. Ecotoxicology and Environmental Safety 198, 1–9. https://doi.org/10.1016/j.ecoenv.2020.110683

Yang, Junxing, Liu, Z., Wan, X., Zheng, G., Yang, Jun, Zhang, H., Guo, L., Wang, X., Zhou, X., Guo, Q., Xu, R., Zhou, G., Peters, M., Zhu, G., Wei, R., Tian, L.,

Han, X., 2016. Interaction between sulfur and lead in toxicity, iron plaque formation and lead accumulation in rice plant. Ecotoxicology and Environmental Safety 128, 206–212. https://doi.org/10.1016/j.ecoenv.2016.02.021

Yi, H., Ravilious, G.E., Galant, A., Krishnan, H.B., Jez, J.M., 2010. From sulfur to homoglutathione: thiol metabolism in soybean. Amino Acids 39, 963–978. https://doi.org/10.1007/s00726-010-0572-9

Zhi, Y., Sun, T., Zhou, Q., Leng, X., 2020. Screening of safe soybean cultivars for cadmium contaminated fields. Scientific Reports 10, 12965. https://doi.org/10.1038/s41598-020-69803-4

Zhou, Q., Cai, Z., Xian, P., Yang, Y., Cheng, Y., Lian, T., Ma, Q., Nian, H., 2022. Silicon-enhanced tolerance to cadmium toxicity in soybean by enhancing antioxidant defense capacity and changing cadmium distribution and transport. Ecotoxicology and Environmental Safety 241, 1–9.

Chapter 3

3 Limitations, future work and concluding remarks

In this chapter, I will discuss the limitations of my experiments as well as the potential future experiments that could come from my research. I will end with a conclusion of my research project and the impact it could have on plant research and soybean knowledge.

3.1 Limitations and future work

Because harvest procedures for soil-grown plants is a lengthy process and can damage roots, hydroponic experiments must be performed for any histological microscopy and root desorption experiments. More replicates should be used in future work to increase statistical power of the ANOVAs (for most analyses, the power was below the critical value of 0.8) and to reduce variance among samples. I would have used more replicates for soil experiments, but I ran out of soil and any new soil purchased would have had different chemical and physical properties.

I wanted to use scanning electron microscopy with energy dispersive x-ray spectroscopy (SEM-EDX) or scanning electron microscopy with wavelength dispersive spectroscopy (SEM-WDS) to image and analyze root cross-sections. Using SEM-WDS would have been better than using SEM-EDX because SEM-WDS is less damaging to the root samples than SEM-EDX as determined by (Akhter et al., 2014). Both methods would have allowed for elemental composition of root cross-sections, and I could have created a heatmap. This heatmap of a root cross-section would show the cross-sectional localization of Cd, S, and perhaps detect Cd-S co-localization, which would indicate Cd-S complexes. While not quantitative, this technique would measure relative concentrations of Cd and S within the root cross-sections. While this in theory sounded like a good idea, I did not have enough time to perform this experiment.

Future work could be to look more closely at histological and microscopy techniques to better assess the effects of S on the distribution of Cd. I was limited in how thinly I could cut the root cross-sections as I was hand-sectioning them all for the

histology images. To get clearer images of root cross-sections, another method for thinly sectioning root samples, such as a microtome or vibratome could be used prior to staining with dithizone (or some other Cd indicator) and viewing with brightfield microscopy.

It might also be important to grow the plants for longer to allow for analysis of mature plants and the seeds they produce. These seeds could be tested for Cd content to determine if the addition of S might reduce Cd concentrations in the seeds of soybean. If this is the case, then the practice of applying additional S to soybean crop soil would have a health benefit for consumers; however, it may have no benefit for the plant itself. Perhaps my experiments were limited by soybean immaturity and a longer experimental duration would yield different results. Increasing the concentration of S added would be very beneficial to determine if higher S concentrations would have a benefit on reducing Cd toxicity in soybean.

More experiments into mechanisms of Cd tolerance could be performed with additional S where there is a known component containing S. For example, fluorescent imaging of root cross-sections could show an increase in suberization of the root endodermis to quantify the influence of additional S addition on cell wall components and thickness.

3.2 Concluding remarks

While S addition to rice and corn have been shown to reduce Cd toxicity and have beneficial effects on plant health in the presence of Cd (Cao et al., 2018; Li et al., 2017), this was not the case in soybean. The addition of S to each of nutrient solution and soil did not reduce Cd toxicity nor did it reduce the amount of Cd in the roots and shoots. Additional S did not increase root plaque formation, nor did it increase root cortical cell sequestration of Cd. Additional S did increase chelator concentrations of γ-Glu-Cys and hPC2, but only at 10 mM added S.

