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Cadmium Uptake in Plants as Influenced by Selenium Uptake and Sulphate Availability

Marnie J. Demand, *The University of Western Ontario*

Supervisor: Macfie, Sheila M, *The University of Western Ontario*

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Abstract

With cadmium uptake by plants posing a risk to plants and consumers alike, strategies to reduce metal uptake are desirable. One strategy may be to apply selenium (as selenate) to the growth medium. I hypothesized selenate would yield greater lignification, with a higher proportion of cadmium bound to root cell walls. Consequently, higher selenium in plants would result in greater tolerance to cadmium. Additionally, since selenate is taken up in place of sulphate, providing the plants with high sulphate would inhibit uptake and translocation of selenium, mitigating selenate's benefits of reducing cadmium uptake and translocation. Experimental results did not support these hypotheses. Selenate did not affect lignification, nor yield lower cadmium uptake and translocation. Rather, shoot selenium and cadmium concentrations were positively correlated. Thus, the safety of consuming plants from where cadmium concentrations are elevated appears unlikely to be improved by applying selenate, and potential for harm may increase.

Keywords

Elemental interactions, lignin, selenium hyperaccumulators, selenium non-hyperaccumulators, *Astragalus* spp., *Brassica napus* subsp. *napus*, *Lactuca sativa*, *Sorghum bicolor*, *Triticum aestivum*, *Zea mays*

Summary for Lay Audience

Polluted soils are sometimes used for agriculture. While problematic and inadvisable, with land scarcity, alternatives may not be feasible. One contaminant of concern is the toxic metal cadmium. Plants grown on cadmium-contaminated soils can pose a health risk to consumers. Another issue pertaining to the food supply is deficiency in selenium, an essential micronutrient for animals. This deficiency is estimated to affect 500 million to 1 billion people worldwide. When selenium is applied to plant cells, it results in structural and chemical changes. Specifically, an increase in lignin concentrations has been reported. Lignin is a molecule that helps to give strength to plant cells. Increased lignin associated with selenium treatments has been reported to reduce the amount of cadmium that enters the cell. Thus, applying selenium in the presence of cadmium was expected to have two benefits. Firstly, the plant was expected to take up less cadmium. Secondly, the plant would have a higher selenium content, providing more of this essential micronutrient to the consumer. Whether this strategy would work was tested in 8 plant species, including 5 that are common agricultural crops or vegetables, all grown hydroponically. Since the amount of another nutrient, sulphate, was expected to impact how much selenium the plant took up, plants were grown in treatments with high sulphate and sufficient sulphate. The amount of lignin in the roots was measured in 3 of the species to test the effect of selenium on the plants. Then, for all 8 species, concentrations of cadmium and selenium in the aboveground portions of the plants were measured. Contrary to expectations, selenium did not result in higher lignin concentrations in the plants' roots, nor did it reduce the amount of cadmium taken up. Rather, the more selenium the plants took up, the more cadmium they tended to take up. Given that lignin concentrations did not increase with selenium, the lack of effect on cadmium was unsurprising, albeit disappointing. These results indicate applying selenium to crops grown in soils with elevated levels of cadmium may increase the risk of harm, rather than providing a benefit.

Co-Authorship Statement

A planned manuscript for submission to a peer-reviewed journal will be co-authored by Marnie Demand, Sheila Macfie, and Sergio Ari Dominguez Romero. Experimental design, data collection, and data interpretation were conducted by Marnie Demand. Data analysis was conducted by Marnie Demand and Sergio Ari Dominguez Romero. Marnie Demand conducted the analysis of the first hypothesis, while Sergio Ari Dominguez Romero provided guidance and recommendations on the statistical testing of the second hypothesis, as well as the R code to do so. Sheila Macfie assisted with development of the experimental design and the interpretation of the data. The manuscript will be drafted by Marnie Demand, and Sheila Macfie will provide assistance in its preparation.

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List of Abbreviations

ANOVA = analysis of variance

Cd = cadmium

DW = dry weight

g = gravitational force

ICP-MS = inductively coupled plasma mass spectroscopy

RO = reverse osmosis

S = high sulphate (indicated in treatment names, including on graphs)

Se = selenate (indicated in treatment names, including on graphs)

SQG_{HH} = soil quality guideline for human health

SULTR = sulphur transporter genes

Chapter 1

1 Introduction

Within plants, essential plant nutrients frequently interact with one another, with an abundance of physiologically synergistic and/or antagonistic relationships possible (Rietra et al., 2017). However, these types of interactions are not restricted to the nutrients that are essential to the plant. Non-essential elements, including cadmium and selenium, can also affect the uptake of one another. This research set out to determine if selenium, an essential nutrient for animals and one in which an estimated 500 million to 1 billion people worldwide are deficient (Combs, 2001), can reduce the uptake and translocation of cadmium, a toxic metal, to the aboveground portion of the plant. If so, it could serve the benefit of both improving the nutritional status of many people, as well as reducing their consumption of cadmium through diet, which is a major source of exposure (IPCS, 1992).

In evaluating the potential for selenium in the growth medium to reduce cadmium concentrations in the plants, I looked at lignification of the roots in response to one form of selenium (selenate) as a possible mechanism for reducing cadmium uptake. Additionally, I addressed the impact of high sulphate levels on the interactions between selenium and cadmium and resulting shoot concentrations thereof. Since selenate can be taken up by plants via sulphate transporters, would high sulphate levels prevent selenate from being able to reduce cadmium concentrations in plants?

1.1 Cadmium in the Environment

Cadmium, while highly toxic, is a naturally occurring element found throughout the environment. Its concentrations vary, being influenced by both natural factors, such as the geology of an area, and by anthropogenic activities. It can be redistributed via anthropogenic emissions, releases to land or water, and natural emissions. Sources of natural cadmium emissions include volcanic eruptions, forest fires, sea spray, and airborne soil particles (UN, 2010). Richardson et al. (2001) estimated annual average

worldwide emissions of cadmium from natural sources at 4,100 tonnes/year. For comparison, the widely cited value for anthropogenic emissions for the year 2001 was approximately 3,000 tonnes (e.g., by Nordic Council of Ministers, 2003 and UN, 2010). However, the original source of this value (Pacyna & Pacyna, 2001), included the caveat that it was underestimate, due to incomplete data on global waste incineration. Recent data on global releases to land and water are limited, with the United Nations (2010) citing values from Nriagu & Pacyna (1988). Nriagu & Pacyna's estimates, which were for 1983, were that between 15,500 and 83,000 tonnes/year of cadmium was released to land (including soil), in addition to 1,200 to 13,400 tonnes/year discharged into aquatic systems.

Anthropogenic activities that result in releases of cadmium to the environment are not uniformly distributed, resulting in elevated concentrations on a local or regional scale (UN, 2010). Contributing activities include the extraction and smelting of non-ferrous metals, waste disposal (including of mining waste), use of sewage sludge, and repeated use of cadmium-containing rock phosphate fertilizers (UN, 2010).

The extraction and refinement of cadmium occurs predominantly as a by-product during the mining and smelting of other non-ferrous metal ores, particularly zinc (Nordic Council of Ministers, 2003). The primary ore of zinc is sphalerite, (Zn,Fe)S, a mineral reported by Cook et al. (2009) to frequently contain 0.2-1% cadmium by mass. Cadmium can replace zinc ions in the crystal lattice of sphalerite. Meanwhile, cadmium-containing minerals such as greenockite (CdS) also frequently occur in zinc deposits (Callaghan, 2020). Globally, demand for cadmium is low, with limited industrial applications. The quantity of cadmium produced as a by-product during the extraction and smelting of other metals is typically sufficient to meet global needs. Consequently, production levels for cadmium are typically a function of zinc refinement, rather than demand for the cadmium itself (UN, 2010).

With cadmium present in zinc deposits, smelter emissions can contain cadmium, resulting in subsequent atmospheric deposition. Mining waste, particularly from zinc

mines, can also be contaminated with cadmium. This includes slag and other solid waste, as well as wastewater.

1.1.1 Cadmium and Human Health

The presence and abundance of cadmium in soils can be problematic, notably when those soils are used as cropland. Consumption of crops is the primary route of exposure to cadmium for the majority of the non-smoking population (IPCS, 1992). Unlike other metals, phytotoxicity due to cadmium can occur at concentrations higher than those regarded as safe for human consumption (Peijnenburg et al., 2000). Thus, potentially harmful concentrations of cadmium can enter the food supply via crops grown on soil contaminated with cadmium (Peijnenburg et al., 2000).

The World Health Organization's International Agency for Research on Cancer (IARC) classifies cadmium as a "Group 1" agent, meaning it is a human carcinogen (IARC, 2019). Specifically, it is known to cause lung cancer, particularly when inhaled, with some evidence from humans that it may also cause kidney and/or prostrate cancers (IARC, 2021). Exposure to cadmium can also causes Itai-itai disease, a painful disease affecting the bones that resembles osteoporosis (reviewed by Pan et al., 2010).

1.1.2 Cadmium in Soils and Croplands

With the health risks posed by cadmium, contamination of soils, particularly those used for agriculture, is concerning. In 2014, The People's Republic of China (PRC) Ministry of Ecology and the Environment released a report on soil contamination. According to their English press release, 16.1% of sites failed to meet their standards for soil contamination. The most common inorganic contaminant found was cadmium, which was reportedly found in 7.0% of all sites (or 43% of contaminated sites). Arable land was even more frequently contaminated than the overall average reported, with 19.4% of these sites failing to meet their standards (PRC, 2014) The guidelines in China for cadmium in agricultural soil vary depending on the pH of the soil. Their agricultural standards for cadmium are 0.3 mg/kg when the pH is 7.5 or lower, and 0.6 mg/kg above a pH of 7.5 (Zhao et al., 2015).

While the PRC (2014) press release highlights the prevalence of cadmium contamination in China, it is possible to overlook the severity of the issue, particularly with their low standards for cadmium of 0.3-0.6 mg/kg. As such, it should be noted that there have been widespread health issues in villages near Chinese mining operations, due to contamination of land and water. The now-abandoned Dabaoshan mine located in Shaoguan City (Guangdong Province, China) provides one example (Liao et al., 2016). Pollution from this singular mine, predominantly in the form of acid mine drainage containing high levels of cadmium, lead, and zinc, has been blamed for widespread contamination. It has polluted the land on which 83 villages are situated, along with rivers, ponds, and over 585 ha of paddy fields (Chan et al., 2021). As a result, local people have experienced extremely high prevalence of cancers, and behavioral issues in children have also been well-documented (Bao et al., 2009).

Similar to the situation in the Dabaoshan mine area, another example of metal-contaminated soils and water being used for agriculture was found in Jos, located in Plateau, Nigeria. Here, industrial wastewater and water from mining ponds has been used for irrigation during the dry seasons, and metal-contaminated lands cultivated for food crops (Gazuwa & Olotuche, 2021). Gazuwa and Olotuche (2021) reported on this situation in their study, in which they analyzed the cadmium, lead, copper and arsenic content of vegetables being grown in three locations: Bassa, Bisichi and Zarmaganda. Unsurprisingly, harmful levels of cadmium, lead, and copper were found in the vegetables. Based on these findings and the health implication, they argued the government should ban the use of contaminated water for cropland irrigation to protect consumers.

Within Canada, routine testing of agricultural soils does not include tests for cadmium. Ministries, such as Ontario's Ministry of Agriculture, Food, and Rural Affairs (OMAFRA), recommend routinely testing all agricultural fields every 2-3 years (2009). However, these tests are for soil fertility, and do not include cadmium or other toxic metals or contaminants. As a result of this lack of testing, a study by Sheppard et al. (2007) provides valuable insight into the cadmium levels in croplands. For their study,

they contacted researchers across Canada to collect samples of soil, either used for agricultural research, or soil that was deemed representative of a region's agricultural soils. The trace element concentrations within these soils, along with those of some previously analysed Canadian agricultural soils were assessed. They reported that 4% of sites had cadmium concentrations in excess of the Canadian Soil Quality Guideline for Human Health (SQG_{HH}) for agricultural soil, as set by the Canadian Council of Ministers of the Environment (CCME, 1999). However, of note, this SQG_{HH} for cadmium is 1.4 mg/kg, which is considerably higher than the guidelines used by China. Additionally, Sheppard et al. (2007) reported a median cadmium concentration in their samples of 0.3 mg/kg, which is equal to the Chinese guideline for soils with a pH of 7.5 or less.

1.1.2.1 Cadmium in Fertilizer

Rock phosphates, which are typically composed of apatite minerals, are an important source of phosphate fertilizer (Mar & Okazaki, 2012). Unfortunately, these minerals can contain elevated concentrations of cadmium, which is not removed during the manufacturing of fertilizer. As a result, the fertilizers applied directly to crops and farmlands can have elevated cadmium concentrations. This has been highlighted as a concern, including by the United Nations Environmental Programme, Chemicals Branch (UN, 2010). However, there is some debate over the significance of cadmium in fertilizer, based on the concentrations at which it is present (e.g., Dharma-wardana, 2018; Roberts, 2014). Dharma-wardana (2018) states that cadmium in fertilizer should not be a concern since the levels added are minimal in comparison to what may already be present in the soil. In contrast, Roberts (2014) argues that, based on calculations made on behalf of The Fertilizer Institute, the risk-based concentration for cadmium in fertilizer is greater than even the highest limits set by governments, at 550 mg/kg phosphorus pentoxide (P_2O_5) versus limits of up to 400 mg/kg phosphorus pentoxide. They thereby conclude that the concentrations of cadmium in fertilizer are sufficiently low to avoid harm. Additionally, Roberts (2014) also argues that cadmium poisoning is "rare", and that the only known case of cadmium poisoning was in an area of Japan where residents developed itai-itai disease from industrial waste. However, this statement contradicts

other papers (e.g., Chan et al., 2021; Liao et al., 2016), which discuss widespread cadmium poisoning in China, resulting in a high prevalence of cancers.

1.1.3 Cadmium in Plants

As noted above in Section 1.1.1, plants can take up cadmium in concentrations that pose a risk to human consumers without the plants experiencing mortality. However, this is not to say there are no consequences to cadmium uptake for the plant. Like humans, plants also suffer from cadmium toxicity, and effects can be lethal.

In plants, adverse effects of cadmium include reduced growth and biomass, inhibition of photosynthesis, and decreased uptake and translocation of essential elements (Gill et al., 2012). Cadmium causes structural damage to the roots, which induces water stress due to limited water uptake, resulting in a reduced rate of water transport, both over short and long distances. This water stress, in combination with decreased stomatal opening, another symptom of cadmium toxicity, can decrease growth, leading to lower biomass (Rucińska-Sobkowiak, 2016). Additionally, cadmium can lead to an increase in reactive oxygen species (ROS), decreased chlorophyll levels, and/or the displacement of essential ions (Ismael et al., 2019; Rodríguez-Serrano et al., 2006). Cadmium ions, which have a charge of 2^+, can replace essential cations, particularly calcium but also iron and zinc ions. When cadmium replaces other ions within the chlorophyll molecule, it interferes with the plant's ability to photosynthesize, further impairing growth.

With cadmium adversely affecting plant health, plants may cope with cadmium in their environment via reducing its toxicity or better yet, reducing uptake. Toxicity can be minimized by plants via sequestration of cadmium in the vacuoles, thus preventing the cadmium from interfering with processes elsewhere in the cell (Parrotta et al., 2015). However, the plant's first line of defence is its cell wall. By binding cadmium to the root cell wall, plants may reduce the amount of cadmium that enters the cell and thus also the amount taken up and translocated to the shoots.

One of the components of the cell wall, specifically secondary cell walls, is lignin. Lignification of roots can make them less penetrable to cadmium, helping to prevent

cadmium uptake and toxicity (Bezrukova et al., 2011). This is because lignin may bind metals, including cadmium (e.g., Liu et al., 2018; Parrotta et al., 2015). However, these suggestions have largely been based on the strong evidence that metal ions bind to isolated lignin, rather than on studies of whole plants.

By preventing cadmium from being translocated to the shoots, the potential for toxicity symptoms in the plant is reduced (Lozano-Rodríguez et al., 1997). Concentrations of cadmium are usually highest in the roots (e.g., Hu et al., 2021; Zhou et al., 2017, review by Ismael et al., 2019). Hu et al. (2021) found on average, 77% of the cadmium in pepper plants was in the roots. However, while roots were consistently the highest in cadmium, the distribution throughout the aboveground portion of their plants varies by cultivar. Typically, leaves have a higher concentration of cadmium than stems, with even lower concentrations in the fruit and seeds (review by Ismael et al., 2019).

1.2 Selenium in the Environment

Like cadmium, selenium is also a naturally occurring element that can cause toxicity at elevated concentrations. However, it differs in that selenium is an essential micronutrient for all animals, with insufficient levels causing deficiencies and resulting in adverse health effects. It is not essential for higher plants; however, plant uptake of selenium is important for human nutrition.

Selenium is found in highly variable concentrations in the environment. The concentration of selenium in the earth's crust has been estimated to average 0.05 to 0.09 mg/kg (CCME, 2009). Yet, concentrations in soil as high as 1200 mg/kg have been reported (Fleming & Walsh, 1956). Of rocks, igneous rocks have the greatest abundance of selenium, and volcanos are responsible for contributing an estimated 400-1200 tons into the atmosphere annually (Saha et al., 2017). This is dwarfed by anthropogenic releases, which were estimated in 1988 to amount to 10,000 to 72,000 tons/year to aquatic ecosystems, with an additional 6,000 to 76,000 tons/year to soils (Nriagu & Pacyna, 1988). Selenium is often associated with coal, and deposits in the USA typically contain concentrations of around 3 mg/kg (Saha et al., 2017). As a result, waste products

from coal processing and burning contain significant amounts of selenium, and the release of this material poses a risk to local ecosystems.

1.2.1 Selenium and Human Health

For humans, the main sources of selenium are the consumption of meats and cereal grains; however, their relative importance differs geographically and based on soil selenium concentrations. In a review, Tamás et al. (2010) reported that cereals, including cereal products, were estimated to contributed 18-24% of total selenium uptake in the United Kingdom, whereas within a low-income population in India, these products contributed an estimated 40-50%.

Two diseases in humans are associated with selenium deficiency: Keshan disease, which causes cardiomyopathy, and Kaschin-Beck disease, which is a form of osteoarthropathy (review by Combs et al., 2011). While exceedingly rare in humans (Ishihara et al., 1999), white muscle disease can be problematic in livestock raised in regions with low selenium levels (Delesalle et al., 2017). The disease, which has a fatality rate of 30-45%, is associated with a deficiency in selenium, and/or a vitamin E deficiency. When identified early on as a concern, it can easily be prevented via supplementation. In addition to identifiable diseases being associated with selenium deficiency, many epidemiological studies have found greater selenium consumption correlated with a lower risk of cancer (Combs, 2001). Concerningly, they also reported that an estimated 500 million to 1 billion people worldwide are deficient in selenium.

Overexposure to selenium, whether chronic or acute, results in what is termed selenosis. Several of the common symptoms of selenosis are non-specific, such as fatigue, diarrhea, nausea, headaches, and joint pain. More identifying symptoms include foul (“garlic”) breath and effects on nails and hair. Hair loss is also common, along with brittle nails that may be discoloured and/or lost (MacFarquhar et al., 2010).

1.2.2 Fortification of Crops with Selenium

To help mitigate selenium deficiencies, different options are available, including but not limited to the utilization of selenium supplements, and fortification of food products or crops. For Finland, a region in which low selenium intake was identified as an issue, the strategy taken was to supplement fertilizers with selenium in the form of sodium selenate, which has been on-going since 1985 (Alfthan et al., 2015).

Finland considered other strategies, such as recommending selenium supplements in pill-form, or fortifying commercial animal feed, prior to settling on supplementing fertilizer (Aspila, 2005). By incorporating selenium into chemical fertilizer, a product that was, and still is, nearly universally used in agriculture, the selenium content of both animal feed and crops directly consumed by humans has been increased. Selenium has a narrow therapeutic range, with consumption of at 55 μg/day recommended for adults, and toxicity anticipated at doses of over 1600 μg/day (review by Tamás et al., 2010). However, risks of inadequate or excess consumption can be reduced by incorporating it into fertilizer. Since selenium is added to fertilizer in an industrial factory setting, rather than on each farm, the amount added can be controlled with relatively high precision (Alfthan et al., 2011). Furthermore, no additional labour is required from farmers, nor is additional effort required from consumers. This reduces the risk of unequal implementation of the program, or non-compliance, as well as overdose, which is a risk with pill-form supplements. As for supplementing animal feed, this was deemed inadequate. Some animal feed was already supplemented with selenium prior to implementing the selenium fertilizer program, which helped to prevent disease in livestock, such as white muscle disease (Alfthan et al., 2011). However, supplementation of animal feed was insufficient to raise the selenium nutritional status of residents to desired levels, hence the fertilizer program. Overall, the inclusion of selenium in fertilizer has been extremely effective; however, one gap has been identified. Certification standards for organically produced foods do not permit the use of chemical fertilizers. As a result, these foods remain unfortified with selenium (Alfthan et al., 2015).

1.2.3 Selenium in Plants

Selenium is not known to be required by vascular plants, and it is toxic in excess. However, the benefits of selenium appear to extend beyond those of fortification for the benefit of consumers. At low doses, selenium has been reported to provide benefits to plants as well (Feng, et al, 2013a). One of these benefits is reduced uptake of metals including cadmium (Cui et al., 2018; Huang et al., 2018a).

Highly variable amounts of selenium are taken up, depending largely on the species of plant as well as the concentration of selenium in the soil. Typically, most plants contain concentrations of less than 25 mg/kg dry weight (DW) (White et al., 2004). Crop species including corn, wheat, and oats typically have less than 30 mg/kg DW, even when grown on selenium-rich soils (Whanger, 2002). However, with application of supplemental selenium, high concentrations can occur in some plants, including common vegetables. Banuelos et al. (1993) reported selenate-supplemented cabbage (*Brassica oleracea* L. var. *capitata*) to have selenium concentrations of 260-450 mg/kg DW in the leaves. Swiss chard (*Beta vulgaris* L. var. *cicla*) had up 750 mg/kg in the leaves when the midribs were excluded, but only 115 mg/kg in the midribs themselves.

Distribution of selenium within plants appears to vary by species, and with the maturity of both the plant tissue and the plant itself. For plants that hyperaccumulate selenium (selenium hyperaccumulators, described below under Section 1.2.3.1), tissue concentrations have been found to be the highest in the fruit, followed by seeds, flowers, leaves, and then roots (Alford et al., 2012; Freeman et al., 2006). In these plants, younger leaves had higher selenium concentrations than older leaves. Plants that do not hyperaccumulate selenium (non-selenium hyperaccumulators) tend to have less variable selenium concentrations, with similar concentrations in their roots and shoots, with slightly more in fruit and flowers (Alford et al., 2012; Saha et al., 2017).

1.2.3.1 Selenium Hyperaccumulators

Plants are classified as selenium hyperaccumulators if their tissue selenium concentrations can exceed 1000 mg/kg DW when found growing on seleniferous soils

(White, 2016). These naturally-occurring soils are also defined based on their selenium concentrations, with the seleniferous designation typically given where the selenium concentration is at least 5 mg/kg DW (review by Saha et al., 2017).

Selenium hyperaccumulation is rare in plants. As of 2018 (Reeves et al., 2018) a mere 39 species, belonging to 15 genera, had been classed as selenium hyperaccumulators, which had increased to 41 species in 16 genera by October 2022 (University of Queensland, 2021, 2022). These genera include *Astragalus* and *Symphyotrichum*. The *Astragalus* species that hyperaccumulate selenium include *Astragalus bisulcatus* (Hook.) A. Gray (two-grooved milkvetch), *Astragalus racemosus* Pursh (creamy milkvetch). *Astragalus canadensis* L. (Canada milkvetch), is also occasionally included; however, it is not generally regarded as being a selenium hyperaccumulator (c.f., Sors et al., 2005). Its status as a possible hyperaccumulator has been attributed to a publication dating back to 1938 when it was reportedly recorded with a selenium concentration of 1110 mg/kg. However, the cited publication, Technical Bulletin 601 by Byers et al. (1938) does not contain any records for *A. canadensis*. Furthermore, in contrast to the oft-cited value of 1110 mg/kg for *A. canadensis*, *A. bisulcatus* has been reported with a selenium concentration of 14920 mg/kg (DW) (Beath & Knight, 1937), and *A. racemosus* with 13685 mg/kg DW (Sura-de Jong et al., 2015); these concentrations are more than an order of magnitude higher than that of the value attributed to *A. canadensis*. The selenium hyperaccumulators in the genus *Symphyotrichum* take up considerably less selenium than *A. bisulcatus* and *A. racemosus. Symphyotrichum ericoides* (L.) G.L. Nesom (heath aster) was recorded with a selenium concentration of 1378 mg/kg DW (El Mehdawi et al., 2015) and *Symphyotrichum lateriflorum* (L.) Á. Löve & D. Löve (calico aster) with 1800 mg/kg DW by Moxon et al., back in 1939.

