DISSERTATION

PLANT SELENIUM ACCUMULATION AND THE RHIZOSPHERE EFFECT

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ABSTRACT

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Hyperaccumulation is a unique phenomenon where large amounts of trace elements are stored in leaves, but it only occurs in less than 1% of all plant species. Plants that hyperaccumulate trace elements are studied as a means to remediate polluted substrates, a process known as phytoremediation. The legume genus *Astragalus* contains the majority of plants that hyperaccumulate Se. My goal was to indentify if and how soil microorganisms may influence *Astragalus* Se accumulation. I examined the root nodule symbiosis in *Astragalus* as a tractable interaction to explore the mechanism by which microorganisms mediate changes in plant Se accumulation. I also investigated plant-based Se removal (phytoextraction) through screening different species.

In the first chapter I summarized literature on how plants hyperaccumulate elements through their root systems. Mechanisms related to physical and chemical characteristics of roots are discussed. The microbial assemblage in the rhizosphere is also important in hyperaccumulation. From this basis I explore how soil microorganisms interact in the rhizosphere of *Astragalus* Se-hyperaccumulators. I tested three hypotheses in the following chapters; (i) soil microorganisms affect Se accumulation in plants, (ii) specifically the root nodule symbiosis has a role in Se-hyperaccumulation, and (iii) the mechanism of root nodule symbiosis affecting hyperaccumulation is through nitrogen allocation into selenoamino acids.

In Chapter 2 I investigated if Se-hyperaccumulators incurred a cost where their symbiotic interactions were disrupted because of their Se tolerance. My experiments did not support evidence of a cost to the symbiosis. I investigated organ [Se] in multiple legume species growing in field conditions, including Se-hyperaccumulators. In general, nodule [Se] were below the threshold used to define Se-hyperaccumulation by leaf concentrations, but they were still notable with some nodules having [Se] near 100 μg Se g^{-1} dry weight. I also detected differences in root and nodule [Se] in the hyperaccumulator *A. bisulcatus*, which may point to a role in Se protecting belowground organs from herbivory. Through x-ray absorption spectra analysis I found that Se was distributed throughout the root nodule in the Se-hyperaccumulators *A. bisulcatus, A.*

praelongus, and *A. racemosus.* The most abundant form of Se in the nodules was organic, C-Se-C.

Following this I conducted further investigations on root nodules. In Chapter 3 I found an effect of root nodule symbiosis in Se accumulation in *A. bisulcatus,* where shoot [Se] was positively correlated with shoot [N] and nodulated plants contained higher shoot [Se] than non-nodulated plants. These effects were not evident in non-accumulators. I determined that a mechanism by which root nodule symbiosis could affect *A. bisulcatus* [Se] was through a 10-fold increase in the selenoamino acid γ-glutamyl-methylselenocysteine.

In Chapter 4 I studied soil microorganisms in general, without focusing on a specific group, and attempted to identify if soil inoculant source affected *Astragalus* Se accumulation. I found a significant increase in root (approximately +200%) and shoot (approximately +70%) [Se] in 6 *Astragalus* species when they were grown in soil inoculant derived from hyperaccumulators compared to inoculant derived from non-accumulators. The 6 species included three Se-hyperaccumulators and three non-accumulators. These results indicate that Se accumulation is mediated by soil microorganisms in some way.

Given the recent explosion of interest in hyperaccumulators as an environmental friendly means of remediating contaminated substrates I investigated *Astragalus* Se-hyperaccumulators for their ability to remove Se from contaminated biosolids produced in Pueblo, Colorado. I found that the Se-hyperaccumulator *A. crotalariae* performs better than the annual crop species *Brassica juncea*, but there is a lack of seed source and agronomic techniques to

move forward at a larger scale with the *Astragalus*. Using the more conventional, fast-growing agronomic species *Brassica juncea* and *B. napus* I found that a dilution of 75% biosolids and 25% sand by volume achieved the highest Se removal potential. Using the information I had gathered in Chapter 4 I attempted to increase plant [Se] in biosolids phytoextraction trials by applying an inoculant derived from soil obtained from the hyperaccumulator *A. bisulcatus*. This approach did not significantly alter plant [Se] in my 13 species trial.

Finally, in Chapter 6 I synthesize the results of my dissertation into my experience conducting scientific research. I have found that often experimental results that are not consistent with research hypotheses can be the most interesting to pursue because they make you continuously wonder why the outcome occurred. I conclude with potential future outlooks for my work. Although my dissertation has focused much on the root-nodule symbiosis in *Astragalus* this work has broader implications for ecological theory of mutualisms, the co-evolution of mutualistic partnerships, and the utilization of rhizosphere communities in phytoremediation.

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This dissertation is dedicated to those who wonder.

Live a Rocky Horror life:

Don’t dream it; be it.

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Chapter 1

Metallophytes - a View from the Rhizosphere

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Summary

Some plants hyperaccumulate metals or metalloids to levels several orders of magnitude higher than other species. This intriguing phenomenon has received considerable attention in the past decade. While research has mostly focused on the above-ground organs, roots are the sole access point to below-ground trace elements and as such they play a vital role in hyperaccumulation. Here I highlight the role of the root as an effective trace element scavenger through interactions in the rhizosphere. I found that less than 10% of the known hyperaccumulator species have had their rhizospheres examined. When studied, researchers have focused on root physical characteristics, rhizosphere chemistry, and rhizosphere microbiology as central themes to understand plant hyperaccumulation. One physical characteristic often assumed about hyperaccumulators is that their roots are small, but this is not true for all species and many species remain unexamined. Transporters in root membranes provide avenues for root uptake, while root growth and morphology influence plant access to trace elements in the rhizosphere. Some hyperaccumulators exhibit unique root scavenging and direct their growth toward elements in soil. Studies on hyperaccumulator rhizosphere chemistry have examined the role of the root in altering elemental solubility through exudation and pH changes. Different interpretations have been reported for mobilization of non-labile trace element pools by hyperaccumulators. However, there is a lack of evidence for a novel role for rhizosphere acidification in hyperaccumulation. As for microbiological

studies, researchers have shown that bacteria and fungi in the hyperaccumulator rhizosphere may exhibit increased metal tolerance, act as plant growth promoting microorganisms, alter elemental solubility, and have significant effects on plant trace element concentrations. New evidence suggests that symbiosis with arbuscular mycorrhizae may not be rare in hyperaccumulator taxa, even in some members of the Brassicaceae. Although there are several reports on the presence of mycorrhizae, a cohesive interpretation of their role in hyperaccumulation remains elusive. In summary, I present the current state of knowledge about how roots hyperaccumulate and suggest ways in which this knowledge can be applied and improved.

Introduction

Metallophytes can be defined by their ability to survive and reproduce on metal-rich soils without suffering toxicity (Baker et al. 2010). Often these plants have been identified by the habitats in which they grow, but high levels of trace elements (defined here as elements other than the 8 most abundant rock-forming elements: Al, Ca, Fe, K, Mg, Na, O, and Si) in plants can also be achieved by growth in amended or contaminated substrates (Reeves 1988; Lombi et al. 2000; Szarek-Lukaszewska and Niklinska 2002). Some metallophytes have a specialization to concentrate elements at levels that would be toxic to non-accumulators. This innate ability to hyperaccumulate trace elements in plant leaves has been observed in species that grow naturally on metal-rich substrates

(Brooks et al. 1977; Baker and Brooks 1989; Wenzel and Jockwer 1999), but the trace element accumulation potential in hyperaccumulators may not be solely a matter of their habitat, when *Thlaspi caerulescens* was subjected to amended substrates ecotypes originating from low-metal soils accumulated more than ecotypes originating from metalliferous soils (Escarré et al. 2000; Dechamps et al. 2005). Clearly hyperaccumulators collect large quantities of soil trace elements and sequester them in their leaves, yet there is not a clear understanding of the ways roots achieve this. Some hyperaccumulators amass large amounts of essential elements (Cu, Mn, Ni, Zn), but other species store non-essential elements (As, Co, Cd, Se). The leaf has been the focus of much study because it serves as a storage site for elements where they are available to livestock and human consumers. Meanwhile, the root, the site of initial and continuous element acquisition, has received far less attention.

Plant access to trace elements is mediated by bioavailability and root location in relation to the element. The rhizosphere, defined as the root-soil interface, where microorganisms, roots, and soil come together (Hiltner 1904), is the micro-ecosystem where roots access soil trace elements. Multiple gradients co-occur in the rhizosphere (bulk density, elemental concentrations, root exudation, microorganisms, moisture, pH, redox potential); thus the rhizosphere is a dynamic system in time and space. Within the rhizosphere additional differences are caused by variation in root activity among root classes (i.e. lateral, primary roots, etc.), root ages, and location (i.e. tip, elongation zone, maturation zone) (Doussan et al. 2003). Given the vast complexity of the

rhizosphere, there is much to explore to explain the belowground aspects of plant hyperaccumulation.

Hyperaccumulators have adapted to life rooted in high-metal soils. Over geologic time the quantity and type of trace elements found in soils change, and in turn, local flora can adapt. Naturally-occurring metalliferous soils foster unique assemblages of tolerant and/or hyperaccumulating plant taxa (Beath et al. 1937; Reeves 2002) and human-influenced metalliferous soils may develop similar types of metal tolerant plant populations (Malaisse and Brooks 1982). Five different explanations for why hyperaccumulators may have evolved have been proposed (Boyd and Martens 1992; Boyd 2007). The hypotheses put forward are (i) plants may hyperaccumulate trace elements because storing large quantities of metals may be a means of metal tolerance and disposal, (ii) hyperaccumulators may use metals as elemental allelopathy against nearby competitors, (iii) metals may serve as osmotic resistance to drought, (iv) accumulated metals may defend the plant against herbivores or pathogens, and (v) metal accumulation may be accidental. This literature review addresses *how* hyperaccumulators work, rather than *why* they hyperaccumulate. I review here what is known about the novel and conventional ways in which hyperaccumulators access and accumulate trace elements.

There is a general lack of ecological knowledge about hyperaccumulators (Boyd and Martens 1992; Whiting et al. 2004), particularly with respect to rhizosphere processes (Abou-Shanab *et al.* 2003a). Metallophytes are botanical curiosities. Their rhizosphere may have unique properties, yet also will retain

similarities to non-accumulating plant species. Hyperaccumulators will continue to have exciting applications if we are able to identify their unique hypertolerance and hyperaccumulation mechanisms, including how they manipulate their rhizospheres. Trace element uptake depends upon plant roots; therefore this region merits extensive study in the context of hyperaccumulation. Current information on rhizosphere conditions in hyperaccumulating plants is based on a small subset (less than 10%) of known hyperaccumulators. Below I group rhizosphere processes into three categories – root physical characteristics, rhizosphere chemistry, and rhizosphere microorganisms. Figure 1.1 illustrates several of the interactions occurring in the rhizosphere that have been described in the literature.

Root Physical Characteristics

Root system development determines the rhizosphere size, configuration, and plant access to soil-borne elements. The root surface area plays a large role in nutrient uptake (Comerford 2005) and it therefore has a significant role in trace element uptake by hyperaccumulators. Researchers have commented on the small root systems characteristic of some hyperaccumulators (Ernst 1996). While it is true some hyperaccumulators are slow-growing and small compared to many annual crop species proposed for phytoremediation, there are perennial hyperaccumulators with well-developed root systems (Kutschera and Lichtenegger 1992; Ernst 1996). Root depth and morphology are important traits

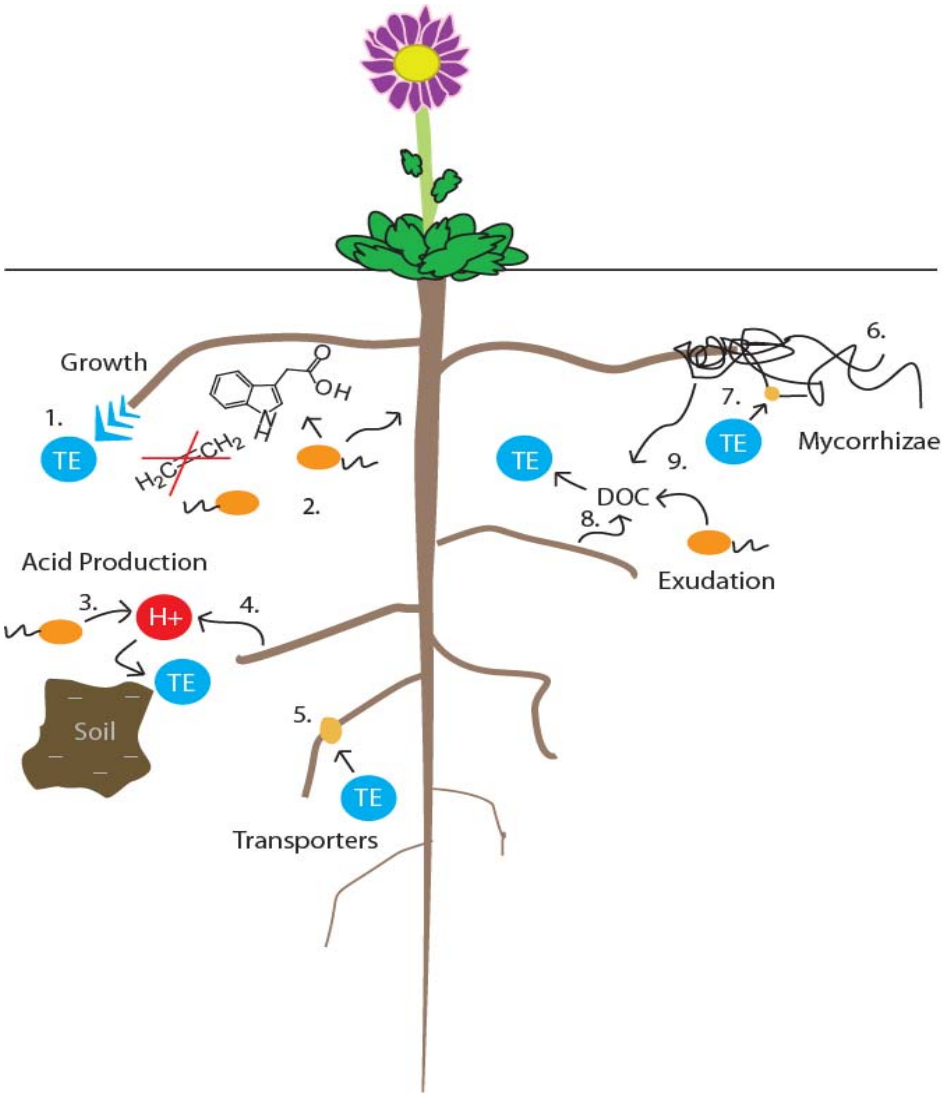

Fig. 1.1 This conceptual diagram shows the many ways that processes in the rhizosphere influence trace element (TE) uptake in hyperaccumulators. Roots search for trace elements and grow towards them by chemotropism (1). Plant growth promoting bacteria can alter root growth by altering hormones such as indole-3 acetic acid or degrade ethylene in the rhizosphere or as endophytes living within the plant (2). Trace element solubility can be altered by acid production (H+) by bacteria (3) or roots (4). Hydrogen ions can then displace trace element cations adsorbed to soil particles. Bioavailable trace elements can enter the plant through transporters located in the root (5). Mycorrhizae can increase plant access to trace elements by increasing belowground surface area (6) where trace elements can enter transporters in the fungus (7). Finally, trace element solubility can be increased in the rhizosphere by complexation with dissolved organic carbon (DOC) that is deposited in the rhizosphere by roots (8) or microorganisms (9).

related to uptake, yet there are very few reports in the literature that compare root morphology between species and how it affects hyperaccumulation. However, it must be noted that comparisons may be challenging to conduct because root structure and length are constrained by soil physical characteristics. Rather than conducting comparisons between species, studies have focused on root length, depth, and surface area within a single species.

Several hyperaccumulators have been described with small, shallow (< 0.5 m) root systems and a high proportion of fine roots that contribute to trace element accumulation (Keller et al. 2003; Himmelbauer et al. 2005). Although these reports featured shallow-rooted plants, deep-rooted (2 m) herbaceous species such as *Biscutella laevigata* (Cd-hyperaccumulator) exist (Kutschera and Lichtenegger 1992), and roots of many hyperaccumulator tree species remain unexamined but could be large and deep.

At the root surface, membrane transporters provide uptake sites for soil borne elements to enter the symplast. Alternatively, trace elements may enter in the plant apoplast and enter the symplast through transporters in the endodermis that surrounds the root vascular cylinder. Several hyperaccumulated metals are essential (Cu, Mn, Ni, and Zn), therefore specific transporters for these elements should be located in root membranes of hyperaccumulators. Zinc (Lasat et al. 2000; Assunção et al. 2001) and Ni (Gendre et al. 2007) transporters have been described for hyperaccumulator species, but transporters for Mn (Mizuno et al. 2008) and Cu have not. Transporters for these elements have been described for non-hyperaccumulator species (Grotz et al. 1998; Clemens 2001; Pittman

2005; Burkhead et al. 2009); although Ni transport is not well understood (Gerendás et al. 1999). The difference in accumulation of some trace elements between hyperaccumulators and non-hyperaccumulators may be related to a higher expression level of the same transporters in hyperaccumulators than in non-hyperaccumulators, or the presence of transporters with different kinetic properties; there is more evidence so far for the former (Pence et al. 2000; Li et al. 2005a). The constitutive upregulation of a root Zn transporter in hyperaccumulator *Thlaspi caerulescens* has been suggested to be caused by the roots sensing the plant to be continuously Zn-starved (Talke et al. 2006) and increased expression of *HMA4* causes a similar response in *Arabidopsis halleri* (Hanikenne et al. 2008). Besides transporters at the root surface, hyperaccumulators have other ways to promote metal accumulation in the shoots, such as reducing sequestration in root vacuoles so metals can enter xylem transport pathways (Lasat et al. 1998; Papoyan and Kochian 2004), and a highly lignified endodermis that prevents metal efflux out of the root vasculature (van de Mortel et al. 2006). In addition, plant species vary in their levels of metal chaperones and chelators such as glutathione and phytochelatins, organic acids, histidine, and nicotianamine (Krämer et al. 1996; Salt et al. 1999; Freeman et al. 2004; Kupper et al. 2004; Raab et al. 2004; Weber et al. 2004; Mari et al. 2006); some of these may facilitate metal transport within hyperaccumulator root cells and root xylem or serve roles in plant metal tolerance. Rather than discussing these processes within the plant in more detail, I turn my attention back to the rhizosphere and the root surface.

Non-essential trace elements can enter roots through transporters for essential elements that have similar valence states and ionic diameters. For instance, As, Cd, and Se resemble the essential elements P, Cu/Fe/Mn/Zn, and S, respectively (Cataldo et al. 1983; Marschner 1995). Given these less specific avenues for entry, there may be ways in which hyperaccumulator species acquire larger amounts of non-essential trace elements than non-hyperaccumulator species. Hyperaccumulators may have higher expression levels for essential element transporters. For example, it was shown that higher S levels occurred in Se-hyperaccumulators than non-accumulators (Galeas et al. 2007), if those transporters do not discriminate against Se this could contribute to higher Se levels in plants. In addition, the transporters in hyperaccumulators could have higher affinities for non-essential elements than transporters in non-accumulators (Bell et al. 1992; Zhao et al. 2002b; Poynton et al. 2004; White et al. 2007). Once inside the hyperaccumulator, trace elements may be complexed and stored in vacuoles. This has been suggested for thiol reductants in the As-hyperaccumulator *Pteris vittata* (Webb et al. 2003), although the amount of As complexed in this manner is small (Zhao et al. 2003; Raab et al. 2004).

In the soil, root transporters need to be in the vicinity of trace elements, so roots should be located in areas of bioavailable elements. Directed root growth towards trace elements in soil (chemotropism) has been reported in some hyperaccumulator species or ecotypes, but the mechanisms are not understood. This response demands an adequate ecological interpretation because current thought on chemotropism relates solely to non-hyperaccumulators that can alter

root growth to dynamically compensate for spatial variability in soil nutrients (Jackson and Caldwell 1996; Robinson 1996; Bloom et al. 2002; Doussan et al. 2003). Many hyperaccumulators exhibit similar root proliferation in soil, but in response to elevated levels of trace elements including Cd, Ni, Se, and Zn (Schwartz et al. 1999; Whiting et al. 2000; Haines 2002; Goodson et al. 2003; Dechamps et al. 2008). Nickel and Zn are essential, but Cd and Se are not; therefore the hypothesis of root-proliferation yielding increased plant nutrition only holds if non-essential elements are co-localized with nutrients.

In contrast, there are known negative effects of high concentrations of some trace elements on root growth in non-hyperaccumulators (Robertson 1985; Barceló and Poschenrieder 1990; Paliouris and Hutchinson 1991; El Kassis et al. 2007), but some hyperaccumulators can direct their root growth, and thus rhizosphere development, in a positive response toward trace elements in ways that non-hyperaccumulators could not (Whiting et al. 2000; Goodson et al. 2003). Additionally, some hyperaccumulator plant ecotypes responded positively to trace elements while other ecotypes within the same species did not (Whiting et al. 2000; Haines 2002; Li et al. 2005a; Dechamps et al. 2008; Li et al. 2009). Positive chemotropism may partially explain the higher trace element content in some hyperaccumulator plants, but not all hyperaccumulators have chemotropic responses to hyperaccumulated elements (Moradi et al. 2009). These ecotype-, population-, and species-specific responses indicate that when possible, care should be taken in choosing seed sources and species for experimental studies. Similar to the ideas of using local ecotypes genetically adapted to specific

restoration settings (McKay et al. 2005), selecting appropriate hyperaccumulator ecotypes that exhibit chemotropic root growth may be important in some phytoremediation settings.

Even though positive root chemotropism is not observed in all hyperaccumulators, these plants may provide a new framework to explain root behavior. Instead of nutrition, what is directed growth in some hyperaccumulators used for? One explanation may be that chemotropic growth causes increased access to trace elements that enhance plant herbivore defense. There is no experimental evidence for this, but chemotropism could be an extension of the elemental defense hypothesis, where hyperaccumulators can reduce herbivore and pathogen attack because their tissues contain toxic amounts of trace elements (Boyd et al. 1994; Martens and Boyd 1994). Preferential root scavenging could increase access to elements and consequently increase concentrations within plants – leading to increased herbivore defense.

Physical characteristics of hyperaccumulator roots have received some attention in the literature, but further work focusing on transport mechanisms, root turnover, trace element partitioning, and root morphology is needed. Investigating more species of hyperaccumulators is important to further our understanding of how roots respond to trace elements. In addition, further studies should be conducted on root uptake and transport by hyperaccumulators; for example Co transport mechanisms have not been described. Further, the study of root turnover in response to trace elements may be useful for studies in

carbon sequestration, but it would be particularly important for phytoremediation where root death could significantly reduce plant uptake. Notably, leaf senescence and turnover from metal toxicity has received attention (Jana and Choudhuri 1982; Fuhrer 1983; Ryser and Sauder 2006), but corresponding analyses belowground are rare (Helmisaari et al. 1999). Finally, describing trace element partitioning in roots can help us understand how hyperaccumulators work and relate to their environment. For example, the fine and coarse roots of *Alyssum murale* (Ni and Zn-hyperaccumulator) have specific Ni and Zn localization where the metals were located in fine root vasculature and on the coarse root exterior (McNear *et al.* 2005). One reason for this type of pattern may be xylem transport in the vasculature, but there could be various explanations for rhizoplane localization, such as an herbivore or pathogen defense strategy, deposition by root exudation, or association within a sheath of rhizosphere microorganisms. Further research on root physical traits should be conducted. If we learn the genetic mechanisms of chemotropic responses in hyperaccumulators there may be potential to transfer this trait to agricultural species to improve plant nutrition or herbivore defense. Currently, we have a better understanding of root growth in hyperaccumulator species than trace element allocation patterns. Root uptake of large amounts of trace elements is responsible for hyperaccumulation. With such high uptake comes high demand for soluble trace elements in the rhizosphere. How then do hyperaccumulators manage trace element dynamics in the rhizosphere?

Rhizosphere Chemistry

There are four distinctive soils in which most hyperaccumulator plants have been discovered: serpentine soils (Ni), seleniferous soils (Se), calamine soils (Zn), and soils of the African copper belt (Co, Cu, Cr, Ni, Zn) (Reeves 2002). While there are characteristic soils in which hyperaccumulators typically evolve, some hyperaccumulators can be found in an array of soil conditions. As an example, *Pteris vittata* (As-hyperaccumulator) has been found in multiple soils with one thousand fold differences in their As concentrations (Liao *et al.* 2004). Additionally, accessions of *Pteris vittata* from uncontaminated soils accumulate similar As concentrations as accessions from contaminated soils (Zhao et al. 2002a). Soil descriptions should be included in hyperaccumulator literature when studies occur in the field, particularly if new hyperaccumulators are described. Once background soil conditions are known we can examine how roots modify the rhizosphere.

Roots alter soil chemistry in many ways, via exudation and uptake. Roots may change soil chemical concentrations, pH, redox conditions, form organic acid complexes with nutrients, and chelate metals (Hinsinger 1998). Hyperaccumulators somehow manage to acquire large amounts of trace elements, likely at least in part through the same mechanisms as their non-hyperaccumulating relatives. Although novel ways of accessing trace elements in the rhizosphere may exist in hyperaccumulators, this remains in debate. There is some evidence for enhanced access of hyperaccumulators to non-labile soil pools, as will be reviewed in the next section.

Root Exudation Effects on Trace Element Solubility

Some reports suggest potentially unique trace element solubilization by hyperaccumulators. For example, it was shown that populations of *Thlaspi caerulescens* grown in pots amended with ZnS accumulated more Zn in their shoots than the calculated total water and ammonium-nitrate extractable Zn in pots, indicating that the plants were accessing less available pools of Zn (Whiting et al. 2001d). Non-hyperaccumulators were not used in this study, so we cannot know whether or not these findings are indeed unique to hyperaccumulators, but due to the lack of high Zn accumulation in the ZnS treatment compared to the other Zn treatments, the mobilizing ability of these plants may not be very strong (Whiting et al. 2001d). Another study found that the mobile Zn fraction in soil accounted for less than 10% of Zn within shoots in a *Thlaspi caerulescens*, while a related non-hyperaccumulator obtained 55% of shoot Zn from the mobile fraction (McGrath *et al.* 1997). The authors suggested that the hyperaccumulator is therefore better able to access Zn from the non-mobile fraction; the route by which the remaining Zn was acquired remains unknown. It is possible that the unaccounted Zn in both species was accumulated by the same undetermined mechanism(s), which could include microbiological activity. In both of these studies it is possible that no novel mobilizing mechanism exists, rather plant depletion of the mobile pool could shift the equilibrium from the less mobile to the mobile pool. Although unique mechanisms of hyperaccumulators can be challenging to demonstrate, some reports of general changes in rhizosphere chemistry exist. A Ni-hyperaccumulator decreased the residual Ni

pool in rhizosphere soil and increased the reducible and oxidizable fractions while a non-hyperaccumulator did not (Kidd et al. 2007). Root exudation should be examined further because it may play a key role in metal solubilization. For example, root exduates (and/or microbial activity) were proposed to be an integral part of Ni accumulation in *Thlaspi goesingense* where organic acids may participate in dissolution of Ni-bearing mineral surfaces (Puschenreiter et al. 2005).