I did not find any publications on the interactions of S and Cd in soybean, let alone studies on whether additional S supply could reduce Cd toxicity in soybean. This is the first research to provide information on the effects of additional S on soybean in the

presence of Cd. The results I have found show that soybean do not benefit from S addition on reducing Cd toxicity as have been shown in rice and corn (Cao et al., 2018; Li et al., 2017). This is surprising because there are no clear explanations for why soybean would respond differently than rice or corn. Perhaps these results are unique to soybean, the cultivar I used, or it could be unique to legumes or other dicots compared to grasses and monocots (Jiao et al., 2004). Different cultivars within a plant species have different responses to Cd (Grant et al., 2000), so these results could be cultivar specific.

One important outcome of my work is that I helped develop a novel technique to enhance chelator signals using NEM for HPLC/LC-MS quantification and analysis. Using NEM to isolate and enhance the S-group of chelators drastically increased the signal of the target chelators compared to not using NEM (Appendix, FigureC1). This is significant because this technique can be used in future research when analyzing chelator concentrations in plants allowing for more precise and stronger signals to be detected.

In conclusion, I found that additional S supply does not mediate Cd toxicity in soybean as seen in rice and corn. However, additional S supply did not harm the plant either by increasing Cd concentration in soybean or increasing Cd toxicity. With the increasing need to add S to fertilizers in agricultural practices in North America, there should be no assumption that additional S could help reduce Cd toxicity in Cd contaminated fields.

3.3 References

Akhter, F., Omelon, C.R., Gordon, R.A., Moser, D., Macfie, S.M., 2014. Localization and chemical speciation of cadmium in the roots of barley and lettuce. Environmental and Experimental Botany 100, 10–19.

Cao, Z.Z., Qin, M.L., Lin, X.Y., Zhu, Z.W., Chen, M.X., 2018. Sulfur supply reduces cadmium uptake and translocation in rice grains (*Oryza sativa* L.) by enhancing iron plaque formation, cadmium chelation and vacuolar sequestration. Environmental Pollution 238, 76–84. https://doi.org/10.1016/j.envpol.2018.02.083

Grant, C.A., Dribnenki, J.P., Bailey, L.D., 2000. Cadmium and zinc concentrations and ratios in seed and tissue of solin (cv Linola^{TM} 947) and flax (cvs McGregor and Vimy) as affected by nitrogen and phosphorus fertiliser and Provide

(*Penicillium bilaji*). Journal of the Science of Food and Agriculture 80, 1735–1743.

Jiao, Y., Grant, C.A., Bailey, L.D., 2004. Effects of phosphorus and zinc fertilizer on cadmium uptake and distribution in flax and durum wheat. Journal of the Science of Food and Agriculture 84, 777–785. https://doi.org/10.1002/jsfa.1648

Li, M., Hao, P., Cao, F., 2017. Glutathione-induced alleviation of cadmium toxicity in *Zea mays*. Plant Physiology and Biochemistry 119, 240–249. https://doi.org/10.1016/j.plaphy.2017.09.005

Appendices

Appendix A: Dose response curves for plants grown in hydroponic culture or soil.

The dose response curves for hydroponically-grown plants (Figure B1) were generated by following the same growth procedures as outlined in sections 2.5.1 and 2.5.2 with modification. No additional S was added to solution and a range of Cd concentrations were added to each solution. Based on curve-fitting analysis, I determined that a concentration of Cd that resulted in a 20-30% growth reduction compared to control was between 0.04-0.05 µM $CdCl_2$ for shoots and roots. The concentration of 0.05 µM $CdCl_2$ was chosen for simpler calculations for future experiments.