1.3 Sulphur in the Environment

Unlike cadmium and selenium, sulphur is abundant in the environment, with an estimated 1.9% of the earth's mass composed of sulphur (EPA, 1991). It also differs in that the toxicity of sulphur in itself is quite low, with the element generally regarded as safe for humans (EPA, 1991). However, some sulphur compounds can pose a risk to the

environment and/or humans and other animals. In particular, sulphur dioxide (SO_2) is a significant atmospheric pollutant that has caused widespread environmental impacts.

Fossil fuel deposits have been reported to contain up to nearly 14% sulphur (Czogalla & Boberg, 1983). Typically, crude oil averages 0.03% to 7.9% sulphur, while coal in the United States ranges from 1% to 5.25% sulphur (Soleimani et al., 2007). While current technology allows for reduced sulphur dioxide emissions, the combustion of fossil fuels was and still is a significant source of sulphur dioxide emissions (Environment and Climate Change Canada, 2022). Sulphur dioxide can cause direct injury to plants and animals. However, more importantly, sulphur dioxide in the atmosphere combines with oxygen and water, forming sulphuric acid (H_2SO_4). This sulphuric acid in the atmosphere, along with nitric acid (HNO_3) formed from nitrogen oxides (NO_X) and water, result in wet acid deposition (e.g., acid rain). Acid deposition's vast, ecosystem-wide effects spurred government action in North America back in 1990 and 1991. Since then, measures to reduce emissions of sulphur dioxide as well as nitrogen oxides have been highly successful in reducing acid deposition, and long-term monitoring indicates that ecosystems have been recovering (EPA, 2022b).

In addition to the issues associated with acid deposition, sulphuric acid is also a key component of acid mine drainage. It is formed when sulphur-rich minerals are exposed to water and the weathering processes at the surface (Luo et al., 2020). Acid mine drainage has been reported to have very high sulphur concentrations. For example, 11,370-18,900 mg/L of sulphate was reported in acid mine drainage from a coal mine near Shandi Village in Yangquan, Shanxi, China, yielding a pH of 2-3 (Wang et al., 2021). In addition to the caustic nature of acid mine drainage on account of its low pH, its acidity also increases the solubility of metals. As a result, toxic concentrations of bioavailable metals frequently occur. These metals may include, but are not limited to, lead, cadmium, copper, zinc, chromium, beryllium, and vanadium (Wang et al., 2021), along with the metalloid arsenic (Luo et al., 2020).

1.3.1 Sulphur in Human Health

Sulphur is essential for all known living organisms. It is a component of amino acids, critically including methionine and cysteine, and sulphur is the 11th most abundant element in humans, at 0.3% by mass (including water) (Reece et al., 2012). As noted above, pure sulphur is generally regarded as safe for humans, despite being harmful to some organisms, enabling its use as a pesticide (EPA, 1991). Common inorganic sulphur compounds include the toxic gas hydrogen sulphide (H_2S), as well as sulphur dioxide (SO_2), a lung irritant, and sulphuric acid (H_2SO_4), which is caustic. As noted above, and arguably of greater importance, sulphur dioxide and sulphuric acid are significant environmental pollutants.

1.3.2 Sulphur in Soils and Croplands

The United States introduced its Clean Air Act in 1970, which was amended in 1990 to address additional pollutants, including those contributing to acid deposition, namely sulphur dioxide and nitrogen oxides. Then, in 1991, the Canada-United States Air Quality Agreement was signed by both countries (Environment and Climate Change Canada, 2020).

Within Canada, the three largest sources of sulphur dioxide emissions are ore and mineral industries, electrical power generation, and oil and gas industries. In 1990, these three sectors produced 49%, 21% and 18% of sulphur dioxide emissions in Canada, respectively, with a total of 3.0 million tonnes released. By 2018, total emissions had dropped to 0.81 million tonnes, with 32%, 27% and 34%, respectively, coming from the three sectors listed above. Total emissions were further reduced in 2020, with a total of 0.65 million tones emitted. By sector, 30% was produced by ore and mineral industries, 26% from electrical power generation, and 38% by oil and gas industries (Environment and Climate Change Canada, 2022). While data on total emissions in the United States are not readily available, the emissions of sulphur dioxide from power plants has dropped drastically. In 1990, emissions from power plants in the United States were 14.3 million tonnes (15.8 million tons). By 2020, this was reduced to 0.67 million tonnes (0.74 million

tons). For 2018, these emissions amounted to 1.1 million tonnes (1.2 million tons) (EPA, 2022a).

With reductions to sulphur dioxide emissions, sulphate deposition to soils and croplands has also dropped drastically. In southern Ontario, soils were receiving up to 44 kg/ha of sulphate from wet acid deposition in 1990. This input of sulphur was reduced to only 8-12 kg/ha by 2018 (OMAFRA, 2018). Previously, it was assumed that sufficient sulphur was present in croplands in southern Ontario, along with the northeastern United States. Throughout the vast majority of the United States, wet sulphate deposition for 2017-2019 was in the range of 0-12 kg/ha. Previously, in 1989-1991, wet sulphate deposition exceeded 32 kg/ha in a significant portion of the northeastern United States.

With decreased atmospheric deposition of sulphate, there is concern over potential sulphate deficiencies in crops, particularly looking to the future. It has been noted that with reduced sulphate deposition from the atmosphere, crop usage of sulphate currently exceeds inputs in parts of the United States, including the Midwest. Where soils are fine-textured and/or high in organic matter, applying sulphate was not found to increase yields at the time of David et al.'s (2016) study. However, the authors noted that due to the depletion of soil sulphur over time, it will likely be beneficial to crop yields in the future Where soils are sandy, sulphur is leached more readily, potentially resulting in sulphur deficiencies with lower atmospheric inputs. As a result of low soil sulphur levels, Wilson et al. (2020) observed increased wheat yields with sulphur fertilization. The Government of Alberta (2013) has also highlighted low sulphate levels as a concern, particularly for an estimated 2.4 to 3.2 million ha of farmland within the province. The soils at the greatest risk again are those that are sandy and low in organic matter, excluding those naturally rich in gypsum (calcium sulphate, $CaSO_4$).

1.3.3 Sulphur in Plants

Like for humans, sulphur is also essential for plants. It is a macronutrient, and typically found in plants at concentrations of around 0.13-0.26%, but may exceed this, depending on the species (Linzon et al., 1979). Sulphur is a component of two of the principal amino

acids found in plants: cysteine and methionine (review by Wirtz & Droux, 2005). As noted under Section 1.3.2, sulphur deposition rates were previously high, due to anthropogenic sulphur emissions. Thus, deficiencies were not previously commonly found, especially in eastern North America where sulphate deposition rates have historically been high. When sulphur deficiencies do occur in plants, symptoms typically include chlorosis and stunting of leaves, with new growth affected first (OMAFRA, 2018). Excess sulphate also does not typically directly affect plants; however, the low pH associated with sulphate deposition can have adverse effects. Specifically, a low pH can mobilize aluminum, leading to aluminum toxicity. This is particularly problematic when the soil pH is below 5.0 (Spectrum Analytical, 2010).

1.4 Uptake of Elements and Interactions Thereof

In plants, essential and unnecessary nutrients can interact. As per the review by Fageria (2001), interactions between elements include the formation of insoluble complexes in the growth medium or may be due to competition for uptake between ions with similar chemical properties.

Interactions between nutrients are common in plants and may be described as physiologically synergistic or antagonistic (Ranade-Malvi, 2011). This categorization is based on whether the addition of two or more elements together yields an increase (synergistic) or decrease (antagonistic) in growth and/or nutrient status when compared to the individual effects of the elements (Ranade-Malvi, 2011). Additionally, interactions between an essential element and a non-essential element can also occur (Khan et al., 2016; Pii et al., 2015), as can interactions between non-essential elements. In addition to these interactions between elements, other soil properties can also affect the availability and uptake of nutrients and other elements within the growth medium. These include, but are not limited to, pH, organic matter, cation exchange capacity, and moisture content (reviewed by Morel, 1997).

1.4.1 Uptake of Selenium and Sulphur

For most plants, the uptake of selenium, a non-essential element, appears inadvertent. Selenium ions are taken up in place of elements that are essential, specifically sulphur (sulphate) and phosphorus (phosphate). In soils, there are two potentially important ions of selenium: selenate and selenite. Selenium in the form of selenate (SeO_4^{2-}) is chemically similar to sulphate (SO_4^{2-}) and can be taken up in its place. Selenite (SeO_3^{2-}) is similar to, and taken up in place of, phosphate (PO_4^{3-}) (reviewed by Gupta & Gupta, 2017). Of the selenium found in agricultural soils, selenate is regarded as being the most significant form for plants (Mayland et al., 1991), with greater prevalence and bioavailability than selenite (Gupta & Gupta, 2017).

The uptake of sulphate, and by extension the uptake of selenate in its place, is an active process, with concentrations of sulphate in the root becoming higher than those of the growth medium (Brown & Shrift, 1982). This uptake is driven predominantly by high affinity sulphate transporters (group 1 SULTRs), with other sulphate transporters such as the Group 2 SULTRs, which are low affinity sulphate transporters playing a far less significant role in uptake (Terry et al., 2000). Once within the plant, sulphate is translocated and metabolized through the sulphur assimilation pathway. When selenate ions replace sulphate ions in this process, selenium is incorporated into cysteine and methionine within proteins (Sors et al., 2005; Tamás et al., 2010).

1.4.2 Interactions between Selenium and Sulphur

As would be expected with a simple replacement of sulphate with selenate, high sulphate in the growth medium has been shown to reduce selenate uptake (e.g., Zayed et al., 1998, review by Tamás et al., 2010). However, a few species, known as selenium hyperaccumulators appear to preferentially take up selenium, yielding high tissue concentrations.

While the mechanism by which these selenium hyperaccumulators are apparently able to selectively take up selenium over sulphur remains unclear, a few suggestions have been made. El Mehdawi et al. (2015) suggested that a sulphate transporter that favours selenate

could be overexpressed in hyperaccumulators. This overexpression, possibly in combination with an evolved increased specificity towards selenate by the transporter could yield a greater potential to take up selenium. However, this remains speculative, including which sulphate transporter might potentially favor selenate. Similarly, White et al. (2004) also suggest that sulphate transporters may vary in their specificity towards selenate and sulphate. Differences in transporter specificity are suggested as an explanation to help account for the variability in the ratio of sulphur to selenium in plant tissue. These ratios differ from species to species, as well as between different ecotypes of a single species, despite plants being grown in identical growth media (e.g., Zayed et al., 1998, review by Tamás et al., 2010).

1.4.3 Uptake of Cadmium

Cadmium ions enter the plant with the bulk flow of water. While some of this cadmium will remain in the apoplast, membrane transport proteins can also facilitate transport from the apoplast to the symplast (Clemens, 2006). As with selenium, cadmium is also taken up in place of elements that are essential. Cadmium ions (Cd^{2+}) replace other divalent metal cations, particularly calcium ions (Ca^{2+}), but also iron ions (Fe^{2+}), and zinc ions (Zn^{2+}) (Clemens, 2006). Cadmium within both the apoplastic and symplastic pathways is transported to the xylem; however, a significant amount is also transported to, and stored in, the vacuoles of root cells (Ismael et al., 2019). Once cadmium is in the xylem, it can be translocated throughout the plant. Additionally, some cadmium is also transported by the phloem in the form of chelates, notably in complexes with glutathione and phytochelatins (Mendoza-Cózatl et al., 2008).

1.4.4 Interactions between Cadmium and Sulphur

In addition to being essential to plants, sulphur has also been found to be beneficial in reducing the toxicity of cadmium to plants. Higher sulphate levels in the growth medium are associated with greater proportion of cadmium in the soluble fraction of root cells (including vacuoles). Zhang et al. (2014) found that in the absence of sulphur in the growth medium, 72% of the cadmium in roots was within the cell wall, with less than 15% in the soluble fraction. Contrasting this, in their high sulphur treatment (720 mg/L),

only 30% of the cadmium in the roots was found in the cell walls, and over 50% was in the soluble fraction. This has been attributed to an increased production of sulfhydryl (thiol) proteins as well as non-protein thiols, both of which bind to cadmium, as discussed by Bashir et al. (2015). Through this chelation process, cadmium can be transported to the vacuoles, reducing its toxic effects on the plant (review by Parrotta et al., 2015).

1.4.5 Interactions between Cadmium and Selenium

Many studies have reported a benefit to selenium in plants when co-applied with cadmium to the plants' growth medium (see review by Ismael et al., 2019). While stress responses and detoxification to the plant have frequently been topics of study, there is also evidence that selenium can reduce cadmium uptake by plants. Such findings have the potential to provide benefits related to food-safety, reducing cadmium intake among consumers, and providing dietary selenium to prevent deficiencies. Nonetheless, the mechanism(s) by which selenium may reduce cadmium uptake are frequently only speculative. Additionally, whether the benefit of selenium is dependent on only the concentration provided in the growth medium, or whether the concentration in plant tissue is important is unclear.

Researchers who have examined the role of selenium on cadmium uptake and concentrations of cadmium in plant tissue, have suggested mechanisms for their results including competition between cadmium and selenium for binding sites on protein carriers (Lin et al., 2012), the formation of insoluble Se-Cd complexes in the roots and/or growth medium (Guo et al., 2021), thickening of root cell walls and increased lignification (Cui et al., 2018). Application of selenium has been found to affect bioavailability of cadmium in soils, including by increasing the pH of the growth medium when applied in the form of selenite (Huang et al., 2018b). Alternatively, selenium may affect the subcellular distribution of cadmium. (Huang et al., 2021; Wan et al., 2019; Y. Zhao et al., 2019). Mitigation of cadmium-induced reductions in biomass has also been observed (Amirabad et al., 2020; Huang et al., 2017).

Amongst these papers, the strongest mechanistic explanation for selenium reducing cadmium uptake comes from Cui et al. (2018). In their study of rice suspension cells, those exposed to selenium and cadmium had increased lignification and thicker cell walls, leading to reduced cadmium diffusion into cells. This thickening was attributed to selenium-induced upregulation of genes associated with lignin synthesis. Cadmium alone had no effect on cell wall thickness compared to controls in their study. Increased lignification and thickening of the cell wall may increase metal adsorption sites, limiting metal absorption and translocation (Krzesłowska et al., 2010; Probst et al., 2009). Whether the same effects would be seen in the roots of whole plants and across different species was not tested. Similar to Cui et al. (2018), Zhao et al. (2019) also reported the cell wall to be important in binding cadmium, thus affecting the subcellular distribution of cadmium and its ability to be translocated to the shoots. However, within the cell wall, lignin was not analyzed, only pectin, hemicellulose 1 and 2, and cellulose. Contrasting this, Huang et al. (2021) found selenium to decrease the amount of cadmium bound to root the cell walls. Huang et al. (2021) found total cadmium in the roots was not affected by selenium, but the proportion of root cadmium in the organelles was near-zero in plants treated with only cadmium, versus about 20% in those treated with cadmium and selenium. Similarly, Wan et al. (2019), also found selenium to reduce cadmium bound to cell walls, and selenium (particularly in the form selenite) was associated with lower cadmium concentrations in the shoots. Thus, selenium does appear to affect subcellular distribution of cadmium across multiple studies, potentially with the cell wall playing an integral role.

An initially compelling mechanism due to its simplicity is competition between selenium and cadmium for uptake. However, this assertion that cadmium and selenium are in competition for binding sites on carrier proteins (Lin et al., 2012) seems improbable on further examination. First, cadmium ions have a charge of 2+, while the common ions of selenium are selenide (Se^{2-}), selenate (SeO_4^{2-}), and selenite (SeO_3^{2-}). Positive and negative ions do not compete. Additionally, the way in which cadmium and selenium bind to thiols differs. While cadmium binds to thiols, selenium replaces the sulphur in these functional groups, forming selenols in their place (Chang et al., 2022). Thus, there

is no competition between the dissimilar cadmium and selenium ions. Alternatively, cadmium and selenium have been suggested to form insoluble complexes in the growth medium (Guo et al., 2021), cadmium selenide (CdSe) for example (American Chemical Society, 2017). Guo et al. (2021) suggests that cadmium selenide may be formed under reducing conditions, either in hydroponics or flooded conditions. Huang et al. (2018b) similarly suggested the solubility of cadmium in the presence of selenium as a factor that may affect cadmium uptake in rice plants. They found the application of selenium to reduce the amount of extractable cadmium in the soil, and to increase soil pH.

Finally, a largely overlooked possible mechanism for reduced cadmium concentrations with selenium is a dilution effect. Co-application of cadmium and selenium can yield greater biomass than treatments with cadmium alone (e.g., Amirabad et al., 2020; Huang et al., 2017). Whether this increase in biomass with the application of selenium is sufficient to dilute the cadmium, reducing concentrations without affecting total uptake, is not typically discussed.

1.5 Study Rationale, Objectives, and Hypotheses

Potentially harmful concentrations of cadmium can, and unfortunately do, enter the human food supply when plants are grown on cadmium-contaminated soil (Peijnenburg et al., 2000). While cadmium-contaminated lands should not be utilized for agricultural crops, this is not the fact of the matter (e.g., Chan et al., 2021; Gazuwa & Olotuche, 2021).

1.5.1 Study Rationale

As a result of the utilization of cadmium-contaminated lands for agriculture, adjusting agronomic practices to minimize the uptake of toxic elements, including cadmium, from these soils is critical. One of the aims of this study was to provide insight on the possibility of using selenate applications to decrease cadmium uptake in plants to improve food safety, while simultaneously preventing selenium deficiencies.

However, sulphate in the growth medium has the potential to decrease the uptake of selenium in the form of selenate by plants (Zayed et al., 1998). Thus, the benefits conferred by selenium in reducing cadmium, as reported by several authors (e.g., Cui et al., 2018; Huang et al., 2018; Pereira et al., 2018), may not be universal. Rather, they may be heavily dependent on the sulphate levels in the growth medium, as well as the ability of the plant species to take up selenium. For selenate applications to be useful in reducing cadmium uptake from soils, the effect would have to be universal, with consistent benefits across species, and occurring regardless other factors, such as sulphate levels.

1.5.2 Objectives

Tissue selenium concentrations are highly variable across different plant species, and despite this, past studies offer limited insight into the effect of tissue selenium concentrations on cadmium uptake. Typically, studies investigated only a singular plant species, if not a singular variety thereof. Additionally, despite evidence for selenium inhibiting cadmium uptake, few have studied the mechanism(s) behind this phenomenon.

This study is intended to provide insight into the possibility of using selenate applications to decrease cadmium uptake in plants, while examining a possible mechanism for the effect: the lignification of root cell walls. Lignification will be quantified in two species of selenium hyperaccumulators and one related non-hyperaccumulator of selenium, grown with or without selenate. The study is also designed to determine if high sulphate levels will limit the usefulness of selenate applications. Alternatively, if selenate is found to increases tolerance to cadmium, this information may potentially be beneficial in designing phytoremediation projects in which cadmium concentrations restricts plant growth.

1.5.3 Hypotheses and Research Questions

After using chemical equilibrium software to rule out the possibility of the formation of insoluble cadmium-selenium complexes in the growth medium, two hypotheses will be tested using an experimental approach. The first hypothesis will be tested in three *Astragalus* species, two that hyperaccumulate selenium and one that does not. The second

hypothesis will be tested in the same three *Astragalus* species, plus 5 crop species (canola, corn, lettuce, sorghum, and wheat).

1.5.3.1 Hypothesis 1

The first hypothesis pertains to the lignification of root cell walls, which are expected to be a barrier to cadmium uptake, the expected mechanism for increased tolerance to cadmium with selenate.

Hypothesis 1:

> *Selenium leads to more lignified cell walls and, because this will increase the amount of cadmium bound to cell walls, higher tissue selenium will be associated with a greater tolerance to cadmium.*

1.5.3.2 Hypothesis 2

The second hypothesis is based on a predicted negative relationship between selenium and cadmium concentrations in the shoots, as had been reported previously by several authors (e.g., Cui et al., 2018; Huang et al., 2018b; Pereira et al., 2018). In essence, the expectation is that the same negative relationship between selenium and cadmium concentrations in the shoots will be present, albeit with lower selenium concentrations, with high sulphate in the growth medium. Multiple species will be used to test Hypothesis 2, including the non-hyperaccumulating and hyperaccumulating *Astragalus* species, capturing a wide range in the concentrations of selenium in the plants.

Hypothesis 2:

> *Because high sulphate in the growth medium inhibits uptake and translocation of selenate, cadmium uptake will increase (as per Hypothesis 1). In essence, the mitigative effect of selenate in reducing cadmium uptake and translocation to the shoots will be diminished by high sulphate.*

Chapter 2

2 Methods and Methodology

Hypotheses were tested using a controlled environment experimental approach. Plants of eight species were grown hydroponically, which maximized control over their growing conditions, including the availability of nutrients. The eight species included five crop species, and three *Astragalus* species, two of which were selenium hyperaccumulators. Following the growth period, lignin in the roots of the three *Astragalus* species was quantified to show the effect of the selenate treatment on lignification, a potential mechanism for decreasing cadmium uptake and translocated to the shoots. Cadmium and selenium concentrations in the shoots of all 8 species were measured to determine if higher shoot selenium concentrations did in fact reduce shoot cadmium concentrations. The relationship between selenium and cadmium in shoots was then compared across species, to determine if the relationship was consistent across the species and across a wide range of tissue selenium concentrations.

2.1 Species Selection and Seed Acquisition

2.1.1 Finding Species with a Broad Range of Selenium Uptake Ability

To test the effects of tissue selenium concentrations on the uptake and translocation of cadmium to the shoots, plant species that would provide a wide range of tissue selenium concentrations were desirable. As discussed in Section 1.2.3.1, some plant species hyperaccumulate selenium, with concentrations exceeding 1000 mg Se per kg DW. Thus, by utilizing one of these species, particularly alongside a related non-hyperaccumulator of selenium, a broad range of tissue selenium concentrations could be anticipated.

Since, as mentioned in Section 1.2.3.1, as of November 2021, there were only 15 genera of plants containing selenium hyperaccumulator species (University of Queensland, 2021), selection was limited. Seeds were acquired from two of the genera: *Astragalus* and *Symphyotrichum*. *Astragalus* was the only genus that had commercially available seeds for both known selenium hyperaccumulators and species not regarded as such.

Symphyotrichum was the only genus that had commercially available seeds for both known selenium hyperaccumulator species, as well as species of unknown selenium hyperaccumulator status.

Prior to the final species selection, preliminary testing was conducted on four *Astragalus* and five *Symphyotrichum* species (Appendix I). Seeds were purchased in August 2018 from Prairie Moon Nursery (www.prairiemoon.com), a retailer specializing in North American native plants and seeds. Seeds were purchased for *Astragalus bisulcatus* (Hook.) A. Gray (two-grooved milkvetch), *Astragalus canadensis* L. (Canada milkvetch), *Astragalus crassicarpus* Nutt. (groundplum milkvetch), *Astragalus racemosus* Pursh (creamy milkvetch), *Symphyotrichum cordifolium* (L.) G.L. Nesom (heart-leaved aster), *Symphyotrichum ericoides* (L.) G.L. Nesom (heath aster), *Symphyotrichum lanceolatum* (Willd.) G.L. Nesom (panicled aster), *Symphyotrichum lateriflorum* (L.) Á. Löve & D. Löve (calico aster), and *Symphyotrichum pilosum* (Willd.) G.L. Nesom (frost aster). Selenium hyperaccumulator candidates included *A. bisulcatus*, *A. racemosus*, *S. ericoides*, and *S. lateriflorum*. The remaining 5 species were candidates for use as a non-selenium hyperaccumulator to provide tissue at the lower range of selenium concentrations.

Based on preliminary testing, the *Symphyotrichum* species were eliminated, along with *A. crassicarpus*. *Symphyotrichum* species were eliminated primarily due to difficulties in their cultivation. The *Symphyotrichum* species had much smaller seedlings, and slower growth, particularly when young, when compared to the *Astragalus* species. Additionally, published values for selenium concentrations in the hyperaccumulator *Symphyotrichum* species were only 1378 mg/kg DW for *S. ericoides,* and 1800 mg/kg DW for *S. lateriflorum* (el Mehdawi et al., 2015, and Moxon et al., 1939, respectively), versus 14920 mg/kg DW for *A. bisulcatus* and 13685 mg/kg DW for *A. racemosus* (Beath & Knight, 1937, and Sura-de Jong et al., 2015, respectively).

For the *Astragalus* species, *A. crassicarpus* was also eliminated primarily based on its growth characteristics. When compared to *A. canadensis*, the other remaining non-hyperaccumulator candidate, it had less vigorous growth, and the control plants were

much less consistent in their biomass (Appendix I). Of the two *Astragalus* hyperaccumulator candidates, preliminary testing found that *A. racemosus* took up approximately twice as much selenium as *A. bisulcatus* on average (Appendix I). While *A. bisulcatus* could have been eliminated, since *A. racemosus* provided the highest shoot selenium concentrations, both species were utilized, yielding a better spectrum of high selenium concentrations. Thus, ultimately, *A. bisulcatus*, *A. racemosus* and *A. canadensis* were selected, with the first two being selenium hyperaccumulators, and the latter being a non-hyperaccumulator of selenium.