Other studies have focused specifically on root exudate amounts and composition. Plant exuded reductants can reduce Mn and Fe oxides that have adsorption sites for metals, increasing metal solubility, but the Ni-hyperaccumulator *Alyssum murale* was found to produce less reductant than a non-hyperaccumulator (Bernal et al. 1994). Twice as much dissolved organic carbon (DOC) and a different composition of exudates was produced by the As-hyperaccumulator *Pteris vittata* than a related non-hyperaccumulator (Tu *et al.* 2004). However, in another study with *Pteris vittata*, DOC in the rhizosphere was shown to be similar in quantity to a non-accumulating plant (Cattani et al. 2009). DOC may have different functions in different plant species, e.g. DOC may immobilize and detoxify metals (Römkens et al. 1999), or rather increase trace element availability (Fitz and Wenzel 2002). Field data coupled with modeling indicated that DOC may create ligand-induced mobilization of Ni, thus improving hyperaccumulation potential (Wenzel et al. 2003). Water-soluble root exudates from hyperaccumulating plants increased trace element extraction (Tu et al. 2004; Li et al. 2005b) and non-water soluble exudates or mucigel production may

influence metal desorption in the rhizosphere as well (Ingwersen *et al.* 2006). Further investigation may provide better insight into differences between species in exudate composition and their importance for hyperaccumulation, for example the role of histidine in Ni-hyperaccumulation in *Alyssum lesbiacum* has been better understood because of complementary studies with the non-accumulator *Alyssum montanum* (Krämer et al. 1996).

Several studies so far have shown no indication that the root activity of Cd/Zn-hyperaccumulator *Thlaspi caerulescens* or Ni-hyperaccumulator *Alyssum murale* have a unique capability to mobilize trace elements in soil (Hutchinson et al. 2000; Zhao et al. 2001; Massoura et al. 2004; Hammer et al. 2006). If hyperaccumulators have a novel mechanism for mobilizing trace elements in soil, they may be expected to increase accumulation in co-cropped plants (if competition is not a factor). This effect was indeed seen in *Hordeum vulgare* co-cropped with *Thlaspi caerulescens* (Gove et al. 2002), but most evidence so far points to the contrary (Whiting et al. 2001b; c; Liu et al. 2005a; Ingwersen et al. 2006).

Lowering rhizosphere pH has also been proposed as a mechanism for increasing metal hyperaccumulation in plants because it generally increases cation bioavailability in soil (Delorme *et al.* 2001). Wenzel *et al.* (2004) reviewed this topic and presented a table showing that many studies that investigated this mechanism rejected this hypothesis, e.g. Cd/Zn-hyperaccumulation in *Thlaspi caerulescens* (Knight et al. 1997; Hutchinson et al. 2000; Luo et al. 2000); and Ni-hyperaccumulation in *Alyssum murale* (Bernal and McGrath 1994), *Alyssum*

serpyllifolium subsp. *lusitanicum* (Kidd et al. 2007), and *Thlaspi goesingense* (Puschenreiter et al. 2003; Wenzel et al. 2003). In contrast, a recent study suggested rhizosphere acidification is important for the Mn-hyperaccumulator *Chengiopanax sciadophylloides* (Mizuno et al. 2006). It must be noted that there are limitations in the interpretation of some of these results. Without the use of microelectrodes or plants growing in agar containing pH indicators rhizosphere sampling can be poor and some cases may have reported differences in bulk soil pH rather than rhizosphere pH. In addition, suitable controls must be used to determine if the amount of acidification by the hyperaccumulator is novel, or the result of typical rhizosphere acidification. When *Thlaspi caerulescens* was compared to the non-hyperaccumulator *Thlaspi ochroleucum*, pH differences did not account for increased Zn uptake by the hyperaccumulator because both plant types had a similar rhizosphere pH (McGrath *et al.* 1997). Although metal mobility in the rhizosphere can increase because of lower pH, from the data collected so far it appears hyperaccumulators are not *novel* drivers of this effect and this may not be a satisfactory explanation for hyperaccumulation (Wenzel et al. 2004).

Amidst differing results and the small number of species investigated, much more work is required to understand the mechanisms by which hyperaccumulators differ from non-accumulators with respect to trace element mobilization. We may find mechanisms used by hyperaccumulators for trace element extraction differ by plant species and elemental species. The function and composition of root exudates should be examined in more detail.

Rhizosphere Microorganisms

While we know very little about how roots work compared to aboveground organs, we know even less about other components of the soil ecosystems. What is the co-evolutionary legacy between roots and rhizosphere microorganisms playing out in the soils beneath hyperaccumulator populations? In general, soil microorganisms influence the rhizosphere by altering nutrient cycling and availability (Gobran and Clegg 1996), but these mechanisms could also influence non-nutrient trace element availability. Table 1 summarizes several studies that have shown that hyperaccumulators grown with inoculated or non-sterilized soil often accumulated different amounts of trace elements aboveground than plants grown in uninoculated or sterilized soil. Of the twenty-six effects shown in Table 1.1, in sixteen instances plants accumulated more trace elements when they were inoculated; compared to seven instances where there was no effect and three instances where inoculation reduced the trace element concentration in plants. Although these effects can be strong, seven of the effects reported increased plant concentrations by > 95%, the mechanisms responsible for these microorganism-associated changes often remain unknown. One possibility could be the effects of sterilization procedures on soil chemistry (Salonius et al. 1967; McNamara et al. 2003), however recent work on Ni-hyperaccumulation has shown that rhizosphere microorganisms affected plant gene expression, as evident from differences in shoot proteome (Farinati et al. 2009). These findings warrant further studies of the interactions between rhizosphere microorganisms and plant hyperaccumulators.

Table 1.1. The magnitude of the effect of microorganism inoculation on plant aboveground trace element concentrations. [§]The equation $([TE]_{inoculated} - [TE]_{control})/[TE]_{control}$ expressed as a percentage was used to determine the magnitude of the effect. Different control conditions were used as described in the original publications.

Plant Species	Microorganism	Effect[§]	Reference
Alyssum murale	*Microbacterium arabinogalactanolyticum*	↑ 32% [Ni]	(Abou-Shanab et al. 2003a)
	M. arabinogalactanolyticum AY509225	↑ 46% [Ni]*	(Abou-Shanab et al. 2006)
	M. liquefaciens	↑ 24% [Ni]	(Abou-Shanab et al. 2003a)
	M. oxydans AY509222	↑ 41% [Ni]*	(Abou-Shanab et al. 2006)
	M. oxydans AY509223	↑ 35% [Ni]*	(Abou-Shanab et al. 2006)
	Non-sterile soil	↑ 95% [Ni]	(Abou-Shanab et al. 2003b)
	Sphingomonas macrogoltabidus	↑ 17% [Ni]	(Abou-Shanab et al. 2003a)
Arabidopsis halleri	Rhizosphere derived inoculant	↑ 100% [Cd], [Zn]*	(Farinati et al. 2009)
Berkheya coddii	*Glomus intraradices*	↑ 167% [Zn]	(Turnau and Mesjasz-Przybylowicz 2003)
	Native AMF	↑ 45% [Zn]	
Pityrogramma calomelanos	Soil derived bacteria	No effect [As]	(Jankong et al. 2007)
	Soil derived fungi	↓ 31% [As]	(Jankong et al. 2007)
Pteris vittata	*Gigaspora margarita*	No effect [As]	(Trotta et al. 2006)
	Gl. mosseae	No effect [As]	(Trotta et al. 2006, Liu et al. 2009)
	Gl. mosseae	↓ 33% [As]	(Liu et al. 2005b)
	Gl. mosseae	↑ 31% [As]	(Wu et al. 2009)
	Soil derived inoculant	↑ 42% [As]*	(Al Agely et al. 2005)
Sedum alfredii	*Burkholderia cepacia*	↑ 243% [Cd]	(Li et al. 2007)
		↑ 96% [Zn]	(Li et al. 2007)
Thlaspi caerulescens	Mixed inoculant containing *Enterobacter cancerogenes, M. sapherdae,* and *Pseudomonas monteilii*	↑ 100% [Zn]*	(Whiting et al. 2001a)
		No effect [Zn]	(Whiting et al. 2001a)
Thlaspi praecox	Soil inoculant - containing *Gl. etunicatum*, *Gl. fasciculatus,* and *Gl. mosseae*	↓ 28% [Cd]*	(Vogel-Mikuš et al. 2006)
		No effect [Pb, Zn]	(Vogel-Mikuš et al. 2006)

* Estimates from graphically presented values in original publications were used to calculate the effect.

Three specific mechanisms of how microorganisms may increase plant hyperaccumulation have been suggested: they may increase root surface area and hair production, increase element solubility, or increase soluble element transfer from the rhizosphere to the plant (Whiting et al. 2001a). In the next section we examine recent findings from the literature regarding rhizosphere bacteria and fungi, and discuss them in the context of these three mechanisms. Knowing more about these mechanisms and their consequences will improve our understanding of plant hyperaccumulation.

Rhizosphere Bacteria

Increasing root surface area and root hair production

Bacteria did increase root hair production and root surface area when the non-hyperaccumulator *Brassica juncea* was grown with Se (de Souza et al. 1999). Rhizosphere bacteria that improve plant growth are known as plant growth promoting rhizobacteria (PGPR) (Benizri *et al.* 2001). PGPR can increase root growth by restricting rhizosphere accumulation of ethylene, which inhibits plant growth; the bacterial mechanism involves ACC-deaminase activity. Also, some bacteria produce plant growth regulators that cause root cell elongation, such as indole-3-acetic acid (IAA, an auxin). Both PGPR mechanisms have been found in association with Ni-hyperaccumulators *Alyssum serpyllifolium* and *Thlaspi goesingense* (Idris et al. 2004; Ma et al. 2009).

Another, highly specialized PGPR interaction occurs where legume growth is increased by symbiotic root nodule bacteria. Although these bacteria have the largest effect on legumes when the bacteria infect the plants and live within the plant nodule, rhizobia also live in the rhizosphere. Root nodules or nodule scars have been observed in several leguminous hyperaccumulators such as Se-hyperaccumulators *Acacia cana* (Beadle 1964), *Astragalus bisulcatus* and *Astragalus pectinatus* (Wilson and Chin 1947), as well as the Ni-hyperaccumulator *Pearsonia metallifera* (Corby 1974); but the metal content of the soil was not recorded at the time. A study of two legumes growing on a copper mine containing 461 μg g^{-1} Cu indicate that nitrogen fixation occurred at similar levels in a copper tolerant population of *Lotus purshianus* compared to a population from a control meadow site, but Cu-tolerant *Lupinus bicolor* had a lower rate of N_2 fixation than the control meadow population (Wu and Kruckeberg 1985). The symbiotic bacteria may be tolerant to trace elements. For example, *Sinorhizobium fredii* and *S. meliloti* have been shown to be Se-tolerant (Kinkle et al. 1994), but further work is necessary to determine the trace element tolerance of rhizobia associated with metallophytes growing on high-metal soils and the effects of the symbiosis on plant metal accumulation.

Other PGPR may also become endophytes within the plant xylem by infecting the plant roots (Gagné et al. 1987), although this infection route has never been verified in hyperaccumulators (Rajkumar et al. 2009). Bacterial genera inside the roots and shoots of hyperaccumulators have been found to be both similar to soil bacteria in *Alyssum bertolonii* (Barzanti et al. 2007). Yet

others have found that endophytes in *Thlaspi caerulescens* and *Thlaspi goesingense* were different from soil bacteria (Lodewyckx et al. 2002; Idris et al. 2004). The difference between habitats of plant roots, shoots, and the soil as well as infection modes may explain some of these observed differences (Idris et al. 2004; Barzanti et al. 2007). The presence of endophytic bacteria in hyperaccumulators may increase plant growth because they can have ACC-deaminase activity (Idris et al. 2004). Other mechanisms may exist to promote plant growth but there are not many studies that focus on hyperaccumulators (Rajkumar et al. 2009). The survival of endophytes within a hyperaccumulator with its high levels of metals indicates that those bacteria may have distinct adaptations for metal tolerance (Idris et al. 2004; Mengoni et al. 2010). Further studies on PGPR could be an effective way to expand our knowledge of the role of bacteria in hyperaccumulation (Glick 2010); research in this area may prove fruitful because many modes of PGPR action have been described in non-hyperaccumulator systems (Glick 1995).

Increasing element solubility

Microorganisms have the ability to improve trace element solubility in the rhizosphere, and thus may affect hyperaccumulation. Indeed, increased acid production and metal solubility was described in the presence of rhizosphere bacteria from hyperaccumulator plants (Abou-Shanab *et al.* 2003b). Besides acids, bacteria can produce other exudates that solubilize metals. Whiting *et al.*

(2001a) found that bacteria solubilized Zn in soil without a change in pH, but specific exudates were not identified. Although the bacteria increased water soluble Zn, only the Zn-hyperaccumulator *Thlaspi caerulescens* achieved higher Zn concentrations with the inoculation while the non-accumulator *Thlaspi arvense* did not (Whiting et al. 2001a). Bacteria can also produce iron-chelating organic molecules called siderophores that may affect availability of Fe and perhaps other trace elements (Lodewyckx *et al.* 2002); the presence of such bacteria alleviated Fe deficiency in Ni-stressed plants (Mishra and Kar 1974). Rhizosphere isolates examined from the Ni-hyperaccumulators *Alyssum serpyllifolium* (Ma et al. 2009) and *Thlaspi goesingense* contained siderophores (Idris *et al.* 2004). Endophytic bacteria of *Alyssum bertolonii* and *Thlaspi goesingense* may also have the potential to produce siderophores (Idris et al. 2004; Barzanti et al. 2007). Bacterial siderophore production can be induced by metals other than Fe (Abou-Shanab *et al.* 2006 and references therein), so there is opportunity for more investigation here.

Another mechanism to increase solubility in the rhizosphere is bacterial phosphatase-mediated dissolution of metal phosphates. However, when examined in the Ni-hyperaccumulator *Alyssum murale* there was no difference in siderophore or phosphatase activity between bulk soil and rhizosphere isolates (Abou-Shanab et al. 2003b). Finally, trace element solubility may be affected by redox changes mediated by bacteria. Di Gregorio *et al.* (2005) have identified Se-reducing bacteria from the rhizosphere of *Astragalus bisulcatus* that reduce selenite to elemental Se; this could influence plant Se uptake because reduced

Se forms are less soluble than oxidized forms. Although mechanisms of solubilization may appear straightforward, it remains to be determined how much of the solubilized trace elements are then encountered by roots or leached away.

Other Mechanisms

In the rhizosphere bacteria are more abundant than in bulk soil, owing to root-released carbon compounds (Rouatt 1959; Grayston et al. 1998; Badri et al. 2009). Hyperaccumulator rhizospheres may provide a niche for specialized, metal-resistant bacteria, since hyperaccumulator roots typically harbor bacteria that are more resistant to metals than bacteria in the bulk soil (Mengoni et al. 2001; Lodewyckx et al. 2002; Abou-Shanab et al. 2003b; Aboudrar et al. 2007; Becerra-Castro et al. 2009). Higher resistance to Cd and Zn was found in rhizosphere bacteria and fungi isolated from *Thlaspi caerulescens* than from a non-hyperaccumulator, even though the hyperaccumulator had fewer rhizosphere microorganisms overall (Delorme *et al.* 2001). Evidence of rhizosphere bacteria that exhibit increased trace element resistance is noteworthy, and the mechanisms driving these observations are unknown and deserve further study.

One evolutionary explanation for the observed increased microbial resistance around hyperaccumulating plants is accumulation of trace elements in soil under hyperaccumulator litter, and subsequent adaptation by the microorganisms (Schlegel et al. 1991; Quinn et al. 2011). Another explanation

may be root release of accumulated elements via excretion and/or root turnover. The mechanisms of bacterial resistance to elevated trace elements in the rhizosphere of hyperaccumulators have not been studied much. Whether they are similar to (Mengoni et al. 2010 and references therein) or differ from known plasmid-borne metal efflux pumps in other bacteria (Idris et al. 2006) remains to be determined in many cases. Bacteria also have the potential for metal uptake (Pal et al. 2007). To learn more from these observations it is important to also determine the effect of these resistant microorganisms on plant hyperaccumulation.

Altered levels of regulatory metabolites may also affect uptake of certain hyperaccumulated elements. Bacteria have been shown to enhance selenate uptake in *Brassica juncea* (a Se accumulating species but not a hyperaccumulator) via a combination of enhanced root hair growth (as mentioned above) and higher rhizosphere levels of the amino acid serine/O-acetylserine. O-acetylserine is a known upregulator of plant sulfate/selenate uptake and assimilation (de Souza et al. 1999).

Increasing soluble element transfer

Although microorganisms affect trace element accumulation in hyperaccumulators, no evidence has been found that bacteria increase trace element movement towards the plant root in the rhizosphere (Whiting et al. 2001a). Hyphal foraging may make fungi better candidates for this.

Mycorrhizae, for instance, are well-known to transport P and other elements toward plant roots (Bolan 1991). Fungi are discussed in the following section.

Rhizosphere Fungi

Arbuscular mycorrhizae are very important in plant nutrition. Fungal hyphae can supply large portions of essential elements to plants, including up to 60% of plant Cu, 80% of plant P, 25% of plant Zn, and have been implicated in transport of S to plants as well (Marschner and Dell 1994). The similarity in ionic radius and charge to P, Cu/Zn, and S make the non-essential elements As, Cd, and Se candidates for hyphal transport also. However, many hyperaccumulators are from the Brassicaceae, which is generally considered to be a non-mycorrhizal family (Leyval *et al.* 1997). Recently, arbuscular mycorrhizae have been found in several non-Brassicaceae hyperaccumulator species (Turnau and Mesjasz-Przybylowicz 2003; Perrier et al. 2006; Trotta et al. 2006; Amir et al. 2007; Wu et al. 2007) and several metallophytes of the Brassicaceae (Orłowska et al. 2002; Regvar et al. 2003; Vogel-Mikuš et al. 2005; Pongrac et al. 2007). Although several hyperaccumulating species are woody, no associations with ectomycorrhizae have been reported yet.

We now know arbuscular mycorrhizal symbiosis occurs in hyperaccumulators, but generally root colonization was found to be low (Trotta et al. 2006; Amir et al. 2007; Pongrac et al. 2007; Wu et al. 2007), and stronger hyperaccumulators have been reported to be less colonized by mycorrhizae

(Amir *et al.* 2007). These findings are ecologically intriguing. Does the incidence of low levels of mycorrhizal colonization in some hyperaccumulators point to a cost of trace element tolerance in these species, where plants can only weakly support mycorrhizal networks? Although some hyperaccumulator species have low colonization, in others moderate to high colonization rates were found (Turnau and Mesjasz-Przybylowicz 2003; Vogel-Mikuš et al. 2006). The amount of mycorrhizal colonization depends upon both plant and fungus; fungal isolate identity affected root colonization rates in the As-hyperaccumulator *Pteris vittata* (Wu et al. 2009).

The factors determining mycorrhizal colonization rates in hyperaccumulators are largely unknown, but in *Thlaspi praecox* and other *Brassicaceae* it may be restricted by plant glucosinolates (Vierheilig et al. 2000; Pongrac et al. 2008), although this evidence does not exclude other factors. In fact, percent root colonization by mycorrhizae has also been found to be related to metal content, but all types of correlations have been found. Negative correlations between metal concentration and root colonization have been observed in some Ni-hyperaccumulators including *Geissois pruinosa*, *Phyllanthus favieri*, *Psychotria douarrei*, and *Sebertia acuminata* (Amir et al. 2007). Positive correlations have been observed in some species where higher metal concentrations were associated with higher root fungal colonization in the As-hyperaccumulator *Pteris vittata* and the Cd/Pb/Zn hyperaccumulator *Thlaspi praecox* (Al Agely et al. 2005; Vogel-Mikuš et al. 2006; Pongrac et al. 2007). No correlation between metal content and mycorrhizal colonization was found in the

As-hyperaccumulator *Pteris vittata* and the Cd/Zn-hyperaccumulator *Sedum alfreddii* (Trotta et al. 2006; Wu et al. 2007). The differences reported in the direction of these correlations may be related to how mycorrhizae respond to metals or plants. Reduced spore germination and counts have been observed in the rhizospheres of several hyperaccumulators (Pawlowska et al. 2000; Amir et al. 2007), but this is not true for all hyperaccumulators (Turnau and Mesjasz-Przybylowicz 2003). Just like their plant hosts, some mycorrhizae have developed tolerance to metals (Adriaensen et al. 2006). This could help them colonize hyperaccumulators, which are found in high-metal soils and contain high levels in their roots (Trotta et al. 2006; Vogel-Mikuš et al. 2006; Amir et al. 2007). In several hyperaccumulators roots meet the concentration criteria (0.01% Cd; 0.1% for As, Co, Cu, Ni, and Se; and 1% for Mn and Zn on a dry weight basis) used to evaluate aboveground parts of plants as hyperaccumulators (Amir et al. 2007; Barzanti et al. 2007; Wu et al. 2007; Barillas et al. 2011).

Like the differences reported in rates of colonization and trace element tolerance, mycorrhizae have varying effects on hyperaccumulator trace element uptake (Table 1). In some cases organs from mycorrhizae-inoculated plants had lower trace element concentrations than uninoculated plants; As concentrations were lower in inoculated *Pteris vittata* and the Cd/Pb/Zn hyperaccumulator *Thlaspi praecox* had less Cd in all plant parts as well as less Zn in roots when inoculated (Liu et al. 2005b; Trotta et al. 2006; Vogel-Mikuš et al. 2006). In other cases higher trace element concentrations were found in inoculated hyperaccumulators, including the Ni-hyperaccumulator *Berkheya coddii* and the

As-hyperaccumulator *Pteris vittata* (Turnau and Mesjasz-Przybylowicz 2003; Al Agely et al. 2005; Wu et al. 2009). In contrast to those effects, no differences were found between inoculated and uninoculated plants with regard to trace element concentrations with As in *Pteris vittata* or Cd, Pb, or Zn in *Thlaspi praecox* (Trotta et al. 2006; Vogel-Mikuš et al. 2006; Liu et al. 2009). One possible mechanism for increased trace element accumulation in inoculated plants is increased absorptive surface area from mycorrhizae, but this mechanism has not been investigated in hyperaccumulators. Differences in trace element concentration from inoculated and uninoculated plants may be explained by the increased growth rate and biomass in inoculated plants (Al Agely et al. 2005; Trotta et al. 2006). Another variable playing a role in accumulation and plant allocation patterns is the translocation factor between roots and shoots in mycorrhizal plants. This type of effect has been observed with Cd and Zn in *Thlaspi praecox* where the shoot: root translocation factor was higher in mycorrhizal plants (Vogel-Mikuš *et al.* 2006) and in *Pteris vittata* where the As translocation factor increased at least five times in inoculated plants (Trotta *et al.* 2006), however this did not increase the concentration of those elements in plants.

From these studies we know mycorrhizal fungi can alter plant trace element accumulation patterns in different ways, but the mechanisms remain obscure. Future research may shed more light on the role of mycorrhizae in trace element hyperaccumulation. This is achievable if a single fungal species is studied, its metal tolerance is noted, and the mechanisms by which it affects

plant uptake are identified. Specifically, we note that mycorrhizae are not all alike; like plants, they differ in metal tolerance (Adriaensen et al. 2006). Mycorrhizal species also occupy different niches in the rhizosphere; some have a majority of their hyphae within the root, while others have a majority outside the root (Maherali and Klironomos 2007).

Fungal species not identified as mycorrhizae have also been found in hyperaccumulator rhizospheres (Jankong et al. 2007; Wangeline and Reeves 2007). The role of these organisms in hyperaccumulation has yet to be determined, but some have the ability to accumulate and volatilize trace elements (Wangeline 2007). Information on hyperaccumulators and their microorganisms is continuing to amass, and findings thus far suggest significant roles for bacteria and fungi in hyperaccumulation (Table 1). A mechanistic approach, as used by Whiting *et al.* (2001a) where alternative microorganism-driven modes of action were investigated, will greatly enhance the applicability of these findings. Reporting inoculation effects with accompanying mechanistic information will be beneficial for advancing phytoremediation because it will enable replication of processes in other systems.

Conclusions

Hyperaccumulators make use of conventional rhizosphere mechanisms to improve their trace element accumulation (i.e. the same mechanisms as other plants) but also may have novel ways in which they manipulate their

rhizospheres. Root surface area and nutrient transporters are known to be important in plant nutrition and these traits are important in hyperaccumulation as well. Some hyperaccumulators possess unique root physical characteristics and are able to exhibit chemotropism towards non-nutrient trace elements. Some have altered expression levels or substrate specificities of trace element transporters. Chemical characteristics in the rhizosphere are similar between hyperaccumulators and non-accumulators in that both types of plants manipulate the solubility of trace elements by root exudates, access labile trace elements in the rhizosphere, and alter rhizosphere pH. In addition, hyperaccumulators may be able to mobilize trace elements that are non-labile. Like the majority of land plants, hyperaccumulators make use of exudates produced by microorganisms, interact with PGPR, and support arbuscular mycorrhizal symbioses, all of which can affect plant trace element uptake in significant ways; yet there are distinctive characteristics of hyperaccumulators because they harbor rhizosphere microorganisms that are very tolerant to metals.

Studies addressing a larger diversity of hyperaccumulator species are needed. While I attempted to generalize patterns, studies of the roots and rhizospheres of less than 10% of known hyperaccumulators have been conducted. From observations on this scant number of plants one cannot assume hyperaccumulator roots are all alike because results differ depending on trace element and plant species. Some hyperaccumulators are quite rare and live in very specialized niches. A greater understanding of how root mechanisms help these plants survive and thrive in such unique soils will contribute greatly to

their utilization. Unfortunately, habitat destruction proceeds at alarming rates near some species (Whiting et al. 2004) and extremely valuable information could be lost. If hyperaccumulator species disappear, so will our chance to elucidate and harness their unique capabilities. Significant effects of soil microorganisms on plant hyperaccumulation have been demonstrated. In the following chapters I focus on microbial effects on Se-hyperaccumulation in *Astragalus* species, with particular emphasis on symbiotic nodulation.