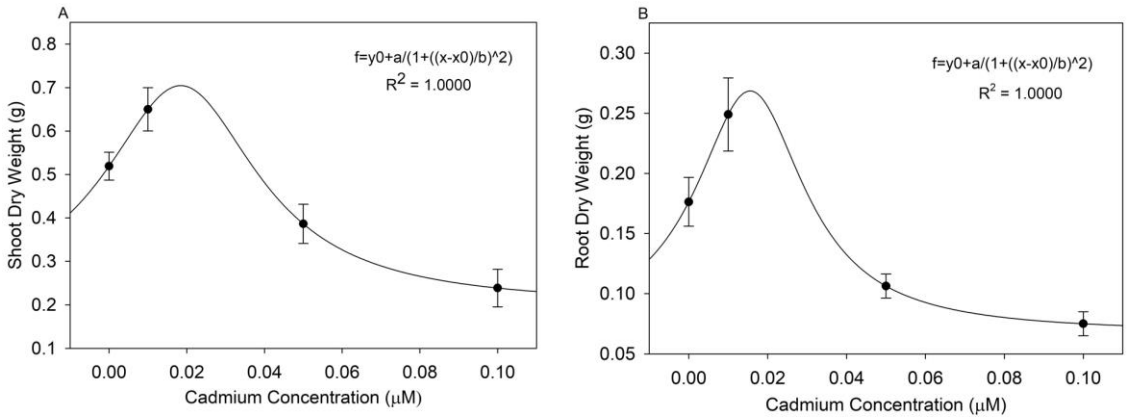

Figure A1. Dose response curves for hydroponically-grown plants. A concentration of Cd that resulted in a 20-30% growth reduction was determined from A) shoot and B) root dry weight relative to control dry weight. Values plotted are mean ± SE with n = 4. The equations represent the line of best fit for each curve.

The dose response curves for soil-grown plants (Figure B2) were generated by following the same growth procedures as outlined in sections 2.5.1 and 2.5.3 with modification. No additional S was added to solution and a range of Cd concentrations were added to each solution. Based on curve-fitting analysis, I determined the concentration of Cd that resulted in a 20-30% reduction in root growth was above 500 mg/kg, which is not environmentally realistic. The concentrations chosen for future experiments were therefore chosen based on the shoot response. It was determined that a concentration of Cd that resulted in a 20-30% growth reduction compared to control was around 300-400 mg/kg $CdCl_2$ for shoots. To account for the poor curve fitting in this range, I opted to run soil experiments using cadmium treatments of 0, 300 or 400 mg/kg.

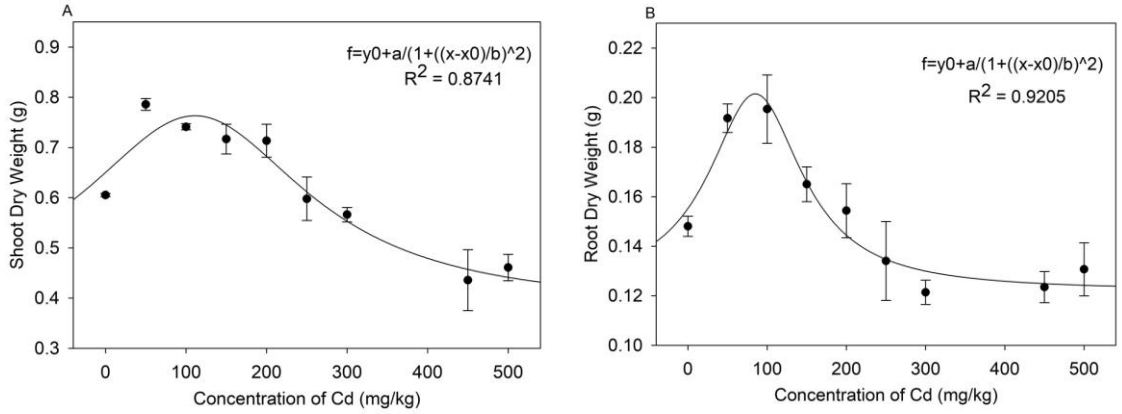

Figure A2. Dose response curves for soil-grown plants. A concentration of Cd that resulted in a 20-30% growth reduction was determined from A) shoot and B) root dry weight relative to control dry weight. Values plotted are mean ± SE with n = 3. The equations represent the line of best fit for each curve.

Appendix B: Experimental set-up for plants grown in hydroponic culture or soil

Three sets of plants were used for the hydroponic experiments, each with a different number of replicates (FigureB1).