2.1.2 Choosing Species in which to Test the Effect of Sulphate on Selenium and Cadmium Uptake

The second hypothesis, examining the effect of high sulphate in the growth medium and how that might inhibit uptake of selenate and translocation of selenium, thus impacting cadmium uptake, was tested using the three *Astragalus* species discussed in Section 2.1.1, along with five common crop species. The crop species were canola (rape, *Brassica napus* L.), corn (*Zea mays* L.), lettuce (*Lactuca sativa* L.), sorghum (*Sorghum bicolor* (L.) Moench), and wheat (*Triticum aestivum* L.). Information on the seeds, including sources, varieties and lot numbers can be found in Table 2.1.

Table 2.1: Supplier information on crop species seeds.

Crop Species	Variety	Seed Supplier	Seed Lot
Canola (rapeseed) (*Brassica napus*)	InVigor L130	BASF Canada Inc.	N/A
Corn (*Zea mays*)	Golden Bantam	West Coast Seeds	361-03-501
Lettuce (*Lactuca sativa*)	Dillon (Organic)	West Coast Seeds	1.169.289
Sorghum (*Sorghum bicolor*)	Mixed Colours Broom	West Coast Seeds	2017MCB
Wheat (*Triticum aestivum*)	Winter Wheat	West Coast Seeds	375-9-145361

Note: N/A indicates information that was not available

Of the selected crop species, the three grasses (family: Poaceae), wheat, corn, and sorghum, were selected on account of their widespread cultivation (Table 2.2). Other crops that occupy a large area worldwide but were eliminated included rice (*Oryza sativa* L.) and barley (*Hordeum vulgare* L.). Rice was not included on account of being semi-aquatic, unlike most other crop species. Between sorghum and barley, which occupy

3.0% and 3.4% (respectively) of the global area used as cropland (Table 2.2) (FAO, 2018) sorghum was chosen. Sorghum is important in arid regions including in Africa, India, and the Middle East, where species such as wheat and rice do not readily grow (Hamman et al., 2001). Barley is also important, particularly in climates where corn does not grow well, due to factors such as a short growing season, cool springs, low rainfall, or high evaporation rates (Akar et al., 2004). However, between sorghum and barley, a higher proportion of sorghum produced globally is used for direct human consumption, at approximately 50% for sorghum versus 30% for barley, as opposed to for animal feed (Hamman et al., 2001, and Akar et al., 2004, respectively).

Another crop that was not selected but occupies a significant area worldwide is soybean (*Glycine max* (L.) Merr.). With the three *Astragalus* species already included in experiments for their selenium hyperaccumulator (or related non-hyperaccumulator) status, soybean was not chosen on account of being a member of the same family: Fabaceae. Non-grass species selected were canola (rape or rapeseed) and lettuce. Canola, of the family Brassicaceae, was selected on account of being widely cultivated and for having applications both as a foodstuff and in non-food uses, particularly for biodiesel. Globally, rapeseed (canola) is the third most used feedstock for biodiesel (20%), after palm oil and soybean oil (Union zur Förderung von Oel, 2019). Thus, if inedible due to high selenium or cadmium concentrations, it may still have applications elsewhere. Finally, lettuce, belonging to the family Asteraceae, was selected. Lettuce is grouped with chicory by the FAO (2018) in their crop statistics, and together they are only the 78th most crop worldwide by area, out of 159 crops (or groupings thereof). As per Table 2.2, some other crops not selected included cabbage and other brassicas, cassava, and potatoes. Cabbage (and other brassicas) were eliminated since canola was already selected from this family. Root vegetables were intentionally excluded, given that a reduction in the cadmium concentrations in the shoots of such vegetables would be of limited benefit from a food safety viewpoint. Lettuce was also of interest in that it is the foliage that is consumed. Based on data in FAO (2018), aside from “cabbage and other brassicas”, lettuce and chicory are the most widely cultivated vegetables among those commonly classified as “leafy greens”.

Table 2.2: Crop Information from the Food and Agriculture Organization of the United Nations.

Crop	Family	Rank by Area Harvested*	Percent of Total Cropland Area Harvested*	Selected?
Wheat	**Poaceae**	**1**	**15.1%**	**Yes**
Maize (Corn)	**Poaceae**	**2**	**13.6%**	**Yes**
Rice, paddy	Poaceae	3	11.7%	No
Soybeans	Fabaceae	4	8.8%	No
Barley	Poaceae	5	3.4%	No
Sorghum	**Poaceae**	**6**	**3.0%**	**Yes**
Rapeseed (Canola)	**Brassicaceae**	**7**	**2.6%**	**Yes**
Cassava	Euphorbiaceae	14	1.7%	No
Vegetables (not elsewhere specified)	N/A	15	1.5%	No
Potatoes	Solanaceae	18	1.2%	No
Cabbage and other Brassicas	Brassicaceae	57	0.2%	No
Lettuce and Chicory	**Asteraceae**	**78**	**0.1%**	**Yes**

*FAO (2018)

2.2 Plant Growth Conditions and Experimental Treatments

2.2.1 Growth Conditions

Plants were grown in a controlled environment chamber using a hydroponic system to maximize control over growing conditions. Five replicates were used, with a total of 235 plants. Following the initial growth phase, each of the 235 experiment plants was grown in a 1 L jar, containing a modified Hoagland solution (see Section 2.2.2) supplemented with the applicable treatment. This nutrient solution was aerated using either a Top Fin Air-8000 (4-port) or an Air-4000 (2-port) Air Pump. Each air outlet port on a pump was connected to up to 6 plants. The growth chamber was maintained at 22°C to 23°C, with a minimum of 60% humidity. The day/night cycle was set at 14 hours of light followed by 10 hours of dark. The average light intensity was 216.5 ±11.5 μmol m^{-2} s^{-1} as measured

using a Field Scout™ Dual Solar/Electric Quantum Meter, model number 3415FSE light meter made by Spectrum Technologies Inc. The light intensity measurements were taken at a height of 18 cm from the shelf on which the plants were situated, corresponding to the height at the base of each plant (top of jar).

2.2.2 Growth Medium

Unless otherwise indicated, reagents were purchased from either VWR Canada or SigmaAldrich Canada. The base growth medium was a modified Hoagland nutrient solution, based on the recipe used by both Cabannes et al. (2011) and White et al. (2007). It contained 2 mM $Ca(NO_3)_2 \cdot 4H_2O$, 2 mM NH_4NO_3, 0.5 mM K_2HPO_4, 0.5 mM KH_2PO_4, 1.0 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 30 µM H_3BO_3, 10 µM $FeCl_3 \cdot 6H_2O$, 10 µM $Na_2EDTA \cdot 2H_2O$ (ethylenediaminetetraacetic acid disodium salt dihydrate), 5 µM $MnCl_2 \cdot 4H_2O$, 1 µM $ZnCl_2$, 0.15 µM $CuSO_4 \cdot 5H_2O$, 0.1 µM $Na_2MoO_4 \cdot 2H_2O$. This solution was supplemented as needed with cadmium, selenate and/or additional sulphate, as detailed in Section 2.2.4. Across all treatments, the pH of the solution was 6.6 to 6.7.

2.2.3 Early Growth of Plants

It was important to establish germination and cultivation timing for each species such that seedlings of similar developmental stage were used for the experimental treatments. Early growth of plants, prior to the application of the experimental treatments (Section 2.2.4) varied slightly by species, as described in Table 2.3 for *Astragalus* spp. and Table 2.4 for crop species.

Seeds of the three *Astragalus* species, *A. bisulcatus*, *A. canadensis* and *A. racemosus*, were scarified using fine grit sandpaper, and then soaked overnight at 4°C in a dilute (0.002%) solution of Vitaflo-280 fungicide (Carbathiin and Thiram) and reverse osmosis (RO) water. Seeds were then cold-stratified by placing them on filter paper moistened with RO water in Petri dishes and kept at 4°C for 10-12 days in the dark. Seeds of crop species did not require scarification, soaking or cold stratification, so these steps were omitted for crop species. Petri dishes, each containing 5-10 seeds (depending on seed size) on moistened filter paper, were then placed at room temperature in the dark to

Table 2.3: Timeline with durations of different growth stages for *Astragalus* spp.

	***Astragalus* Species**		
	A. canadensis	***A. bisulcatus***	***A. racemosus***
Scarified	Yes	Yes	Yes
Seed Soak Duration	Overnight	Overnight	Overnight
Duration of Cold/Moist Stratification	10 days	11-12 days	11-12 days
Duration of Germination (in Petri Dishes)	2 days	1 day	1 day
Duration of Early Growth (in Coarse Sand)	12 days	8 days	8 days
Duration in 100 mL Jars	7-8 days	6-7 days	6-7 days
Duration in 1 L Jars Prior to Treatment	2 weeks	2 weeks	2 weeks
Duration 1 L Jars with Treatments Applied Weekly	30 days = 4 weeks and 2 days	69 days = 9 weeks and 6 days	83 days = 11 weeks and 6 days
Duration in 1 L Jars with Treatments Applied every 3rd Day	16 days = 2 weeks and 2 days	N/A	N/A
Number of Times Treatments Applied	11 (5 times weekly, 6 times every 3rd day)	10	12

Note: N/A is used where a treatment was not utilized for the specified species, thus the duration is not applicable. Specifically, duration with treatments applied every 3rd day are not applicable in species with slower growth, as these plants were harvested prior to needing more frequent replacement of their nutrient solution containing the treatments.

promote germination for all species except lettuce, which was placed in the growth chamber at 23° to germinate in the light. Seeds were watered daily for 1-4 days, depending on the species and its respective rate of germination. Seedlings were then planted in well gravel WP #1, a coarse to very coarse silica sand (US sieve size of 12-20 mesh, or 0.841 mm to 1.68 mm). This substrate was moistened with nutrient solution

(described in Section 2.2.2), without the addition of the experimental treatments. At this point, seedlings not already in the growth chamber (i.e., those germinated in the dark) were moved to the chamber, where they remained until harvest. Growth chamber conditions are described above, in Section 2.2.1.

Table 2.4: Timeline with durations of different growth stages for crop species

	Crop Species				
	Canola	**Corn**	**Lettuce**	**Sorghum**	**Wheat**
Scarified	No	No	No	No	No
Seed Soak Duration	N/A	N/A	N/A	N/A	N/A
Duration of Cold/Moist Stratification	N/A	N/A	N/A	N/A	N/A
Duration of Germination (in Petri Dishes)	3 days	3-4 days	2 days	3-4 days	2-3 days
Duration of Early Growth (in Coarse Sand)	2 days	2 days	4 days	2 days	2 days
Duration in 100 mL Jars	3 days	3 days	7 days	3 days	3 days
Duration in 1 L Jars Prior to Treatment	0	0	0	0	0
Duration 1 L Jars with Treatments	20 days = 2 weeks and 6 days	20 days = 2 weeks and 6 days	20 days = 2 weeks and 6 days	20 days = 2 weeks and 6 days	20 days = 2 weeks and 6 days
Number of Times Treatments Applied	3	3	3	3	3

Note: N/A is used where a treatment was not utilized for the specified species, thus the duration is not applicable.

After 2-12 days in the well gravel, seedlings were moved to 100 mL jars containing the nutrient solution, again without the addition of the experimental treatments. The variation in timing was due to having to wait until the seedlings were large enough to be suspended above the nutrient solution. Three to 8 days later, uniformly sized seedlings were moved to 1 L jars, again containing the nutrient solution, and aerated. *Astragalus* spp. were grown in these 1 L jars for 2 weeks prior to the application of the treatments, while crop

species were given the treatments when they were transplanted to 1 L jars. Preliminary testing had found *Astragalus* spp. to have much less uniform growth among plants than was desirable. Allowing additional time for growth prior to the applications of treatments permitted more uniformly sized plants to be selected at a later time point. From the initial scarification or sowing of seeds to the selection of uniformly sized plants for treatments, 67-96% of the initial batch of seeds or their resulting seedlings were eliminated, with an average of 86%.

2.2.4 Experimental Treatments

Up to 8 different treatments were used (Table 2.5) depending on the species (Table 2.6). These were based on the species' relative selenium tolerance, and the hypothesis being tested. To produce the treatments, the base nutrient solution was supplemented with nothing (control), cadmium, selenate and/or additional sulphate. Cadmium treatments were produced by adding a volume of stock solution to achieve 1.6 µM of $CdCl_2$•2½ H_2O in the growth medium. Selenate in the form Na_2SeO_4•$10H_2O$ was used for both 'selenate' and 'high selenate' treatments, to achieve concentrations of 5 µM and 40 µM of selenate, respectively. Treatments containing high sulphate received an additional 4.5 mM of $MgSO_4$ on top of the 1.00015 mM in the base growth medium, yielding a total sulphate concentration of 5.50015 mM, hereafter rounded to 1.0 mM and 5.5 mM, respectively.

Modeling of the growth medium (Appendix II) with all experimental treatments across a wide pH range was conducted to verify that cadmium, sulphate and selenate in the experimental treatments were bioavailable and did not precipitate. For sulphate and selenate, 100% was in solution within the pH range modelled, and at least 97.6% of cadmium was in a bioavailable form. Additionally, the application of the experimental treatments had negligible effects on the availability of essential nutrients in the solutions.

Table 2.5: Concentrations of cadmium, selenate, and sulphate in experimental treatments.

Treatment Names		Treatment Concentrations		
Full Name	**Abbreviated Name**	**Cadmium (μM)**	**Selenate (μM)**	**Sulphate (mM)**
Control	Control	0	0	1.0
Cadmium	Cd	1.6	0	1.0
Selenate	Se	0	5	1.0
Cadmium + Selenate	Cd + Se	1.6	5	1.0
Cadmium + high Sulphate	Cd + S	1.6	0	5.5
Cadmium + Selenate + high Sulphate	Cd + Se + S	1.6	5	5.5
Cadmium + high Selenate	Cd + Se (40)	1.6	40	1.0
Cadmium + high Selenate + high Sulphate	Cd + Se (40) + S	1.6	40	5.5

Note: Values for sulphate have been rounded to 1 decimal place.

To evaluate the effect on cadmium uptake as influenced by selenate and high sulphate, all 8 species were grown in 5 of the treatments: 'control', 'cadmium', 'cadmium + selenate', 'cadmium + high sulphate', and 'cadmium + selenate + high sulphate' (Table 2.5). To evaluate the relative selenium accumulation in *Astragalus* species, they were grown in one additional treatment, 'selenate', while the two selenium hyperaccumulators, *A. bisulcatus* and *A. racemosus*, were also grown in two other treatments: 'cadmium + high selenate', and 'cadmium + high selenate + high sulphate'. Unlike the other two *Astragalus* species, *A. canadensis* was not grown in the high selenate treatments, due to its lower tolerance to selenium toxicity. Preliminary testing had found a dose of 60 μM selenate to be lethal in this species, while a dose of 20 μM reduced biomass by an average of 85% compared to control.

Thus, in total, 235 plants were grown, which included 5 replicates per treatment. The breakdown was 30 plants of *A. canadensis*, 40 plants of *A. bisulcatus*, 40 plants of *A. racemosus*, and 25 plants for each of canola, corn, lettuce, sorghum, and wheat.

Table 2.6: Utilization of experimental treatments by species.

	Treatment Name							
Species	**Control**	**Cadmium**	**Selenate**	**Cadmium + Selenate**	**Cadmium + high Sulphate**	**Cadmium + Selenate + high Sulphate**	**Cadmium + high Selenate**	**Cadmium + high Selenate + high Sulphate**
A. bisulcatus	X	X	X	X	X	X	X	X
A. canadensis	X	X	X	X	X	X		
A. racemosus	X	X	X	X	X	X	X	X
Canola	X	X		X	X	X		
Corn	X	X		X	X	X		
Lettuce	X	X		X	X	X		
Sorghum	X	X		X	X	X		
Wheat	X	X		X	X	X		

An "X" denotes that the species was grown in the associated treatment. Full descriptions of the treatments were provided in Table 2.5.

The nutrient solution, including the experimental treatments, was replaced every 7th day for the duration of the experiment for all species except *A. canadensis*. For *A. canadensis*, the nutrient solution was replaced every 7th day for the first 30 days, and then replaced every 3rd day for the remaining 16 days. The change in frequency for *A. canadensis* was due to their large size relative to the other *Astragalus* species, resulting in more rapid uptake of the nutrient solution. For all species, solution volumes were topped up to 1 L daily (as needed) with RO water.

The *Astragalus* species varied in growth rates, so *A. canadensis* plants were grown in the treatments described above for 46 days, *A. bisulcatus* for 69 days, and *A. racemosus* for 83 days (Table 2.3). Resultingly, treatments were applied 11, 10 and 12 times, respectively. The total mass of cadmium applied to each plant (in applicable treatments) was 2.0 mg, 1.8 mg and 2.2 mg, for *A. canadensis*, *A. bisulcatus* and *A. racemosus*, respectively. The total sulphur (in the form sulphate) applied in treatments with high sulphate was 2.9 g, 2.6 g, or 3.2 g, respectively. Treatments with sufficient sulphate received 0.53 g, 0.48 g, and 0.58 g of sulphate, respectively. In selenate treatments, each

plant received 4.3 mg, 3.9 mg or 4.7 mg, or selenium respectively. In “high selenate” treatments, *A. bisulcatus* plants received 32 mg of selenate each, and *A. racemosus* plants 38 mg.

Crop species, which grew quickly relative to even *A. canadensis*, were grown in the experimental treatments for only 20 days (Table 2.4). Treatments were applied every 7th day, resulting in 3 applications. The total doses of selenium and cadmium administered (in applicable treatments) were 1.2 mg and 0.54 mg, respectively. Sulphur amounted to 0.53 g in treatments with ‘high sulphate’, and to 0.096 g in the other treatments.

2.3 Harvest Procedure

Upon completion of the growth period, plants were harvested, at which point roots and shoots were divided and processed according to the analysis for which they were to be used.

First, roots were separated from the aboveground portion (hereafter called shoots). The shoots were weighed for fresh mass, placed in paper bags, and placed in an oven at 60°C to dry to constant weight. Roots were blotted dry with paper towel and then weighed for their fresh mass. Then, to remove (unabsorbed) nutrient solution from the surface of the roots, roots were rinsed in a bath of 3 L of RO water, which was changed between plants. Plants that underwent desorption of cadmium from their root surface, as detailed in Section 2.3.1, had their roots separated into two portions at this point, one of which was used for the desorption. Roots, including both those that underwent desorption and those that did not, were then separately blotted dry with paper towel and placed in paper bags in the oven at 60°C.

For the lignin analysis two small subsamples, each of approximately 0.5 g (fresh weight), were taken and weighed. One sample was taken from the large diameter roots, located near the base of the plant, while the second was taken from the small diameter root near and including the root tips. There were two exceptions to the collection of two root samples per plant, with two plants each only yielding one sample. These plants, both of which were *A. canadensis* in the ‘cadmium + selenate’ treatment, had uniformly textured

roots throughout and, more importantly, did not have sufficient root mass to yield two samples of approximately 0.5 g each, given the need for root tissue for ICP-MS analysis as well.

The processing of the samples for lignin analysis is detailed in Section 2.4.1, and the analysis itself is described in Section 2.4.2.

The desorbed and non-desorbed roots were used for quantification of cadmium content using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Once dry, shoots and roots were re-weighed, and the total root mass calculated, factoring in the proportion of the roots removed for the lignin analysis. The dried tissue was then prepared for ICP-MS analysis, as detailed in Section 2.5.

2.4 Cadmium Desorption

Cadmium desorption was conducted to test the distribution of cadmium within the roots to evaluate the first hypothesis. This hypothesis examined whether higher selenium concentrations in plant root tissue was associated with higher lignin concentrations, allowing a greater amount of cadmium to be adsorbed to the cell wall. In testing this hypothesis, *Astragalus* spp. in the 'control', 'cadmium', 'cadmium + selenate', and 'selenate' treatments were employed. For these plants, approximately one half of the roots were collected during the harvest process and underwent cadmium desorption.

The cadmium desorption was utilized to estimate the proportion of the cadmium in the roots that was bound to the outer portion of the roots, particularly the epidermal root cell walls and possibly the apoplast of outer cortical cells, as opposed to the amount in the interior of the roots. The portions of the roots that did not undergo cadmium desorption provided data on the total cadmium concentration in the roots. To estimate the amount of cadmium bound to the epidermal cell walls, the difference between the non-desorbed roots (total cadmium in root) desorbed roots (cadmium in the interior of the root) was used.

During harvest, the roots were divided into two portions with similar root size distributions, taking care to minimize root breaking in the process, with one portion allocated for cadmium desorption. These roots were suspended in 1 L of 5 mM calcium chloride for 10 minutes, as per the methods recommended by Buckley et al. (2010). The calcium chloride solution was situated over an ice bath and changed between plants. Roots were suspended such that the base of the roots, where they had been severed from the rest of the plant, was kept slightly above the surface of the calcium chloride solution. After removing the roots from the calcium chloride solution, roots were blotted dry with paper towel, placed in paper bags, and dried at 60°C until they reached a constant mass. Both desorbed and non-desorbed parts of the root system were analyzed for cadmium via ICP-MS as described in Section 2.5.

2.5 Lignin Analysis

Along with the cadmium desorption, the lignin content of root tissue was needed to test the first hypothesis. Consequentially, the same plants that underwent cadmium desorption were also analyzed for lignin. These plants were the *Astragalus* spp. in the 'control', 'cadmium', 'cadmium + selenate', and 'selenate' treatments.

2.5.1 Cell Wall Extraction

To quantify the lignin in the roots, the cell wall component was first extracted following the methods of Fukuda & Komamine (1982). This entailed immediately grinding the fresh tissue into powder in liquid nitrogen. The sample was then rinsed by placing it in a 15 mL centrifuge tube and 95% ethanol was added to make the volume up to 15 mL. Samples were then centrifuged at 1000*g* for 5 minutes, after which the supernatant was removed. Samples were then rinsed three more times, each time by adding 10 mL of 95% ethanol, centrifuging for 5 minutes at 1000g, and removing the supernatant. Sample were then rinsed twice in 10 mL of 2 parts hexane to 1 part 95% ethanol, again centrifuging at 1000g for 5 minutes and removing the supernatant after each centrifugation. The centrifuge tubes containing the samples were then uncapped and allowed to air dry until they reached a constant mass.

2.5.2 Lignin Quantification

For extraction and quantification of lignin from within the cell wall component of the roots, acetyl bromide was used to digest the tissue. Following comparison of several methods (including Chang et al., 2008; Fukuda & Komamine, 1982; Fukushima & Hatfield, 2001, 2004; Hatfield et al., 1999; and Moreira-Vilar et al., 2014), the methods of Fukuda & Komamine, 1982 were followed with slight modification. Their paper analysed tissue from a herbaceous plant, *Zinnia elegans* L., and their protocol was similar to the methods of other papers.

First, where possible, approximately 10 mg of the dried cell wall component (extracted as described above in Section 2.4.1) was weighed and placed in a glass scintillation vial. Next, 1 mL of a solution of 25% acetyl bromide and 75% glacial acetic acid was added to each vial and contents swirled to mix. Vials were capped, placed within a second lidded glass container, and then placed in an oven at 70°C for 30 minutes. Vials were removed from the oven every 10 minutes to swirl the mixture to ensure mixing and then returned to the oven. After 30 minutes, vials were removed from the oven, swirled again, and placed in a bath of ice and water to cool. Once cooled to room temperature, 0.9 mL of 2 M sodium hydroxide was added to each vial. After thorough swirling, 0.1 mL of 7.5 M hydroxylamine hydrochloride was added. Glacial acetic acid was then added, and the contents of each vial transferred to a 15 mL centrifuge tube and the volume made up to 10 mL with glacial acetic acid (or proportionally less, for samples where less than 10 mg of root cell wall material was available). Samples were then centrifuged for 5 minutes at 1400g (force as per Moreira-Vilar et al., 2014, and due to centrifuge tubes' ability to withstand force).

Alkali lignin (purchased from Millipore Sigma) was pre-treated in the same manner as root tissue and was digested alongside the root cell wall samples to aid in the interpretation of spectrophotometry results and to standardize across batches, as per Yang et al. (2007). For pure lignin samples, a mass of approximately 1 mg was weighed and used. Since the lignin content of pure alkali lignin is obviously significantly higher than the lignin content of cell walls, a lower mass was used to yield a similar lignin

concentration as the plant samples. These samples, despite their lower mass, were still made up to a volume of 10 mL with glacial acetic acid prior to centrifuging.

Blanks, which contained no tissue or lignin, were similarly processed alongside samples, adding the acetyl bromide, sodium hydroxide, hydroxylamine hydrochloric, and glacial acetic acid in the same manner as the root cell wall samples and alkali lignin samples.