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Chapter 2

Observations of the Root Nodule Symbiosis in *Astragalus* Species with Emphasis on Selenium Hyperaccumulators

Summary

A survey of the presence of the root nodule symbiosis and its interaction with selenium (Se) in *Astragalus* has not been conducted before. Here I report the presence of root nodules in four species including *A. crotalariae, A. praelongus, A. preussi,* and *A. racemosus*. In a greenhouse study I found that the hyperaccumulator *A. bisulcatus* concentrated 101% more Se in the nodule than in the root, yet there was no difference in the non-accumulator *A. drummondii.* To assess these if these patterns occur under natural conditions I examined organ [Se] in Se-hyperaccumulators and non-accumulators from field collections. While [Se] were routinely above the hyperaccumulator threshold level of 1,000 μg Se g^{-1} dry weight (DW) in leaves, flowers, and fruits from Se-hyperaccumulators, root samples rarely contained that amount, and nodules never exceeded 110 μg Se g^{-1} dry weight. Selenium concentrations in non-accumulator legumes never reached 100 μg Se g^{-1} DW in any organ. An evaluation of the nodulation index and nodule weights in Se-hyperaccumulators and non-accumulators indicated that there was no cost of Se tolerance on nodulation characteristics in hyperaccumulators. Rather, I found that in Se-hyperaccumulators under greenhouse conditions higher levels of Se treatment corresponded with higher nodule counts, indicating a potential role for dinitrogen fixation in Se-hyperaccumulation. The effect was not found in non-accumulator *Astragalus* species. Finally, Se localization and molecular Se speciation of root nodules was determined by x-ray absorption spectra and x-ray analysis of near

edge spectra for the Se-hyperaccumulators *A. bisulcatus, A. praelongus,* and *A. racemosus*. All nodules had Se throughout with a majority of it stored as C-Se-C molecules. These findings suggest that as the evolution of Se hyperaccumulation in *Astragalus* developed, root nodule symbiosis has played an integral role.

Introduction

Several hyperaccumulator taxa and metallophytes are members of the legume family (Fabaceae). Many of these species are within the Papilionoideae subfamily, which has been reported to have more than 98% of its members form nodules (Allen and Allen 1981). Hyperaccumulators within this group may be expected to form root nodules symbioses as well. However, to date our knowledge of if and how leguminous Se-hyperaccumulators interact with symbiotic rhizobia is incomplete. For metal tolerant symbiotic legumes to evolve, tolerance needs to occur in both the plant and the bacterial partner (Antonovics et al. 1971). Bacteria within root nodules will be exposed to consistent environmental conditions and may have some protection from stress, but free-living rhizobia will be more susceptible to stress and environmental fluctuations (Chalk et al. 2010).

Some *Astragalus* species native to western North America hyperaccumulate Se. The species that hyperaccumulate Se manage to amass large concentrations, but co-occurring congeners do not accumulate Se to any

large extent (Shrift 1969; Galeas et al. 2008). Is there a co-evolutionary legacy of plant hyperaccumulation and root nodule microorganisms? Three alternative pathways could have developed: plants that have evolved to hyperaccumulate Se may associate with rhizobia that have evolved to interact with high [Se] within hyperaccumulator plants and rhizospheres, the presence of Se in the system could disrupt the symbiosis entirely where Se-hyperaccumulators rarely nodulate or form ineffective partnerships, or there could be no effect (Fig 2.1).

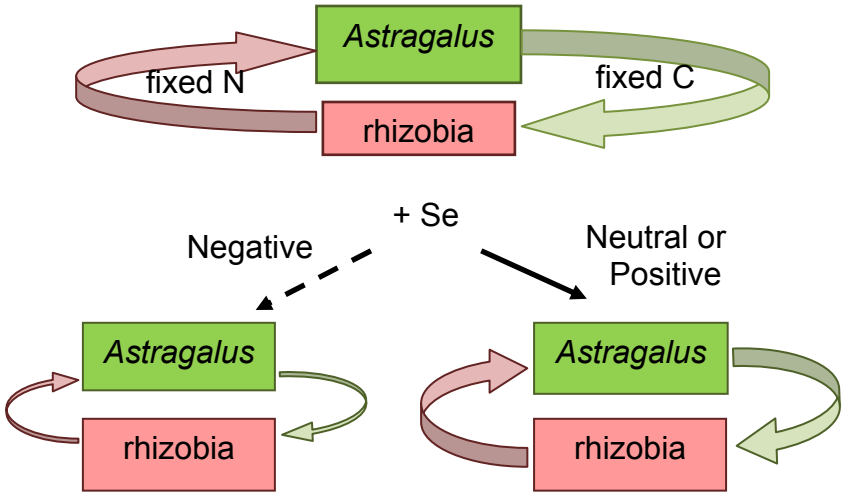

Fig. 2.1 At the top the curved arrows represent the strong, positive feedback between organisms in root nodule symbiosis in the absence of stress. When Se is introduced to the system it may exert a negative effect (dashed arrow) on the interaction. The negative effects, Se toxicity in non-tolerant organisms or Se-induced inhibition of the symbiosis could result in similar effects where the magnitude of the positive feedback between organisms is diminished. Alternatively, as shown on the right, Se may exert a neutral or positive effect (solid arrow) on the interaction by not changing the interaction or by increasing the specificity of the positive feedback between organisms.

Plants have been shown to require symbiotic associations under conditions of environmental stress. As an example, thermal tolerance and salt tolerance were only achieved in plants growing in symbiosis with mycorrhizae (Rodriguez et al. 2008). Some metal tolerant legumes have effective symbioses under stress conditions, as was observed for *Lotus purshianus* and its symbiont growing in copper mine waste (Wu and Kruckeberg 1985; Wu and Lin 1990). Alternatively, an example of the disruptive influence of abiotic stress has been shown in the legume genera *Acacia* where more salt tolerant plant species responded less to rhizobial inoculation than did salt sensitive host species; therefore plant salt tolerance results in less dependence on the symbiotic mutualism (Thrall et al. 2008). In addition, cases of metal inhibition of nodulation and nitrogen fixation have been shown in other legumes (Chen et al. 2003; Manier et al. 2009; Saraswat and Rai 2011). A recent analysis of symbiotic interactions in Ni-hyperaccumulators could indicate that a pattern of disrupted symbiosis is common across the hyperaccumulator taxa. The strongest Ni-hyperaccumulators were found to be the least infected by mycorrhizae (Amir et al. 2007).

In relation to Se-hyperaccumulation, it is known that some rhizobia isolates are tolerant to 200 mM Se (IV) and 400 mM Se (VI) (Kinkle et al. 1994). However these strains are not known to form root nodules with *Astragalus* species. Successful root nodule symbioses exist in both *Astragalus* hyperaccumulators and non-accumulators (Allen and Allen 1981). Previous reports document that the Se-hyperaccumulators *A. bisulcatus* and *A. pectinatus*

are nodulated (Wilson and Chin 1947) , but no one knows how those interactions react to Se**.** In *A. bisulcatus* root [Se] can reach levels that are used to define plants as Se-hyperaccumulators based on shoot [Se] (>1,000 *μ*g Se g^{-1} DW) (Barillas et al. 2011). With roots having high [Se], nodules of hyperaccumulators potentially experience similar [Se]. I wanted to compare the role of Se in nodulation of *Astragalus* species that have evolved to hyperaccumulate Se and those that have not. I address the question; does plant Se-hyperaccumulation negatively affect root nodule symbiosis?

I checked for the presence of nodulation and investigated the [Se] in organs of several *Astragalus* hyperaccumulators and leguminous non-accumulators. I hypothesized that Se-hyperaccumulation incurred a cost on the symbiotic interaction, where hyperaccumulators have lost some of their ability to effectively interact with rhizobia. I expected nodulation in Se-hyperaccumulators and non-accumulators to be reduced with Se addition. To address these issues I conducted field surveys and examined differences in nodulation between Se-hyperaccumulators and non-accumulators in greenhouse studies. My observational studies also included Se localization and speciation studies within root nodules from the three Se-hyperaccumulators *A. bisulcatus, A. praelongus,* and *A. racemosus* to assess how bacteria in nodules are subjected to Se within these plants.

Materials and Methods

Observational Study 1: Nodulation Occurrence in Se-hyperaccumulators

Seeds from *A. crotalariae* were obtained from the Desert Legume Program (Tucson, AZ) and the USDA Western Regional Plant Introduction Station (Pullman, WA) provided *A. preussi.* The seeds were mechanically scarified, soaked overnight, and then sown into soil collected from underneath *A. bisulcatus* growing near Fort Collins, CO. After 2 months of growth, 50 μM sodium selenate solution (20 mL) was applied weekly for the remaining 4 months, after which plants were removed from pots and examined for nodules. I also excavated root systems of *A. praelongus* (Uravan, CO), *A. racemosus* (Pueblo, CO), and *A. bisulcatus* (Fort Collins, CO) growing in their native habitats to search for root nodules during the growing season.

Experiment 1: Root and Nodule [Se] Under Greenhouse Conditions

This study was conducted in Fort Collins, CO at the Plant Growth Facilities in the University Greenhouse at Colorado State University. Plants were grown from seed in 164 mL Ray Leach "Cone-tainers" (Stewe & Sons, Oregon) containing a HCl-washed, 1:1 (volume) mixture of sand and fritted clay. The experimental design consisted of 2 species (*A. bisulcatus* and *A. drummondii*), a Se treatment of 20 μM sodium selenate (Na_2SeO_4), and an inoculation treatment (live soil slurry) with 10 replications for a total of 20 experimental units.

Astragalus seed was purchased from Western Native Seed (Coaldale, CO). Seeds were scarified with sand paper, sterilized with bleach for 2 minutes followed by 2 minutes in 90% ethanol, and soaked overnight in sterile water before planting in pots. The seedlings were allowed to establish for 1 month before fertilizer treatments were applied. Fertilizer solution (1.6 mM K_2SO_4, 0.358 mM NH_4NO_3, 17.9 μM $FeCl_3$, 16.9 μM Na_2-EDTA, 2 mM $MgSO_4$, 0.169 mM KH_2PO_4, 0.833 mM K_2HPO_4, 0.6 mM $CaSO_4$, 0.5 mM $CaCl_2$, 23.1 μM H_3BO_3, 4.6 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.2 μM $NaMoO_4$, and 0.5 μM $CoSO_4$) was applied twice a week. The plants grew for 2 months with fertilizer additions, I then suspended fertilizer application for 2 weeks before inoculation and selenium treatments were applied.

Two soil samples, one from each *Astragalus* species, were collected from plants growing in a grassland community in Fort Collins, CO (40^0 33' 43.01" N, 105^0 8' 13.1" W) to make soil slurries for inoculation (20 g soil in 1 L sterile, distilled water). The inoculant (20 mL) was added to pots directly by matching species with soil type. Six days after inoculation the fertilizer treatments were resumed with ¼-strength fertilizer twice per week. After another 6 days, Se treatments were started. Sodium selenate solutions were mixed in distilled water and supplied as 50 mL applications once every week. One month after Se treatments began the plants began to brown so fertilizer was reduced to once a week and Se treatments were reduced to once every 2 weeks for the duration of the experiment (10 more weeks). Plants were harvested and separated into root, shoot, and nodule. Each sample was dried at 50° C for one week before

analysis. Twenty mg of dried plant samples were digested in 1 mL nitric acid for 2 hours at 60° C and then 130° C for 6 hours. Organ [Se] was determined by inductively coupled atomic emission spectra (ICP-AES) on this digest diluted into 9 mL water. For statistical analysis I compared root and nodule [Se] in each species by *t*-test in Systat v.12 (Systat Software, Chicago, IL).

Observational Study 2: Organ [Se] From Field Collections

To examine organ [Se] I excavated root systems of non-accumulators and Se-hyperaccumulators during the growing season (Table 2.1). I collected the non-accumulators *A. argophyllus* and *A. convallarius* in non-seleniferous soil from the Piceance Basin near Meeker, CO. In Pueblo, CO I collected samples of the non-accumulator *Melilotus alba* from seleniferous soil along with the hyperaccumulator *A. racemosus*. The hyperaccumulators *A. praelongus* and *A. rafaelensis* were collected from seleniferous soils in Uravan, CO. From Fort Collins, CO I collected the non-accumulators *A. missouriensis, M. officinalis,* and *Oxytropis sericeus* along with the hyperaccumulator *A. bisulcatus* growing on seleniferous soil. Aboveground organs including leaves, flowers, and fruits were separated from the belowground organs in the field. Within one day of collection the samples were returned to the lab and the belowground organs were washed and separated into roots and nodules. All parts were dried at 40 ^{0}C, weighed, and ground for ICP-AES analysis following the previously described conditions.

Table 2.1. Collection locations of the plants including the closest Colorado town, the degrees latitude and longitude, and site elevation (m).

Site and Species	Latitude	Longitude	Elevation (m)
Fort Collins	40º 42' 37.45" N	105º 06' 39.28" W	1597
A. bisulcatus			
A. missouriensis			
M. officinalis			
O. sericea			
Meeker	39º 34' 35.24" N	108º 24' 45.67" W	2466
A. argophyllus			
A. convallarius			
Pueblo	38º 19' 41.88" N	104º 33' 07.79" W	1536
A. racemosus			
M. alba			
Uravan	38º 22' 28.93" N	108º 44' 40.93" W	1504
A. praelongus			
A. rafaelensis			

Experiment 2: Se Effect on Nodulation

I grew *A. bisulcatus, A. convallarius, A. drummondii, A. praelongus, A. shortianus* obtained from Western Native Seed (Coaldale, CO), and *A. racemosus* from Prairie Moon Nursery (Winona, MN) in a mixture of 2:1 volume washed sand and field soil (sieved 2 mm). The soil collection locations are shown in Table 2.2. The plants received 0, 50, or 100 µM sodium selenate (Na_2SeO_4) and an N-free fertilizer solution (0.4 mM K_2SO_4, 4.475 μM $FeCl_3$, 4.225 μM Na_2-EDTA, 0.5 mM $MgSO_4$, 0.042 mM KH_2PO_4, 0.208 mM K_2HPO_4, 5.775 μM H_3BO_3, 1.15 μM $MnSO_4$, 0.2 μM $ZnSO_4$, 0.075 μM $CuSO_4$, 0.05 μM $NaMoO_4$, and 0.125 μM $CoSO_4$) weekly starting at 4 months of age. After 2 months of treatment shoots were harvested and dried at 50 ^{0}C while roots with nodules were immediately frozen at -20 ^{0}C until subsequent harvesting could be conducted. After freezing, roots were harvested by washing to remove soil and

separating nodules. Nodules were counted at harvest, then dried at 40 ^{0}C, and weighed to determine dry weight.

Table 2.2. Soil collection locations for the 6 species including the closest Colorado town, the degrees latitude and longitude, and site elevation (m).

Species	Location	Latitude	Longitude	Elevation (m)
A. bisulcatus	Fort Collins	40º 42' 37.45" N	105º 06' 39.28" W	1597
A. convallarius	Meeker	39º 34' 35.24" N	108º 24' 45.67" W	2466
A. drummondii	Livermore	40º 45' 59.77" N	105º 21' 06.21" W	1946
A. praelongus	Uravan	38º 22' 28.93" N	108º 44' 40.93" W	1504
A. racemosus	Limon	39º 21' 00.59" N	103º 51' 27.43" W	1749
A. shortianus	Livermore	40º 46' 00.57" N	105º 21' 04.29" W	1940

A comparison of the nodulation index (number of nodules per gram shoot) where hyperaccumulators were expected to have a lower value than non-accumulators was conducted using a one-way *t*-test. Similarly, a comparison of nodule size based on the average dry weight of each nodule in hyperaccumulators and non-accumulators was conducted using a one-way *t*-test where hyperaccumulators were expected to have a lower value. Statistical analyses were conducted with Systat v.12 (Systat Software, Chicago, IL).

Observational Study 3: Se Localization and Molecular Speciation

Root nodules were obtained from *A. bisulcatus* growing in the field along with *A. praelongus* and *A. racemosus* growing under greenhouse conditions with 50 µM sodium selenate (as described in Observational Study 1). Nodules were separated from the majority of the root, washed in water to remove external Se,

frozen in liquid N_2, and then sliced in half. The samples were kept frozen until analysis was complete to prevent changes in Se distribution and speciation. Nodule Se localization and speciation were determined using micro-focused X-ray fluorescence (μXRF) mapping and X-ray absorption near-edge structure (μXANES) spectroscopy at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Lab (Marcus et al. 2004). Frozen nodule samples were placed onto a Peltier stage kept at −33°C to minimize beam radiation damage. I recorded μXRF elemental maps at 13 keV, using a 15 μm (H) × 6 μm (V) beam, 15 μm × 15 μm pixel size, and 50 ms dwell time per pixel. The chemical forms of Se in selected areas were investigated using Se K-edge XANES. This analysis can determine the oxidation state and can be used to identify chemical speciation when compared with well-characterized Se standard compounds (Pickering et al. 1999). A seven-element germanium (Ge) solid-state detector (Canberra, ON, Canada) was used to record μXRF maps and μXANES spectra. Standard procedures were used to correct the spectra for deadtime, pre-edge background, and post-edge normalization (Kelly et al. 2008). The spectra were calibrated with red elemental Se (white line position set at 13074.73 eV). A library of standard selenocompounds were compared to Se XANES spectra using least square linear combination (LSQ) fitting in the 12,630–12,850 eV range. Se standards included: Na_2SeO_4 (S8295), Na_2SeO_3 (S1382), SeCystine (S1650) and SeMet (S3132) purchased from Sigma-Aldrich, and MeSeCys, γGMSC, SeCysth and $SeGSH_2$ purchased from PharmaSe (Austin, TX, USA). SeCys was obtained by reducing SeCystine at 25°C overnight in 100 mM sodium

borohydride at a 1:1 molar ratio. Gray and red elemental Se were provided by Amy Ryser and Dan Strawn. LabVIEW (National Instruments, Austin, TX, USA) programs were used for data processing and analyses at the beamline.

Results

Observational Study 1: Nodulation Occurrence in Se-hyperaccumulators

I provide the first report of nodulation in 4 *Astragalus* Se-hyperaccumulators. Nodules were observed in *A. crotalariae* and *A. preussi* grown with *A. bisulcatus* field soil under greenhouse conditions. I also observed nodulation in *A. praelongus* (Fig 2.2B) and *A. racemosus* growing in their native habitats. Nodulation in *A. bisulcatus* was confirmed in plants growing in the field (Fig 2.2A).

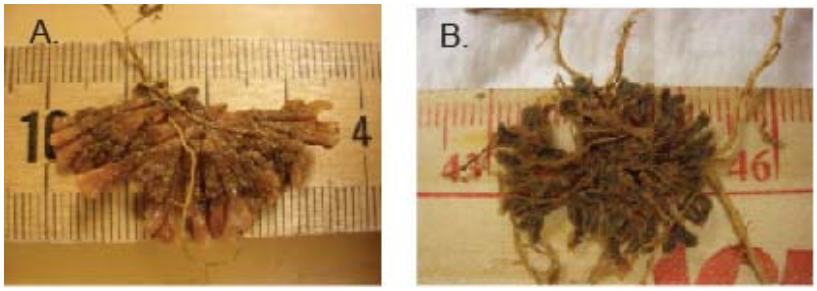

Fig. 2.2 Large root nodules (> 2 cm wide) were collected from *A. bisulcatus* (A) and *A. praelongus* (B) growing in the field.

Experiment 1: Root and Nodule [Se] Under Greenhouse Conditions

Only in the Se-hyperaccumulator *A. bisulcatus* did organ [Se] significantly differ between roots and nodules (p = 0.013). Nodules had a 101% higher [Se] than roots (Fig 2.3A). There was no difference in organ [Se] in the non-accumulator *A. drummondii* (Fig 2.3B).

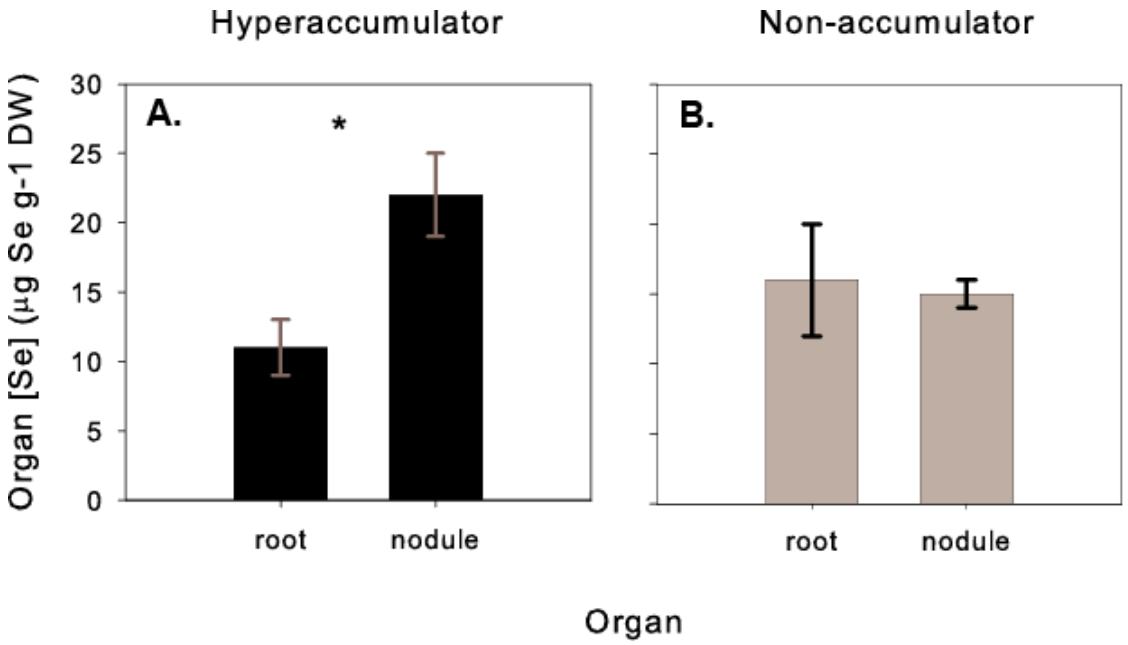

Fig. 2.3 Mean [Se] of roots and nodules with standard error are shown for *A. bisulcatus* (n_{root} = 6; n_{nodule} = 4), a Se-hyperaccumulator (A) and *A. drummondii* (n_{root} = 6; n_{nodule} = 5), a non-accumulator (B). The asterisk indicates a significant difference between belowground organs.

Observational Study 2: Organ [Se] From Field Collections

Se-hyperaccumulator, non-accumulator *Astragalus*, and co-occurring legume species were collected from Colorado field sites (see Table 2.1) and

analyzed for [Se] in leaves, flowers, fruits, roots, and nodules. Histograms show that the [Se] in both accumulators and non-accumulators was variable (Fig 2.4). No non-accumulator surpassed an organ [Se] of 100 μg Se g^{-1} DW. In contrast, all organs except for nodules in hyperaccumulators had at least one case where the [Se] was higher than 1,000 μg Se g^{-1} DW. One nodule [Se] of a Se-hyperaccumulator exceeded 100 μg Se g^{-1} DW in this study. The maximum nodule [Se] found among all the non-accumulators was 29 μg Se g^{-1} DW.

Table 2.3 shows the maximum organ [Se] recorded for each species investigated. The highest leaf (83 μg Se g^{-1} DW), pod (48 μg Se g^{-1} DW), and nodule (29 μg Se g^{-1} DW) [Se] in non-accumulators were recorded for *A. missouriensis* and *O. sericea* that occurred on the seleniferous site in Waverly, CO. In contrast *A. argophyllus* growing on a non-seleniferous site had the highest flower (58 μg Se g^{-1} DW) and root (66 μg Se g^{-1} DW) [Se] of the non-accumulators. *A. praelongus* had the highest leaf (2,925 μg Se g^{-1} DW), flower (2,999 μg Se g^{-1} DW), pod (5,405 μg Se g^{-1} DW), and root (1,281 μg Se g^{-1} DW) [Se] of all the Se-hyperaccumulators. The maximum nodule [Se] in the hyperaccumulators was in *A. racemosus* at 109 μg Se g^{-1} DW. Data for the mean organ [Se] with standard errors in each species is shown in Appendix 1.

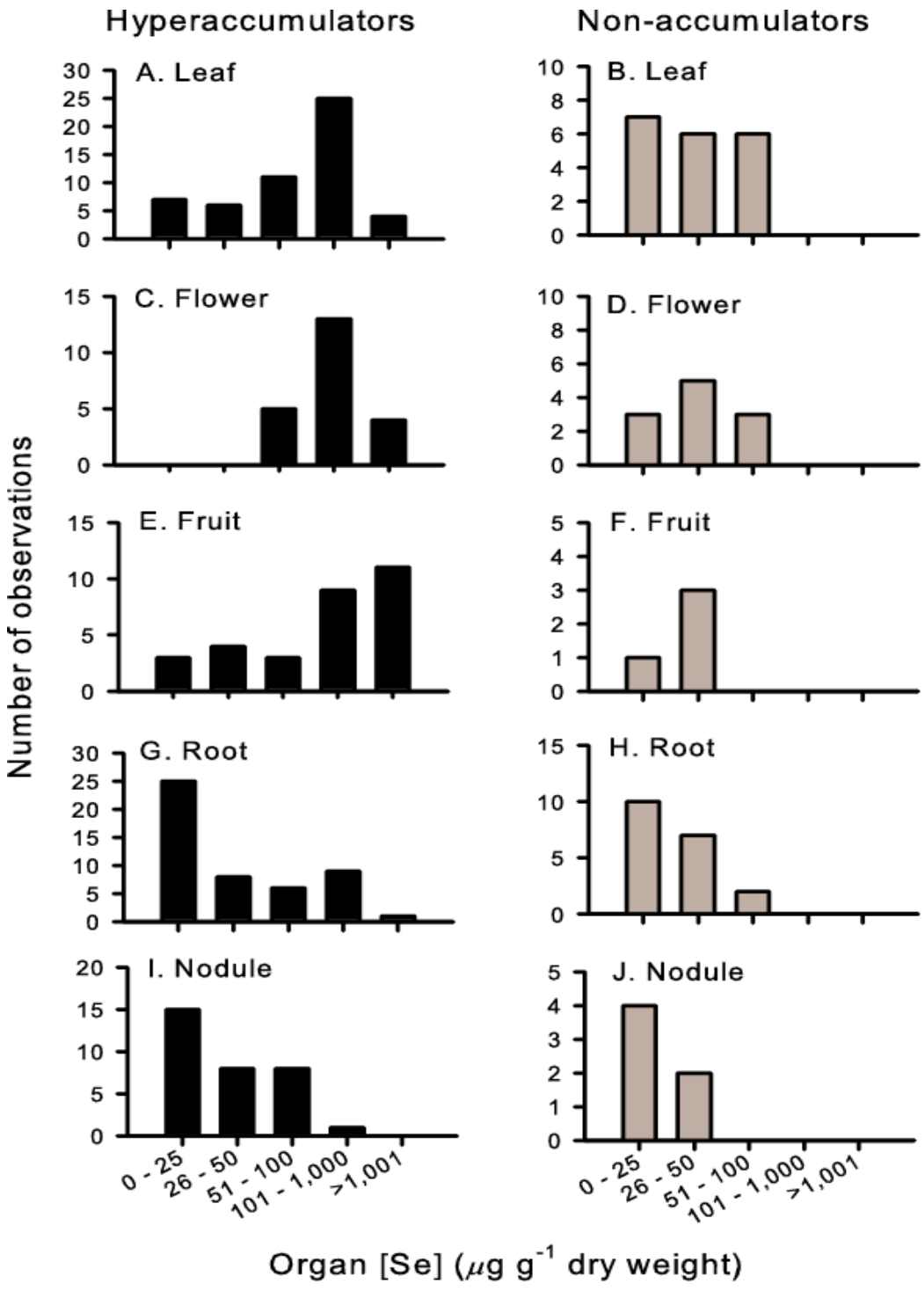

Fig. 2.4 Histograms of organ [Se] are shown for Se-hyperaccumulator leaves (A), flowers (C), fruits (E), roots (G), and nodules (I). The organ [Se] in non-accumulator legumes are shown for leaves (B), flowers (D), fruits (F), roots (H), and nodules (J).