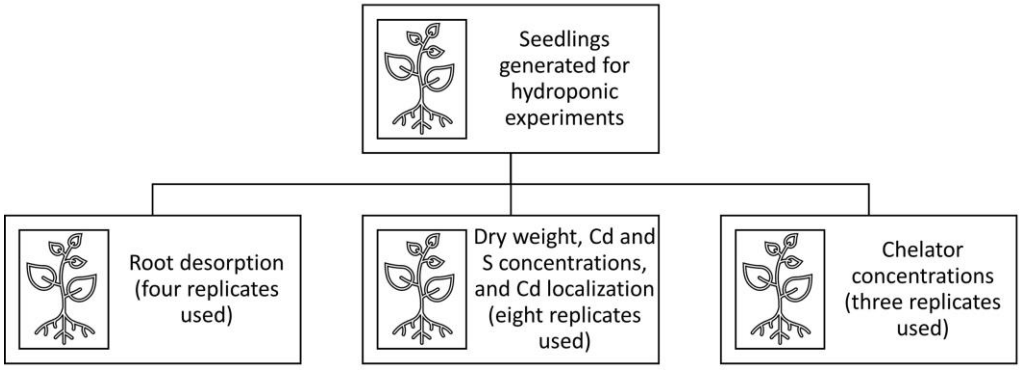

Figure B1. Diagram of hydroponically-grown plant sets. Seedlings prepared for hydroponic culture were divided into three sets, the number of replicates for reach experimental treatment varied from 3 to 8 depending on the variable(s) to be measured.

One set of seedlings was used for soil-grown plants (Figure B2). Only three replicates were used due to limited mass of soil that was available. More than enough soil was purchased to run 5 or more replicates but after it was sieved to remove debris, clumps of clay, sticks, pieces of vegetation, worms, and insects, I was limited to having only three replicates for each experimental treatment.

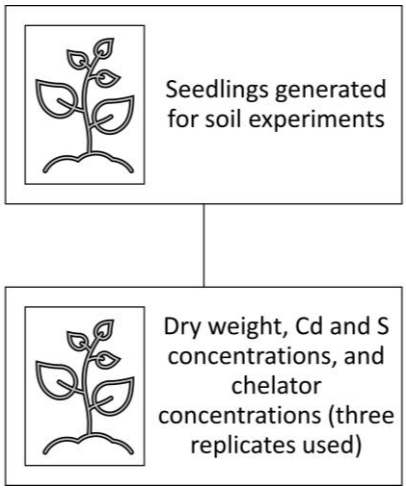

Figure B2. Diagram of soil-grown plant set. One set of seedlings was prepared for soil culture, with 3 replicates of each experimental treatment

Appendix C: NEM-derivatization increases peak area of chelator signals.

The effect of NEM derivatization on increasing peak area of analyzed chelators measured in this thesis (Figure D1) was evaluated by using the same plant material from section 2.5.8 and following the same procedures from section 2.5.8 with modification. Instead of having samples from individual replicates, replicates with the lowest concentrations of hPC were used to form a homogenous sample for roots and leaves. This combined sample was then divided into replicates and processed the same as section 2.5.8. One set of replicates used H_2O instead of DTT and NEM, one set used DTT as normal and H_2O instead of NEM, and the final set was processed the same as section 2.5.8. The addition of DTT increased the hGSH peak area by more than a factor of 500 compared to using only water in leaves (Figure D1A). The addition of DTT increased the hGSH peak area by more than a factor of 200 compared to using only water in roots. The addition of NEM further increased the hGSH peak area by a factor of 7 compared to adding only DTT in leaves and roots. The addition of NEM increased the hPC2 peak area by a factor of 11 compared to adding only DTT in leaves (Figure D1B); the values for roots were too small to plot. The addition of NEM increased the hPC2 peak area by a factor of 3 compared to adding only DTT in roots.