Following the acetyl bromide lignin extraction, the optical densities of samples (blanks, alkali lignin and plant samples) were read in an Ultrospec 2000 spectrophotometer at a wavelength of 280 nm. The samples were further diluted if needed to yield absorbance readings greater than 0.1 (transmittance = 79.4%) and less than 1.0 (transmittance = 10%). Blanks were read at the same dilutions as the samples.

2.6 ICP-MS Analysis

Once dry, the shoots, desorbed roots, and non-desorbed roots were processed for inductively coupled plasma mass spectroscopy (ICP-MS) analysis. As described below, dried plant tissue was processed based on the SW-846 Test Method 3010A (EPA, 1992), with slight modification.

Samples were chopped then powdered. For shoot samples, the entire aboveground portion of the plant was coarsely chopped, enabling a representative subsample to be taken. This subsample was then finely chopped and powdered manually, ensuring no pieces were over 1 mm in any dimension. Roots for analysis were similarly prepared, with the entire portion (e.g., either desorbed or non-desorbed roots) coarsely chopped, subsampled and the subsample powdered (<1 mm pieces).

Dried powdered tissue was acid-digested in preparation for ICP-MS analysis. For each sample, approximately 0.1 g of powdered tissue was weighed, placed in a test tube, capped with a marble, and set in a test tube rack. The exact mass was recorded for later calculations. Each rack also contained one blank sample with no plant tissue, and two standards, which were treated the same as the samples and utilized to determine the efficiency of the acid digestion. For cadmium, the National Institute of Standards and

Technology, Standard Reference Material 1570a Trace Elements in Spinach Leaves was used. For selenium, BCR-402 White Clover (trace elements) was used. It was obtained from the European Commission, Joint Research Centre, Institute for Reference Materials and Measurements (the letters BCR refer to the Community Bureau of Reference, which is the former reference material program for the European Commission). These standards, which also consisted of powdered plant tissue, were treated the same as the samples, again with approximately 0.1 g used for each.

To each test tube, 1.0 mL MilliporeSigma OmniTrace® Ultra nitric acid (67-70%) was added, and samples began digesting overnight at room temperature. The following day, the test tubes were placed on a hotplate, and brought to a gentle boil to complete the reaction. Once the reaction had completed, as indicated by the absence of colour in the vapor, test tubes were removed from the heat and allowed to cool. The contents were then filtered using VWR 415 filter paper, transferred to 50 mL centrifuge tubes, and diluted to 50 mL. Samples were then analyzed via ICP-MS by either the Water Quality Centre at Trent University (crop species) for selenium, cadmium and sulphur, or the Biotron at the University of Western Ontario (*Astragalus* spp.), for selenium and cadmium. The choice of laboratory was based on their availability and processing times.

2.7 Statistical Analysis

Statistical analysis was conducted for the different components of the hypotheses using 1-way and 2-way ANOVAs (Analysis of Variance), t-tests, linear regressions and using multiple linear regression models. The first hypothesis, which was tested in the *Astragalus* species, was that selenium leads to thicker, more lignified cell walls and, because this will increase the proportion amount of cadmium bound to cell walls, higher tissue selenium will be associated with a greater tolerance to cadmium. This hypothesis was assessed using a two-way ANOVAs, and linear regressions. Additionally, t-tests were used when a comparison between only 2 treatments was of interest. These analyzes were run using SigmaPlot (Version 14.5, Systat Software, 2020).

The second hypothesis, tested using the *Astragalus* and crop species, stated that high sulphate in the growth medium would inhibit uptake and translocation of selenate, increasing cadmium uptake, especially in non-selenium hyperaccumulator plants. This hypothesis was tested using multiple linear regressions with R (Version 4.1.0, The R Foundation for Statistical Computing, 2021) in R Studio (Version 1.4.1717, RStudio PBC, 2021).

Where necessary to meet the assumptions of the statistical tests, transformations were used. Specifically, logarithmic transformations were utilized to achieve equal variance (Brown-Forsythe test), and normality (Shapiro-Wilk test).

Chapter 3

3 Results

Two hypotheses were tested, with the first examining the roots of three *Astragalus* species for the effects of selenate on lignification, cadmium localization and tolerance. For the second hypothesis, five crop species were used in addition to the three *Astragalus* species. In testing this hypothesis, the impact of sulphate on the relationship between cadmium and selenium concentrations was evaluated.

3.1 Hypothesis 1: Lignification of Roots, Cadmium Distribution, and Tolerance

The first hypothesis was that the application of selenate to the growth medium would result in greater lignification of root cell walls, leading to a greater tolerance to cadmium by binding the cadmium to root cell walls. This binding of the cadmium to the lignified cell walls would reduce the amount translocated to the shoots. Consequentially, selenate treatments would mitigate cadmium-induced reductions in biomass. This was tested in three species, including two selenium hyperaccumulators, *A. bisulcatus* and *A. racemosus*, and one related non-selenium hyperaccumulator, *A. canadensis*.

3.1.1 Lignin Content of Roots

Application of selenate to the growth medium was not associated with increased lignification of the root cell wall fraction (Figure 3.1). The lignin concentrations were analyzed via a two-way ANOVAs for each species to determine the effects of treatment and sampling location. Since plant species are known to differ in their lignin contents, the effect of species was not evaluated. Data for *A. canadensis* and *A. racemosus* was logarithmically transformed, since untransformed data failed normality (Shapiro-Wilk test). No differences were found between treatments with cadmium, cadmium and selenate, nor selenate in any of the *Astragalus* species tested ($P=0.139$ for *A. canadensis*, $P=0.163$ for *A. bisulcatus*, and $P=0.322$ for *A. racemosus*). The lignin content did not vary with location within the root system. Root samples collected from the thickest roots, being either the tap root or main laterals roots, were not significantly different from

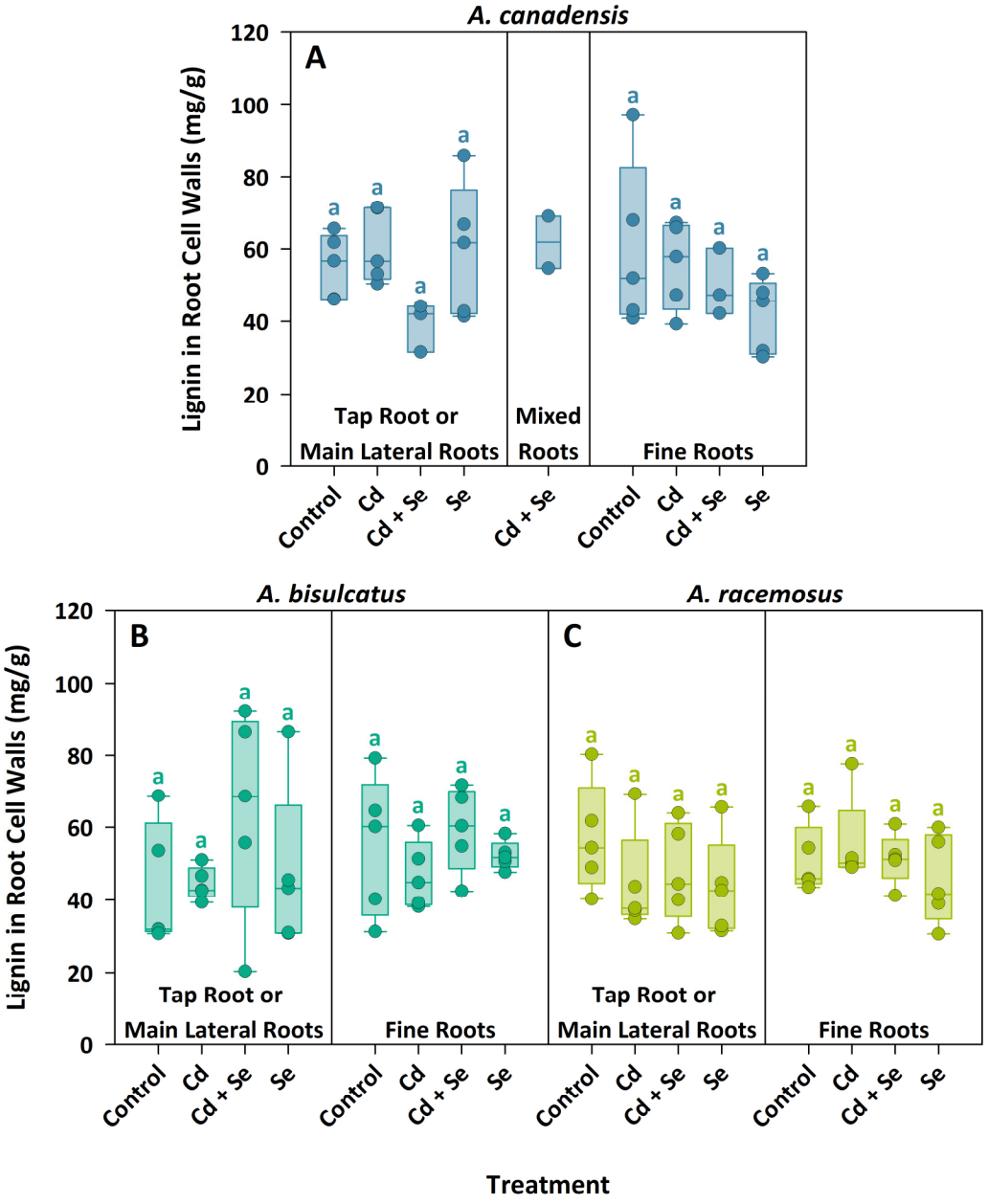

Figure 3.1: Lignin content of root cell wall fraction for the Astragalus species. Cell walls from three Astragalus species (Panels A-C) were extracted, digested in acetyl bromide, and lignin quantified via spectrophotometry. Five replicates were grown, samples were taken from the tap root/main lateral roots and from the fine roots, except for two A. canadensis (Panel A) plants in the 'Cd + Se' ('cadmium + selenate') treatment, which had insufficient root mass, so only one sample was collected from each ('Mixed Roots'). Boxplots show the minimum, maximum, median, 75th, and 25th percentiles, and are overlain by the individual data points. The mean is not shown. No differences were found between treatments or root size.

samples of the fine roots (P=0.641 for *A. canadensis*, P=0.521 for *A. bisulcatus*, and P=0.347 for *A. racemosus*, interactions also P>0.05).

While greater lignification in selenate-treated plants was not observed, there was some variability in the lignin content of root cell walls. Averaging the values for the two root samples for each plant yielded a range of lignin concentrations in root cell wall extract from 31.1 mg of lignin per g cell wall extract (*A. racemosus* in 'Se' treatment) to 80.2 mg/g (*A. bisulcatus* in 'Cd + Se' treatment). Among plants treated with cadmium, the lowest concentration was 40.4 mg/g (*A. bisulcatus* in 'Cd' treatment).

3.1.2 Cadmium Bound to Root Cell Walls

Within the range of lignin concentrations found in root cell walls, only one statistically significant relationship was found between concentrations of lignin and cadmium bound to cell walls (Figure 3.2A). This correlation was in *A. canadensis* in the 'Cd' treatment (P=0.041; for other species-treatment combinations, P=0.111 to P=0.992). However, within the *A. canadensis* 'Cd' treatment, 4 out of 5 plants had more cadmium measured in desorbed roots than in non-desorbed roots, which was problematic, yielding a calculated cell wall cadmium concentration of 0 μg/g. This suggests that during the cadmium desorption process, rather than cadmium being exclusively removed from the epidermis root cell wall, cadmium was also removed from the roots' interiors in some samples. In light of this issue, the total cadmium in the roots was also compared to the lignin concentration in the root cell wall extract (Figure 3.2B). No statistically significant correlations were found (P=0.196 to P=0.681). However, given the high variance and limited sample size (n=5), the lack of correlations found should be interpreted with caution.

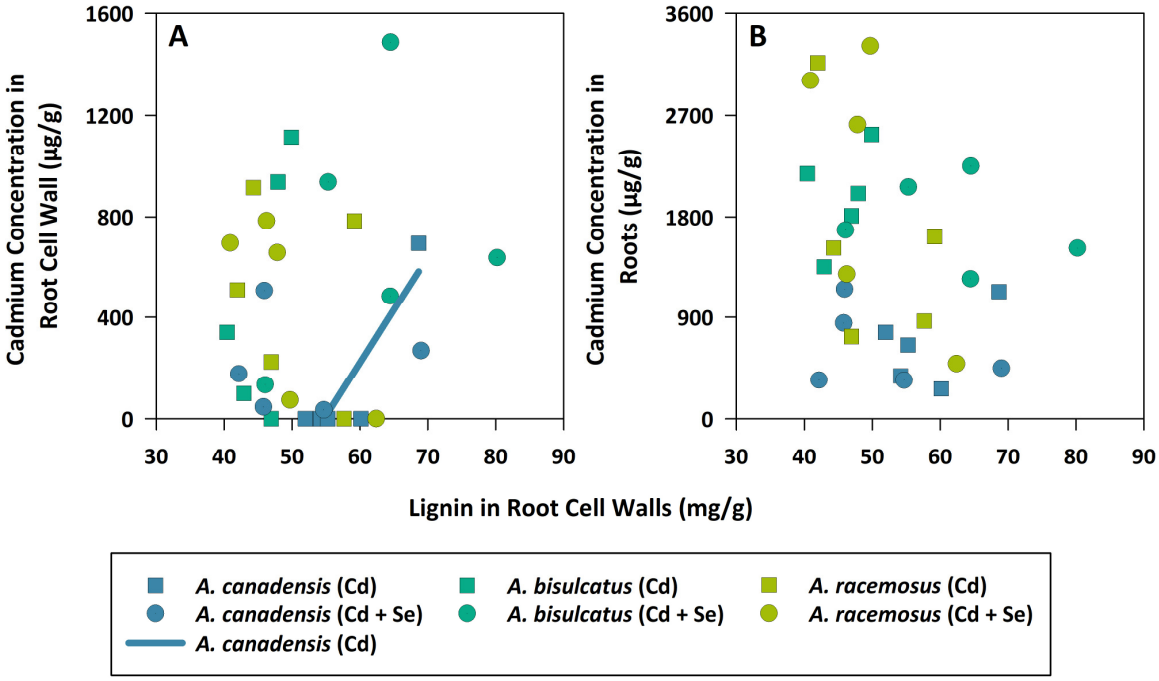

Figure 3.2: Relationship between root cell wall lignin content and estimated concentrations of cadmium, either bound to cell walls of the root epidermis, or total cadmium in roots, in the three *Astragalus* species.

To estimate root cell wall cadmium (Panel A), cadmium concentrations were measured via ICP-MS in both whole roots (Panel B) and those that had undergone cadmium desorption with calcium chloride to remove cadmium from the epidermis cell wall and apoplast. The difference between cadmium concentrations in the non-desorbed roots and desorbed roots yielded an estimate root cell wall cadmium. Lignin was quantified via spectrophotometry following digestion in acetyl bromide. Lignin values are the median of the two sub-samples taken from each plant's root system. Legend colours indicate species, and symbols indicate treatment ('cadmium' or 'cadmium and selenate').

3.1.3 Cadmium Toxicity with Selenate

Plants treated with cadmium and selenate in combination were expected to have greater biomasses than plants that received cadmium without selenate. However, a protective effect was not observed (Figure 3.3). Rather, the combination of cadmium and selenate (circles) caused equal or greater toxicity, measured as lower biomasses relative to control

(stars) and cadmium (squares) treatments and there was no significant correlation between biomass and concentration of selenium in roots.

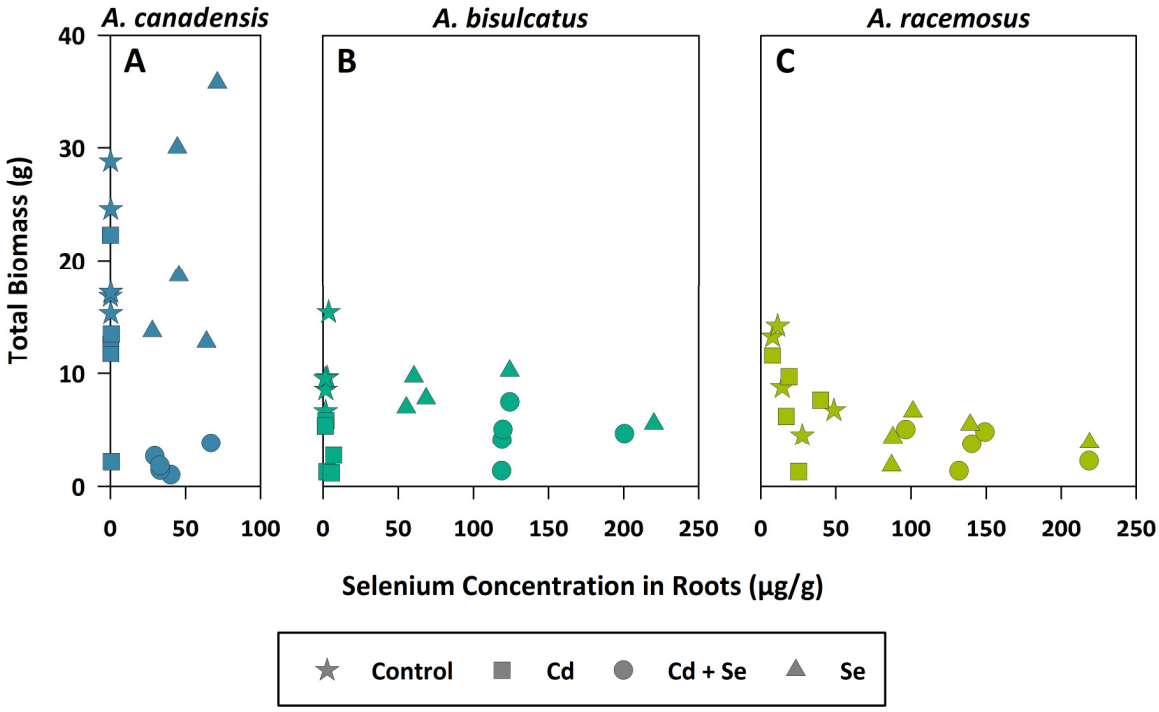

Figure 3.3: Effect of selenate and cadmium treatment on total plant biomass. Total dry biomass was weighed for each plant. Selenium concentrations in the roots were quantified via ICP-MS analysis in *A. canadensis, A. bisulcatus,* and *A. racemosus* (Panels A-C, respectively). Legend symbols, shown in grey, pertain to the treatments ('control', 'cadmium', 'cadmium and selenate', and 'selenate') and apply to all species (colours).

A one-way ANOVA was run for each species (differences between species were not of interest). Since significant difference were detected, post-hoc testing was conducted, using Tukey's post-hoc test for multiple pair-wise comparisons. In *A. canadensis* (Figure 3.3A), a non-selenium hyperaccumulator, cadmium alone did not reduce biomass compared to the control (P=0.289) (stars), nor did selenate (triangles) alone have an impact on biomass (P=0.979). However, the combination of cadmium with selenate (circles) yielded in a 90% reduction in the average biomass compared to both the control (P=0.003 for there being a treatment effect) and compared to the plants given selenate (P=0.002 for a treatment effect). Thus in *A. canadensis*, rather than conferring an

advantage, selenate in combination with cadmium resulted in greater toxicity symptoms, reducing biomass.

In *A. bisulcatus* (Figure 3.3B), one of the selenium hyperaccumulators, selenate alone did not have an effect on biomass when compared to the control (P=0.624). However, cadmium (squares) reduced the average biomass by 67% relative to the controls (P=0.003 for a treatment effect), and by 59% relative to selenate-treated plants (P=0.033 for a treatment effect). Biomass in plants given cadmium and selenate in combination (circles) reduced average biomass by 55% biomass relative to the control (P=0.014 for a treatment effect). There was no significant difference between plants given the 'Cd + Se' treatment and the 'Cd' treatment (P=0.850). Thus, in *A. bisulcatus*, cadmium reduced biomass, but this was not mitigated by selenate treatment.

The other selenium hyperaccumulator, *A. racemosus* (Figure 3.4C) also had a significant reduction in biomass, 64%, in the 'Cd + Se' (circles) treatment compared to the 'Control' (stars) (P=0.032 for a treatment effect). No other significant differences were found, with no effect of 'Se' versus 'Control' (P=0.085), or between 'Cd' and 'Control' (0.681). However, *A. racemosus* plants were highly variable and only 5 replicates were grown, so while it is clear that cadmium and selenate in combination had a negative effect, the failure to detect other significant differences should be interpreted with caution.

3.2 Hypothesis 2: Shoot Selenium and Cadmium Concentrations, as Impacted by Sulphate

The second hypothesis examined the effect of high sulphate in the growth medium on selenate and cadmium uptake and translocation. High sulphate was expected to inhibit uptake and translocation of selenate, reducing the capacity of selenate to inhibit cadmium translocation in the plants. The relationship between selenate and cadmium was expected to occur regardless of species. Included in the experimental design were eight species, including 5 crops as well as the three *Astragalus* species used in testing the first hypothesis. The inclusion of the *Astragalus* species, including the two hyperaccumulators, allowed for a wide range of tissue selenium concentrations to be

captured. It was expected that the hyperaccumulators would be less affected by high sulphate, but for the relationship between cadmium and selenium to hold true across species.

3.2.1 Impact of High Sulphate on Selenate Uptake

Due to competition for transporters, high sulphate in the growth medium was anticipated to lead to less selenate being taken up and translocated, yielding lower selenium concentrations in the tissues. The inverse relationship was also expected, with selenate predicted to decrease uptake of sulphate. High sulphate was not predicted to affect sulphur concentrations in the plants, since all plants received at least a sufficient amount.

As expected, high sulphate in the growth medium reduced selenate uptake and translocation to the shoots (Figure 3.4). In terms of the differences among treatments, the first noteworthy comparison from Figure 3.4 is between treatments with sufficient and high sulphate in plants given cadmium and low (5 µM) selenate, with sufficient versus high sulphate ('Cd + Se' versus 'Cd + Se + S'). With the exception of the hyperaccumulator *A. racemosus* (Figure 3.5C) (P=0.090), all species took up less selenate in high sulphate treatments with low selenate. The difference was smallest in *A. bisulcatus* (Figure 3.4B), the other hyperaccumulator, with a 31% reduction in the median (P=0.048). The effect was more pronounced in the non-hyperaccumulators, including crop species. In *A. canadensis* (Figure 3.4A), there was a 90% reduction in the median selenium concentration in the shoots (P<0.001). In the crop species (Figure 3.4D-H), reductions in the median shoot selenium concentrations ranged from 92% to 97% (P<0.001 to P=0.005) in all species except sorghum, which had an 82% reduction (P<0.001). To examine the effects of high (40 µM) selenate, only the two selenium hyperaccumulators (Figure 3.4B-C) were grown due to the toxicity of selenium to other species. High selenate combined with cadmium and high sulphate yielded a 53% reduction in the median selenium concentration in *A. bisulcatus* (P=0.008 for a treatment effect) and a 61% reduction in *A. racemosus* (P=0.015 for a treatment effect). These results for the *Astragalus* species were based on one-way ANOVAs with post-hoc testing using Tukey's test for multiple pair-wise comparisons. Data for *A. bisulcatus* and *A.*

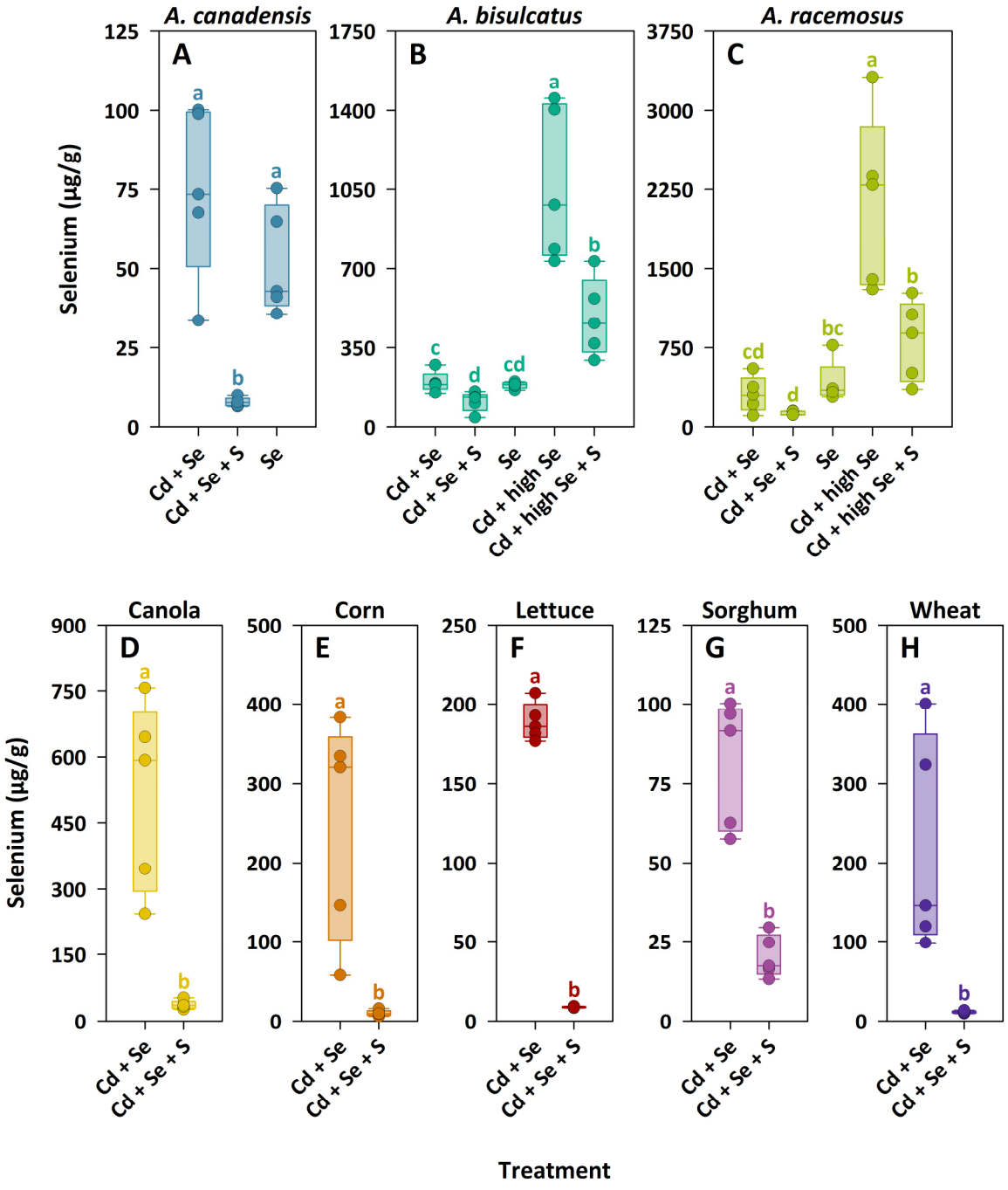

Figure 3.4: Selenium concentrations in shoots.
Selenium concentrations in the shoots of the 3 *Astragalus* species (Panels A-C) and 5 crops (Panels D-H). Selenium concentrations were measured via ICP-MS analysis. Only treatments in which selenate was applied are plotted. In the x-axis labels, 'Se' indicates selenate, while 'S' is used to denote high-sulphate treatments. Boxplots show the minimum, maximum, median, 75th, and 25th percentiles, and are overlain by the individual data points. The mean is not shown.

racemosus were logarithmically transformed since untransformed data failed both equal variance (Brown-Forsythe) and normality tests (Shapiro-Wilk). For crop species, t-tests were used to make the comparison between the two treatments. All species passed the normality Test (Shapiro-Wilk), but canola and lettuce both failed the test for equal variance (Brown-Forsythe). As a result, the Student's t-test was used for corn, sorghum and wheat, and Welch's t-test, which does not assume equal variance, for canola and lettuce.