Table 2.3. Maximum [Se] (μg Se g^{-1} DW) in different legume organs collected from the field. Some organs were not collected and elemental concentrations were not determined (--).

Plant type	Species	Leaf	Flower	Pod	Root	Nodule
Non-accumulator	*A. argophyllus*	47	58	--	66	0
	A. convallarius	77	--	--	37	--
	A. missouriensis	83	52	48	48	--
	M. alba	39	40	28	17	26
	M. officinalis	61	--	--	56	--
	O. sericea	12	20	5	25	29
Hyperaccumulator	*A. bisulcatus*	436	606	291	65	77
	A. praelongus	2,925	2,999	5,405	1,281	91
	A. racemosus	422	81	83	87	109
	A. rafaelensis	689	2,151	1,263	--	--

Experiment 2: Se Effect on Nodulation

The three hyperaccumulators did not exhibit a cost of Se tolerance on nodulation characteristics compared to the three non-accumulators. I compared the nodulation index, which was determined by the number of nodules produced per gram of shoot biomass, and the weight of each nodule. No reduction in either trait was found in the hyperaccumulators compared to the non-accumulators (Fig 2.5). Data for each individual species is shown in Appendix 2.

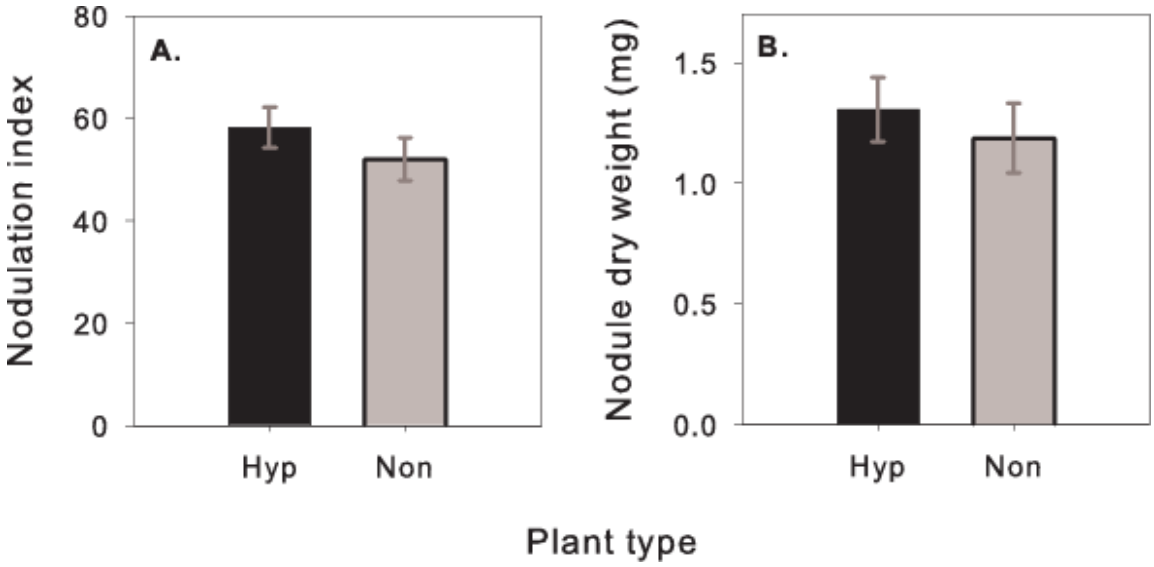

Fig. 2.5 There is no cost of Se tolerance associated with nodulation traits measured in three *Astragalus* Se-hyperaccumulators compared to three *Astragalus* non-accumulators. Selenium-hyperaccumulators had a similar nodulation index (determined by the number of nodules produced per gram of shoot dry weight) as non-accumulators (A). No difference was detected between the Se-hyperaccumulators and the non-accumulators in terms of the dry mass per nodule (mg) (B).

Adding Se to the three Se-hyperaccumulators in my greenhouse experiment significantly increased (p = 0.017) the number of nodules formed by those plants, but there was no effect in the three non-accumulators (Fig 2.6).

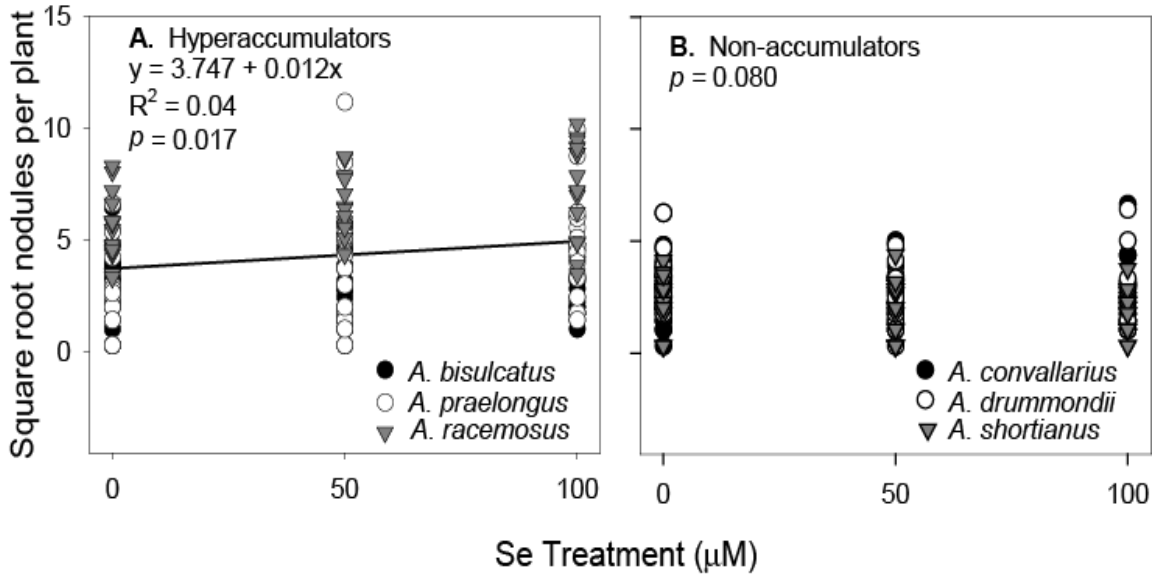

Fig. 2.6 The effect of Se addition on the square root number of root nodules produced per plant growing under greenhouse conditions in Se-hyperaccumulators (A) and non-accumulators (B) is shown by regression analysis. Regression lines were included only if the statistical analysis results were significant.

Observational Study 3: Se Localization and Molecular Speciation

From the μXAS results I found that Se was located throughout root nodules in *Astragalus* hyperaccumulators (Fig 2.7). Molecular speciation by XANES showed that the majority (> 40%) of Se in the nodule of each species was C-Se-C, which was most similar to the methylselenocysteine standard (Table 2.6). Each species also contained selenite (SeO_3). Both *A. bisulcatus* and *A. praelongus* contained some form of elemental Se (red or gray), but *A. racemosus* did not (Table 2.6). Nodules from *A. praelongus* contained some selenocysteine while nodules from *A. bisulcatus* and *A. racemosus* contained some selenoglutathione.

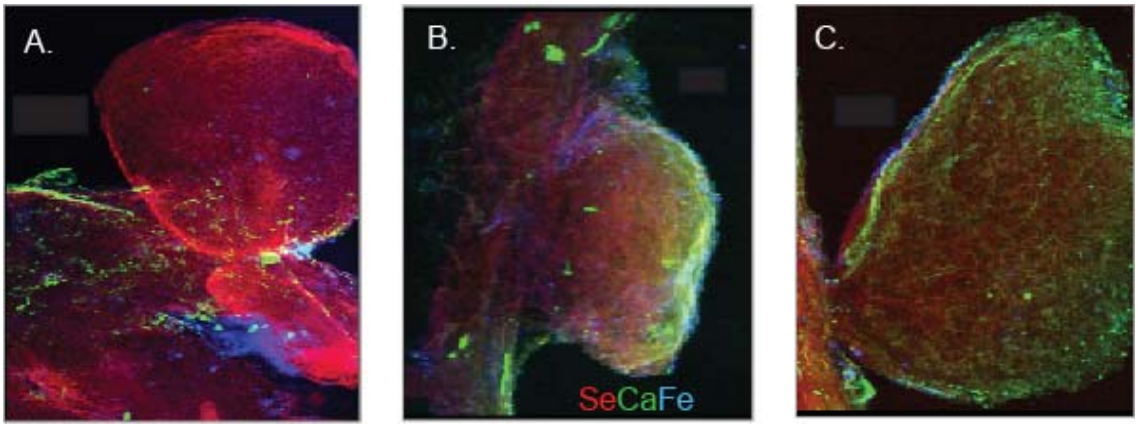

Fig. 2.7 Images of nodules depicted by μXAS scans from hyperaccumulators *A. bisulcatus* (A), *A. praelongus* (B), and *A. racemosus* (C) are shown. Selenium presence is indicated by red, calcium is indicated by green, and iron is indicated by blue.

Table 2.6. The percent of Se (%) in each molecular species from nodules in three *Astragalus* hyperaccumulators as determined by x-ray absorption near edge spectra (XANES).

	SeO_3	C-Se-C	SeCys	$SeGSH_2$	Gray Se	Red Se
A. bisulcatus	3.8	88.9	0.0	1.5	3.0	2.7
A. praelongus	2.4	69.5	21.6	0.0	6.0	0.0
A. racemosus	19.3	44.8	0.0	31.7	0.0	0.0

Discussion

For the first time four Se-hyperaccumulators were found to form nodules under greenhouse or field conditions, including *A. crotalariae, A. praelongus, A. preussii,* and *A. racemosus*. I also found nodules in *A. bisulcatus* under greenhouse and field conditions, which is consistent with reports from Wilson and Chin (1947). I had the most success finding root nodules in the field from *A.*

bisulcatus and *A. praelongus* when the roots grew under rocks, in locations where moisture was retained.

When I compared Se-hyperaccumulators to non-accumulators grown under greenhouse conditions I found no evidence of a cost of Se tolerance in the root nodule symbiosis in hyperaccumulators. These findings are in contrast to reduced symbiotic dependence found in some hyperaccumulators. Strong Ni-hyperaccumulators native to New Caledonia had reduced mycorrhizal colonization compared to species that are moderate or weak accumulators of Ni (Amir et al. 2007). When I added Se to hyperaccumulators under greenhouse conditions the number of nodules per plant increased with increasing Se doses. This was not the case in the non-accumulators where there was no significant effect of adding Se. These effects for non-accumulator legumes agree with the finding that Se had no effect on nodule number in *Melilotus indica*, although Se additions did reduce mean nodule dry weight in that study (Wu et al. 1994). However, the Se-hyperaccumulators made more nodules when they were given more Se, indicating that in these species this belowground symbiosis is linked in some way to Se. *A. bisulcatus* stores up to 99% of Se in young leaves as the selenoamino acid methylselenocysteine (Sors et al. 2005). Perhaps increased nodule numbers increases plant N content, which and could help Se-hyperaccumulators store more Se in organic forms. At the very least this indicates that the symbiosis is tolerant to Se, similar to the findings of a successful symbiotic interaction in the metallophyte *Lotus purshianus* growing on an abandoned copper mine (Wu and Lin 1990).

While mostly investigated aboveground, the protective effect of trace element hyperaccumulation may also occur belowground. To my knowledge the elemental defense hypothesis has never been demonstrated in belowground organs, although in some species belowground organs do satisfy the concentration criteria used to classify plants as hyperaccumulators (Amir et al. 2007; Barzanti et al. 2007; Wu et al. 2007, Barillas et al. 2011). My finding that nodule [Se] was higher than root [Se] under greenhouse conditions in *A. bisulcatus* suggests a possible role for Se in nodule defense. No difference was found in these organs in the non-accumulator *A. drummondii*. These results suggest that nodules, which are expensive organs for the plant to construct and maintain, are protected by Se in a hyperaccumulator, but not in a non-accumulator. Larval weevils of *Sitona lepidus* (Coleoptera: Curculinidae) feed on root nodules of clover (Johnson et al. 2004), but no studies have investigated root nodule herbivory in *Astragalus*. While this study was not designed to test the elemental defense hypothesis, the results suggest new aspects to investigate.

In contrast with my greenhouse findings, field observations showed that root and nodule [Se] were similar in Se-hyperaccumulators. The maximum organ [Se] for each species tested was shown to provide a reference to the 1,000 *μ*g Se g^{-1} DW threshold for Se-hyperaccumulator leaves described in the literature (Brooks 1998). I did not find nodule [Se] in hyperaccumulators at that level, but all other organs surpassed the 1,000 μg Se g^{-1} DW threshold. Nodule [Se] did surpass the 100 *μ*g Se g^{-1} DW level in a Se-hyperaccumulator. The leguminous

non-accumulators that I investigated never breached the 100 μg Se g^{-1} DW level in any organ, even when co-occurring with hyperaccumulators on seleniferous soil. Nodule [Se] levels in *Astragalus* could relate to the promiscuity of rhizobia that associate with *Astragalus* hyperaccumulators and non-accumulators. I did not address this issue, but different nodule [Se] between Se-hyperaccumulators and non-accumulators could be a selective force for rhizobia symbionts.

Tolerance to inorganic Se forms at levels that well exceed the nodule [Se] in all my specimens have been observed in some rhizobia isolates (Kinkle et al. 1994). Inside the root nodules of *Astragalus* Se-hyperaccumulators the majority of Se was stored in organic forms, with the majority being C-Se-C compounds. The maximum nodule [Se] found under field conditions in this study was 109 μg Se g^{-1} DW. This is much lower than the 200 mM Se (IV) or 400 mM Se (VI) minimum inhibitory concentrations (MIC) determined by Kinkle (1994) for *Rhizobium leguminosarum* bv. viciae. Therefore it may be the case that Se-hyperaccumulator and non-accumulator *Astragalus* may associate with the same bacterial isolates. If that is the case, co-occurring *Astragalus* species may not have segregated symbionts, but rather each host species may promote the growth of the bacterial symbiont, thereby enhancing the inoculant size within sites where the two *Astragalus* groups co-occur. Whether or not Se serves as a selective force, microsymbiont identity remains to be investigated by molecular methods.

Selenium cycles through the plant and is proposed to move from the roots into the leaves in the spring, from the leaves into the flowers and fruits, and then

back to the roots during dormancy (Galeas et al. 2007). This proposed mechanism does not account for root nodules. The data I collected here was from actively growing plants when Se should be translocated from roots to aboveground organs. There may be potential to detect differences in belowground organs when Se moves back belowground. Perennial legumes are expected to have perennial nodules (Gurusamy et al. 2000), so Se cycling within plants could manifest in nodules as well. If nodules are inactive during the overwintering process Se may not affect nodule physiology, but may rather affect their susceptibility to herbivory. Even if nodule [Se] are elevated in hyperaccumulators bacteria inside nodules are separated from the plant cell by the peribacteroid membrane (Brewin 1991). Therefore, nodule Se may be isolated from the bacteria. However, free-living rhizobia must cross the Se-enriched rhizosphere soil from Se-hyperaccumulators to infect the host root. Even though *Astragalus* Se-hyperaccumulators do not show evidence of reduced reliance on symbiotic root nodule interactions, the co-evolutionary effects of Se-hyperaccumulation on bacterial symbionts remain to be determined.

Conclusions

Just like their non-accumulator congeners, *Astragalus* Se-hyperaccumulators form root nodule symbioses. They differed from their congeners in the fact that their symbiotic relationship is related to Se treatment and accumulation, while these effects did not occur in non-accumulators.

Selenium concentrations in nodules and the consequences of those levels for segregation of symbionts between co-occurring plant congeners remain to be determined in a more conclusive way. From my findings I hypothesize that the increased number of nodules in Se-hyperaccumulators treated with increased Se levels may result in the symbiotic interaction helping plants acquire more N, which in turn helps the plant to store more Se as selenoamino acids in their shoots.

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Chapter 3

Plant Selenium Hyperaccumulation - With a Little Help from Microbial Friends

Summary

Root nodule symbioses exist to combat nitrogen limitation in plants, but nitrogen (N) obtained through fixation may also be used to construct defensive chemicals within the plant. I investigated the influence of the rhizobia symbiosis in both selenium (Se)-hyperaccumulator and non-accumulator *Astragalus* species subjected to Se. Using a group of 3 hyperaccumulators (*A. bisulcatus, A. praelongus,* and *A. racemosus*) and 3 non-accumulators (*A. convallarius, A. drummondii,* and *A. shortianus*) I investigated if shoot N levels were related to shoot Se levels. In hyperaccumulators, shoot [Se] was positively correlated with shoot [N] ($p < 0.05$; Pearson's r = 0.403), indicating a potential role for dinitrogen fixation in Se-hyperaccumulation. No such correlation was found for the non-accumulator *Astragalus* species. In a comparative greenhouse study using the Se-hyperaccumulator *A. bisulcatus* and the non-accumulator *A. drummondii* I observed that in both species nodulated plants had increased growth and Se:S ratios. Although there were similar effects on Se:S ratios in the two species, the nodulation effect was manifested in different ways. The hyperaccumulator, *A. bisulcatus,* contained 74% higher [Se] in the shoots when it was nodulated, but there was no nodulation effect on shoot [S]. In contrast, in the non-accumulator *A. drummondii* nodulation did not alter shoot [Se], and reduced shoot [S] by 39%. To elucidate the mechanism of the observed positive effects of nodulation and N supply on shoot [Se] in hyperaccumulators I measured the levels of selenoamino acids in *A. bisulcatus* shoots. I found that nodulated plants had a 10-fold higher

concentration of the amino acid γ-glutamyl-methylselenocysteine (γGMSC) than non-nodulated plants. Selenium from γGMSC comprised 32.3% of the total Se in nodulated plants and only 2.3% in non-nodulated plants. There was no difference in methylselenocysteine (MeSeCys) concentration between nodulated and non-nodulated *A. bisulcatus.* My findings indicate that the root nodule mutualism can profoundly affect Se-hyperaccumulation. No evidence of a similar effect was found in non-accumulator *Astragalus* species. From my results I hypothesize that N contributed by the root nodule symbiosis in *A. bisulcatus* increases shoot [Se] through incorporation of this N (mainly as glutamate) into γGMSC. These findings are of interest because they give better insight into the mechanisms of hyperaccumulation, particularly the importance of symbiotic interactions. My findings may also be applicable in Se phytoremediation and biofortification, as selected hyperaccumulator symbionts may affect Se accumulation and speciation in related crop species.

Introduction

Astragalus is the largest genus in the legume family, with an estimated 3,000 species of annual and perennial herbaceous plants (Osaloo et al. 2003). Livestock and wild animals commonly use members of this genus as forage, and 32 species are utilized by humans for food, medicine, or cosmetics (Uphof 1968). Despite the many beneficial uses of *Astragalus* species, toxins are present in some species. The three major toxic principles in the *Astragalus* genus are (1)

the aliphatic nitro compounds, (2) the indolizidine alkaloids including swainsonine, and (3) selenium compounds (Rios and Waterman 1997).

Selenium hyperaccumulation occurs in several plant species from North America and Australia with the majority of them being species of *Astragalus* (Brooks 1998). Shoot [Se] above 0.1% dry weight (1,000 μg Se g^{-1} DW) designate plants as Se-hyperaccumulators, but normal concentrations in plants are less than 2 μg g^{-1}, and 100 μg g^{-1} may be considered extreme (Reeves and Baker 2000). *Astragalus* species that hyperaccumulate Se manage to amass large concentrations; however co-occurring congeners do not accumulate Se to any large extent (Shrift 1969; Galeas et al. 2008; Sors et al. 2009). Given the low concentrations of trace elements in neighboring plants and the ability of hyperaccumulators to amass trace elements at concentrations higher than that of the soil in which they grow, the ability to hyperaccumulate may serve a beneficial function (Boyd and Martens 1992; Boyd 2004). The reason why plants hyperaccumulate Se remains in question, but the most evidence collected to date has focused on and found support for the elemental defense hypothesis (Boyd 2007) where Se is thought to protect plants against herbivores and pathogens (Hurd-Karrer and Poos 1936; Hanson et al. 2003; Galeas et al. 2008; Quinn et al. 2008). In addition Se has recently been implicated in elemental allelopathy where Se enrichment in soil around hyperaccumulators inhibits Se-sensitive competitors (El Mehdawi et al. 2011).

Sulfur and Se have similar physical and chemical properties, thus they follow similar metabolic pathways in plants including root uptake of inorganic

forms, transformation into amino acids, and methylation (Barceloux 1999; White et al. 2004; Sors et al. 2005a; 2005b). However, unlike S plants do not require Se (Fu et al. 2002). In bacteria and animals Se is essential in low concentrations, but it can become toxic to organisms at higher concentrations (Barceloux 1999). In fact, concentrations of 8.1 $\mu g\ g^{-1}$ in feed can cause toxicosis and death in pigs (Stowe et al. 1992). In addition, Se poisoning in humans resulting from foods produced in seleniferous regions have been described (Yang et al. 1983; Dhillon and Dhillon 1997; Fordyce 2007). Despite the risks that seleniferous flora present, a lack of Se in the human diet also poses a threat to human health (McLaughlin et al. 1999). To combat deficiency and to serve as anti-carcinogenic foods, Se-fortified plants have been developed (Lyons et al. 2003; Broadley et al. 2006). Hyperaccumulators are particularly interesting in this respect, because of their capacity to accumulate high levels of Se in a form that is particularly anti-carcinogenic (Ellis and Salt 2003). Most plants accumulate selenate (the most bioavailable form of Se in oxic soils, and therefore the form that is taken up), but hyperaccumulators accumulate predominantly organic Se, particularly methylselenocysteine (MeSeCys) (Freeman et al. 2006). To be able to use hyperaccumulators more efficiently, we need to know more about their Se accumulation mechanisms. In particular, very little is known about the belowground processes that allow hyperaccumulators of Se and other elements to amass large quantities of these trace elements from soil (Alford et al. 2010; Barceló and Poschenrieder 2011).

Interactions between soil microorganisms and root systems can have significant impacts on the ability of plant hyperaccumulators to concentrate trace elements in their shoot systems (Whiting et al. 2001; Abou-Shanab et al. 2003; Turnau and Mesjasz-Przybylowicz 2003; Liu et al. 2005; Vogel-Mikuš et al. 2006; Jankong et al. 2007; Li et al. 2007; Farinati et al. 2009; Alford et al. 2010). Both strong reductions and strong increases in plant trace element concentrations have been found in hyperaccumulators interacting with soil microorganisms. Li et al (2007) found that *Burkholderia cepacia* increased [Cd] by 243% in *Sedum alfredii.* A reduction of 33% was found in *Pteris vittata* [As] when it was inoculated with *Glomus mosseae* (Liu et al. 2005). At times no effects of soil microorganisms on plants were found, as was found in [As] in *Pityrogramma coddii* inoculated with soil derived bacteria (Jankong et al. 2007).

Many legumes form mutualistic relationships with dinitrogen fixing rhizobia in root nodules. Adaption to stress in legumes is expected to be complex because successful tolerance requires adaptation of both the plant and bacterial symbionts (Antonovics et al. 1971). In addition, plant survival during stress may be dependent on symbiotic interactions (Rodriguez et al. 2008). Although symbionts can have strong effects on their partners, we do not always understand the role of symbionts in host adaptation to stress. In *Astragalus,* nodulation has been found in nearly every species that has been investigated (Allen and Allen 1981), but the influence of mutualistic rhizobia on Se-hyperaccumulators has never been investigated.

I hypothesized that symbiotic rhizobia would increase plant [Se] in both Se-hyperaccumulators and non-accumulators. The mechanism could be through increased root surface area in nodulated plants, which controls root access to soil elements. An alternative and/or complementary mechanism would be that rhizobia alter plant Se metabolism by affecting selenoamino acids through N inputs.

Materials and Methods

Experiment 1: Relationship of N and Se

A. bisulcatus, A. convallarius, A. drummondii, A. praelongus, A. shortianus were obtained from Western Native Seed (Coaldale, CO), and *A. racemosus* from Prairie Moon Nursery (Winona, MN). The seeds were scarified, soaked overnight in tap water, and then sown in a mixture of 2:1 volume washed sand and field soil collected from each species (sieved 2 mm). Sixteen plants of each species received 100 µM sodium selenate (Na_2SeO_4) and an N-free fertilizer solution (0.4 mM K_2SO_4, 4.475 μM $FeCl_3$, 4.225 μM Na_2-EDTA, 0.5 mM $MgSO_4$, 0.042 mM KH_2PO_4, 0.208 mM K_2HPO_4, 5.775 μM H_3BO_3, 1.15 μM $MnSO_4$, 0.2 μM $ZnSO_4$, 0.075 μM $CuSO_4$, 0.05 μM $NaMoO_4$, and 0.125 μM $CoSO_4$) at 4 months old and continued through the duration of the experiment (2 additional months). Shoots and roots that were separated from nodules were harvested, and then dried at 50 ^{0}C.