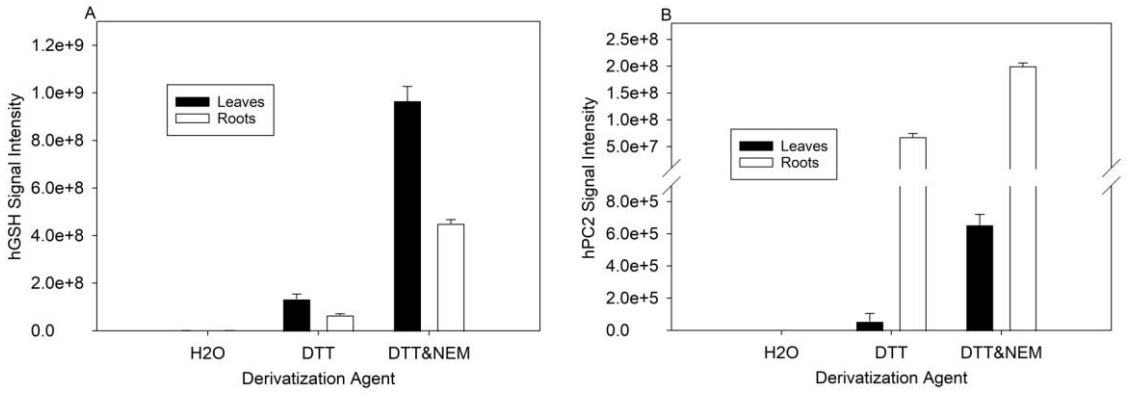

Figure C1. Effect of N-ethylmaleimide (NEM) on increasing peak area of chelators. The peak areas of (A) hGSH and (B) hPC2 in non-derivatized extractions (water) and those derivatized with DTT or DTT and NEM. Values plotted are mean ± SE with n = 3.

Appendix D: Regression analysis for all provided graphs.

Regression analysis (using Microsoft Excel) was performed for each data set in this thesis to confirm the effects of Cd and/or S. The significance (P) values associated with the regression analyses are shown in Table C. None of the regressions altered the interpretation of the data based on the results of two-way ANOVA. I believe the power of the data is still too low for a regression to detect significant treatment effects that ANOVA did not. My recommendation is that more replicates are grown to determine if there are significant effects for any of the treatments.

Table D. Regression analysis of cadmium (Cd) and sulfur (S) treatments. For each variable measured, the p-values associated with the main effects of Cd and S are shown for plants grown in hydroponic culture (H) and soil (S) .

Variable	[Cd]	[S]
Leaf γ-Glu-Cys (H)	$P < 0.001$	$P = 0.092$
Leaf γ-Glu-Cys (S)	$P = 0.255$	$P = 0.679$
Leaf hGSH (H)	$P < 0.001$	$P = 0.536$
Leaf hGSH (S)	$P = 0.288$	$P = 0.982$
Leaf hPC2 (H)	$P < 0.001$	$P = 0.294$
Leaf hPC2 (S)	$P < 0.001$	$P = 0.353$
Root γ-Glu-Cys (H)	$P < 0.001$	$P = 0.001$
Root γ-Glu-Cys (S)	$P < 0.001$	$P = 0.315$
Root hGSH (H)	$P = 0.258$	$P = 0.585$
Root hGSH (S)	$P = 0.016$	$P = 0.847$
Root hPC2 (H)	$P < 0.001$	$P = 0.081$
Root hPC2 (S)	$P < 0.001$	$P = 0.181$
Shoot DW (H)	$P < 0.001$	$P = 0.846$

Shoot DW (S)	P = 0.457	P = 0.617
Root DW (H)	P < 0.001	P = 0.446
Root DW (S)	P = 0.274	P = 0.832
Root desorption (S)	P < 0.001	P = 0.737
Root desorption (R)	P < 0.001	P = 0.111
Shoot Cd (H)	P < 0.001	P = 0.256
Shoot Cd (S)	P < 0.001	P = 0.603
Root Cd (H)	P < 0.001	P = 0.685
Root Cd (S)	P < 0.001	P = 0.930
Shoot proportion Cd (H)	P = 0.059	P = 0.001
Shoot proportion Cd (S)	P < 0.001	P = 0.275
Shoot S (H)	P = 0.500	P < 0.001
Shoot S (S)	P = 0.012	P = 0.103
Root S (H)	P = 0.017	P < 0.001
Root S (S)	P = 0.960	P = 0.006
Shoot proportion S (H)	P = 0.200	P = 0.021
Shoot proportion S (S)	P = 0.278	P = 0.204

Curriculum Vitae

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Honours and Awards:	Dean's Honor List 2018-2019, 2019-2020
Related Work Experience	Teaching Assistant The University of Western Ontario 2020-2022
Conference Presentations	Ontario Biology Day Conference 10 min Presentation 2019 —Uptake and Translocation of Cadmium in Soybean (*Glycine max*) in response to sulfite addition Plant Canada Conference Poster 2019 —Uptake and Translocation of Cadmium in Soybean (*Glycine max*) in response to sulfite addition Canadian Society of Plant Biologists Conference 3 min Talk 2021 — Effects of Sulfur on Cadmium Uptake and Translocation in Soybean (*Glycine max*) Biology Graduate Research Forum Poster 2021 — Effects of Sulfur on Cadmium Uptake and Translocation in Soybean (*Glycine max*) International Plant Sulfur Workshop 2022 — Excess Sulfur Addition does not Mediate Cadmium Toxicity in Soybean