Additionally, it should be noted that selenate uptake was unaffected by cadmium treatment (Figure 3.4A-C). Treatments with only selenate ('Se') (sufficient sulphate) did not differ from those with cadmium and selenate (sufficient sulphate) ('Cd + Se') (P=0.697 *for A. racemosus*, P=0.999 for *A. bisulcatus*, and P=0.172 for *A. canadensis*, based on the above-described Tukey's post-hoc testing of one-way ANOVAs). This was tested only in the three *Astragalus* species, since the 'Se' treatment was not utilized in evaluating the second hypothesis, for which the crop species were grown.

3.2.1.1 Relationship between Sulphur and Selenium in Shoots

Crop species were analyzed for sulphur concentrations in the shoots. This analysis was conducted purely to confirm that the lower sulphate treatments did in fact contain sufficient sulphate. Since plants were not grown in a high sulphate treatment without cadmium, the sulphur concentrations in shoots were compared between plants from the cadmium and sufficient sulphate ('Cd') and cadmium and high sulphate ('Cd + S') treatments. Additionally, a comparison was conducted between plants from the cadmium and selenate with sufficient sulphate ('Cd +Se') and cadmium, selenate and high sulphate ('Cd + Se + S') treatments. Since the crop species were used only to test the second hypothesis, they were not grown in the selenate only ('Se') treatment.

As shown in Appendix III, for 4 out of 5 crop species, there was no significant difference in tissue sulphur concentrations between the 'Cd' and 'Cd + S' treatments. (P=0.826 for canola, P=0.939 for corn, P=0.823 for sorghum, and P=0.473 for wheat). Only lettuce had higher sulphur in 'Cd + S' treatment versus the 'Cd' treatment (P=0.044). These

results are based on one-way ANOVAs for each species, with data for corn and wheat logarithmically transformed. Prior to transforming the data, corn failed testing for equal normality (Shapiro-Wilk), and wheat failed testing for equal variance (Brown-Forsythe). Untransformed data for canola, lettuce and sorghum passed both equal variance and normality tests.

While conducting this analysis for sufficient sulphate (Appendix III), it was noted that shoot sulphur concentrations were typically highest in plants from treatments with cadmium and selenate ('Cd + Se'). There were significant differences in sulphur concentrations in the shoots between the 'Cd + Se' and 'Cd + Se + S' treatments in all crops except sorghum, which did not differ (P=0.158 for sorghum, P=<0.001 for other crops). Contrary to expectations that selenate treatment would result in less sulphate uptake, in the 4 crops in which there was a significant difference, those that received selenate and cadmium with sufficient sulphate had the highest sulphate concentrations. These four crops in the 'Cd + Se' treatment had median sulphur concentrations 155% (canola) to 232% (lettuce) higher than those of plants in the 'Cd + Se + S' treatment.

In response to these unexpected trends in sulphur concentrations when plants were provided with selenate, correlations were plotted between tissue sulphur and selenium concentrations (Figure 3.5). Of the five crop species, only in wheat was the regression significant (R^2=0.860, P=0.023) between the concentrations of sulphur and selenium in the shoot tissue (Figure 3.5A). However, its slope was minimal, at 0.026 mg/g sulphur per μg/g selenium. In the treatment with high sulphate (Figure 3.5B), the regressions were significant in canola (R^2=0.900, P=0.014), corn (R^2=0.954, P=0.004), sorghum (R^2=0.874, P=0.020), and wheat (R^2=0.878, P=0.019). All of the relationships found were positive correlations, with higher selenium concentrations associated with higher sulphur concentrations in the shoots. However, as discussed above in Section 3.2.1, the plants that received high sulphate had lower selenium concentrations than those that received sufficient sulphate (note the different x-axis scales in Figure 3.5). Thus, high sulphate in the growth medium reduced overall selenate uptake and translocation;

however, higher selenate uptake and translocation was correlated with higher sulphate uptake and translocation.

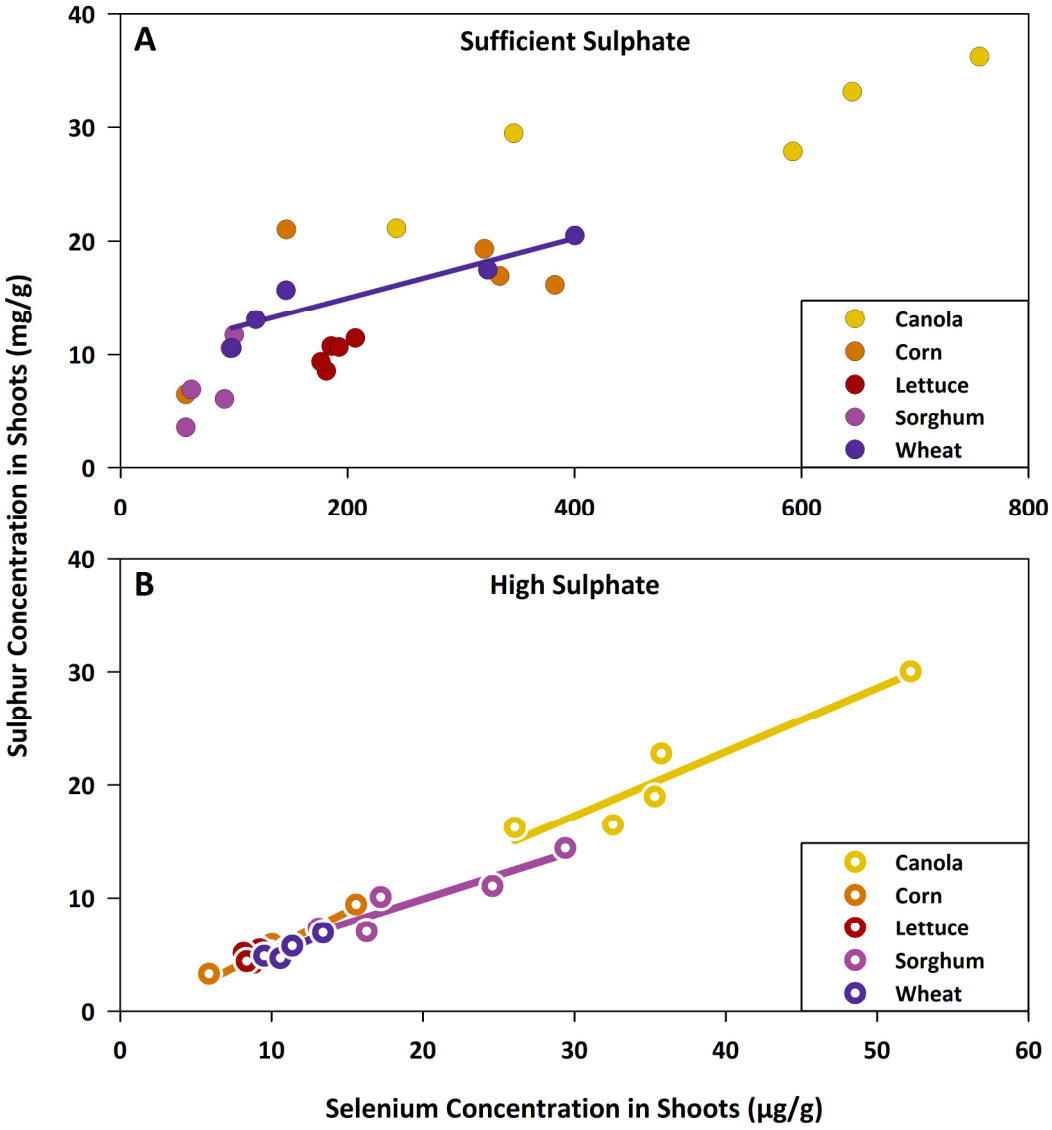

Figure 3.5: Correlation between sulphur and selenium concentrations in shoots of crop species treated with cadmium and selenate.
Includes plants grown in the 'cadmium and selenate' treatment (Panel A), and those grown in the 'cadmium + selenate + high sulphate' treatment (Panel B). Concentrations of sulphate and selenate were determined via ICP-MS. Note that scales on the x-axes differ between panels.

Of note, a multiple linear regression model with selenium and sulphur concentrations in combination did not better explain the trends in cadmium uptake (Section 3.2.2). As a

result, shoot sulphur and shoot selenium were not used as covariates in the general linear model.

3.2.2 Relationships Between Selenium and Cadmium Concentrations in Shoots

It was expected that selenate would reduce the uptake and translocation of cadmium, with higher concentrations of selenium in plant tissue associated with lower cadmium concentrations. Additionally, with high sulphate expected to decrease shoot selenium concentrations, higher cadmium concentrations were expected in treatments with high sulphate. However, the patterns between selenium and cadmium concentrations in the shoots were expected to hold constant, regardless of the concentrations present, as well as among species. As discussed above in Section 3.2.1, high sulphate did have the anticipated effect on shoot selenium concentrations; however, other expected trends were not observed.

The date for hypothesis 2 was analyzed via an estimated marginal means model (Lenth, 2021) within a general linear model, utilizing robust covariance matrix estimators (Zeileis, 2006; Zeileis et al., 2020). Post-hoc tests for comparisons within each of the two sulphate levels were conducted using Tukey's method with the P-values adjusted according for comparing the 8 families (species). For additional comparisons (i.e., between sulphate levels), the Sidak method was used, again with P-value adjustments for the number of species.

3.2.2.1 Selenium and Cadmium Concentrations in Shoots by Sulphate Level

Contrary to expectations, a strong negative relationship between cadmium and selenium concentrations (both measured in μg/g) was not found when plants were provided with sufficient sulphate. This relationship between cadmium and selenium concentrations, represented by the slope, was generally weak, yielding wide confidence intervals, particularly in some of the crop species (Figure 3.6A). The confidence intervals for crop species were especially wide in lettuce, sorghum, and corn, with slopes and their

confidence intervals, 2.31 ±4.53 Cd/Se, 2.13 µ±3.48 Cd/Se, and 0.51 ±1.07 Cd/Se, respectively, with both elements measured in concentrations in the shoot tissue (µg/g). For the *Astragalus* species, the confidence intervals were narrower, allowing a difference to be detected between the slopes of *A. canadensis* and *A. bisulcatus*. The correlation between cadmium and selenium in shoots in these two species differed (P=0.015), with *A. canadensis* having a negative slope, at -0.16 ±0.14 Cd/Se (P=0.021), and *A. bisulcatus* having a slightly positive slope, 0.11 ±0.07 Cd/Se (P=0.003) (concentrations measured in µg/g). While the correlation for *A. racemosus* had the narrowest confidence interval of all species within this treatment, ±0.02 Cd/Se, its slope was near zero, at 0.03 Cd/Se (concentrations measured in µg/g). It did not differ significantly from any other species. In addition to the differences between the correlations for *A. canadensis* and *A. bisulcatus*, *A. canadensis* also differed from canola (P=0.031), the latter of which had a slope of 0.16 ±0.14 Cd/Se (P=0.028) (concentrations measured in µg/g). Overall, these slopes and the difference between them indicate that even where a relationship could be found between the concentrations of cadmium and selenium, it was not consistent across species.

As presented in Section 3.2.1.1, plants that were provided with high sulphate had lower selenium concentrations in their shoots, as expected. However, when the relationships between selenium and cadmium concentrations in the shoots were plotted (Figure 3.6B) and analyzed, they were not consistent across species. The relationship, represented by the slope, differed among species, as shown in Figure 3.6B. In total, 9 differences between species were found (P-values are listed in Appendix IV). These differences all occurred between canola, corn, sorghum, *A. canadensis*, *A. bisulcatus* and *A. racemosus*. Lettuce and wheat did not differ significantly from one another, nor any other species. In addition to the lack of a universal trend across the species tested, the slopes (relationship between cadmium and selenium concentrations in the shoots) also tended to be positive, rather than the negative slopes that were predicted. Slopes of canola (3.62 ±1.40 Cd/Se), corn (30.29 ±12.67 Cd/Se) and sorghum (7.93 ±5.08 Cd/Se) differed significantly from

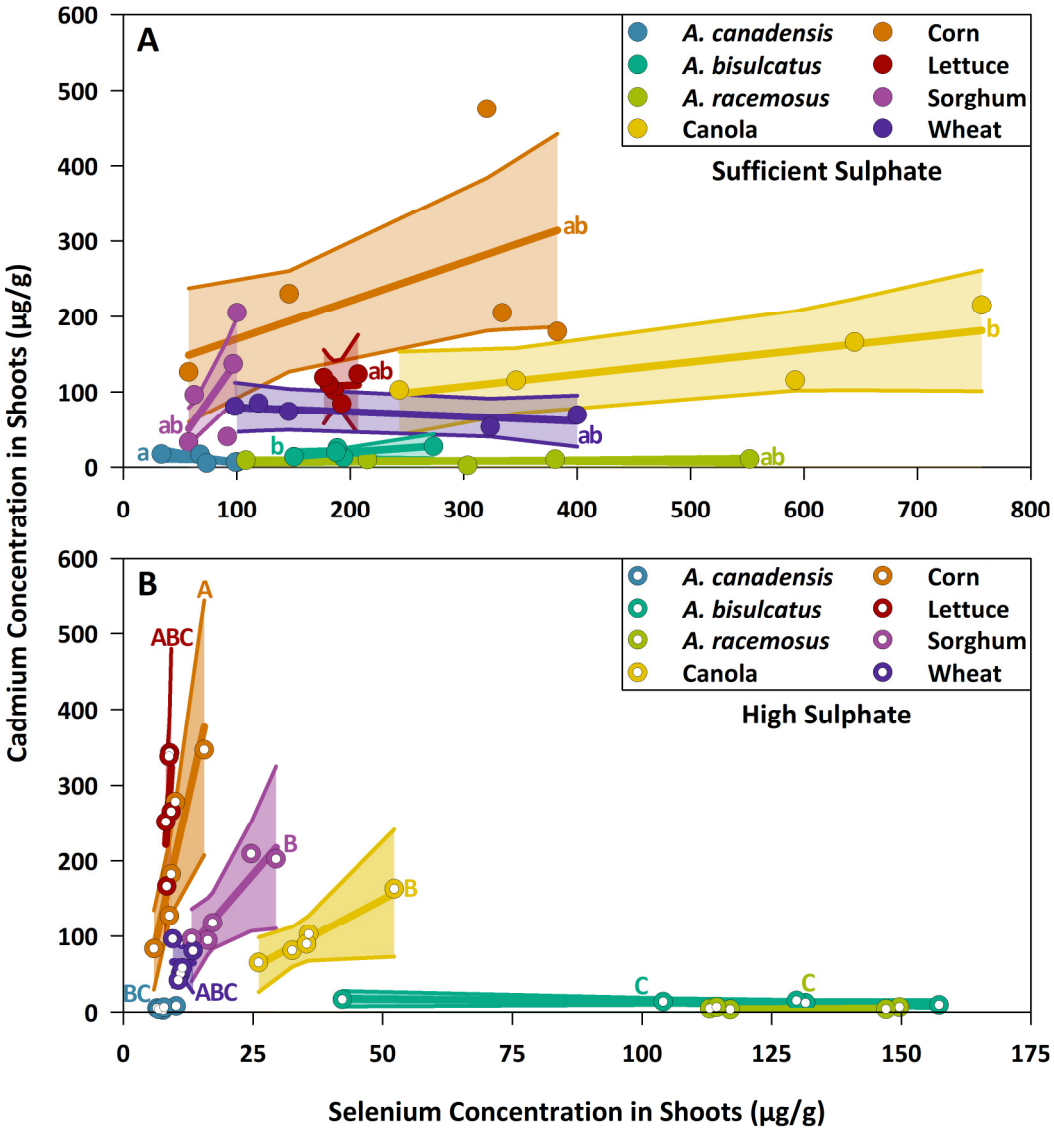

Figure 3.6: Relationship between cadmium and selenium concentrations in shoots. Includes plants grown with cadmium and selenate with sufficient sulphate (Panel A), and high sulphate (Panel B), excluding high selenate treatments. Note that letters identify differences in the slopes, rather than in the means.

zero (P<0.001, P<0.001, and P=0.002, respectively), with all three being positive. The two other crop species had very large confidence intervals, with slopes and their confidence intervals of 96.12 ±224.98 Cd/Se for lettuce and -0.20 ±51.96 Cd/Se for wheat (concentrations of both elements measured in µg/g). Also of note, while differences between the *Astragalus* species and the crop species were detected, all three *Astragalus* species had slopes near zero, indicating a lack of relationship between the

concentrations of selenium and cadmium in their shoots. Specifically, *A. bisulcatus* had a slope of -0.06 ±0.08 Cd/Se, *A. canadensis* 0.79 ±2.23 Cd/Se. and *A. racemosus* 0.01 ±0.12 Cd/Se (concentrations of both elements measured in μg/g).

Not only were slopes typically either positive (or not significantly different from zero) and different between species, but high sulphate levels also yielded inconsistent effects on the slopes. These differences in slope between plants grown in the sufficient (Figure 3.6A) and high sulphate (Figure 3.6B) treatments were found in three species: *A. bisulcatus* ($P=0.018$), canola ($P<0.001$), and corn ($P<0.001$). Similar differences were not detected in the remaining five species. This was predominantly an artifact of either insufficient range in the cadmium (e.g., *A. racemosus*) or selenium (e.g., lettuce) concentrations, or wide confidence intervals.

Selenium hyperaccumulators, *A. bisulcatus* and *A. racemosus*, were also grown in a high selenate treatment. Other species were not grown in this treatment, due to lower tolerance to selenate at high concentrations. Within these high selenate treatments, the effect of selenium on cadmium was also examined; however, no relationships were found between tissue selenium and cadmium concentrations (Figure 3.7). None of the slopes, either with sufficient or high sulphate differed significantly from zero ($P=0.410$ to 0.964), nor did any of the slopes differ within species by sulphate level ($P=0.523$ for *A. bisulcatus*, $P=0.853$ for *A. racemosus*), or between the two species ($P=0.575$ with sufficient sulphate, $P=0.886$ with high sulphate).

Of note, while there were correlations, as discussed above, between cadmium versus selenium concentration, there were minimal treatment effects (data shown in Appendix V). The inclusion of selenate in the growth medium had no effect on the amount of cadmium plants took up and translocated to their shoots, with one exception. This exception applied only in *A. racemosus*, where the 'Cd + high Se' and 'Cd' treatments differed, with the high selenate (40 μM) treatment yielding higher cadmium concentrations ($P=0.011$).

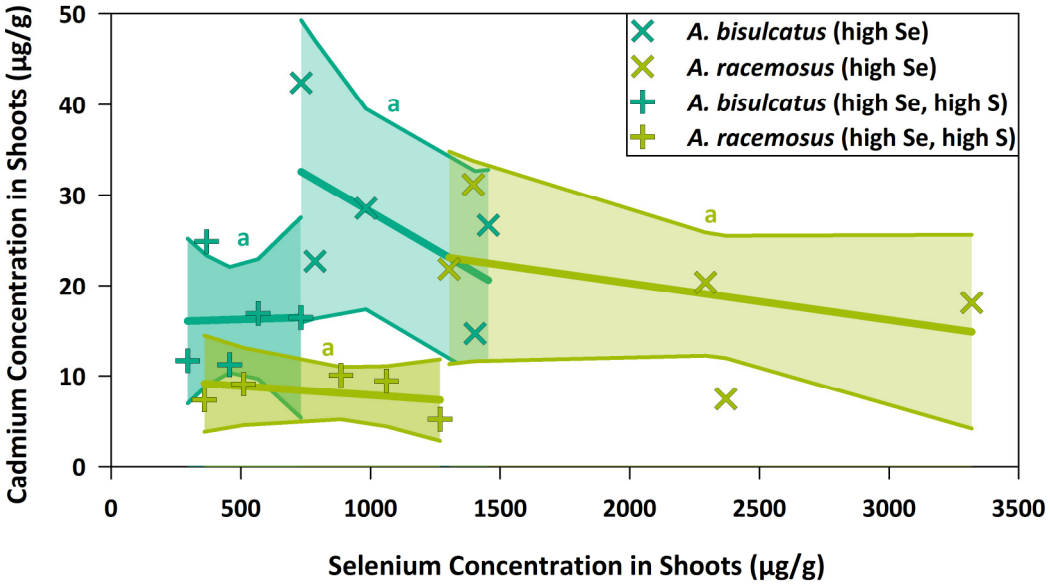

Figure 3.7: Relationship between cadmium and selenium concentrations in shoots in high selenate treatments.
Includes plants grown with cadmium and high selenate (40 µM) with sufficient (×) or high (+) sulphate. Note that letters identify differences in the slopes, rather than in the means.

No differences in cadmium concentrations were found between the treatments containing cadmium within canola, corn, sorghum, wheat, or *A. canadensis*. In lettuce, the inclusion of sulphate affected cadmium when in combination with cadmium and selenate. In the other two species, *A. bisulcatus* and *A. racemosus*, the only differences in cadmium concentrations with treatment were between the 'cadmium + high selenate' and 'cadmium + selenate + high sulphate' treatments. Thus, plants that took up more selenium may take up more cadmium but, overall, plants given selenate did not take up more cadmium than plants that were not given selenate.

Chapter 4

4 Discussion

Neither of the proposed hypotheses regarding the influence of selenium on cadmium toxicity and uptake were supported. First, the ability of selenate treatments to increase lignification of roots, and the effect thereof on binding cadmium to the roots and reducing toxicity to the plant was examined. Instead, increased lignification was not found, and selenate did not provide any apparent reduction of cadmium toxicity. Second, while high sulphate did reduce shoot selenium concentrations as expected, higher shoot selenium was not associated with lower cadmium concentrations. Considering the results of testing the first hypothesis, which failed to find the expected mechanism, the lack of benefit to selenate in reducing cadmium is not entirely surprising.

4.1 Hypothesis 1: Lignification of Roots, Cadmium Distribution, and Tolerance

Lignin was quantified in the root cell walls of the three *Astragalus* species, for evaluation of the first hypothesis. This hypothesis was that treatments with selenate would yield greater lignification of root cell walls, which in turn was expected to result in higher cadmium concentrations bound to the root cell walls. Thus, cadmium would be immobilized, resulting in plants having a greater tolerance to cadmium.