Experiment 2: Nodulation Effect on Shoot [Se]

Scarified and surface sterilized seed (2 minutes with bleach and 2 minutes with 90% ethanol) of *A. bisulcatus* and *A. drummondii* (Western Native Seed, Coaldale, CO) were grown in 164 mL Ray Leach "Cone-tainers" (Stewe & Sons, Oregon) containing a HCl-washed, 1:1 (volume) mixture of sand and fritted clay. Fertilizer solution (1.6 mM K_2SO_4, 0.358 mM NH_4NO_3, 17.9 μM $FeCl_3$, 16.9 μM Na_2-EDTA, 2 mM $MgSO_4$, 0.169 mM KH_2PO_4, 0.833 mM K_2HPO_4, 0.6 mM $CaSO_4$, 0.5 mM $CaCl_2$, 23.1 μM H_3BO_3, 4.6 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.2 μM $NaMoO_4$, and 0.5 μM $CoSO_4$) was applied twice a week beginning when the plants were one month old. The plants grew for 2 months with fertilizer additions, then fertilizer application was suspended for 2 weeks to induce N deficiency before inoculation and selenium treatments were applied. Plants were treated with 20 µM sodium selenate (Na_2SeO_4) and were subjected to one of two inoculation treatments (live soil slurry or autoclaved soil slurry) with 10 replications. I did not use specific strains of rhizobia in my experiment because there were no cultures available from *A. bisulcatus* or *A. drummondii* hosts in the USDA-ARS National *Rhizobium* Germplasm Collection. In addition, I have been unsuccessful in isolating rhizobia from *A. bisulcatus* nodules. Instead two soil samples, one from each *Astragalus* species, were collected from plants growing in a grassland community in Fort Collins, CO (40^0 33' 43.01" N, 105^0 8' 13.1" W) to make soil slurries for inoculation (20 g soil in 1 L sterile, distilled water). The inoculant was added to pots directly or was autoclaved for 30 minutes prior to application. Twenty mL of inoculant was added to each pot by

matching species with soil type. Six days after inoculation the fertilizer treatments were restarted with ¼-strength fertilizer twice per week. After another 6 days, Se treatments were started. One month after Se treatments began the plants began to brown so fertilizer was reduced to once a week and Se treatments were reduced to once every 2 weeks for the duration of the experiment (10 more weeks). Shoots were harvested and dried at 50° C for one week before analysis.

Experiment 3: Selenoamino Acids Study

Astragalus bisulcatus was grown from seed collected near Fort Collins, CO. The seed was scarified and sterilized for 2 minutes in 95% ethanol followed by 2 minutes in 50% bleach. After several rinses with sterile water the seeds were soaked overnight before planting. The seeds were planted the next day in pots containing a 1:1 mixture by volume of sand and sieved (2 mm) field soil collected from the same *A. bisulcatus* population. The soil in the pots was from one of 2 treatments, autoclaved soil microorganisms (treatment) or live soil microorganisms (control). The treatment soil underwent three consecutive rounds of autoclaving and immediate freezing of the mixture. The control soil was not manipulated. Seeds were allowed to germinate over 2 months on a mist bench. After the germination period the plants were allowed to acclimate to greenhouse conditions for 10 days. Then plants were treated with Se (100 μM sodium selenate, Na_2SeO_4) and a low N fertilizer (0.08 mM NH_4NO_3) on a weekly basis for 9 weeks. Shoots were harvested and two leaflets per leaf were

removed and immediately frozen in liquid N_2. The leaflets were stored at -80 ^{0}C until amino acid extraction could be performed. The remaining shoot was dried at 50 ºC. Roots were frozen and stored at -20 ^{0}C until further analysis for nodule counts and root surface area could be conducted. Frozen roots were thawed and subsequently washed. Nodules were counted and removed. Root surface area was immediately determined by scanning and analysis by WinRHIZO (Regent Instruments, Canada).

To extract amino acids from leaflets, the frozen leaf tissue was ground in a mortar and pestle with liquid nitrogen. Two mL of 50 mM HCl was added to the ground sample and it was left to thaw. The sample was then centrifuged for 20 minutes at 13,200 rpm. The resulting supernatant was run through a Waters C18 Sep-Pak® (Milford, MA) that had been previously charged with 5 mL acetonitrile and rinsed with 20 mL mass spectrometry grade water. The sample was stored at -80 ^{0}C until analysis. Samples were run on a Waters (Milford, MA) quadrupole time of flight mass spectrometer coupled with ultra performance liquid chromatography (Q-TOF UPLC) at the Proteomics and Metabolomics Facility at Colorado State University. Chemical standards of methylselenocysteine (MeSeCys) and γ-glutamyl-methylselenocysteine (γGMSC) were included to verify peak retention times and calibrate concentrations.

Plant Elemental Analysis

Twenty or 50 mg of dried and ground shoot samples from the previous 3 experiments were digested in 1 mL nitric acid for 2 hours at 60° C and then 130°

C for 6 hours. Organ [Se] and [S] was determined by inductively coupled atomic emission spectra (ICP-AES) on this digest diluted into 9 mL water at the Soil, Water, and Plant Testing Laboratory at Colorado State University. For shoot [N] 50 to 100 mg dried and ground shoot material was combusted in a Leco Tru-Spec (St. Joseph, MI) at the Colorado State University Natural Resource Ecology Laboratory.

Data Analysis

Data analysis was conducted in Systat v.12 (Systat Software, Chicago, IL). In experiment 1: the relationship of N and Se, correlation analyses were conducted separately for the 3 Se-hyperaccumulators and 3 non-accumulators using physiological attributes (log shoot [Se] and log shoot [S]), fitness attributes (log shoot weight and log root weight), and nodulation attributes (log nodule weight, square root nodule number, and log shoot [N]). Comparisons of log transformed shoot [Se], shoot [S], and shoot [N] were conducted between Se-hyperaccumulators and non-accumulators. In experiment 2: the nodulation effect on shoot [Se], *A. bisulcatus* and *A. drummondii* nodulation effects were compared by *t*-test for log transformed shoot dry weights, the log ratio of Se:S, log shoot [Se], and shoot [S]. Finally, in experiment 3: the selenoamino acids study, *A. bisulcatus* plants in different soil treatments (autoclaved or live soil) were compared by *t*-test for log shoot dry weight, root surface area, log shoot [Se], log shoot [MeSeCys], and log shoot [γGMSC]. The amount of total shoot

Se derived from selenoamino acids was estimated by converting the fresh weight amino acid concentration to dry weight concentrations for each treatment.

Results

Experiment 1: Relationship of N and Se

Shoot [N] was positively correlated with shoot [Se] in hyperaccumulators, but there was no correlation in non-accumulators (Table 3.1). In both the Se-hyperaccumulator species and the non-accumulator species, shoot [S] was positively correlated to shoot [Se]. Significant negative correlations of shoot [S] to shoot root, and total nodule weights were detected in both hyperaccumulators and non-accumulators. However, in the non-accumulators shoot, root, and nodule weights were also negatively correlated to shoot [Se] but no significant correlations were detected for those attributes in the hyperaccumulators. In both plant types there were significant positive correlations of shoot, root, and nodule weights.

Figure 3.1 shows a comparison of the shoot [Se], shoot [S], and shoot [N] between the three hyperaccumulator and the three non-accumulator species. Hyperaccumulators had a significantly higher (p = 0.010) shoot [Se] than non-accumulators (Fig 3.1A). No differences in shoot [S] (Fig 3.1B) or shoot [N] (Fig 3.1C) were detected between the hyperaccumulators and non-accumulators.

Table 3.1. Pearson correlation coefficients for physiological, fitness, and nodulation traits are shown for the three hyperaccumulator species (n = 40) and the three non-accumulator species (n =33). Significant coefficients are shown in bold.

Hyperaccumulators	**[Se]**	**[S]**	**Shoot wt**	**Root wt**	**Nodule wt**	**No. nodules**
[S]	**0.529**					
Shoot wt	-0.199	**-0.574**				
Root wt	-0.166	**-0.447**	**0.873**			
Nodule wt	-0.049	**-0.452**	**0.869**	**0.857**		
No. nodules	0.045	0.145	-0.083	-0.051	0.124	
%N	**0.403**	0.165	-0.169	-0.263	0.039	0.133
Non-accumulators						
[S]	**0.639**					
Shoot wt	**-0.423**	**-0.577**				
Root wt	**-0.636**	**-0.363**	**0.450**			
Nodule wt	**-0.395**	**-0.534**	**0.660**	**0.503**		
No. nodules	0.081	0.300	-0.268	-0.130	-0.199	
%N	0.159	-0.083	0.045	0.335	0.047	0.094

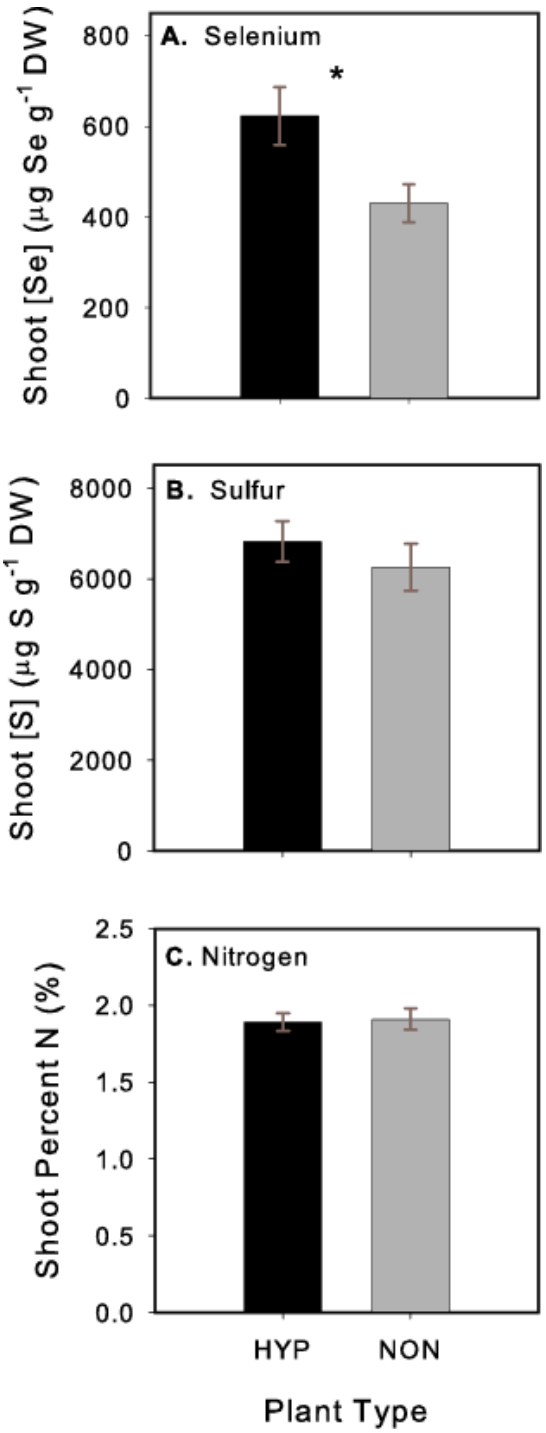

Fig 3.1. A comparison of the three Se-hyperaccumulators (HYP; n = 40) and three non-accumulators (NON; n = 33) for the levels of the trace element Se (A) and the essential nutrients S (B) and N (C) in shoots is shown with standard error. Significant differences are shown by asterisk.

Experiment 2: Nodulation Effect on Shoot [Se]

The hyperaccumulator *A. bisulcatus* and the non-accumulator *A. drummondii* were used to determine if nodulation status affected shoot [Se]. As shown in Figure 3.2, both species produced nodules and in both nodulation was associated with increased shoot biomass ($p < 0.001$ for both species).

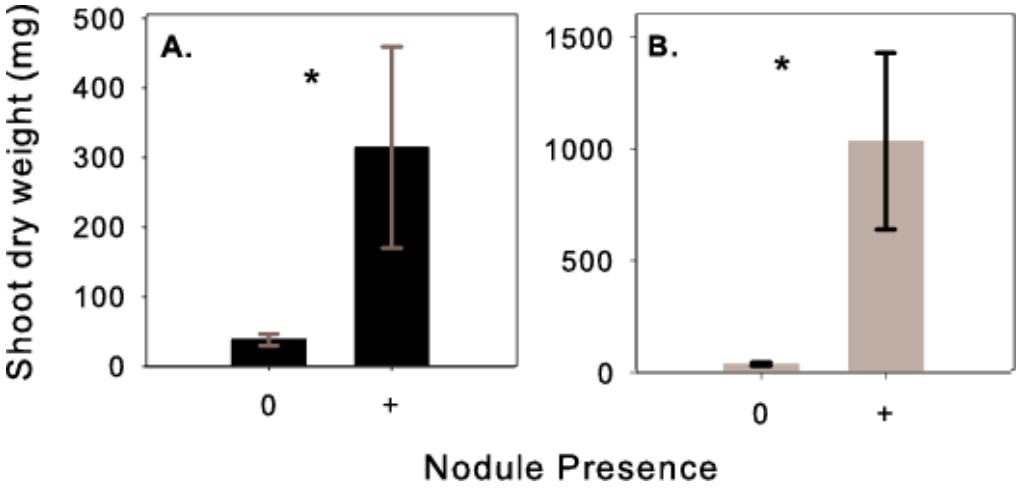

Fig 3.2. The effect of nodulation is shown for shoot dry weights with standard error in the hyperaccumulator *A. bisulcatus* (A) and the non-accumulator *A. drummondii* (B). Nodule absence (0) and nodule presence (+) were compared with significant differences indicated by different asterisk. (*A. bisulcatus* $n_0 = 12$, and $n_+ = 6$; *A. drummondii* $n_0 = 10$, and $n_+ = 6$).

Both *A. bisulcatus* ($p = 0.007$) and *A. drummondii* ($p = 0.044$) had significantly higher shoot Se:S ratios when nodulated (Fig 3.3A, 3.3B). Although the effect of nodulation was similar overall, the changes in elemental concentrations were different between the two species. While in *A. bisulcatus* shoot [Se] was higher in nodulated *A. bisulcatus* plants than non-nodulated plants (+74%, $p = 0.033$, Fig 3.3C), there was no such difference in *A.*

drummondii (Fig 3.3D). Shoot [S] did not differ with nodulation in *A. bisulcatus* (Fig 3.3E), but it was significantly lower in nodulated *A. drummondii* plants than in non-nodulated plants (-39%, $p = 0.048$, Fig 3.3F).

Experiment 3: Selenoamino Acids Study

To obtain better insight into the contribution of bacterial N_2 fixation to Se hyperaccumulation *A. bisulcatus* was grown in the presence or absence of rhizosphere inoculant obtained from the same species growing in its natural habitat; the treatment received autoclaved (dead) inoculum. As shown in Table 2 the live inoculum soil resulted in substantial nodulation, while the autoclaved inoculum did not give any nodules. The nodulated Se-hyperaccumulator did not differ in shoot [Se] from plants without nodules, but did reach a significantly higher shoot dry weight ($p < 0.001$), and root surface area ($p < 0.001$, Table 3.2). Interestingly, the shoot concentration of the seleno-di-amino acid [γGMSC] was 10-fold higher in nodulated plants ($p = 0.009$) than in plants without nodules (Table 3.2): in contrast, shoot concentration of the selenoamino acid MeSeCys was not significantly different between nodulated and non-nodulated plants. Differences between nodule numbers were not statistically tested because plants in the autoclaved soil did not have any variance in that attribute (Table 3.2).

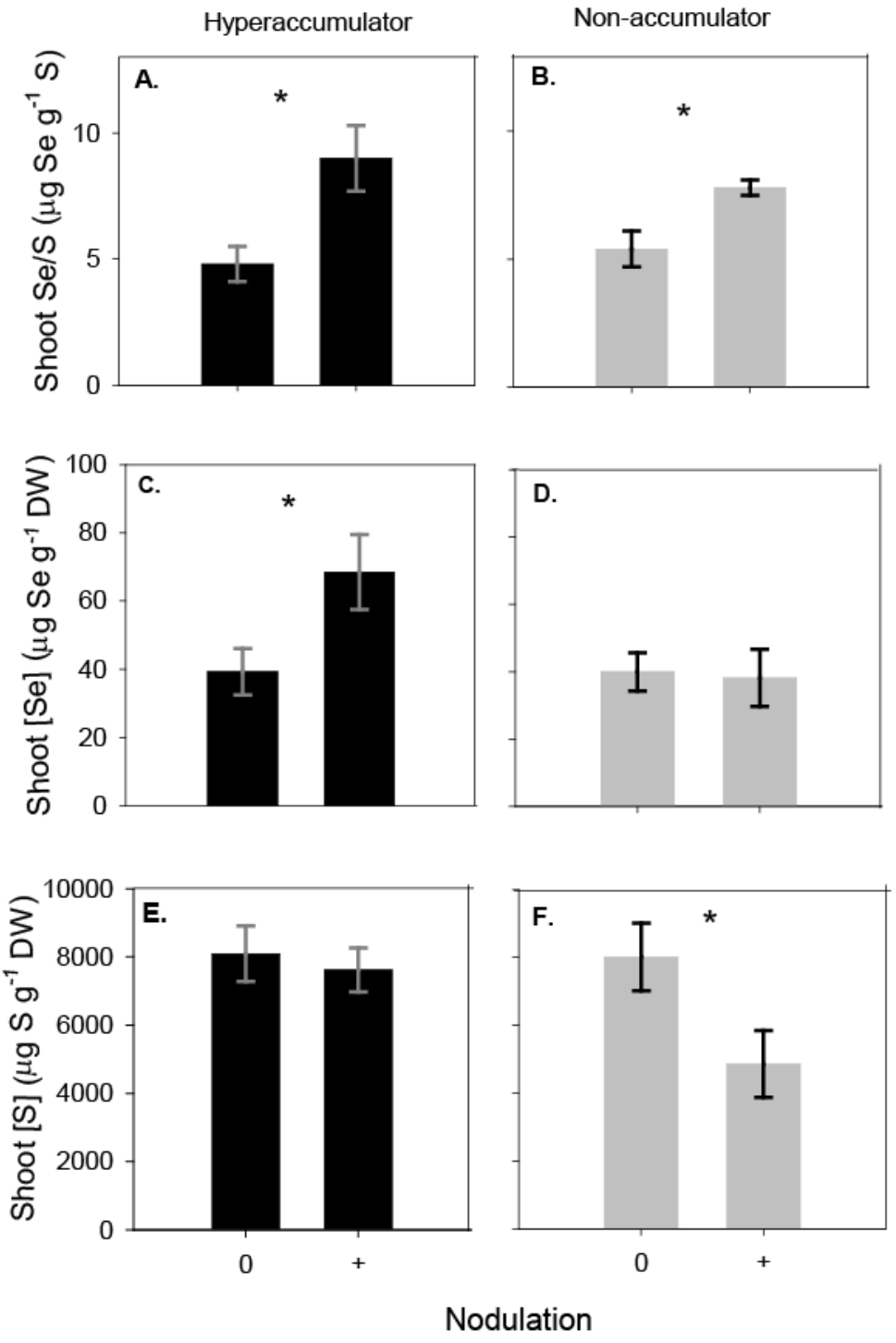

Fig. 3.3 Mean elemental concentrations with standard error are shown for shoots of two legume species. The panels show the effect of nodule absence (0; n = 8) or presence (+; n = 6) on shoot concentrations in the Se-hyperaccumulator *A. bisulcatus* and the non-accumulator *A. drummondii*.

Table 3.2. The average value with standard error is presented for plants growing with autoclaved or soil live microorganisms. Different letters across the rows indicates a significant difference between the two soil treatments.

	n	Autoclaved soil	Live soil
Shoot weight (mg)	21	79.8 ± 4.8 [a]	231.4 ± 11.9 [b]
Root surface area (cm^2)	10	70.1 ± 8.1 [a]	123.5 ± 9.5 [b]
Number of nodules	21	0 ± 0	21.3 ± 2.7
[Se] ($\mu g\ g^{-1}$ DW)	21	3,179 ± 1,646 [a]	2,402 ± 566 [a]
[MeSeCys] ($\mu g\ g^{-1}$ FW)	7	589.5 ± 102.2 [a]	413.0 ± 42.8 [a]
[γGMSC] ($\mu g\ g^{-1}$ FW)	7	15.3 ± 9.9 [a]	152.8 ± 21.0 [b]

To estimate what fraction of total shoot Se was γGMSC and MeSeCys I used the percentage dry weight of samples and converted the γGMSC and MeSeCys concentrations from a fresh weight basis to a dry weight basis. I then calculated what fraction of total, acid-digestible Se was γGMSC or MeSeCys (Fig 3.4). A similar fraction of Se was MeSeCys in nodulated plants grown in live soil (50.3%) compared to non-nodulated plants grown in autoclaved soil (56.9%). The fraction of Se present as γGMSC was approximately 13 times higher in nodulated plants (32.3%) than in non-nodulated plants (2.3%). The selenocompounds that made up the remaining Se were not identified, but accounted for 17.4% in nodulated plants and 40.8% in non-nodulated plants.

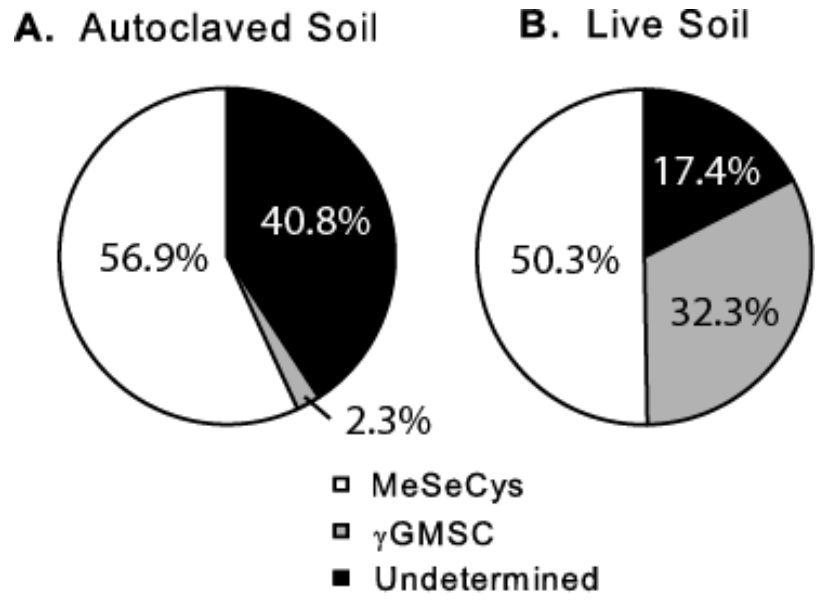

Fig. 3.4. The percent of shoot [Se] on a dry weight basis is shown for different forms in *A. bisulcatus* (n=7) grown in autoclaved (A) or live (B) field collected soil. The fractions were calculated by setting the total acid-digestible Se to 100% and LC-MS was used to measure MeSeCys and γGMSC. The percent of total Se unaccounted for by either amino acid is denoted as undetermined.

Discussion

Plant symbioses with rhizobia affect metabolism in both partners (Colebatch et al. 2004). Since *Astragalus* species hyperaccumulate Se and predominantly store Se in the form of N-containing amino acids, I investigated to what extent rhizobia contribute to hyperaccumulation. Using 3 species of Se-hyperaccumulators and 3 non-accumulators I found that shoot [N] was correlated to shoot [Se] in the hyperaccumulators, but not in the non-accumulators. Shoot [Se] in Se-hyperaccumulator *A. bisulcatus* was 74% higher when nodulated; there was no effect of nodulation on shoot [Se] in the non-accumulator *A. drummondii*. Thus, the hypothesized positive effect of nodulation on [Se] was

observed, but was specific to Se-hyperaccumulators and did not occur in the non-accumulators.

It is possible that symbiotic rhizobia increase N-based defenses in the Se-hyperaccumulator *A. bisulcatus* because Se is stored in the leaf in several forms, including selenoamino acids (Shrift 1969; Pickering et al. 2003; Freeman et al. 2006). In the *A. bisulcatus* leaf Se is stored as MeSeCys and γGMSC, with minor fractions of SeO_4^{2-}, SeO_3^{2-} (Freeman et al. 2006). I did not find differences between nodulated and non-nodulated plants for total shoot [Se] or [MeSeCys], but differences were observed in shoot [γGMSC], a product of the enzymatic coupling of MeSeCys to glutamate (Ellis and Salt 2003; Freeman et al. 2006). Both MeSeCys and γGMSC can safely be accumulated since they cannot be non-specifically incorporated into proteins (like SeCys or SeMet) and do not cause oxidative stress (like inorganic forms of Se), explaining the extreme tolerance of hyperaccumulators. Nodulation-derived N could contribute to plant Se levels through incorporation into selenocysteine (SeCys, the precursor of MeSeCys), glutamate (the other substrate for γGMSC), or through the enzyme γ-glutamyl-cysteine synthetase (ECS) that likely catalyzes the formation of γGMSC (Fig 3.5). Even though the *A. bisulcatus* plants did not differ in shoot [Se] in the experiment where selenoamino acid levels were determined, the presence or absence of nodulation did affect the metabolic profile of Se in *A. bisulcatus.* Nodulated plants were estimated to derive 32% of their shoot Se from γGMSC while in plants without nodules this fraction was only 2%. This finding that nodulation affected [γGMSC] but not [MeSeCys] suggests that the contribution of

the rhizobial N to overall Se accumulation in *A. bisulcatus* is primarily through providing N in the form of glutamate. The rhizobia may also provide N for ECS synthesis, but this is likely a more minor contribution since the enzyme ECS is likely present at a much lower level than the metabolite γGMSC.