4.1.1 Lignin Contents of Roots and Variability Thereof

Contrary to expectations, treatments in which selenate was provided in the growth medium did not yield greater lignification of root cell walls. There were no significant differences in lignification among any of the experimental treatments for which lignin was measured. This differs from what Cui et al. (2018) found, with rice suspension cells having over double the lignin content in their highest cadmium and selenium treatment (192.3 ± 9.5 mg/g in cell wall material) versus control, at just over 75 mg/g lignin (exact value not reported). However, this difference could also result from type of the tissue used, with Cui et al. (2018) using the cell wall extract of rice suspension cells, as opposed to that of intact roots.

The lignin concentrations I measured were highly variable, particularly in *A. bisulcatus*. Individual measurements in *A. bisulcatus* ranged from 20.3 to 92.2 mg/g of lignin in the cell wall extract yielding a 4.5-fold difference, and an average of 51.7 mg/g. The other two species had narrower ranges, with a 3.2-fold difference and an average of 54.5 mg/g in *A. canadensis* (range: 30.2-97.1 mg/g) and 2.6-fold difference with an average of 49.5 mg/g in *A. racemosus* (range: 30.6-80.3 mg/g). However, these differences were not a consequence of cadmium and/or selenate treatments, nor root sampling location.

Data on the lignin content of other *Astragalus* species is scarce; however, Moussaoui et al. (2011) did assess the roots of one species, *Astragalus armatus* Willd., for papermaking. They reported the pulp averaged 16.75% lignin (167.5 mg/g). They unfortunately did not discuss the variance of lignin values within *A. armatus* roots. For contrast, Fukushima and Kerley (2011) assessed the lignin contents of a variety of plants, with lignin values up to 232 mg/g in the mature wood of slash pine (*Pinus elliottii* Engelm.). However, the legumes they tested had lignin contents of just over 50 mg/g, at 55 mg/g for alfalfa (*Medicago sativa* L.), 52 mg/g for red clover (*Trifolium pratense* L.), and 50 mg/g for lespedeza (*Lespedeza bicolor* Turcz.). Thus, the three *Astragalus* species I tested had similar lignin concentrations to other legumes.

Variability among lignin contents might be explained by the inconsistencies in the growth rate of plants of all three of the *Astragalus* species grown. Abiven et al. (2011) reported that lignin in the roots of corn increased as the plants aged; however, the rate of change was not uniform over time. In corn, they found relatively constant slow increase in lignin contents from an age of about 50 days to 122 days, and then as plants reached maturity (day 166), lignin contents abruptly increased by a factor of 3.3. The wheat plants they tested did not undergo a similar change in lignification rate, with a fairly steady increase in lignin content as they aged and matured, so the effect of age appears likely to depend on species. While age is different from maturity, I noted that the *Astragalus* species grown for testing the first hypothesis were far less consistent in their growth rates than the crop species (including both wheat and corn), which were grown only for the second hypothesis. There were notable differences among plants for all three *Astragalus* species

in their growth rate and resulting biomass. I employed methods to minimize the variability in size among young plants, with the culling of plants at multiple time points prior to commencing the application of experimental treatments. Yet, despite all plants within each species being the same age at the time of harvest, they varied in widely in their biomass within treatments (Figure 3.3). In *A. canadensis*, there were qualitative differences in sexual maturity within treatments, with plants at varying stages of bloom. While neither *A. racemosus* nor *A. bisulcatus* had flower buds evident by the time of harvest, they differed in size.

Given the high variability in the lignin contents within treatments, it appears that the application of selenate is unlikely to be the main driving factor. However, with the low sample size and high variability within the data, the influence should not definitively be ruled out. If selenate treatment did in fact affect lignification of roots, the effect was lesser than that of another, unknown, parameter that was not included in the study.

4.1.2 Lack of Effect of Lignin on Cadmium

Not only was total lignin in the roots highly variable within treatments, but it was also not correlated with cadmium concentrations in the roots. Neither the estimated cadmium bound to the cell wall, nor the total cadmium concentration in the roots, were correlated with lignin concentrations. These results were unexpected and did not support the hypothesis.

Ahsan et al. (2012) suggested increased lignification reduces cadmium translocation to the shoots by binding the cadmium. This mechanism was supported by the work by Cui et al. (2018), who found selenium treatment to induce increased lignification, enabling the retention of cadmium in cell walls. Cadmium alone was also associated with increased lignification by Zagoskina et al. (2007) in tissue cultures of tea (*Camellia sinensis* L.); however, Cui et al. (2018) did not find this for rice suspension cells. Thus, a correlation was expected between lignin and cadmium in the roots of plants in this experiment. Nonetheless, no correlations were found, neither with selenate nor cadmium treatments, as discussed above, nor cadmium concentrations.

The lack of an observed effect of lignin on cadmium could be a consequence of the high variability and small sample size (n=5), and thus the relationship should not definitively be ruled out. Additionally, while lignin was highly variable, as discussed above, the highest lignin content I recorded was 97.1 mg/g. This was roughly half of the 192.3 ± 9.5 mg/g reported by Cui et al. (2018) in their rice suspension cells treated with selenium and cadmium. In addition to differences in the species of plants used, as well as whole plants versus suspension cells, I also used a different form of selenium (selenate) than Cui et al. (2018), who used selenite. Selenate is reduced to selenite in the plant, which is then further reduced into selenide and then converted into selenocysteine. The selenocysteine can then be converted into selenomethionine and then dimethylselenide, or in the case of selenium hyperaccumulator plants, from selenocysteine into dimethyl diselenide (review by Saha et al., 2017). As a result, I was expecting the selenate to be converted to selenite in my plants, yielding similar results despite the form of selenium used. Zayed et al. (1998) studied the volatilization of different forms of selenium, and reported that when plants were supplied with selenite, it was entirely converted to organic forms, with no inorganic selenium remaining in shoot or roots. Contrasting this, when applied in the form of selenate, it was taken up in 4-5-fold higher quantities, but much of it remained in this form within the plant.

Overall, with limited sample size and high variability, and perhaps insufficient lignification, the lignification of roots cannot be ruled out as a factor affecting root cadmium concentrations. Nonetheless, the results did not indicate it to be of significance for these plants. Again, this may be due to insufficient lignification, possibly due to the form of selenium used.

4.1.3 No Benefit to Selenium with Cadmium

For the plants studied, there was no benefit to selenate in the presence of cadmium. This was again contrary to my hypothesis, however, since the predicted mechanism was not found, not surprising. Since the selenate treatments did not affect lignification, and lignification was not associated with retention of cadmium in the roots, no support for the proposed mechanism to mitigate cadmium toxicity was found. Additionally, treatments

with selenate alone did not yield greater biomass when compared to controls, so selenate had no apparent benefit. While many studies (e.g., Cui et al., 2018; Huang et al., 2018b; Pereira et al., 2018) have reported a physiologically synergistic effect of selenium and cadmium, Feng et al. (2013b). Feng et al. (2013b) reported that the two elements in combination could reduce biomass, but only at high concentrations. Low concentrations (1.27 µM) of selenite increased biomass of rice and mitigated the negative effects of moderate amounts of cadmium; however, this did not hold true at higher concentrations. When selenite was supplied at 12.7 µM or 63.5 µM, with 89 µM or 178 µM cadmium, biomass was reduced. Similarly, when cadmium was at 178 µM, selenite had a negative effect, even at a concentration of merely 1.27 µM.

4.2 Hypothesis 2: Shoot Selenium and Cadmium Concentrations, as Impacted by Sulphate

The second hypothesis examined the relationship between shoot selenium and cadmium concentrations. Here, a negative relationship was expected. The relationship was expected to hold true, regardless of the species and the concentrations of selenium within the plants. High sulphate in the growth medium could inhibit uptake and translocation of selenate, particularly in non-selenium hyperaccumulators, thus lessening the benefit provided by selenate in reducing cadmium uptake. Nonetheless, the trend between shoot selenium and cadmium was expected to remain the same, albeit at different tissue concentrations.

4.2.1 Impact of High Sulphate on Selenate Uptake

The impact of sulphate on selenate uptake (and vice-versa) was not a direct component of the hypotheses being tested. However, while verifying the concentration of sulphate provided in the “sufficient” sulphate treatments was, in fact, sufficient, shoot selenium and sulphur concentrations were found to be positively correlated. Sulphur concentrations were also found to be much higher in plants that were treated with selenate.

With selenate taken up via high-affinity sulphate transporters and metabolized via the sulphate assimilation pathway, competition between the ions was expected to yield a

negative correlation between concentrations of sulphur and selenium in the plant tissue, as reported by Zayed et al. (1998). However, in some cases, a positive correlation was found, corroborating my results. Boldrin et al. (2016) also found selenate to increase sulphate concentrations in the shoots of wheat plants. They reported increased expression of sulphur transporter genes (SULTR), particularly SULTR1;1, and to a lesser degree, SULTR1;3 and SULTR4;1. With SULTR1;1 regulated in response to sulphur starvation (Rouached et al., 2008). Boldrin et al. (2016) attributed the effect of selenium to a mimicking of a sulphate deficiency within the plant. The other two sulphate transporters, SULTR1;3 and SULTR4;1, influence sulphate distribution within the plant, including phloem loading and transport and storage in vacuoles, respectively (review by Gigolashvili & Kopriva, 2014). Since Boldrin et al. (2016) did not vary sulphate doses, the effect of sulphate on selenate was not reported.

4.2.2 Relationships Between Selenium and Cadmium Concentrations in Shoots

With Hypothesis 1 quashed, and the second hypothesis being based on the mechanism of Hypothesis 1, it is not surprising that the results found did not align with initial expectations. However, Hypothesis 2 was still evaluated.

Based on Hypothesis 1, reduced uptake of selenate with high sulphate levels was expected to result in less lignification, with selenate causing increased lignification. By extension, cadmium uptake and translocation to the shoots would be less inhibited with the lesser amount of lignin. However, with selenate having no impact on lignification, as discussed in Section 4.1.1, it is not surprising that Hypothesis 2 was unsupported as well.

In testing hypothesis 2, it was found that first, the relationship between shoot cadmium and selenium concentrations tended towards being positively correlated. This was in contrast to the expected negative correlations. In cases where the slopes did appear to be negative, they did not differ significantly from zero, indicating no relationship between shoot cadmium and selenium concentrations. When high and low sulphate levels were compared, the response differed in three species (*A. bisulcatus*, canola, and corn). In the remaining 5 species, no significant differences were found. However, it should not be

concluded that the relationship between cadmium and selenium shoot concentrations was constant in these species across sulphate levels. Within each of these 5 species, the slope was not significantly different from zero in at least one of the two sulphate levels. Thus, there was no relationship between selenium and cadmium concentrations in these species. Whether the lack of a relationship is consistent between the two sulphate levels is not of interest. All in all, no universal relationship was found between shoot cadmium and selenium concentrations.

When Hypothesis 2 was evaluated, resulting findings did not follow initial expectations. Rather, higher selenium concentrations were associated with higher cadmium concentrations on a plant-by-plant basis. With high sulphate, less selenate was taken up, as expected. However, again, there was a tendency towards a positive correlation between cadmium and selenium. Plants which took up more of one element either took up more of both elements, or about the same amount of one element, regardless of how much of the other was taken up and translocated to the shoots. As a result, not only was Hypothesis 1 not supported by the lignification data (as discussed above in Section 4.1), but an alternative mechanism for the predicted results seems improbable, since the predicted results were not found. Possible rationales for the results I found are speculative, since potential mechanisms for increased, rather than decreased, uptake of cadmium with selenate were not tested.

There was no treatment effect detected of applying selenate in combination with cadmium. Overall, plants that received selenate and cadmium did not take up more cadmium than plants that did not receive selenate. However, within the treatments with cadmium, selenate, and sufficient sulphate, there was the above-noted positive correlation in *A. bisulcatus* and canola. In plants that received cadmium, selenate, and high sulphate, a positive correlation was found in canola, corn, and sorghum. Only in *A. canadensis* with sufficient sulphate was there a negative correlation. In all other cases, there was no relationship (slope of zero).

Again, why individual plants that took up and translocated more selenium to their shoots also took up more cadmium was not tested in the experimental design, as it was an

unanticipated result. It was qualitatively noted that plants that received cadmium and selenate frequently had different root morphology from other plants. These plants that received cadmium and selenate typically had shorter and fewer fine roots, and roots were somewhat darker in colour. Based on the differences in root morphology and colour, evident on a macroscopic scale, damage to the roots appeared extensive. This structural damage may have given constituents in the hydroponic growth medium, including the cadmium and selenate, direct access to the xylem, bypassing the need for selective uptake. While the analysis using Visual MINTEQ (Gustafsson, 2018) (Appendix II) showed that the cadmium and selenium did not form insoluble complexes in the growth medium, by extension it also showed that they were both present in solution.

Alternatively, the correlation between cadmium and selenium in the shoots of canola, corn and sorghum with high sulphate may have been due to an increase in sulfhydryl proteins in the tissue. There was a treatment effect whereby selenate treatments were associated with increased sulphur concentrations in the shoots. However, more importantly, there was also a positive correlation between the shoot concentrations of selenium and sulphur. This correlation was significant in wheat with sufficient sulphate, and in canola, corn, sorghum, and wheat with high sulphate. As noted in section 4.2.1, Boldrin et al. (2016) suggested that selenate increases sulphur uptake by mimicking sulphate deficiency. Thus, when selenate was applied, shoot sulphur concentrations increased, and the more selenium that was taken up, the greater these shoot sulphur concentrations were in these species. Sulphur has been found to stimulate the production of sulfhydryl proteins, which bind to cadmium to reduce its toxicity to the plant (Zhang et al., 2014). Thus, the increase in these proteins with higher tissue sulphur concentrations may increase the number of potential cadmium binding sites within the plant. Thus, it is possible selenium may indirectly lead to increased cadmium uptake on a plant-by-plant basis, dependent on how much selenium and, by extension, how much sulphur the plant takes up. However, since this was not observed in wheat, it weakens the argument. There was a significant correlation between shoot selenium and sulphur in wheat with sufficient sulphate, but the slope was near zero. With high sulphate, there was a limited range in the selenium concentrations (9.52-13.41 μg/g DW), so the limited ability of wheat to take up

selenium with high sulphate present may have led to the lack of a correlation between selenium and cadmium.

Overall, my results showed that tissue selenium concentrations were not negatively correlated with shoot cadmium concentrations. This is contrary to the work of many others (e.g., Amirabad et al., 2020; Cui et al., 2018; Guo et al., 2021; Huang et al., 2017; Huang et al., 2021; Huang et al., 2018b; Wan et al., 2019) who have suggested that selenium provides a benefit, reducing cadmium uptake. This difference may be largely a consequence having used selenate, rather than the more commonly tested selenite (review with meta-analysis by Affholder et al., 2019). However, the results I found were somewhat similar to those of Yang et al. (2021), who also found that selenium did not reduce cadmium uptake. Their paper differed from the majority of the others that examined cadmium and selenium in that their testing utilized paddy fields with naturally occurring elevated selenium and cadmium concentrations. While this is more realistic than studies (including mine) that supplement the growth medium with cadmium and selenium, there was less control over other variables (e.g., soil characteristics). For example, Yang et al. (2021) collected rice and soil samples from paddies located near two different villages, yielding soil pH values ranging from 4.3 to 5.9, and organic matter ranged from 21 to 39 g/kg, among other parameters reported. Like Yang et al. (2021), Chang et al. (2022) also studied paddy soils in a seleniferous region of China. However, unlike the former, Chang et al., (2022) found selenium and cadmium concentrations in (polished) rice to be negatively correlated. Thus, there does not appear to be a clear divide of results whereby paddies differ from controlled experiments. Rather, the relationship between cadmium and selenium concentrations typically appears negative but, as found here, not necessarily consistent across studies.

Chapter 5

5 Conclusion

I anticipated finding support for implementing selenium fortification of plants grown on cadmium-contaminated croplands. Mechanistically, selenium (in the form selenate) was predicted to yield increased lignification of root cell walls, thus decreasing cadmium uptake by plants. Meanwhile, the selenium content of the plants would be increased, reducing the potential for selenium deficiencies in consumers. Overall, a trend was predicted, whereby the tissue selenium concentration in plants would be negatively correlated with their cadmium concentration. Additionally, high sulphate in the growth medium was predicted to reduce selenate uptake. Nonetheless, the trend between shoot cadmium and selenium concentrations was predicted to hold true, albeit at lower shoot selenium concentrations with high sulphate provided. Yet nearly all of this was unsupported in the end. Selenium was not negatively correlated with cadmium in shoot tissue. Rather, higher shoot selenium was associated with more cadmium in the shoots, where there was any correlation at all. No differences in lignification were found with selenate treatments. While sulphate reduced uptake of selenate, selenate increased shoot sulphur concentration, again, contrary to predictions.

The experiment was conducted in hydroponic culture, maximizing control over the growing environment and consistency. Thus, its conclusions may not directly relate to conditions in croplands. Nonetheless, the data from these highly controlled conditions suggest selenium fortification in the presence of cadmium to be unsafe. Without any evidence of a benefit, much less clear evidence of an overwhelming benefit, experiments on croplands are not indicated. Likewise, without abundant support for the safety of such practices, and evidence of potential increased harm, selenium fortification on cadmium-contaminated croplands appears ill-advised.

5.1 Implications

Despite the limitations of this study, and the lack of overwhelming evidence supporting the safety of selenium fortification of crops in the presence of cadmium, the practice

appears ill-advised. Generally, within species, plants that had higher shoot tissue selenium concentrations tended to have higher cadmium shoot tissue concentrations. While it is somewhat good news that this did not appear to be a treatment effect whereby selenium increased cadmium, caution should still be exercised. The higher concentrations of cadmium in shoot tissue associated with higher shoot selenium concentrations could have negative consequences to consumers of the plants, whether humans or other animals.

Not only was the increased cadmium uptake with selenium potentially problematic for consumers, but it also had negative consequences for the plant. Plants suffered, with increased toxicity symptoms observed, including a reduction in biomass. Given these effects, application of selenate is also not recommended for cadmium phytoremediation sites. Should the relationship between shoot cadmium and selenium concentrations be causal and not strictly a correlation, it may appear as though applying selenate could enhance phytoremediation efficiency, increasing the concentration of cadmium taken up. However, it appears unlikely to be causal. Additionally, note that the higher concentration of selenate came at a high cost to the plant in terms of growth and health when in combination with cadmium.

Further to this cautionary statement, when sulphate levels are elevated and/or sulphate is applied, selenium fortification fares even worse. Elevated sulphate decreases the effectiveness of selenate fortification significantly. In my plants, the selenium concentrations with high sulphate were a small fraction of those in plants with sufficient sulphate (Figure 3.4). This raises issues of cost-effectiveness of fortification efforts, as well as the risk of selenate not taken up by the plant leaching into the environment. The high sulphate also had no benefit in terms of reducing cadmium concentrations in the shoots.

5.2 Future Directions

Further testing in native soils with elevated cadmium concentrations would be the next logical step in assessing the interactions between cadmium, sulphate, and selenate in

plants. However, my findings in hydroponics do not support selenate fortification of crops as an unequivocally safe practice (Figure 5.1). Thus, further work in this direction appears relatively futile. Given that other studies (e.g., Cui et al., 2018) have found that selenite may be beneficial in reducing cadmium in suspension cells via lignification, a finding I was not able to collaborate with selenate, there may still be potential for further work focused on selenite. However, one of the main reasons that I did not use selenite was that rather than being taken up in place of sulphate, it is taken up in place of phosphate (reviewed by Gupta & Gupta, 2017). Phosphate fertilizers are typically liberally applied in agricultural settings. As a result, an analogous study with sufficient and high phosphate may provide results of limited value, with few unfertilized croplands present, aside from those on organic farms.

Selenium has a relatively narrow therapeutic range for humans. In Canada, the United States, and Europe, the recommendation is 55 μg/day of selenium for adults, and toxicity is associated with doses of over 1600 μg/day (review by Tamás et al., 2010). As a result, selenium fortification of crops should be monitored over time. Thus, there may be other, better mechanisms of inducing lignification that could be explored in its place, and which may require less monitoring. For example, Bezrukova et al. (2011) explored the impact of lectins on lignification as a mechanism to reduce cadmium uptake. Furthermore, from the work of Cui et al. (2018), selenite resulted in lower cadmium in rice suspension cells, with increased expression of several genes. Among these were three associated with lignin synthesis in rice (*OsPAL*, *OsCoMT*, and *Os4CL3*). Non-selenium induced upregulations of these genes may provide a similar benefit, which could be explored.

Additionally, it is worth noting the results, particularly for Hypothesis 2, were contrary to expectations. Thus, not only was Hypothesis 1 unsupported, with increased lignification not found to be a mechanism, but the there did not appear to be a mechanism for reduced cadmium uptake whatsoever. However, why selenate tended to be positively correlated with higher cadmium concentrations on a plant-by-plant basis could be investigated. This may include whether plants that took up more selenate and cadmium also take up more of other potentially-harmful elements (e.g. lead and arsenic). If so, they may pose a greater-

than-anticipated food safety concerns, with compounded toxicity for consumers. However, if plants that take up more cadmium and selenate take up more of many elements, including those that are beneficial to humans (e.g., iron and magnesium), would mechanisms that would reduce cadmium uptake potentially also negatively affect the nutritional value of the crops?

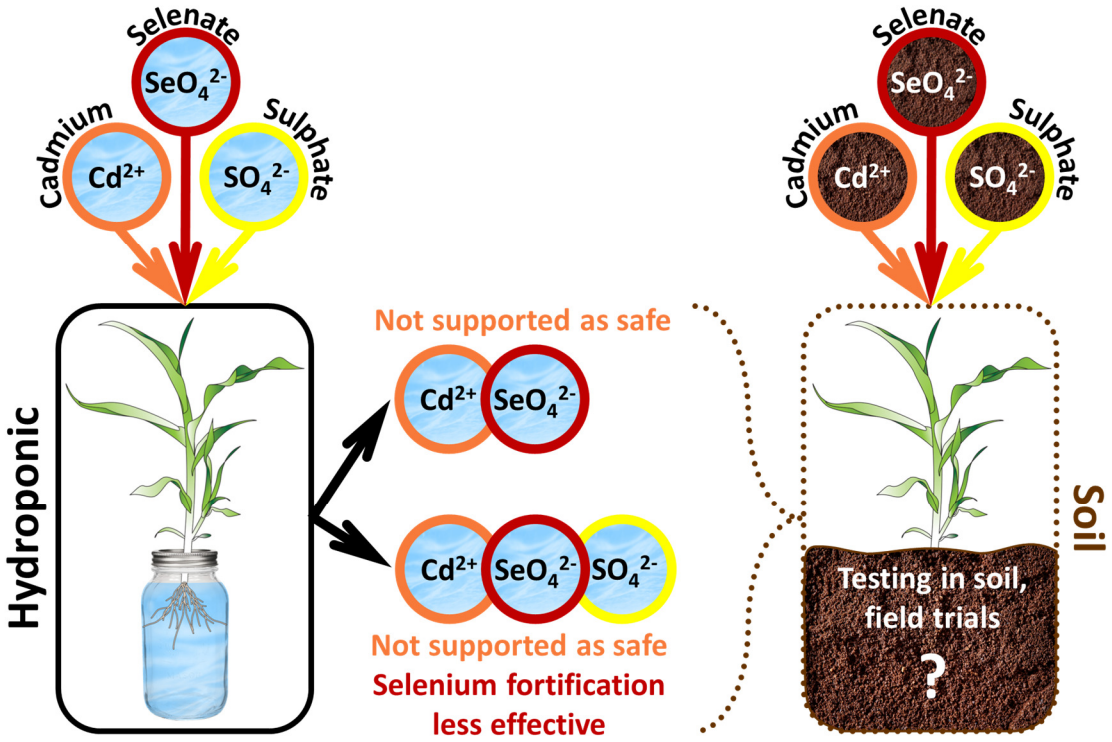

Figure 5.1: Implications of Study Results.
Based on the results of this study in hydroponic conditions, the application of selenate to reduce cadmium uptake was not supported as a safe practice, either with sufficient or high sulphate present. Selenium fortification of crops with selenate was also less effective under conditions with high sulphate. While these results may not directly translate to soil and field conditions, whether future testing is even warranted is debatable. Without overwhelming support for the safety of fortification with selenate in the presence of cadmium, further research efforts are recommended to explore alternative mechanisms. (Sorghum plant image: Kansas State University Extension, https://express.adobe.com/page/B5g3d/ Mason jar image: Walmart, https://www.walmart.ca/en/ip/bernardin-regular-mason-jar-with-standard-lid-1-l-clear/6000016937532)

Finally, the wildflower species I selected are not recommended for future experiments! Theses *Astragalus* species had very high variability between plants. To observe a significant difference in a parameter, such as biomass or selenium concentrations, the effect had to be very large to be statistically significant. As a result, the concentrations of cadmium used may have been too high, resulting in severe toxicity in some plants. If instead only mild toxicity was induced, selenium may have been able to provide a benefit. Furthermore, since the combination of cadmium and selenium resulted in greater toxicity symptoms than they did individually, the selenium dose may also have been excessive. This was further complicated by the wide range of species utilized, and the desire to utilize the same concentrations of cadmium and selenium in the growth medium for all species, including the selenium hyperaccumulators. If the experiments were to be revised and rerun, I would suggest eliminating the *Astragalus* species from the experimental design all together. While more replicates could be utilized, what should have served as a warning in my preliminary experiments were the selenium concentrations in the three *A. bisulcatus* plants: 1386 mg/kg, 2734 mg/kg, and 3542 mg/kg. Despite the same treatment and conditions, they varied very widely in their selenium uptake abilities. With this level of variability, the number of replicates needed would likely not be feasible, due to their slow and inconsistent growth.