My results suggest that symbiotic rhizobia play a role in Se-hyperaccumulation in *Astragalus*, but not in accumulation of Se by non-accumulators. If rhizobia increase [Se] in Se-hyperaccumulators it could indicate that those microorganisms have played a role in evolution of Se-hyperaccumulation in this species. The nodulation effect on shoot [Se] may be critical during the early periods of growth in a young N_2 –fixing hyperaccumulator because increased shoot [Se] helps defend plants from herbivores and pathogens (Hurd-Karrer and Poos 1936; Hanson et al. 2004; Galeas et al. 2008; Quinn et al. 2008). Previous work has shown that the leguminous symbiosis enhanced plant defenses in other species (Johnson et al. 1987; Briggs 1990; Valdez Barillas et al. 2007; Dean et al. 2009; Kempel et al. 2009), especially N-containing defensive compounds. Nitrogen fixing symbioses are known to contribute to plant secondary metabolites, such as alkaloid production in *Oxytropis sericea* (Valdez Barillas et al. 2007). Linkage of Se hyperaccumulation to N nutrition, and thus symbiotic N_2 fixation, was further supported by the finding that shoot [Se] was positively correlated with shoot [N]. This correlation was not significant in the non-accumulator *Astragalus* species. Thus N_2 fixation may facilitate Se sequestration in hyperaccumulators but not in non-accumulators. The nodule number and total nodule weight were not significantly correlated to

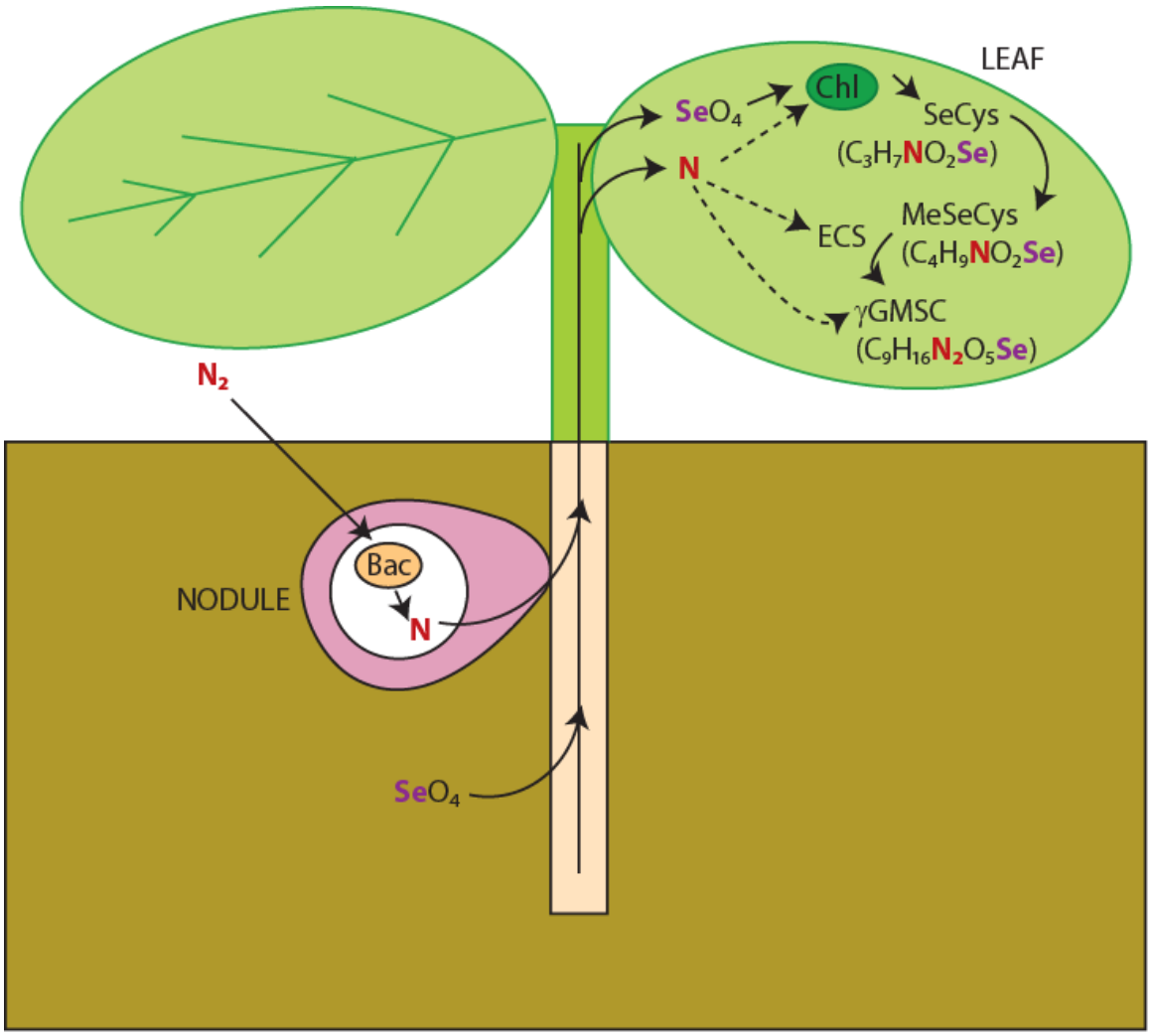

Fig. 3.5. Model of how rhizobia in *Astragalus* hyperaccumulators may contribute to Se hyperaccumulation. Bacteroids (Bac) within the nodule symbiotically fix N_2 and inorganic Se enters the plant through the root system. Although N can enter through the root system, the pathway is not represented because it is not mediated by symbiotic rhizobia. The elements are transferred through the xylem and enter the leaf where they can be used as building blocks for selenoamino acid synthesis in the mesophyll chloroplast (Chl) and cytosol. Both Se and N can be incorporated into selenocysteine (SeCys) in the chloroplast. This selenoamino acid can subsequently be methylated to form methylselenocysteine (MeSeCys). Another N atom could be added when glutamate is added to MeSeCys to form γ-glutamyl-methylselenocysteine (γGMSC). The likely enzyme mediating this process is γ-glutamyl-cysteine synthetase (ECS), which occurs in both the cytosol and the chloroplast. N may also be utilized to produce ECS itself.

shoot [Se], and therefore shoot [N] may be a better predictor of shoot [Se] in *A. bisulcatus*, than the degree to which the plant is colonized by bacteria.

To determine if the positive correlation found between shoot [Se] and shoot [N] in the hyperaccumulators was a result of differences in nutrient or trace element levels between Se-hyperaccumulators and non-accumulators I compared these attributes. The two groups of *Astragalus* species did not differ in nutrient (S or N) concentrations, indicating that plant nutrition levels were similar between the two groups. Rather they only differed in shoot [Se], where as expected the Se-hyperaccumulators had a higher concentration than the non-accumulators. In the nodulation study the ratio of Se:S increased with nodulation in both *A. bisulcatus* and *A. drummondii,* but the change in the ratios was driven by different elements. An increase in shoot [Se] was found to cause the enhanced Se:S ratio in the hyperaccumulator *A. bisulcatus,* while a decrease in shoot [S] caused the enhanced Se:S ratio in the non-accumulator *A. drummondii*

A. bisulcatus and *A. drummondii* occurred on the same site and the soil beneath these plants was similar in total and water-extractable Se. I used soil slurry to inoculate the plants in the greenhouse experiment; therefore soil properties other than the microorganisms and water soluble compounds should not have had much influence on these results. The use of fertilizer in the experiment was intended to minimize effects from other nutritional root symbioses such as mycorrhizae. However, I cannot rule out that the specific symbiotic interactions reported here showing differences in patterns of nodulation and [Se] in the two species resulted from different microorganism identities in the

two soil inocula. It has been shown that different strains of rhizobia produced different effects in nodulated legumes, for example swainsonine production differed in *Oxytropis sericea* when it was nodulated with different strains (Valdez Barillas et al. 2007). Rather, I demonstrated that in both *Astragalus* species nodulation created the traditional nutritional effect by increasing plant biomass, but only in *A. bisulcatus* did nodulation influence the accumulation of the non-essential element Se. In the experiment where six *Astragalus* species were compared with respect to the relationship between N and Se all plants were grown in the same soil, which was a composite derived from each species. The Se-hyperaccumulators were found to differ from the non-accumulators in that they showed a positive correlation between shoot [Se] and shoot [N].

In view of my finding that rhizobial symbionts appear to play an important and potentially limiting role in Se hyperaccumulation in *Astragalus* species by providing N for γGMSC synthesis, future studies on hyperaccumulators ought to consider the influence of belowground root-microbe symbioses on the uptake and accumulation of trace elements by plants. This could also lead to finding rhizobia that have the ability to enhance phytoremediation or biofortification potential. Some rhizobia isolates have been shown to have high Se tolerance (Kinkle et al. 1994); rhizobial isolates from Se-hyperaccumulators have never been tested in this regard. These unique *Astragalus* species may provide further biotechnological benefits by yielding new Se-resistant rhizobial isolates.

Mutualisms have a large role in the ecology of ecosystems because they provide vital services such as nutrient exchange, pollination, and seed and spore

dispersal (Boucher et al. 1982). Exploring mutualisms within the context of stress and tolerance are increasingly important because ecosystems are subjected to rising levels of stress as human-induced pressures build. Therefore, it would be beneficial to learn how mutualisms persist under extreme conditions in natural ecosystems as well as understand how environmental stress has contributed to differentiation among sympatric mutualists.

Conclusions

Traditionally, the legume-rhizobia symbiosis is viewed as a nutrient exchanging mutualism where bacteria supply the plant with fixed nitrogen in exchange for fixed carbon. I have found that in symbiosis with rhizobia the Se-hyperaccumulator *A. bisulcatus* concentrated more of the non-nutrient trace element Se than non-nodulated plants. This effect of the symbiosis did not occur in the non-accumulator *A. drummondii.* In addition Se-hyperaccumulators had a significant positive correlation of shoot [Se] and shoot [N]. The mechanism by which the root nodule symbiosis contributes to increased shoot [Se] in *A. bisulcatus* appears to be partially attributable to selenoamino acid production. These results indicate that symbiotic interactions with rhizobia are involved in *Astragalus* adaptation to Se-rich soils, and that similar interactions should be considered in applied contexts.

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Chapter 4

Soil Origin Mediates Plant Selenium Levels in *Astragalus*

Summary

A few species of *Astragalus* hyperaccumulate selenium (Se) and are restricted to seleniferous soils. The other species in this large genus do not hyperaccumulate Se and are not restricted to seleniferous soils. I investigated if soil origin and its associated microorganisms influence plant Se levels in 3 Se-hyperaccumulators, *A. bisulcatus, A. praelongus,* and *A. racemosus,* along with 3 non-accumulators, *A. convallarius, A. drummondii,* and *A. shortianus*. When plants were amended with 100 µM sodium selenate and grown in soil originating from hyperaccumulators both the Se-hyperaccumulator species and the non-accumulator species had higher shoot and root [Se] than plants grown in soil originating from non-accumulators. A 71% increase in shoot [Se] and 237% in root [Se] was observed in the Se-hyperaccumulators. In non-accumulator soil the Se translocation factor from roots to shoots was higher for hyperaccumulator *Astragalus* species, indicating that transport of Se within the plant is affected by the soil and its associated microorganisms. When growing in soil originating from hyperaccumulators non-accumulator *Astragalus* species had a 69% increase in shoot [Se] and a 178% increase in root [Se], but there was no difference in their Se translocation factors in the two soils. No difference was observed in shoot or root [S] between soil origins in either *Astragalus* group, indicating that the effect was specific to Se. The difference in organ [Se] was not driven by soil [Se] because when the plants were grown in each soil and no Se was added they showed no difference in shoot or root [Se]. Finally, dosing plants

with 100 μM Se increased shoot [S] by approximately 2,000 μg S g^{-1} dry weight (DW) and decreased root [S] by approximately 900 μg S g^{-1} DW compared to the 0 μM Se control in both hyperaccumulator and non-accumulator *Astragalus* species. In summary, soil origin and associated microorganisms can affect plant Se levels, indicating that microorganisms associated with seleniferous soils have a unique ability to increase Se uptake by Astragalus.

Introduction

Selenium hyperaccumulation occurs in approximately 20 species (Brooks 1998). The majority of these species are from the large legume genus *Astragalus* and they occur only on seleniferous soils in western North America (Barneby 1964). Other non-accumulator species of *Astragalus* co-occur on seleniferous soils, but are not restricted to these soils and do not hyperaccumulate Se, even when co-occurring with hyperaccumulators (Shrift 1969). Since Se-hyperaccumulators are restricted to seleniferous soils even though Se is not essential for plant growth (Terry et al. 2000), the soil microorganisms that have evolved with Se-hyperaccumulators may contribute in some way to Se accumulation. Selenium tolerant microorganisms found in rhizospheres from Se-hyperaccumulators and crop plants growing in seleniferous soils have the potential to alter Se availability to plants (Di Gregorio et al. 2005; Gupta et al. 2010). If such an effect occurs, the microorganisms may be useful

for understanding the ecological basis and evolution of Se hyperaccumulation in addition to advancing rhizosphere-assisted phytoremediation.

Soil microorganisms can significantly alter trace element uptake into hyperaccumulators through redox reactions, methylation, demethylation, complex formation, and biosorption (Adriano et al. 2004). Researchers have found that inoculation with microorganisms can alter trace element levels in both hyperaccumulators and non-accumulators. Abou-Shanab et al. (2003a; 2006) found that bacteria isolated from the rhizosphere of *Alyssum murale* were able to increase shoot [Ni] in the plant compared to an un-inoculated control. Copper accumulation in *Elsholtzia splendens* was altered by addition of bacterial isolates (Chen et al. 2005). Others have shown that plant Se accumulation and volatilization in non-accumulator plants could be enhanced by bacteria (Zayed and Terry 1994; de Souza et al. 1999a; de Souza et al. 1999b). Becerra-Castro et al. (2009) found that the Ni-hyperaccumulator *Alyssum serpyllifolium* fostered a different rhizosphere community than a non-accumulator and that the solubility of Ni in the soil could be increased.

Effects of soil microorganisms on plants can be driven by fungi as well as bacteria. Mycorrhizae have been shown to increase As in *Pteris vittata* (Al Agely et al. 2005; Wu et al. 2009). Nickel concentrations in *Berkheya coddii* were increased by mycorrhizal inoculation (Turnau and Mesjasz-Przybylowicz 2003). It is thought that mycorrhizae increase plant accumulation of immobile elements in soil because fungal infection of the root increases the absorptive surface area (Sanders and Tinker 1971). However, mycorrhizal colonization was noted to be

negatively correlated with leaf [Ni] and infection was low in strong Ni-hyperaccumulators (Amir et al. 2007). Researchers have found that mycorrhizae can also reduce trace element concentrations in some hyperaccumulators (Liu et al. 2005; Vogel-Mikuš et al. 2006; Jankong et al. 2007).

I wanted to conduct a comparative study to determine if soils and their associated microorganisms that support hyperaccumulators (seleniferous) and those that do not (non-seleniferous) affect Se accumulation in *Astragalus*. I hypothesized that plants would grow best in their home soils and that plant [Se] would be higher in soil inoculant originating from hyperaccumulators. Since Se shares similar physical and chemical properties with the essential element S I expected plant [S] to mirror what was found with plant [Se]. To see if I could find an inoculation effect I compared 6 species of *Astragalus* native to Colorado, USA. Some of the species are Se-hyperaccumulators (*A. bisulcatus, A. praelongus* and *A. racemosus*) and some of the species are non-accumulators (*A. convallarius, A. drummondii,* and *A. shortianus*). If there are differences in how these soils and their associated microorganisms affect plant [Se] it may indicate that there are unique characteristics of microorganisms from seleniferous soils that help plants hyperaccumulate Se.

Materials and Methods

Soil Collection

Soil was collected to a depth of 15 cm directly underneath the canopy of each of 6 *Astragalus* species (Table 4.1) growing in Colorado during the summer of 2009. Soils were sieved (2 mm) and were then mixed according to their plant host type, Se-hyperaccumulators (*A. bisulcatus, A. praelongus*, and *A. racemosus*) or non-accumulators (*A. convallarius, A. drummondii,* and *A. shortianus*) in a 1:1:1 volume basis. The composited soils were sent to the Soil, Water, Plant Testing Laboratory at Colorado State University for routine analysis along with total and AB-DPTA extractable Se (Table 4.2). These soils became the inoculant source in the following greenhouse experiment.

Table 4.1. Soil collection locations for the 6 species including the closest Colorado town, the degrees latitude and longitude, and site elevation (m).

Species	Location	Latitude	Longitude	Elevation (m)
A. bisulcatus	Fort Collins	40º 42’ 37.45” N	105º 06’ 39.28” W	1597
A. convallarius	Meeker	39º 34’ 35.24” N	108º 24’ 45.67” W	2466
A. drummondii	Livermore	40º 45’ 59.77” N	105º 21’ 06.21” W	1946
A. praelongus	Uravan	38º 22’ 28.93” N	108º 44’ 40.93” W	1504
A. racemosus	Limon	39º 21’ 00.59” N	103º 51’ 27.43” W	1749
A. shortianus	Livermore	40º 46’ 00.57” N	105º 21’ 04.29” W	1940

Table 4.2. Soil characteristics from composite soils used as hyperaccumulator and non-accumulator soils in the greenhouse experiment with *Astragalus* species. Elements are presented as mg kg^{-1}.

	Origin	
	Hyperaccumulator	**Non-accumulator**
Texture	sandy clay loam	clay loam
pH	7.6	6.6
EC	1.1	0.4
% OM	2.2	3.4
NO_3-N	33.4	9.8
P	19.9	6.5
K	609	261
Zn	5.11	1.32
Fe	7.05	18.4
Mn	6.36	8.20
Cu	3.12	1.93
Total Se	2.02	1.21
Extractable Se	0.71	0.90

Greenhouse Experiment

Starting in January 2010 through July 2010 I grew 6 *Astragalus* species from seed in the University Greenhouse at Colorado State University. Seeds of *A. bisulcatus, A. convallarius, A. drummondii, A. praelongus,* and *A. shortianus* were obtained from Western Native Seed (Coaldale, CO). *A. racemosus* seeds were obtained from Prairie Moon Nursery (Winona, MN). To start germination, seed coats were scarified by nicking with a razor blade and the seeds were then soaked overnight in tap water. The following day seeds were planted into pots containing a 2:1 volume mixture of washed sand and composited field soil (as an inoculum). My intention with this 2:1 mixture was to compare soil microorganisms from Se-hyperaccumulators and non-accumulators by

attempting to control for other differences in soil properties. Use of the sand should create a potting mixture that would not interfere with nutrient and Se availability in the treatments. Plants were grown in their own soil inoculant (home) or soil inoculant from the other plant type (foreign). In May 2010 Se treatments and N-free fertilizer treatments began. Control treatments of 0 µM Se were applied as water only and treatments of 100 µM sodium selenate (Na_2SeO_4) were applied twice a week for the duration of the experiment. A N-free fertilizer solution (0.4 mM K_2SO_4, 4.475 *µ*M $FeCl_3$, 4.225 *µ*M Na_2-EDTA, 0.5 mM $MgSO_4$, 0.042 mM KH_2PO_4, 0.208 mM K_2HPO_4, 5.775 *µ*M H_3BO_3, 1.15 *µ*M $MnSO_4$, 0.2 *µ*M $ZnSO_4$, 0.075 *µ*M $CuSO_4$, 0.05 *µ*M $NaMoO_4$, and 0.125 *µ*M $CoSO_4$) was applied once a week. Additionally, a Ca-only fertilizer (0.15 mM $CaSO_4$ and 0.125 mM $CaCl_2$) was applied once a week. Shoots were separated from the roots and harvested in July 2010. Shoots were dried at 40 °C. Samples of roots were immediately frozen and stored at -20 °C until they could be analyzed. Root samples were thawed, rinsed, and separated from root nodules. These samples were dried at 40 °C for further analysis. Dry weight was recorded for shoots and roots.

Elemental Concentrations

Dried and ground plant samples of 20, 50, or 100 mg were digested in 1 mL nitric acid for 2 hours at 60° C and then 130° C for 6 hours. This was then

diluted to 10 mL with deionized water. Organ [Se] and [S] was determined by inductively coupled atomic emission spectra (ICP-AES) on the digest.

Data Analysis

Data analysis was conducted in Systat v.12 (Systat Software, Chicago, IL). I conducted separate *t*-tests to determine the effect of soil type and Se treatments on log-transformed shoot and root biomass in hyperaccumulators and non-accumulators. Separate *t*-tests were used to compare the effect of soil origin on the log-transformed shoot and root [Se] and [S] in hyperaccumulators and the non-accumulators in the 100 µM Se treatment. Selenium translocation in the 100 µM Se treatment was compared for effects of soil origin by *t*-test in both hyperaccumulators and non-accumulators. Effects of soil origin were compared for log-transformed shoot and root [Se] in the 0 µM Se treatment by separate *t*-tests. The Se-hyperaccumulators and non-accumulators were combined because no difference was detected in organ [Se] between the plant groups when no additional Se was used. To test for the effect of Se addition on shoot [S] in the two plant groups I used a *t*-test to compare log-transformed values from the 0 µM Se and 100 µM Se treatments.

Results

There was no difference in shoot or root biomass between the two soil origins (pooled Se treatment) in either the Se-hyperaccumulators or the non-accumulators (Table 4.3). No effect of Se treatment (pooled soil origin) on shoot or root biomass was detected in either plant group (Table 4.3). Data for each species shoot and root dry weights are shown by individual soil and Se treatments in Appendix 3 and Appendix 4, respectively.

Table 4.3. The effect of soil type and Se treatment are shown for mean organ dry weight (mg) with standard error (n =48) for the three hyperaccumulators and three non-accumulators. No significant differences for each organ or treatment were found.

	Hyperaccumulators		**Non-accumulators**	
	Shoot	**Root**	**Shoot**	**Root**
Soil origin				
Home	558 ± 64	393 ± 48	187 ± 16	165 ± 13
Foreign	449 ± 51	383 ± 46	246 ± 36	169 ± 16
Se treatment				
0 µM	519 ± 60	371 ± 44	232 ± 33	176 ± 14
100 µM	538 ± 57	405 ± 50	201 ± 22	158 ± 15

Inoculation with soil originating from Se-hyperaccumulators significantly increased shoot and root [Se] in both hyperaccumulators (p_{shoot} = 0.004; p_{root} < 0.001) and non-accumulators (p_{shoot} = 0.006; p_{root} = 0.001) compared to soil inoculation originating from non-accumulators when plants were amended with 100 µM Se (Fig 4.1). Shoots from hyperaccumulators and non-accumulators had higher [Se], with a difference of 327.1 and 220.1 µg Se g^{-1} DW, respectively,

when grown in soil originating from hyperaccumulators. Belowground hyperaccumulator and non-accumulator roots had more Se, 409.3 and 299.5 µg Se g^{-1} DW, respectively, when grown in soil from hyperaccumulators. This was a 71% increase in shoot [Se] and a 237% increase in root [Se] in the hyperaccumulators. Similarly, a 68% increase in shoot [Se] and a 178% root [Se] was found in the non-accumulators.

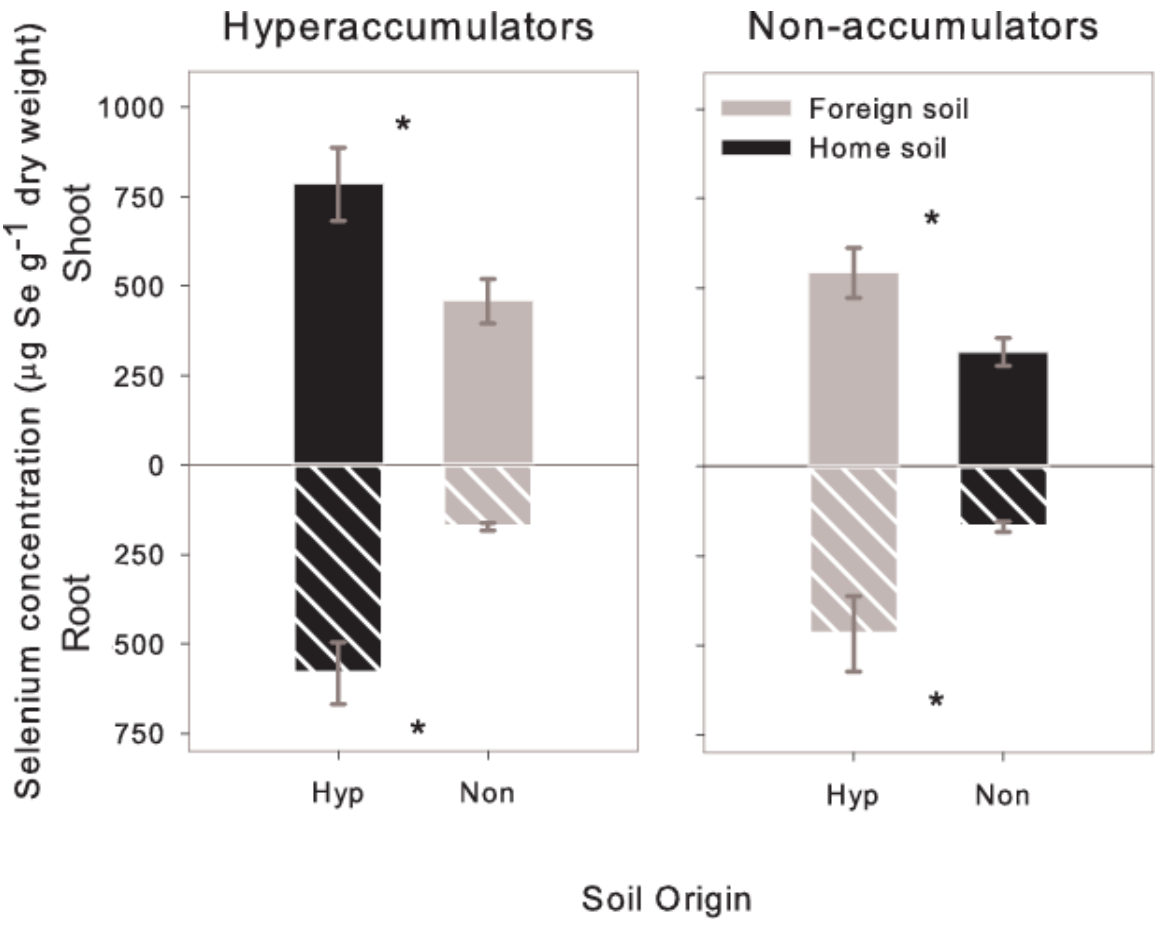

Fig. 4.1 Mean shoot and root [Se] is shown with standard errors by soil inoculant origin for both hyperaccumulators and non-accumulators. Shoots are shown in solid bars and roots are shown by hatched bars. Home soils are depicted in black and foreign soils are depicted in gray. Significant differences (α = 0.05) in each organ are shown with an asterisk.

Although shoot and root [Se] were higher in hyperaccumulator plants growing in inocula derived from hyperaccumulator soil amended with 100 μM Se, the Se translocation factor from roots to shoots, measured as the ratio of shoot [Se] to root [Se], was significantly higher (p = 0.005) in non-accumulator soil inocula (Fig 4.2). There was no effect of soil inoculant source on the root to shoot translocation factor in non-accumulators (Fig 4.2). In the same treatments I found no effect of soil origin on shoot or root [S] in either plant group (Fig 4.3).

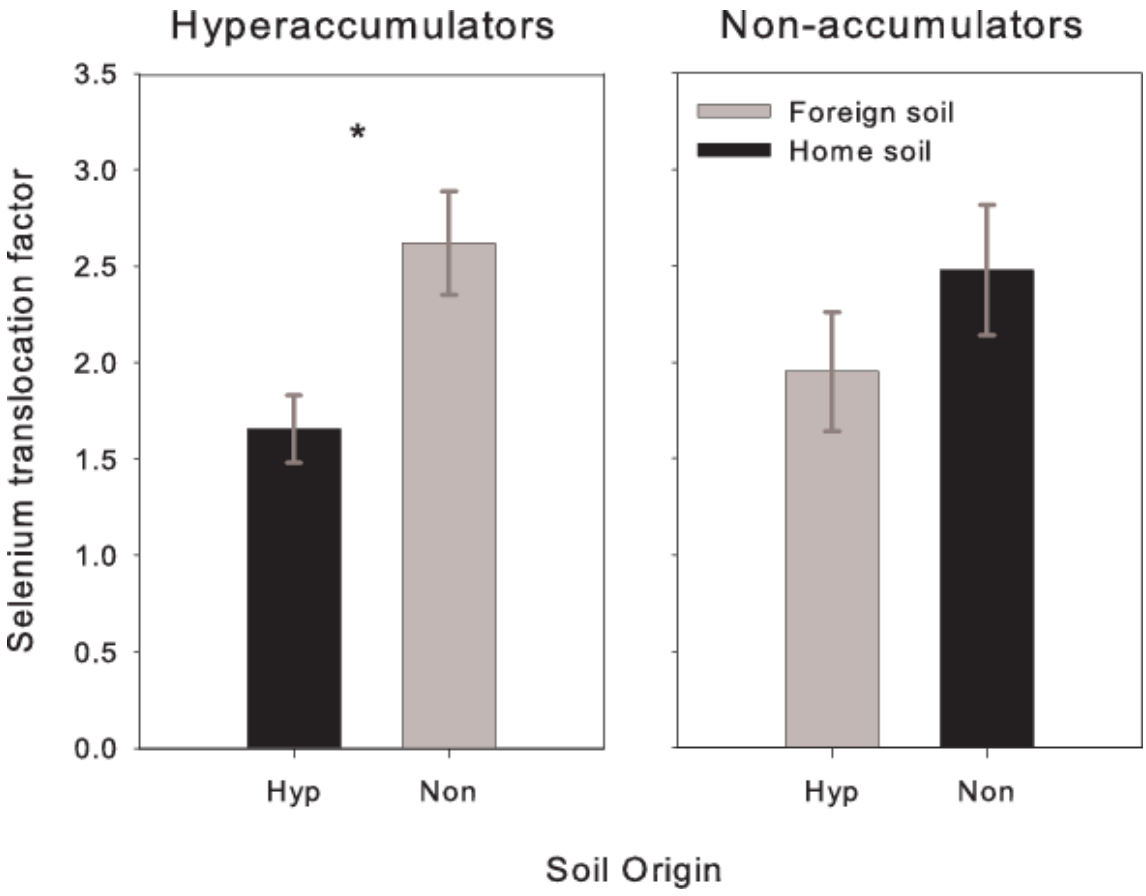

Fig. 4.2 The effect of soil inoculant origin on mean Se translocation (shoot [Se] divided by root [Se]) with standard error is shown for hyperaccumulators and non-accumulators. Significant differences (α = 0.05) are shown by asterisk. Black bars indicate home soil and gray bars indicated foreign soil.

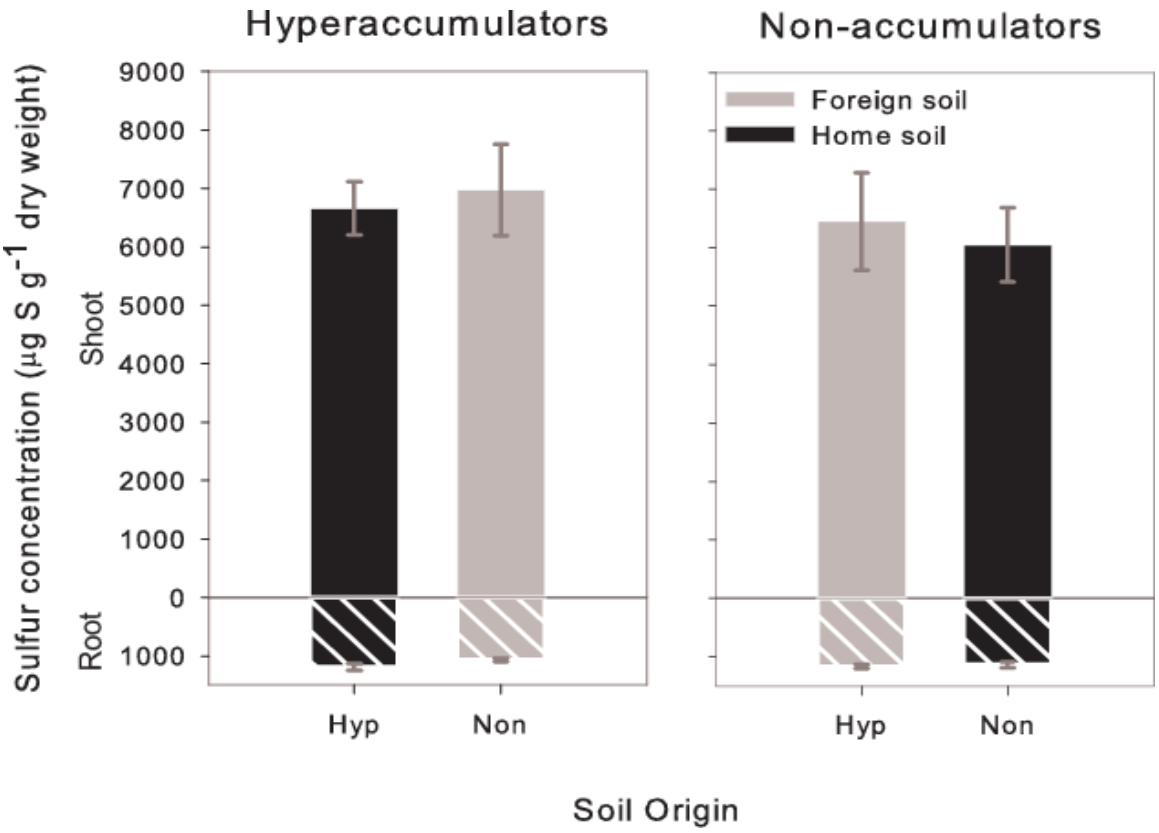

Fig. 4.3 Mean shoot and root [S] are shown with standard errors by soil inoculant origin for hyperaccumulators and non-accumulators. Shoots are shown by solid bars with black indicating plants growing in their home soil inoculant and gray indicating plants growing in a foreign soil environment. Roots are shown by hatched bars with the same color pattern.

Plants grown with no additional Se did not differ in shoot or root [Se] by soil inoculant origin (Table 4.4). As shown in Fig 4.4 the addition of Se significantly increased shoot [S] ($p < 0.001$) by 1,953 µg S g^{-1} DW in hyperaccumulators and by 2,107 µg S g^{-1} DW in non-accumulators ($p < 0.001$). Selenium addition significantly decreased ($p_{hyp} < 0.001$; $p_{non} < 0.001$) root [S] by 845 and 986 µg S g^{-1} DW in hyperaccumulators and non-accumulators respectively (Fig 4.4).

Table 4.4. The mean Se concentration within the shoot and root (μg Se g^{-1} dry weight) of plants (hyperaccumulators and non-accumulators combined) growing in soil without added Se is shown with standard error (n = 48). No significant differences in organ [Se] by soil origin were detected.

Soil	Shoot	Root
Hyperaccumulator	13.5 ± 2.1	25.0 ± 2.0
Non-accumulator	11.6 ± 2.2	22.5 ± 2.2

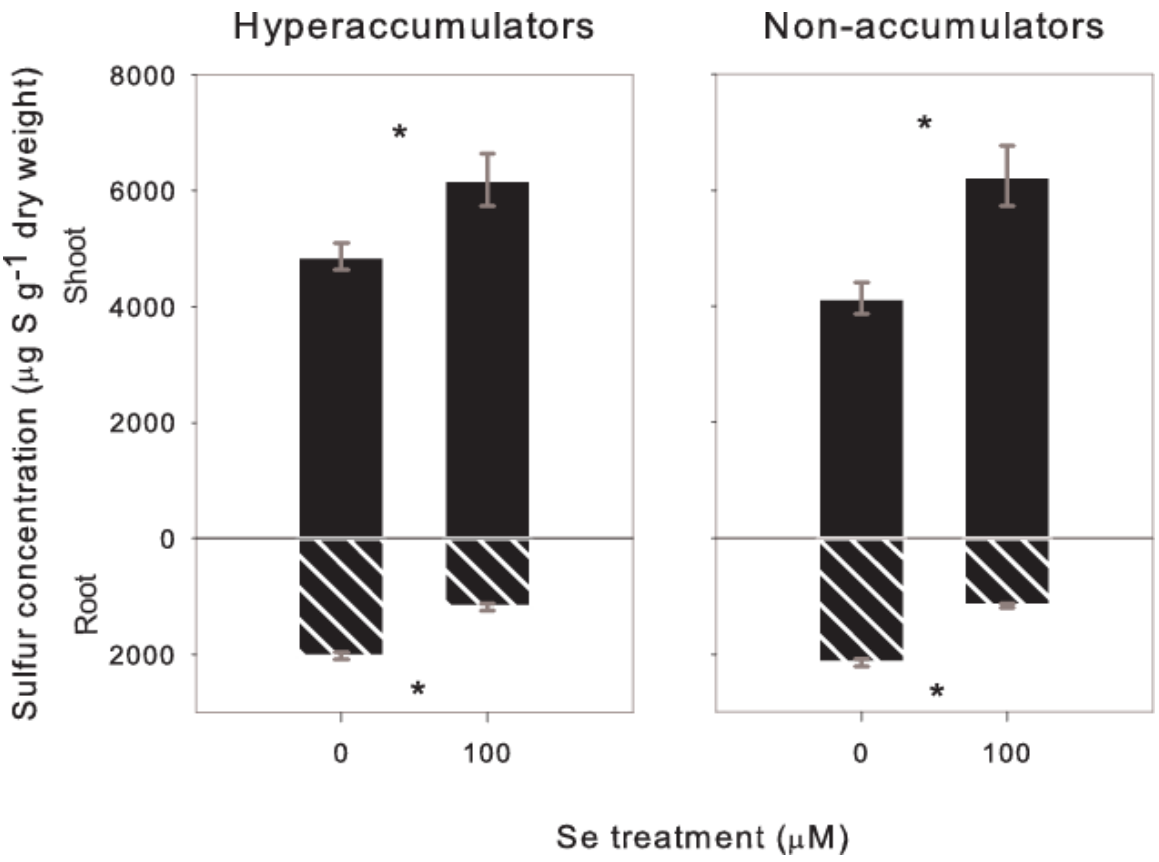

Fig. 4.4 The effect of Se addition on mean shoot and root [S] with standard error is shown for hyperaccumulators and non-accumulators. Shoots are shown in solid bars and roots are shown in hatched bars. Significant differences (α =0.05) between organ [S] by Se treatment are shown by asterisks.

Discussion

Soil microorganisms can have strong effects on plant trace element levels by either increasing or decreasing plant trace element concentrations (Alford et al. 2010 and references therein). In this experiment Se-hyperaccumulators had a 71% increase, corresponding with a difference of 327.1 μg Se g^{-1} DW, in the 2:1 mixture of sand and soil originating from hyperaccumulators compared to the 2:1 mixture of sand and soil originating from non-accumulators. A similar pattern was evident in non-accumulators where they had a 69% increase of 220.1 μg Se g^{-1} DW in soil from hyperaccumulators compared to soil from non-accumulators. Roots showed even stronger increases in [Se]. Roots from hyperaccumulators growing with a soil inoculant derived from hyperaccumulators had a 237% increase (409.3 μg Se g^{-1} DW) compared to plants growing in soil inoculant derived from non-accumulators. Likewise, roots from non-accumulators growing with inoculant from hyperaccumulators had a 178% increase (299.5 μg Se g^{-1} DW) compared to the plants grown in non-accumulator soil.

Differences in shoot or root [Se] are not likely to be attributable to the baseline [Se] in the two soils because those concentrations were similar. In fact, the soil originating from non-accumulators had a higher concentration of extractable Se than the soil originating from Se-hyperaccumulators. No difference in shoot or root [Se] was detected in plants growing without Se amendment in inoculant derived from hyperaccumulator or non-accumulator soils. The differences in shoot and root [Se] that were found when Se

amendment was added were not an artifact of plant growth differences because no effects of soil type or Se treatment on plant biomass were detected.

In my experiment soil inocula originating from Se-hyperaccumulators increased shoot and root [Se] in both hyperaccumulator and non-accumulator *Astragalus* species. In other studies several mechanisms have been proposed for the ability of bacteria to increase plant Se accumulation; bacteria may increase production of compounds that enhance trace element accumulation, bacteria may increase root surface area, bacteria may transform selenate into forms that are more readily taken up by plants, or bacteria may stimulate the plant sulfate transporters which can also take up selenate (de Souza et al. 1999b). There is evidence that the interaction of plants inoculated with bacteria produced a heat-labile compound that could cause an increase in Se accumulation in axenic plants subsequently treated with the compound (de Souza et al. 1999a). Although I did not test for such a compound in this experiment, my results could be consistent with that mechanism.

My results also do not refute the proposition that bacteria may transform selenate in the rhizosphere into forms that are more readily taken up by plants. As an example of different preferences for Se molecules, *Brassica oleracea* took up more selenomethionine than selenate, particularly when sulfate levels in the media were elevated (Zayed et al. 1998). Although this may be a mechanism at work here, it must be noted that roots of *Astragalus* species have been shown to not differ much in their Se uptake potentials. No difference was detected in root uptake of selenite or low concentrations of selenate between the Se-

hyperaccumulator *A. crotalariae* and the non-accumulator *A. lentiginosus* (Ulrich and Shrift 1968). Roots of the non-accumulator did not take up as much selenate at high selenate concentrations, which the authors attributed to possible Se toxicity (Ulrich and Shrift 1968). Still, differences in plant uptake may be at work.

An explanation of the effects I observed may lie in differences in Se translocation within the plants. Different Se molecules have different rates of translocation inside plants, where more Se moved into shoots when supplied as selenate than as selenite (Arvy 1993; Hopper and Parker 1999; Li et al. 2008; Bitterli et al. 2010) and it has been shown that mycorrhizal infection can reduce shoot [Se] (Munier-Lamy et al. 2007). Although, in *Pteris vittata* where the As translocation factor was increased at least five times in inoculated plants, it did not increase the [As] in plants (Trotta *et al.* 2006). I did find that Se translocation factors differed between the two soil origins in Se-hyperaccumulators. This may account for some differences in plant [Se]. However, it would be insufficient for describing the difference in organ [Se] in non-accumulators.

A more detailed study of soil microorganisms would be required to determine the mechanism causing my observations. However, my results do refute some of the potential mechanisms proposed by de Souza et al. (1999b). Increased root surface area may not explain the enhanced [Se] in plants in this study because no difference in other elements such as [S] was observed between soil origins in the 100 μM treatment. In addition I did not detect any differences in root biomass between the two soil treatments. Root surface area

was not shown to influence accumulation of other forms of Se such as selenite and selenomethionine in *Brassica juncea* (de Souza et al. 1999a) or selenate in *A. bisulcatus* as I showed in the previous chapter.

Based on my findings it is not likely that bacteria were responsible for increased [Se] in *Astragalus* by increasing activity of sulfate transporters because there was no difference in [S] in plants grown in the two soil types, even though differences in [Se] were detected. Rather than bacteria causing an increase in sulfate transporter activity in plant roots, it may be that higher [Se] in media may increase plant S uptake as seen in Bell et al. (1992). Adding 100 μM Na_2SeO_4 to the plants increased the shoot [S] compared to the control where no Se was added. Interestingly, the addition of Se also reduced root [S] in the two plant types. Total plant S (μg) increased and the root and shoot allocation patterns changed in both plant types where more S was put into the shoots in the 100 μM Se treatment.

Differences in soil properties may have contributed to effects observed in this experiment. The ability of soil to fix Se, soil pH, and microbial activity strongly influence Se availability (Gissel-Nielsen 1971; Neal et al. 1987; Masscheleyn et al. 1990; Wu 2004; Eich-Greatorex et al. 2007). Both of the soil inoculants used were composited mixtures from collections underneath three different *Astragalus* species. A routine soil analysis of the mixtures determined them to be clay loams, with the soil originating from Se-hyperaccumulators being a sandy clay loam. However, soil fixation of Se in the two soils could differ because plants growing on sandier soils would have had more Se available to

them since oxidized forms of Se are less likely to bind to sand particles than clay particles (Gissel-Nielsen 1971). The soil inoculants also differed in organic matter content with the non-accumulator soil having a higher percent organic matter. Organic matter can also fix selenite (Wu 2004), particularly at a pH > 6 (Eich-Greatorex et al. 2007), but different forms of organic matter have been shown to have different effects on Se in soil. Dissolved organic matter has been positively correlated with grass [Se], possibly through microbial Se mineralization of organic Se (Weng et al. 2011). It is also known that the rhizosphere effect influences Se availability in some soils. When Mn-oxides were present with organic acids typically exuded by roots, approximately 33% of selenite was oxidized to selenate, increasing Se availability to plants (Blaylock and James 1994). In addition, over a range of pH from 6.5 to 7.5 it has been shown that higher pH resulted in higher ryegrass Se uptake when selenite was added to soil (Gissel-Nielsen 1971). Although the Se added in this experiment was in the form of selenate, which is generally bioavailable, factors mediated by the soil conditions and root exudates have the potential to have influenced the results.

No difference in biomass was detected between the two Se treatments, however, visual signs of toxicity were evident, particularly in the non-accumulators. These included leaf chlorosis, leaf tip burn, and leaf necrosis. It is likely that these effects did not contribute to biomass differences because Se treatments were not started until the plants had been growing for 5 months. After Se additions began the plants did not add much more biomass. Rather, I did find that soil inoculant originating from Se-hyperaccumulators significantly increased

plant [Se] in both Se-hyperaccumulator and non-accumulator *Astragalus* species in this greenhouse experiment. Microorganisms that have contributed to the strong increase in plant [Se] in this experiment may help elucidate the ecological and evolutionary basis of Se hyperaccumulation and be useful in rhizosphere-assisted phytoremediation to increase phytoextraction potential by increasing plant [Se].

Conclusions

Shoot and root [Se] were elevated in both hyperaccumulators and non-accumulators that grew in soil inoculant derived from hyperaccumulators compared to inoculant derived from non-accumulators. This effect was specific to Se and not evident in shoot and root [S]. Soil microorganisms from Se-hyperaccumulators should be investigated further to determine the mechanism responsible for this observation. Such information will be beneficial to our understanding of the ecology and evolution of Se-hyperaccumulation as well as providing techniques to improve rhizosphere-assisted phytoremediation.

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Chapter 5

Screening Plants and Inoculant for Selenium Phytoextraction Potential from Municipal Biosolids

Summary

I undertook a study to evaluate the use of plants to remove excess selenium (Se) from municipal biosolids. Initially, biosolids were examined by x-ray absorption spectroscopy (XAS) to determine Se localization and molecular speciation within the sample. Selenium was distributed uniformly with the three major components being organic C-Se-C (similar to methylselenocysteine), elemental gray Se, and selenite. I then began phytoextraction trials by growing plants in the municipal biosolids to create aboveground biomass enriched in Se, which can be harvested to reduce Se in the substrate. In the first trial I compared four Se-hyperaccumulator *Astragalus* species (*A. bisulcatus, A. crotalariae, A. preussi,* and *A. racemosus*) with five non-accumulator legumes (*A. cicer, A. convallarius, A. drummondii, A. utahensis,* and *Oxytropis sericea*). From this group I found that the most effective species for phytoextraction was the Se-hyperaccumulator *A. crotalariae.* In the second trial I compared plant growth and [Se] in three different dilutions of municipal biosolids to determine optimal phytoextraction conditions for *Brassica juncea* and *B. napus*. Dilutions were 50%-50%, 75%-25%, and 100%-0% mixtures of biosolids and sand by volume. Optimal Se phytoextraction occurred in the 75%-25% biosolids and sand mixture because both shoot weight and shoot [Se] were maximized. Finally I used an inoculant of soil microorganisms from *A. bisulcatus* as a factor and tested 13 species (*Amaranthus retroflexus, Astragalus racemosus, Brassica napus, Carex pendula, Cortaderia selloana, Cyperus papyrus, Helianthus annuus, Melilotus*

officinalis, Nicandra physalodes, Robinia neomexicana, Sorghum sudanese, Typha latifolia, and *Zea mays*) for their Se phytoextraction potential. Inoculation did not affect plant phytoextraction potential in this trial. The top 4 candidates for further studies on phytoextraction in that trial were *Brassica napus, Melilotus officinalis, Helianthus annuus,* and *Astragalus racemosus*.

Introduction

Biosolids are nutrient-rich organic material remaining after sewage sludge treatment. They are disposed of in multiple ways in the United States through land application, incineration, or landfilling. In Region 8 of the United States Environmental Protection Agency (Colorado, Montana, North Dakota, South Dakota, Utah, and Wyoming) 85% of biosolids are land applied (US-EPA 2011). Land application of biosolids is akin to addition of a slow-release fertilizer because of the high amounts of organic matter and plant nutrients such as N, P, and K. However, composted wastes have higher metal concentrations than soils (García et al. 1990; Ciavatta et al. 1993) and there is risk of contaminating soil when biosolids are applied because of their elevated levels of trace elements. Biosolids with elevated levels of trace elements are restricted from land application in the United States according to the Resource Conservation and Recovery Act (RCRA).

In Pueblo County, Colorado the average soil [Se] is 5.1 mg kg^{-1} (USGS 2010). This soil is thought to contribute to elevated Se levels in the municipal

biosolids generated in the city of Pueblo, CO. Selenium is the only pollutant in the Pueblo municipal biosolids that is above the US-EPA regulatory limit for biosolids land application (Table 5.1). Reduction of the amount of Se that occurs in excess in these biosolids could permit their beneficial reuse.

Table 5.1. Description of Pueblo, CO municipal biosolids analysis and US EPA 40 CFR §503.13 Table 1 pollutant ceiling concentrations (maximum allowable concentrations for land application). All values are expressed on a dry weight basis. The 2008 sample was used in the x-ray analysis, the *Astragalus* trial, and the dilution trial. The 2010 sample was used in the inoculation trial only.

Attribute	2008	2010	Ceiling levels
Paste pH	6.9	7.2	NA
Total solids (%)	90.7	88.7	NA
Volatile solids (%)	42.9	54.6	NA
K (%)	0.266	0.318	NA
P (%)	1.91	2.52	NA
Organic N (%)	2.45	3.00	NA
NH_4-N (mg kg^{-1})	5,826	8,546	NA
NO_3-N (mg kg^{-1})	16.3	71.4	NA
As (mg kg^{-1})	10.5	5.0	75
Cd (mg kg^{-1})	16.3	19.4	85
Cr (mg kg^{-1})	8.7	23.3	NA
Cu (mg kg^{-1})	531	813	4,300
Hg (mg kg^{-1})	0.151	< 0.05	57
Mo (mg kg^{-1})	6.2	11.4	75
Ni (mg kg^{-1})	31.1	37.2	420
Pb (mg kg^{-1})	54.3	70.3	840
Se (mg kg^{-1})	154.7	211.0	100
Zn (mg kg^{-1})	83.4	1,141	7,500

Plants have been suggested as a means to remediate contaminated substrates (Baker et al. 1994; Salt et al. 1995). This approach has been utilized for Se contaminated soils in central CA (Bañuelos 2001). Hyperaccumulators, or plants that accumulate and tolerate high levels of trace elements under natural

conditions, have been proposed for use in phytoremediation trials (Whiting et al. 2004). However, their slow growth rates and low biomass may prevent their wider use. High biomass producing annuals may provide alternatives that perform better than the hyperaccumulators that amass large quantities of trace elements. As an example, *Brassica juncea* and *B. napus* have been shown to be more efficient at Zn phytoextraction than the Zn-hyperaccumulator *Thlaspi caerulescens* (Ebbs et al. 1997).

Microbial inoculation has been shown to improve plant uptake of trace elements. Inoculating *Alyssum murale* with bacteria increased the shoot [Ni] in plants compared to an un-inoculated control (Abou-Shanab et al. 2003; Abou-Shanab et al. 2006). Microbial inoculants may increase plant biomass or tolerance to trace elements (Glick 2003) and alter plant trace element availability and toxicity (Ma et al. 2011). Burd et al. (1998) showed that inoculation with a PGPR strain protected canola from Ni toxicity. This was not through any change in plant [Ni], but possibly through alteration of plant stress hormones. Bacterial inoculants altered Cu accumulation in the Cu-accumulator *Elsholtzia splendens* (Chen et al. 2005). Most notable for this study is that plant Se accumulation and volatilization was shown to be increased by inoculation (Zayed and Terry 1994; de Souza et al. 1999a, 1999b). However, inoculants can also immobilize trace elements in the rhizosphere (Di Gregorio et al. 2005) and reduce plant trace element concentrations (Liu et al. 2005; Vogel-Mikuš et al. 2006; Jankong et al. 2007).

Plants have been grown in raw, undigested sewage sludge to try to reduce the elevated levels of contaminants that occur in those substrates. Wu et al. (2007) have shown that *Sedum alfredii* could phytoextract Zn from sewage sludge. This effect was significantly increased when *S. alfredii* was co-cropped with *Alocasia macrorrhiza* (Wu et al. 2007). Others have examined crop plants for their ability to accumulate trace elements from biosolids to investigate risks of contamination in agricultural crops. Liphadzi and Kirkham (2006) found that *Helianthus annuus* leaves had higher concentrations of Cd, Ni, and Pb without addition of EDTA compared to plants with EDTA because EDTA increased metal availability and thus plant toxicity.

I attempted to reduce Se contamination in biosolids originating from Pueblo, CO through a phytoextraction approach. To assess the feasibility of this the biosolids Se distribution and composition was measured by x-ray absorption spectra (XAS) and x-ray absorption near edge spectra (XANES). Initially I screened Se-hyperaccumulators in the *Astragalus* genus along with non-accumulator congeners for phytoextraction potential. Then different mixtures of biosolids with sand were evaluated to determine which mixture would maximize Se recovery in *Brassica juncea* and *B. napus*. Finally, I screened a large group of plants to determine if inoculation with soil microorganisms that naturally associate with the Se-hyperaccumulator *Astragalus bisulcatus* could improve plant Se extraction.

Materials and Methods

Biosolids Characterization

Biosolids were collected from the city of Pueblo, CO wastewater treatment facility in October 2008 and June 2010. The 2008 samples were used for the *Astragalus* and *Brassica* phytoextraction trials while the 2010 sample was used in the inoculant trial. The 2008 biosolids sample was also used to determine Se localization and molecular speciation. A subsample of the biosolids was frozen in liquid N_2 and shipped on dry ice to the Lawrence Berkeley Laboratory Advanced Light Source for analysis on Beamline 10.3.2 (Marcus et al. 2004) in May 2009. Determination of the Se within the sample was conducted in a manner similar to Freeman et al. (2006). Briefly, Se distribution in a few grains of the frozen sample was imaged by scanning in the focused beam at 13,085 eV. Selenium K-edge XANES were used to provide molecular speciation of the Se within the sample (Pickering et al. 2000). The LabVIEW programs (National Instruments) available at Beamline 10.3.2 were used to analyze data.

Samples of the 2008 and 2010 biosolids were analyzed by the Soil, Water, and Plant Testing Lab at Colorado State University in the same manner as Ippolito et al. (2010). Briefly, the elemental composition was assessed by $HClO_4$–HNO_3–HF–HCl digestion and subsequent analysis with inductively coupled plasma – atomic emission spectrometry (ICP-AES).