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Appendices

Appendix I: Preliminary Testing of Selenium Tolerance

Preliminary testing was conducted on four *Astragalus* species and five *Symphyotrichum* species. The species tested were *Astragalus bisulcatus* (Hook.) A. Gray (two-grooved milkvetch), *Astragalus canadensis* L. (Canada milkvetch), *Astragalus crassicarpus* Nutt. (groundplum milkvetch), *Astragalus racemosus* Pursh (creamy milkvetch), *Symphyotrichum cordifolium* (L.) G.L. Nesom (heart-leaved aster), *Symphyotrichum ericoides* (L.) G.L. Nesom (heath aster), *Symphyotrichum lanceolatum* (Willd.) G.L. Nesom (panicled aster), *Symphyotrichum lateriflorum* (L.) Á. Löve & D. Löve (calico aster), and *Symphyotrichum pilosum* (Willd.) G.L. Nesom (frost aster).

In the preliminary testing, it was found that the *Symphyotrichum* species were difficult to grow in a hydroponics system relative to the *Astragalus* species. Initial growth was slow, and fungal spores from *Chromelosporium fulvum* were present in the environment. The growth rate of *C. fulvum* exceeded that of the *Symphyotrichum* seedlings, blocking light from reaching the seedlings. As a result, growth trials for *Symphyotrichum* species were unsuccessful, and biomass data was not collected.

Preliminary testing of *Astragalus* species was conducted to verify their ability to hyperaccumulate selenium within the growth parameters to be utilized. In these preliminary trials, the nutrient solution contained 60 µM selenate. The concentration of 60 µM was estimated to be sufficient to allow plants to take up more than 1000 mg of selenium per kg dry weight, which is the threshold used (albeit in native soil) for defining a selenium hyperaccumulator.

The selenate does used in the preliminary trials, 60 µM, was found to be more toxic than anticipated, being lethal in *A. canadensis*, and near-lethal in *A. crassicarpus*. The *A. canadensis* plants in the 60 µM selenate treatment died relatively quickly, enabling additional plants to be tested in a lower selenate concentration, 20 µM, unlike *A. crassicarpus* plants, which were slightly more tolerant. *Astragalus crassicarpus* plants in the 60 µM selenate treatment had a median biomass of only 3.8% that of the control, with

a standard deviation of 4.6% of control (Table A1.1). Thus, these plants were both extremely small, and highly variable. For the controls, the coefficient of variation was 77%, highlighting the lack of uniformity in their growth. As a result of the extremely low biomass of selenate-treated *A. crassicarpus* plants, the plants were pooled into one sample for ICP-MS analysis. This pooled sample found the plants to have an average shoot selenium concentration of 461 mg/kg DW (Figure A1.1). For contrast, *A. canadensis* plants in 20 µM selenate had a similar average shoot selenium concentration of 416 mg/kg DW. Since both had concentrations well under 1000 mg/kg, *A. canadensis* was selected for the main experiment, based on its less inconsistent growth (Table A1.1).

Table A1.1: Biomass of plants treated with selenate, as percent of control
Data is show with plus/minus the standard deviation (percent of control)

Species	Selenium Hyperaccumulator?	Selenate Treatment	Biomass with Selenate Treatment (% of control)
A. canadensis	No	20 µM	15.3 ± 7.8
		60 µM	(Lethal)
A. bisulcatus	Yes	60 µM	14.4 ± 10.7
A. racemosus	Yes	60 µM	114.7 ± 112.7
A. crassicarpus	No	60 µM	3.8 ± 4.6

The two potential options for a selenium hyperaccumulator, *A. bisulcatus* and *A. racemosus* had average shoot selenium concentrations of 2554 mg/kg, and 4791 mg/kg, respectively (Figure A1.1). These were both above the target 1000 mg/kg DW, but since they were quite different, both were utilized rather than selecting one over the other. They also differed greatly in their biomass under the same 60 µM selenate treatment (Table A1.1), with *A. bisulcatus* having a biomass of much less than the control (14.4% of control on average), while selenate-treated *A. racemosus* plants were slightly larger than the controls on average (114.7% of control). However, these plants were also highly variable, as indicated by their standard deviations shown in Table A1.1.

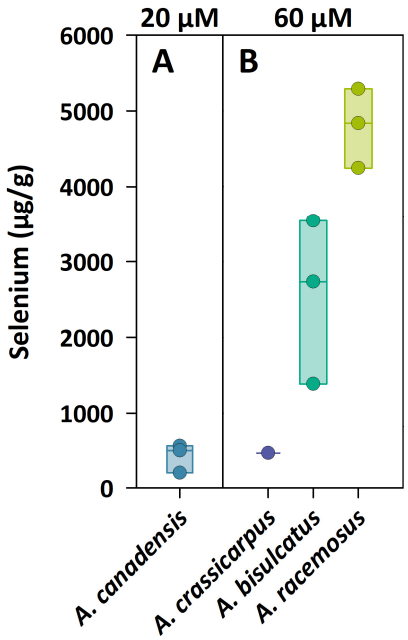

Figure A1.1: Selenium concentrations in shoots of four *Astragalus* species, as found during preliminary testing. In *A. canadensis*, 60 µM selenate was lethal, so 20 µM was used instead for this species (Panel A), while 60 µM was used for the other three species (Panel B).
Plants were analyzed via ICP-MS for shoot selenium concentrations. Due to low biomass, *A. crassicarpus* plants were pooled, yielding only one data point for this species. For the three other species, three replicates of each were grown and separately analyzed. Boxplots are overlain with the individual data points.

Appendix II: Nutrient Solution Modelling

The plant growth medium was modelled for all 8 treatments. Particular attention was paid to selenate and cadmium, ensuring that insoluble complexes did not form in the growth solution. Essential nutrients were also modelled for solubility, verifying that there were no large differences between the treatments in nutrient availability. The 8 treatments were: control ('Control'), cadmium ('Cd'), cadmium and selenate ('Cd + Se'), cadmium with high sulphate ('Cd + S'), cadmium, selenate and high sulphate ('Cd + Se + S'), selenate ('Se'), cadmium and high selenate ('Cd + high Se'), and cadmium and high selenate and high sulphate ('Cd + high Se + S').

Modelling was conducted using Visual MINTEQ 3.1, and the 8 treatments were modelled across a wide pH range, spanning 4.0 to 8.0, with intervals of 0.25. A wide range in pH was utilized since plants can affect the pH of the growth medium via the production of root exudates. Thus, simply modelling the nutrient solution at its initial pH, 6.5, (check value in lab notebook, but ~6.5) was deemed insufficient. For each experimental treatment, the concentrations of the ions that made up that growth medium were used as the inputs for the software (refer to Sections 2.2.2 and 2.2.3 for nutrient solution composition). A list of possible solid phases was also set within the software. The pH was set manually, the software run, and the data exported to Excel (Microsoft 365). The pH was then set to the next value, and the process repeated. Data was then imported to SigmaPlot and plotted.

Results of the growth medium modelling are shown in Figures A2.1-A2.7. Figure A2.1 shows the percentage total cadmium dissolved in the growth medium for all 8 treatments, with a range of 97.6% to 100% in solution. Figures A2.2 to A2.7 show the percentages of total calcium, chloride, iron, molybdenum, manganese, and phosphorus, respectively, that were dissolved. Figures are not show for selenate, nor the following nutrients: ammonium, boron, copper, magnesium, nitrate, potassium, sodium, sulphate and zinc. These 9 nutrients and selenate were all 100% dissolved across the full pH range modelled.

Throughout the experiments, the control plants appeared healthy, and did not appear to suffer any nutrient deficiencies. This is of note, since the pH of the nutrient solution, approximately 6.6 to 6.7 was close to the pH at which iron becomes insoluble. The main symptom of both iron and manganese deficiencies is interveinal chlorosis. While some plants treated with cadmium and selenate did suffer chlorosis, it was uniform across their new leaves, not interveinal. For iron, less than 1% is dissolved at pHs of 6.75 or higher (Figure A2.4), despite the inclusion of EDTA, a chelating agent.

Overall, modeling the co-application of selenate and cadmium did not show the formation of insoluble complexes in any of the treatments. Cadmium was highly available across the full range of pH values tested for all treatments, and selenate was 100% dissolved. The slight differences between the 8 treatments in the solubility of calcium, chlorine, iron, molybdenum, manganese, and phosphorous were not expected to have major consequences on plant growth. Additionally, as noted above, no deficiency symptoms were observed in any of the control plants.

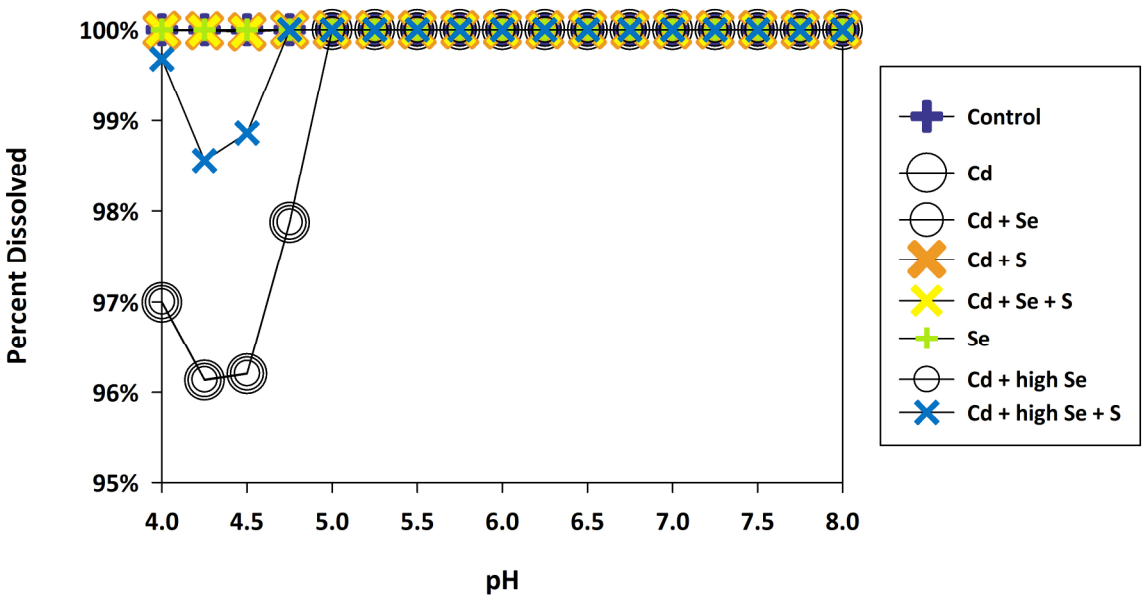

Figure A2.1 Percent of cadmium dissolved in each of the 8 experimental treatments.
Note: The y-axis scale on this graph ranges from 95% to 100%

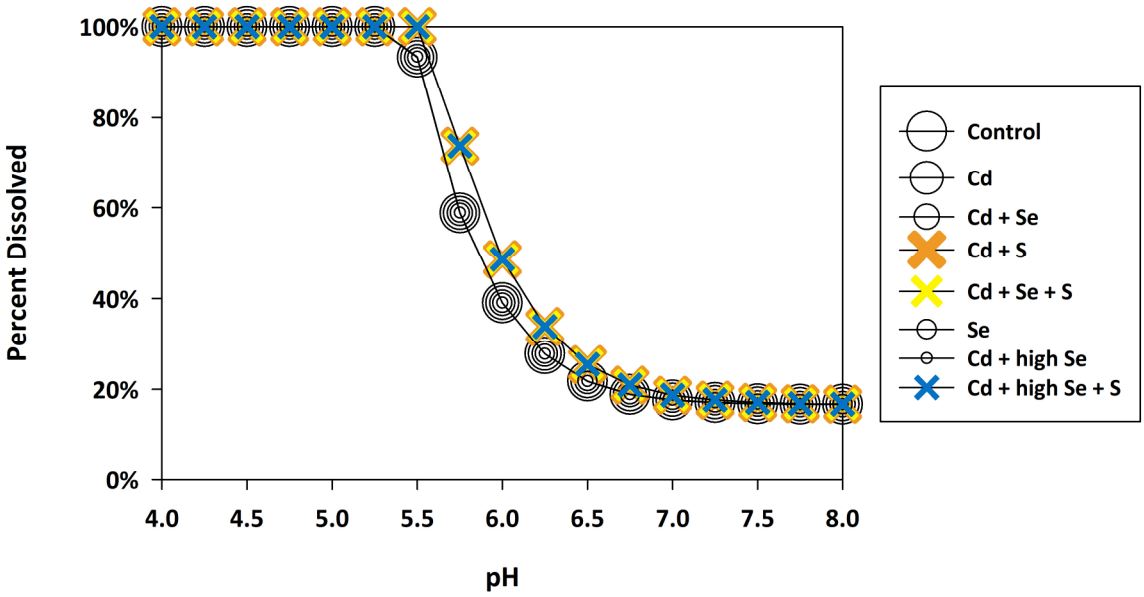

Figure A2.2 Percent of calcium dissolved in each of the 8 experimental treatments.

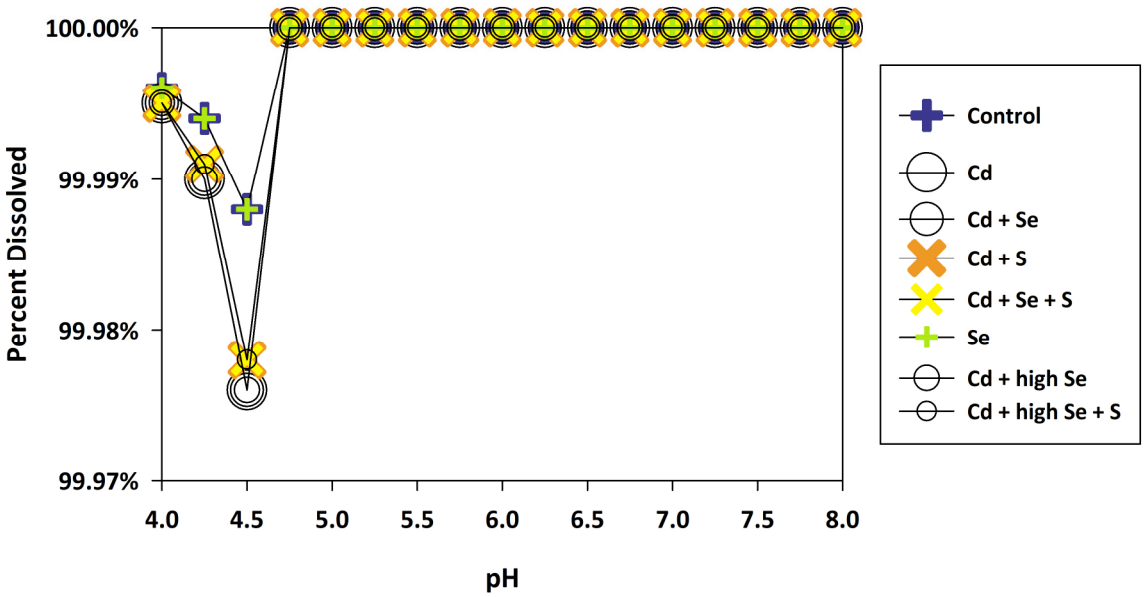

Figure A2.3 Percent of chloride dissolved in each of the 8 experimental treatments.
Note: The y-axis scale on this graph ranges from 99.97% to 100.00%

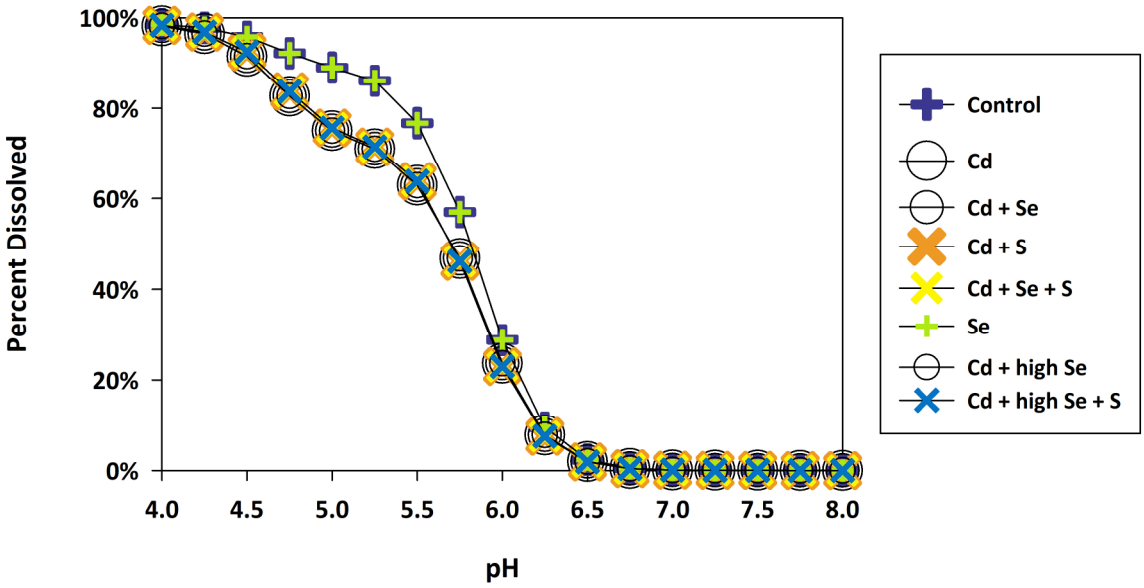

Figure A2.4 Percent of iron dissolved in each of the 8 experimental treatments.

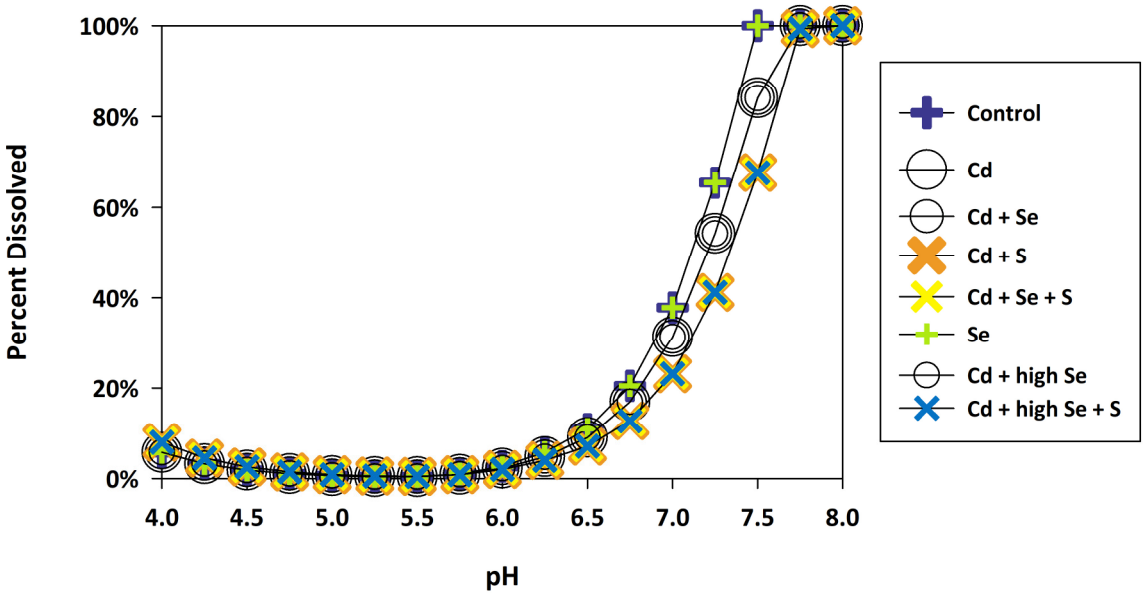

Figure A2.5 Percent of manganese dissolved in each of the 8 experimental treatments.

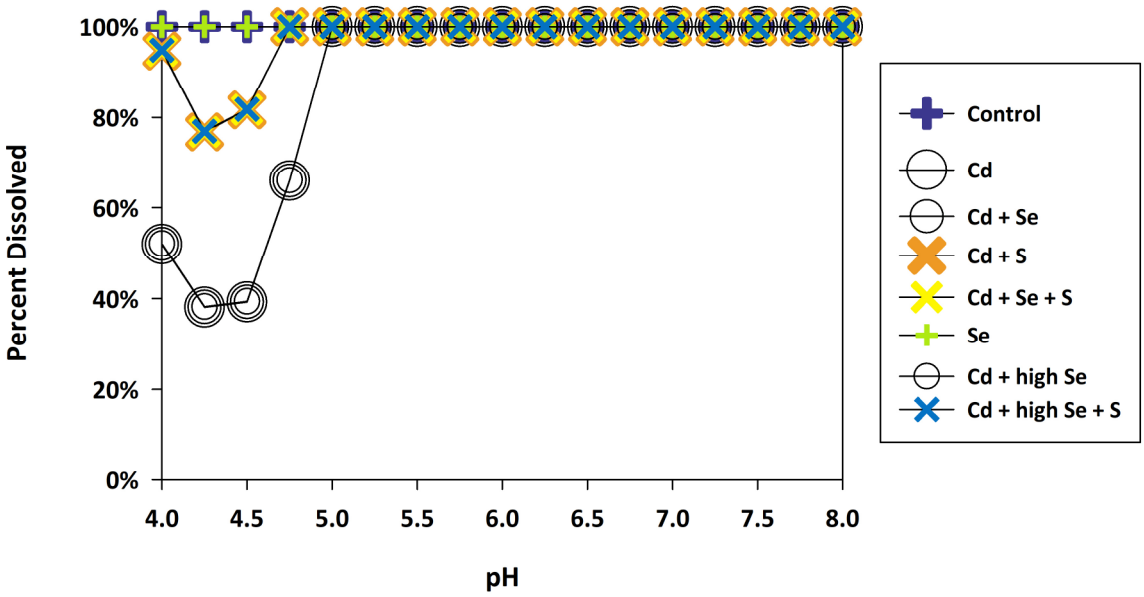

Figure A2.6 Percent of molybdate dissolved in each of the 8 experimental treatments.

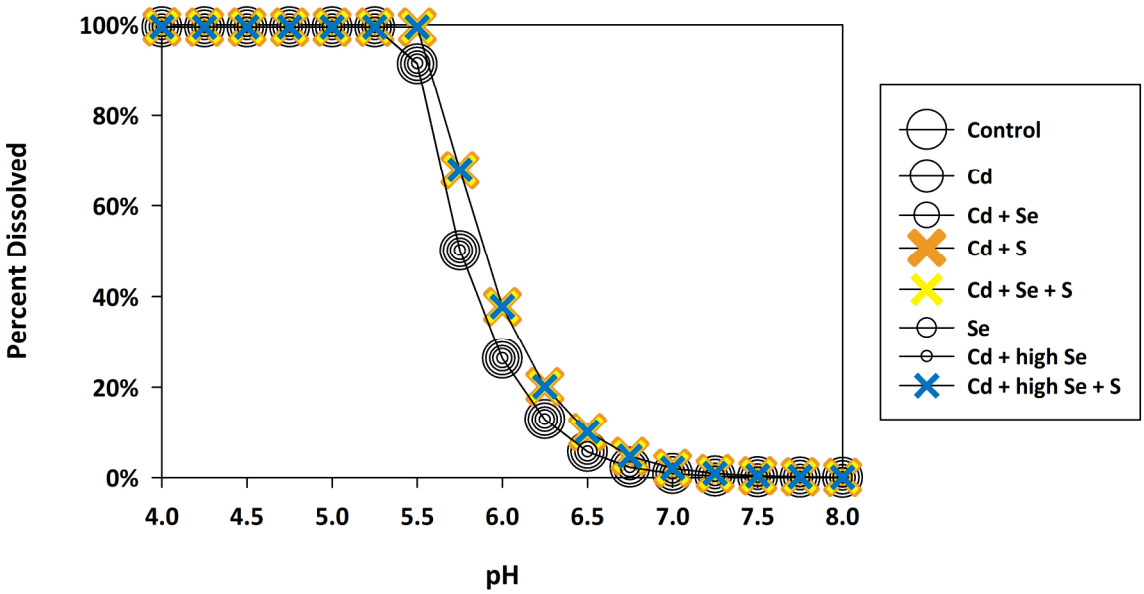

Figure A2.7 Percent of phosphate dissolved in each of the 8 experimental treatments.

Appendix III: Verification of Sufficient and High Sulphate Categorization

Shoot and root samples of the *Astragalus* species were analyzed via ICP-MS for cadmium and selenium by the Biotron at the University of Western Ontario. However, at the time crop samples needed to be analyzed, there were delays anticipated by the Biotron in their timelines. Consequently, these samples were instead sent to the Water Quality Centre at Trent University. The Water Quality Centre was able to analyze the samples for cadmium, selenium, and sulphur all via ICP-MS. As a result, the samples sent to the Water Quality Centre were analyzed for all three elements, rather than just selenium and cadmium.

Testing of sulphur concentrations in the plants' shoots was intended only to verify that the treatment containing "sufficient" sulphate did in fact have sufficient sulphate. If sulphate had been insufficient (i.e., deficient), the sulphur content in plants provided with higher sulphate would be expected to be significantly higher than in plants that were deficient. This trend was not observed (Figure A3.1), with no significant differences in the sulphur concentrations between the 'Cd' treatment (cadmium with sufficient sulphate) and the 'Cd + S' treatment (cadmium + high sulphate), in 4 of the 5 species (all crops except lettuce). When comparing the 'Cd + Se' (cadmium + selenate, with sufficient sulphate) to 'Cd + Se + S' (cadmium + selenate + high sulphate), it was again expected that the high sulphate would not result in greater sulphur concentrations in the shoots. These results are based on a one-way ANOVAs for each species, with data for corn and wheat logarithmically transformed. Prior to transforming the data, corn failed testing for equal normality (Shapiro-Wilk), and wheat failed testing for equal variance (Brown-Forsythe). Untransformed data for canola, lettuce and sorghum passed both equal variance and normality tests.

When comparing the 'Cd + Se' and 'Cd + Se + S', treatments, an unanticipated result was found. The expected results were that if sulphate was sufficient in the "sufficient" sulphate treatments, there would be no difference in shoot sulphur concentrations

between the two treatments. Likewise, if that dose of sulphate was insufficient, sulphate concentrations would be expected to be greater in the high sulphate treatments. However,

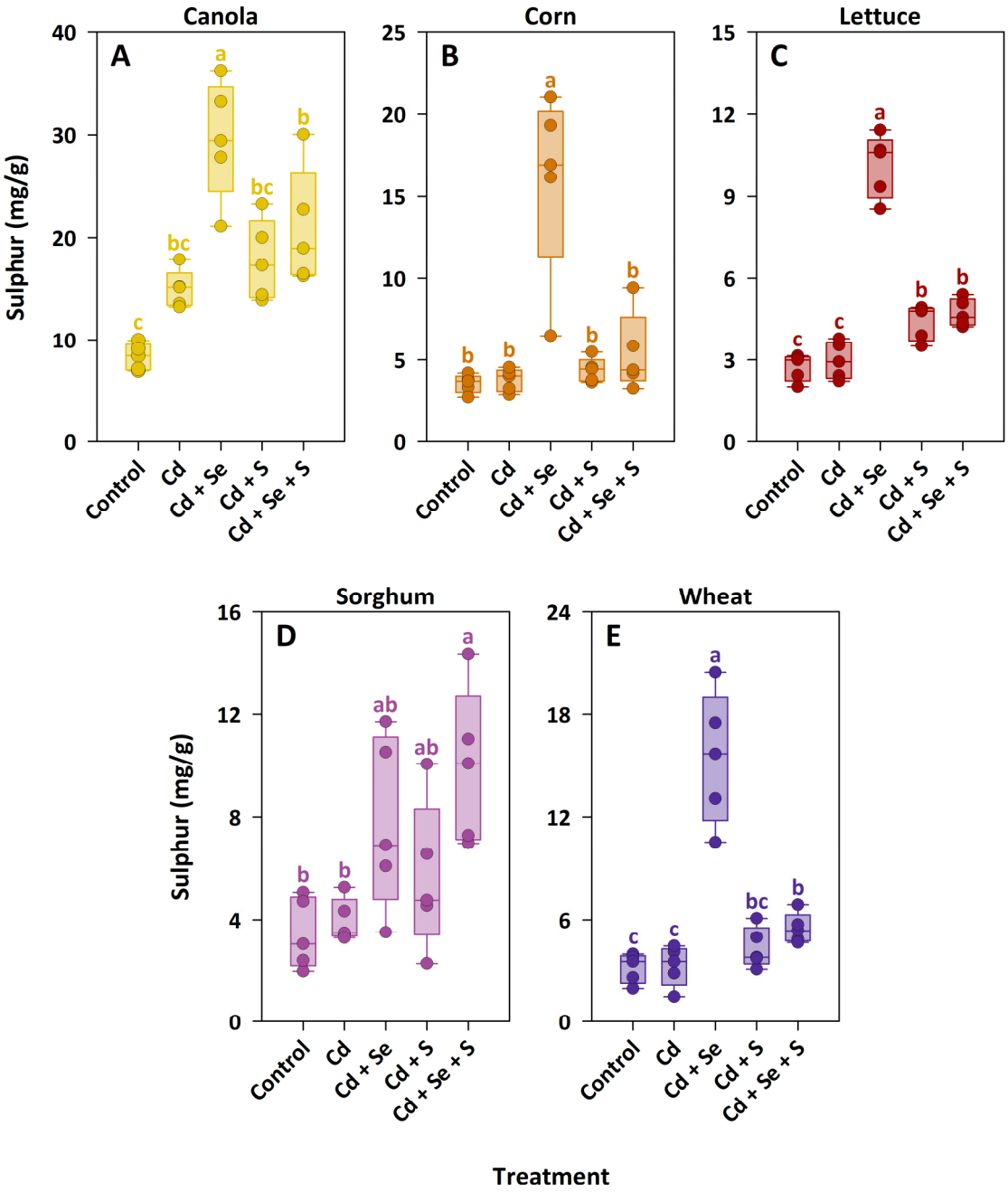

Figure A3.1: Sulphur concentrations in the shoots of the five crop species (Panels A-E) by treatment.
Boxplots show the minimum, maximum, median, 75th, and 25th percentiles, and are overlain by the individual data points.

in four of the five crop species (all species tested except sorghum), a completely different result was found. In these species, the presence of selenate in the growth medium was associated with a much higher shoot sulphate concentration. The amount of sulphur in the ‘Cd + Se’ (cadmium + selenate) was much higher than the amount of sulphur in any of the other treatments. This unexpected result sparked the creation of Section 3.2.1.1, which shows the correlation between sulphur and selenium concentrations in the shoots of the five crop species.

Appendix IV: Impact of Tissue Concentrations of Selenium on those of Cadmium: P-Values

Figures 3.6A-B in Section 3.2.2.1 show the relationship between cadmium and selenium concentrations in the shoots of the 8 species tested, when provided with sufficient sulphate (3.6A) and high sulphate (3.6B). On these graphs, letters are used to show significant differences in the slopes of the lines between the different species. Rather than showing the P-Values on the graph, they are presented in below Figures A4.1 for Figure 3.6A, and Figure A4.2 for Figure 3.6B.

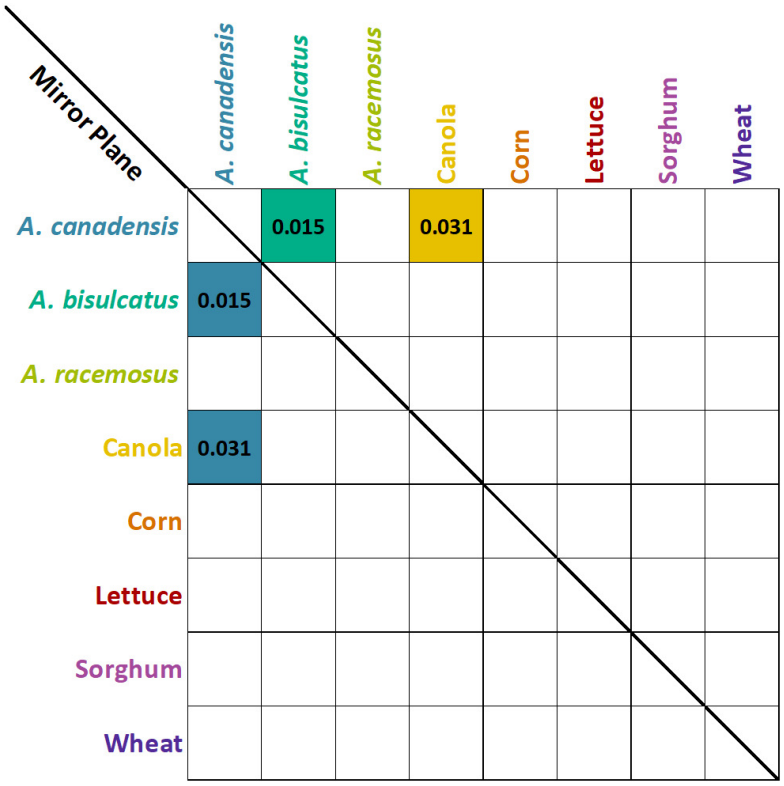

Figure A4.1: P-Values from Figure 3.6A (Located in Section 3.2.2.1). Figure 3.6A shows the relationship between cadmium and selenium concentrations in the shoots of the 8 experimental species when grown with sufficient sulphate concentrations. Note that the P-Values show differences in the slopes of the lines, not the means. For ease of reading, a mirror plane is included, and each P-Value is shown twice.

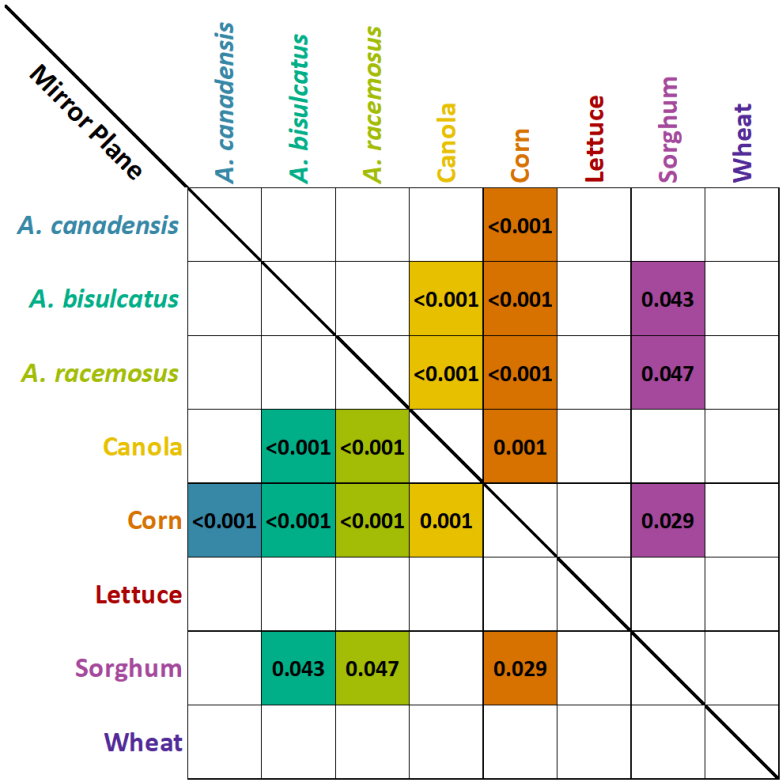

Figure A4.2: P-Values from Figure 3.6B (Located in Section 3.2.2.1). Figure 3.6B shows the relationship between cadmium and selenium concentrations in the shoots of the 8 experimental species when grown with high sulphate.
Note that the P-Values show differences in the slopes of the lines, not the means. For ease of reading, a mirror plane is included, and each P-Value is shown twice.

For ease of reading, Figures A4.1 and A4.2 have a diagonal mirror plane down the middle diagonal of each, so each of the P-Values showing the difference between the slopes is included twice on the figure. Only P-Values that were significant are shown ($P<0.05$), with all other cells left blank. Figures A4.1 and A4.2 can be read left-to-right (or alternatively top-to-bottom). By selecting one species from the list and following it left to right (or top to bottom), all of the species from which it was significantly different are shown. For example, in Figure A4.2, which shows the sufficient sulphate treatments, to determine which species had a different slope from *A. bisulcatus*, read across the row labelled *A. bisulcatus* and find the P-value of <0.001, <0.001, and 0.043 in the cell corresponding to canola, corn, and sorghum. Thus, the slope of *A. bisulcatus* was

different from that of canola (P=<0.001), corn (P=<0.001), and sorghum (P=0.043) in treatments with high sulphate.

Overall, when sulphate was provided at sufficient concentrations (Figure A4.1), there were two significant differences in the slopes: *A. canadensis* differed from canola and from *A. bisulcatus*. When high sulphate was present in the growth medium (Figure A4.2), there were nine significant differences in the slopes, which occurred amongst 6 of the 8 species. Only lettuce and wheat had slopes which never differed from those of another species tested.

Appendix V: Cadmium Concentrations in Shoots

Shoot tissue of all 8 plant species (*Astragalus bisulcatus*, *A. canadensis*, *A. racemosus*, canola, corn, lettuce, sorghum, and wheat) was analyzed for cadmium concentrations via ICP-MS. For the treatments containing both cadmium and selenate this data was utilized in Figure 3.6 and Figure 3.7. Shoot selenium concentration data for the remaining two treatments with cadmium, 'Cd' and 'Cd + S', was not utilized or presented elsewhere in this thesis. The data for shoot cadmium concentrations for all treatments containing cadmium, including those in Figure 3.6 and Figure 3.7, are presented in Figure A5.1 (*Astragalus* spp.) and Figure A5.2 (crop species).

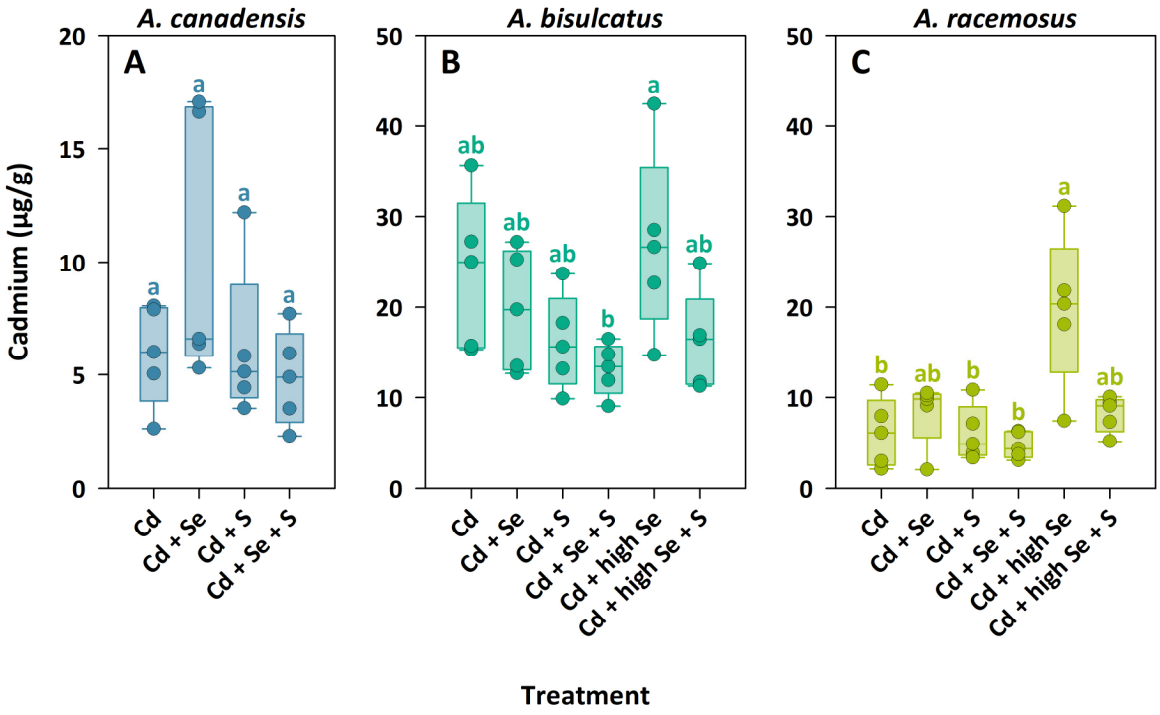

Figure A5.1: Cadmium concentrations in the shoots of the three *Astragalus* species. Cadmium concentrations in the shoots were quantified via ICP-MS analysis in *A. canadensis, A. bisulcatus,* and *A. racemosus* (Panels A-C, respectively). Only treatments in which cadmium was applied are plotted. In the x-axis labels, 'Se' indicates selenate, while 'S' is used to denote high-sulphate treatments. Boxplots show the minimum, maximum, median, 75th, and 25th percentiles, and are overlain by the individual data points.

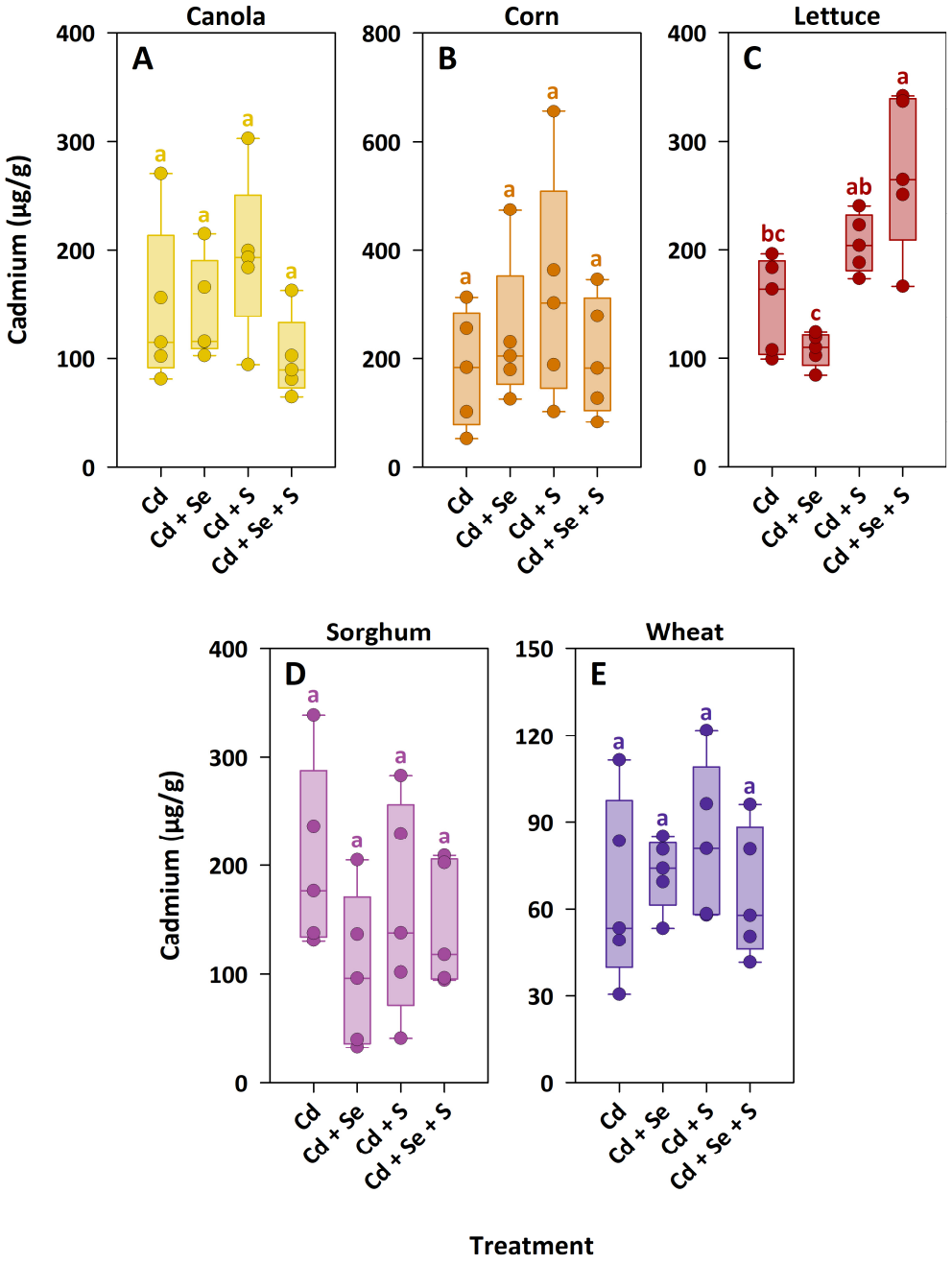

Figure A5.2: Cadmium concentrations in the shoots of the five crop species. Cadmium concentrations in the shoots were quantified via ICP-MS analysis in canola, corn, lettuce, sorghum, and wheat (Panels A-E, respectively). Only treatments in which cadmium was applied are plotted. In the x-axis labels, 'Se' indicates selenate, while 'S' is used to denote high-sulphate treatments. Boxplots show the minimum, maximum, median, 75th, and 25th percentiles, and are overlain by the individual data points.

For treatments without cadmium ('Control' and 'Se') cadmium concentrations are not plotted, but were generally low. Among the 40 'Control' plants (5 replicates of 8 species), the maximum cadmium concentration found in the shoots was only 0.13 μg/g. For the 'Se' treatments (15 plants, *Astragalus* spp. only), the maximum was 3.26 μg/g.

Significant differences between treatments, as indicated on Figures A5.1 and A5.2 were determined via a one-way ANOVA for each species (differences between species were not of interest). All species except lettuce passed equal variance tests (Brown-Forsythe), and all species except *A. racemosus* passed normality testing (Shapiro-Wilk). As a result, the data for lettuce and *A. racemosus* were logarithmically transformed.

With the exception of the data also presented in Figure 3.6 and Figure 3.7, the data in Figure A5.1 and Figure A5.2 was not utilized in testing the hypotheses. However, it was found to be relevant when discussing the results found (Section 4.2.2). A positive correlation between shoot cadmium and selenium concentrations was found in some canola, corn, and sorghum, when they were provided with high sulphate. This correlation between cadmium and selenium does not appear to be due to a treatment effect, but rather plants that took up more of one element took up more of both.

If there was a treatment effect with selenate increasing cadmium uptake, a significant difference in the cadmium concentrations would be expected when selenate was included in the treatments versus the comparable treatments without selenate. Thus, the 'Cd + Se + S' and 'Cd + S' treatments in Figure A5.2 would be expected to differ, at least in Panels A, B, and D (canola, corn, and sorghum). No such differences were found in any of the species between these two treatments ($P>0.05$ in all cases). Additionally, differences between the 'Cd' and 'Cd + Se' treatments in Figure A5.1 and Figure A5.2 would show a treatment effect of selenate. The only occurrence of a difference with the addition of selenate was in *A. racemosus* between the 'Cd' and 'Cd + high Se' (40 μM selenate) treatments (Figure A5.1C) ($P=0.011$). Worth noting, the *A. racemosus* plants in the 'Cd + high Se' treatment experienced high toxicity, with three of the five replicates having biomasses under 0.5 g DW, with the average biomass being 1.63 g DW. For comparison, *A. racemosus* plants in the 'Cd' treatment had an average biomass of 7.29 g DW, with a

minimum of 1.30 g DW. While a higher biomass could dilute the concentrations of selenium and cadmium in the plant, plants with low biomass were in severe decline, with minimal growth following the applications of the experimental treatments.

Curriculum Vitae

Name: Marnie Demand

Post-secondary Education:

University of Western Ontario, London, Ontario
Master of Science in Biology with Collaborative Specialization in Environment and Sustainability
2018-Present

University of New Brunswick, Fredericton, New Brunswick
Bachelor of Science in Forestry, Major in Urban Forestry
2015-2018

Fleming College, Lindsay, Ontario
Ontario College Diploma, Urban Forestry Technician
2012-2015

Lakehead University, Thunder Bay, Ontario
Environmental Studies and Earth Science
2006-2008, 2010, 2011

Honours and Awards:

Department of Biology Graduate Student Teaching Award
University of Western Ontario, London, Ontario
2021

AER Graduate Scholarship in Environment and Sustainability
University of Western Ontario, London, Ontario
2021 and 2019

Ontario Graduate Scholarship
2019-2021

Natural Sciences and Engineering Research Council of Canada
Canada Graduate Scholarship Masters (NSERC CGS-M)
2018-2019

Natural Sciences and Engineering Research Council of Canada
Undergraduate Student Research Award (NSERC USRA)
University of Western Ontario, London, Ontario
2017

President's Honor Roll
Fleming College, Lindsay, Ontario
2015

Natural Sciences and Engineering Research Council of Canada
Undergraduate Student Research Award (NSERC USRA)
Lakehead University, Thunder Bay, Ontario
2008

Related Work Experience:

Forestry Technologist
City of London, London Ontario
2021-Present

Graduate Teaching Assistantship: Ecology (Bio 2483)
University of Western Ontario, London, Ontario
2020

Graduate Teaching Assistantship: Environmental Issues (ES1021)
University of Western Ontario, London, Ontario
2019

Student Fellowship
International Society of Arboriculture, Champaign, Illinois
2015

Publications:

Demand, Marnie J., (2021) Killing One Plant With Two Stones: Unsuccessful Mitigation of Pollution and Nutritional Deficiencies. Inspiring Minds - Showcasing Western's Graduate Research, Scholarship and Creative Activity. 150. https://ir.lib.uwo.ca/inspiringminds/150/

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