Astragalus Phytoextraction Trial

Seeds of *A. bisulcatus, A. cicer, A. convallarius, A. drummondii, A. utahensis,* and *O. sericea* were obtained from Western Native Seed (Coaldale, CO). Prairie Moon Nursery (Winona, MN) provided *A. racemosus* seed. The Desert Legume Program (Tucson, AZ) supplied seed of *A. crotalariae* and *A. praelongus.* Seed from *A. preussi* (accession W6 26876) was obtained from the USDA-ARS Western Regional Plant Introduction Station (Pullman, WA). Seed coats were nicked with a razor blade and soaked overnight in tap water prior to germination. Plants were germinated in a sand layer overlying a mixture of 1:1 volume biosolids and sand in SC10, Ray Leach Cone-tainers ® (Stewe & Sons, Tangent, OR) where they grew for the duration of the experiment. Prior to mixing with sand, the biosolids were sieved (5.6 mm) to remove the large flocculated component. Two types of plants were compared, Se-hyperaccumulators (*Astragalus bisulcatus*, *A. crotalariae*, *A. praelongus*, *A. preussi*, *A. racemosus*) were used for their ability to amass large quantities of Se in their tissues; and related non-accumulators (*A. cicer*, *A. convallarius*, *A. drummondii*, *A. utahensis*, *Oxytropis sericea*) that could potentially produce higher amounts of biomass. No fertilizer treatments were applied; the plants received only water or recirculated leachate from the pots. After five months of growth the aboveground biomass was harvested, dried at 45 ºC, weighed, and ground for subsequent elemental analysis.

Brassica Phytoextraction Trial

The Se removal potential of *B. juncea* and *B. napus* growing in 50%, 75%, and 100% volume mixtures of biosolids (sieved 5.6 mm) combined with sand was compared. Seeds of *B. juncea* (accession PI 173874) and *B. napus* (accession PI 649150) were obtained from USDA-ARS North Central Regional Plant Introduction Station (Ames, IA). The seeds were soaked overnight in tap water before being planted in the biosolids mixtures in SC10, Ray Leach Cone-tainers ® (Stewe & Sons, Tangent, OR). The plants were grown for 2.5 months and received water only until they were harvested, dried at 45 ºC, weighed, and ground for Se analysis.

Inoculation Trial

A trial was established to examine the effect of soil microorganism inoculation on plant phytoextraction potential. Thirteen species were used in the trial. Fourteen replicate plants per species were grown in a 75% biosolids (sieved 5.6 mm) and 25% sand by volume mixture in SC10, Ray Leach Cone-tainers ® (Stewe & Sons, Tangent, OR). Seed of *Amaranthus retroflexus* was obtained from Azlin Seed Service (Leland, MS). *Astragalus racemosus* seed was obtained from Prairie Moon Nursery (Winona, MN). Seed of *Brassica napus* (accession PI 649150) came from the USDA-ARS North Central Regional Plant Introduction Station (Ames, IA). *Carex pendula, Cortaderia selloana, Cyperus papyrus,* and *Nicandra physalodes* were from Pase Seed (North Collins, NY).

Seed from *Helianthus annuus, Melilotus officinalis, Robinia neomexicana, Sorghum sudanese,* and *Typha latifolia* was obtained from Granite Seed (Lehi, UT). *Zea mays* seed was obtained from Jax Farm and Ranch (Fort Collins, CO)

Astragalus racemosus and *Robinia neomexicana* seed coats were nicked with a razor blade prior to germination. All seed was soaked overnight in tap water to increase germination before planting. Seed was planted in SC10, Ray Leach Cone-tainers ® (Stewe & Sons, Tangent, OR) containing a 75% biosolids (sieved 5.6 mm) and 25% sand by volume mixture. Plants were grown for 2 months and received water or recirculated leachate. Half of the pots (n = 7) were inoculated with 25 mL each of inoculant. The inoculant was created by collecting field soil from a seleniferous soil site supporting the Se-hyperaccumulator *Astragalus bisulcatus*, sieving the soil through a 2 mm sieve, and mixing 20 g of soil with 2 L tap water. Plants from each of the 13 species receiving inoculant were kept separate from the plants that were not inoculated. After an additional one month of growth the plants were harvested, dried at 45 ºC, weighed, and ground for subsequent Se analysis. Two-way ANOVAs examining the effects of species and inoculation on log transformed shoot mass, log transformed shoot [Se], or log transformed total shoot Se were performed.

Plant Selenium Analysis

The dried and ground plant samples of 20, 50, or 100 mg were digested in 1 mL nitric acid for 2 hours at 60° C and then 130° C for 6 hours. This was then

diluted to 10 mL with deionized water. Shoot [Se] was determined by inductively coupled atomic emission spectroscopy (ICP-AES) of the digest.

Results

Biosolids Characterization

The 2008 and 2010 biosolids samples contained 155 and 211 mg kg^{-1} Se, respectively (Table 5.1). Except for Se, the elemental composition of the two biosolids samples were all below the United States EPA 40 CFR §503.13 ceiling concentration limits for land application of biosolids (US-EPA 1993). The μ-XAS mapping and XANES analyses showed that Se was uniformly distributed within the biosolids (Fig 5.1A) and the three major forms of Se were C-Se-C (58%), which most closely matched methylselenocysteine, elemental gray Se (24%), and selenite (17%) (Fig 5.1B).

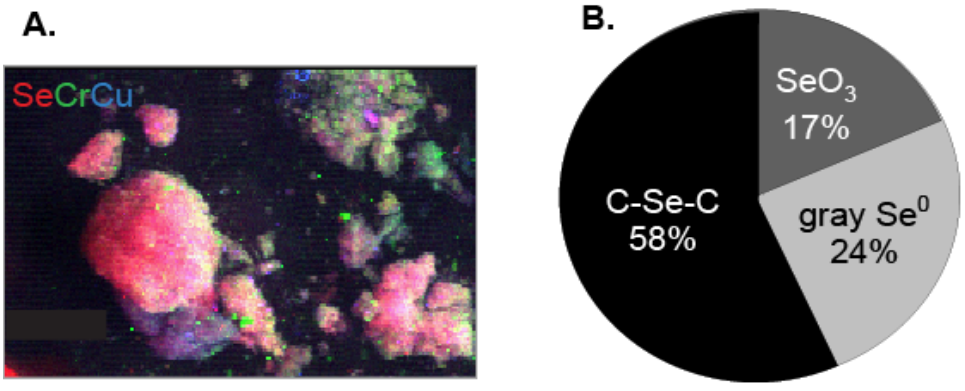

Fig. 5.1 Selenium (indicated by red) is uniformly present throughout the 2008 Pueblo, CO municipal biosolids sample (A). Chromium content is indicated by green and copper is indicated by blue. The three main Se-containing compounds in the municipal biosolids were C-Se-C, elemental gray Se (Se^0), and selenite (SeO_3^{2-}) (B).

Astragalus Phytoextraction Trial

Figure 2 shows the Se phytoextraction potential, which was determined by multiplying shoot [Se] (Fig 5.2B) by shoot dry weight (Fig 5.2C) to yield the total amount of Se (*μ*g) removed per plant (Fig 5.2A). The species that removed the most Se per shoot (113.8 *μ*g) was *A. crotalariae.* The species that removed the next highest amount of Se was *A. preussi* with an average of 78.4 *μ*g Se removed per shoot. *A. racemosus* removed the third highest amount of Se in the trial at 56.8 *μ*g per plant. Three species (*A. bisulcatus, A. convallarius,* and *A. drummondii*) failed to germinate in the biosolids and yielded no data for Se phytoextraction.

A. preussi had the highest shoot [Se] at 145.5 *μ*g Se g^{-1} dry weight (DW), then *A. crotalariae* at 85.6 *μ*g Se g^{-1} DW, and all other species had less than 70 *μ*g Se g^{-1} DW (Fig 5.2B). *A. praelongus* had the third highest shoot [Se] (66.2 *μ*g Se g^{-1} dry weight) and was slightly higher than *A. racemosus* (65.3 *μ*g Se g^{-1} DW). The species with the highest amount of shoot biomass was *A. cicer* (1.78 g) followed by *A. crotalariae* (1.33 g), *A. racemosus* (0.97 g), and *A. preussi* (0.86 g).

Brassica Phytoextraction Trial

Both *B. juncea* and *B. napus* germinated and grew at all levels of biosolids. Dilution of municipal biosolids with sand altered the amount of Se that the shoots were able to extract (Fig 5.3A). These differences were a product of

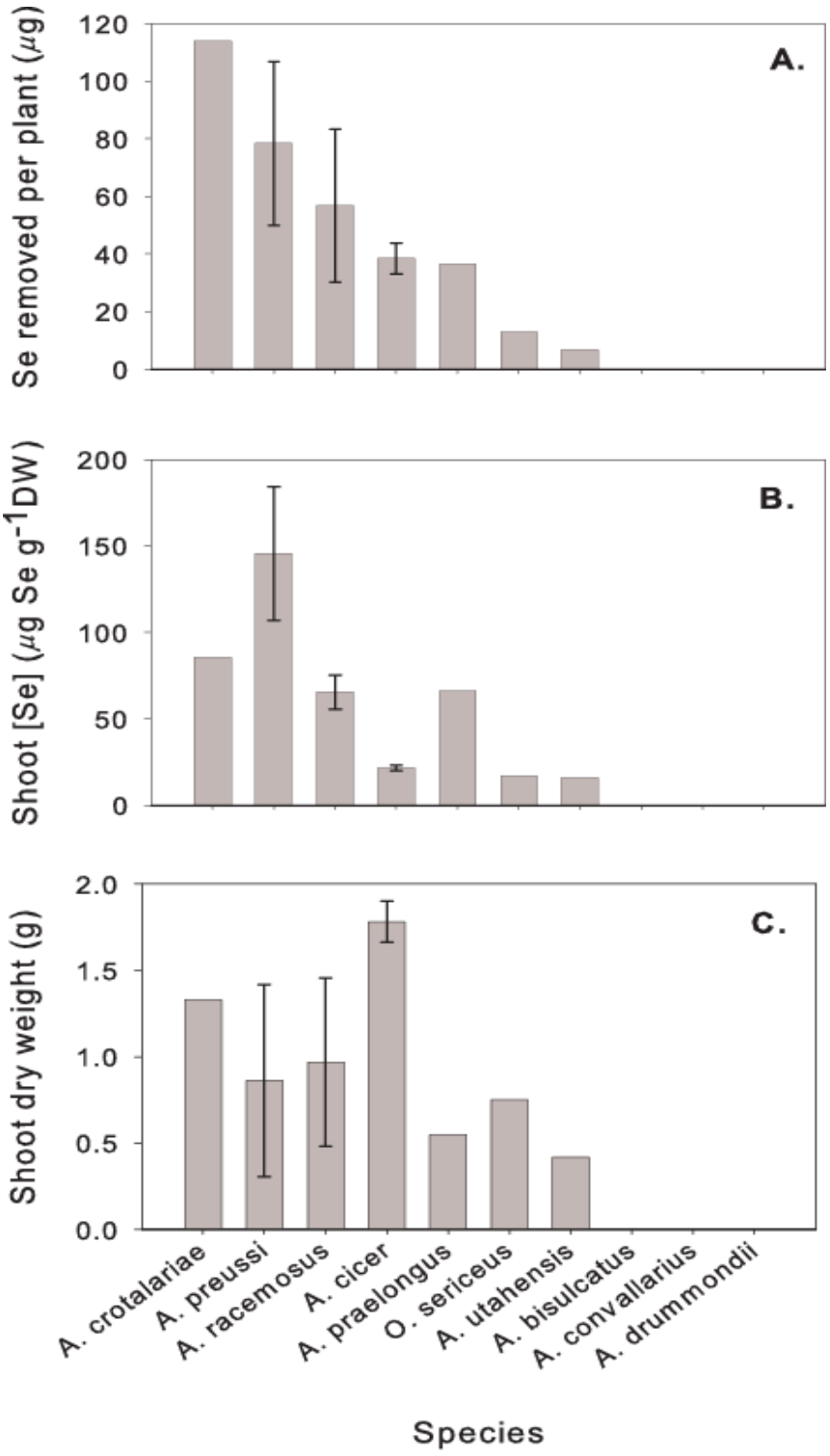

Fig.5.2 Total amount of Se in shoot (A), shoot [Se] (B), and shoot dry weight (C) for each species from the trial to evaluate *Astragalus* Se-hyperaccumulators. In this trial plants were grown in the 2008 Pueblo, CO municipal biosolids.

both altered shoot [Se] (Fig 5.3B) as well as biomass production in the species (Fig 5.3C). Maximal shoot biomass for *B. juncea* was 4.2 g in 75% biosolids. *B. napus* had similar results with a maximal shoot biomass of 4.0 g in 75% biosolids. The highest shoot [Se] for *B. napus* was 24.9 *μ*g Se g^{-1} DW in the 75% biosolids. *B. juncea* reached the highest shoot [Se] at 25.1 *μ*g Se g^{-1} DW in 100% biosolids. The largest amount of Se removed by the two species was in the 75% biosolids mixture, where *B. juncea* extracted 90.1 *μ*g Se and *B. napus* extracted 98.0 *μ*g Se.

Inoculation Trial

Inoculation with soil microorganisms from *A. bisulcatus* did not alter the phytoextraction potential in any of the 13 species tested, nor did it alter shoot biomass or [Se]. Since inoculation did not show an effect I combined data from the two inoculant treatments. *Brassica napus* removed 29.0 *μ*g Se per shoot, followed by 17.8 *μ*g Se per shoot in *Melilotus officinalis*, and 13.4 *μ*g Se was removed per shoot in *Helianthus annuus* (Fig 5.4A). *Astragalus racemosus* removed 13.3 *μ*g Se per shoot (Fig 5.4A) and had the highest shoot [Se] of 64.1 *μ*g Se g^{-1} DW (Fig 5.4B). *Brassica napus* had the highest shoot dry weight at 1.64 g of any species in the trial and was followed closely by *Sorghum sudanense* at 1.63 g (Fig 5.4C).

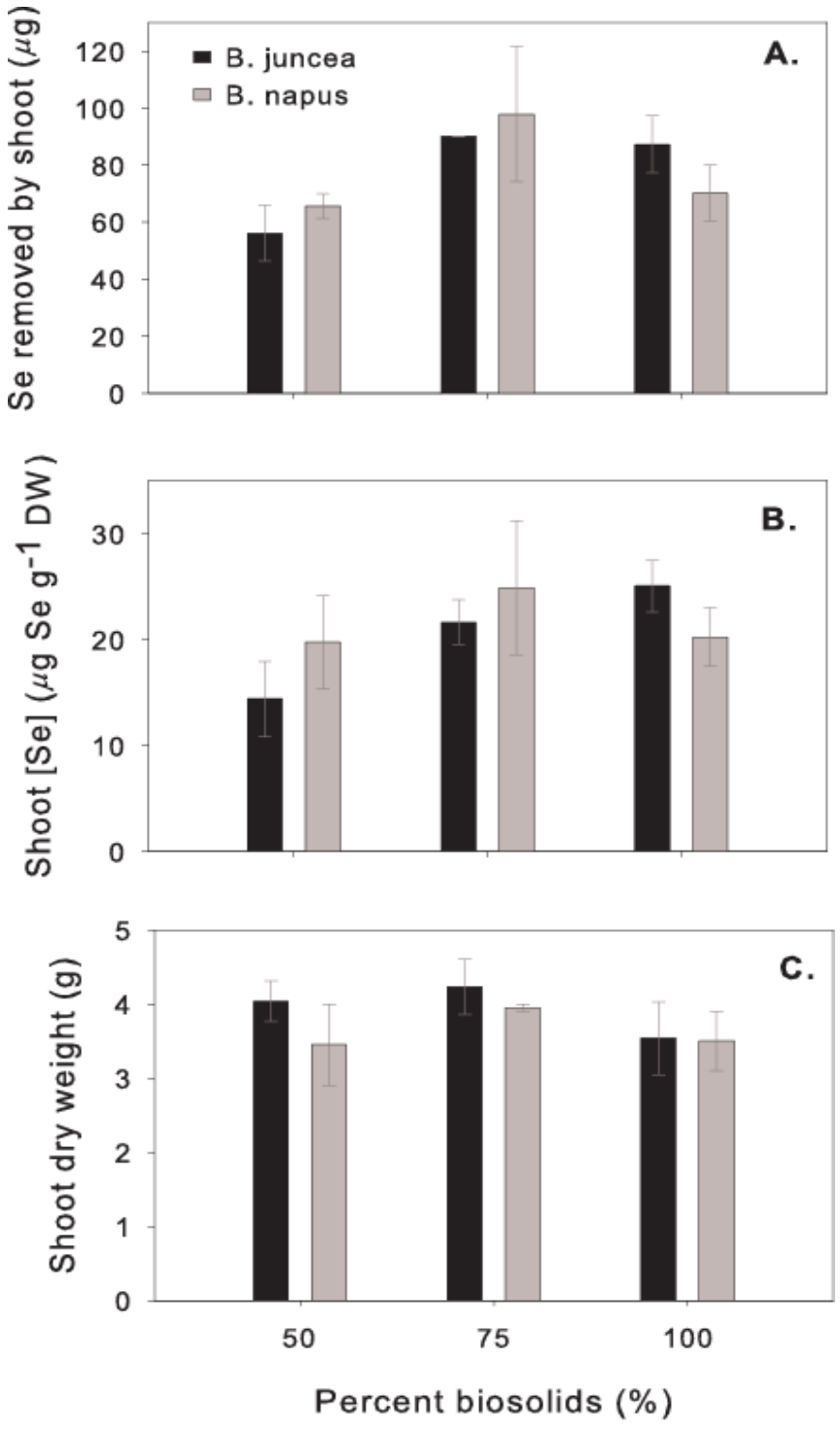

Fig. 5.3 Mean with standard error for the total amount of Se in the shoots (A), shoot [Se] (B), and shoot dry weight (C) of *B. juncea* (black bars) and *B. napus* (gray bars). Plants were grown in the Pueblo, CO municipal biosolids collected in 2008.

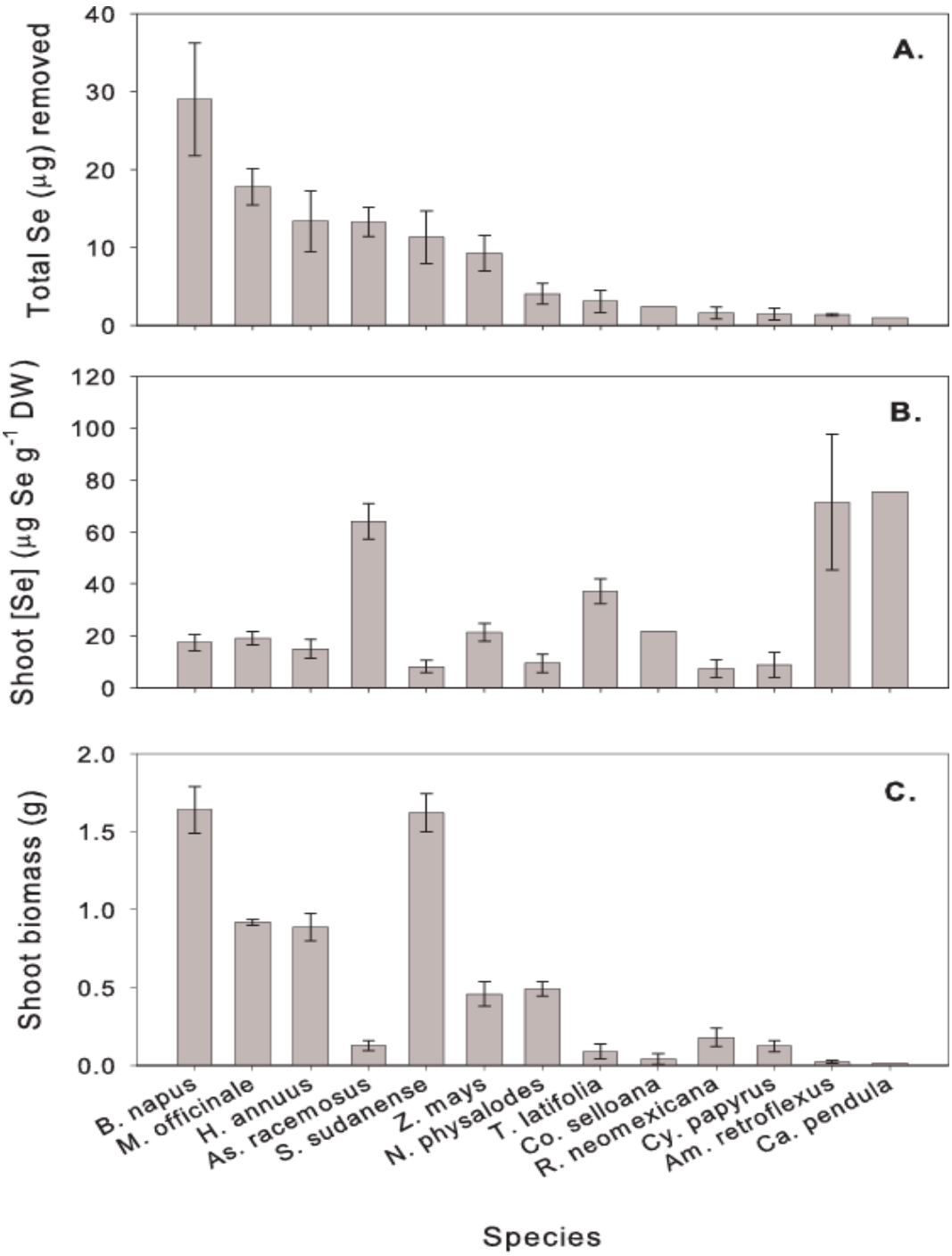

Fig. 5.4 Mean with standard error of the total amounts of shoot Se (A), shoot [Se] (B), and shoot dry weight (C). The data is presented here with soil inoculation treatments combined (n = 14) and plants were grown in the 2010 sample of municipal biosolids from Pueblo, CO.

Discussion

Since Se was present throughout the municipal biosolids phytoextraction could be an appropriate approach to removing Se. Anywhere that plant roots grow within the biosolids they will encounter Se. The three most abundant molecular species of Se were identified as organic C-Se-C, elemental gray Se, and selenite. Plants are able to take up organic Se forms and selenite, although the amounts of Se reaching the shoot differs between forms (Zayed et al. 1998). Without oxidation, elemental Se will likely not be accumulated by plant roots. Redox reactions occurring in the rhizosphere can alter Se availability to plants (Blaylock and James 1994) and microorganisms can slowly oxidize elemental Se to selenite and selenate in soils (Losi and Frakenberger 1998). Oxidation of elemental Se could enhance phytoextraction potential by increasing Se bioavailability.

Astragalus hyperaccumulators could be utilized effectively as species for Se-phytoextraction. In general the *Astragalus* hyperaccumulators removed more total Se per plant than the non-accumulators. In particular, *A. crotalariae* extracted the most Se from biosolids in any of the three trials. This species produced the highest amount of biomass by a Se-hyperaccumulator. Its high biomass production was coupled with a high [Se], resulting in removal of over 100 *μ*g Se. Sors et al. (2005) observed that *A. crotalariae* was a stronger hyperaccumulator than *A. bisulcatus* or *A. racemosus* in their experiments. Given their success in my trial several *Astragalus* hyperaccumulators, including *A. crotalariae, A. preussi,* and *A. racemosus*, have potential for future

development for phytoremediation. Currently a lack of available seed source precludes the large-scale use of *A. crotalariae* and *A. preussi*. Seed from *A. racemosus* is more readily available, which is why only that species was used in the subsequent inoculation experiment. However, use of any of the *Astragalus* species, like all hyperaccumulators, would require development of agronomic techniques to increase biomass production and harvestiblity to attempt phytoextraction on larger scales than greenhouse trials (Chaney et al. 2007).

Sufficient biomass production coupled with high trace element concentrations have been discussed as prerequisites for successful phytoextraction (Chaney et al. 2000; Krämer and Chardonnens 2001). Results from *A. cicer* show that high biomass production in *Astragalus* without a moderately high [Se] did not provide strong phytoextraction potential. If *A. cicer* had the same [Se] in its shoot (21.5 μg Se g^{-1} DW), it would have to amass 5.3 g of shoot biomass to surpass the Se phytoextraction performance of *A. crotalariae.* *A. preussi* removed the second highest amount of Se per plant after *A. crotalariae*. This performance was achieved by the species amassing the highest [Se] of any hyperaccumulator, 170% more Se than *A. crotalariae*. *A. racemosus*, which removed the third highest amount of Se per plant in the *Astragalus* trial, had a similar shoot [Se] to *A. praelongus*, but *A. racemosus* grew larger in the biosolids and was therefore able to acquire more Se. Alternatively, *A. cicer* acquired more total Se than *A. praelongus* because *A. cicer* produced more biomass even though *A. praelongus* had a higher shoot [Se].

In the *Brassica* phytoextraction trial I attempted to establish optimum mixtures of biosolids and sand to facilitate the highest amount of plant Se removal. In both *B. juncea* and *B. napus* the highest amount of Se removed per shoot was in the 75% biosolids and 25% sand mixture. This was achieved by the two species each growing largest in this mixture. *B. napus* had its highest shoot [Se] in this mixture, but *B. juncea* had the highest shoot [Se] in the 100% biosolids mixture. Mixing in sand at a rate of 75% volume of biosolids to sand provides a high level of Se phytoremediation potential balanced with a lower amount of additional inputs (sand). Of these two species *B. napus* may be a more attractive phytoremediation crop because its seed can be harvested for oil production including non-food uses such as biofuel production (Bañuelos et al. 2010).

Inoculants that have increased plant trace element concentrations have often been conducted with single isolates (Abou-Shanab et al. 2003; Turnau and Mesjasz-Przybylowicz 2003; Abou-Shanab et al. 2006; Li et al. 2007; Wu et al. 2009) that have often been ascribed a mode of action. A potential risk of this approach is that the establishment of the isolate in the rhizosphere is not known. As an example, establishment of new rhizobial strains can be difficult when rhizobia populations were already present in soils (Brockwell and Bottomley 1995). In addition, the complex characteristics of the rhizosphere community as a whole cannot be mimicked with a single organism (Andrews and Harris 2000). Since we do not fully understand the role of the rhizosphere community, or how it influences Se-hyperaccumulation I opted for a more general approach where I

applied a mixed inoculant slurry from soil collected where the hyperaccumulator *A. bisulcatus* grew. This approach has the same risk of not knowing if the microorganism community that was introduced produced an effective presence in the rhizosphere, plus the mode of action of the consortia is unknown. The benefit is that this approach would be easy to implement and would require minimal laboratory preparation to apply at larger scales.

Three distinct effects of microorganisms in Se cycling have been discussed in the literature. Inoculation with soil microorganisms could reduce the bioavailability of Se because some bacteria are known to reduce Se oxyanions (Di Gregorio et al. 2005; Di Gregorio et al. 2006; Gupta et al. 2010). Alternatively, bacteria have been shown to increase plant Se levels *in vitro* (de Souza et al. 1999a; de Souza et al. 1999b). In the rhizosphere Se bioavailability can be increased by oxidizing reduced forms of Se (Oram et al. 2011). Finally, soil microorganisms can volatilize Se (Doran and Alexander 1976).

I found no effect of inoculation on plant phytoextraction potential. This could be a result of insufficient time to produce differences in the two treatments. Although I found effects of soil inoculant altering plant [Se] earlier as described in Chapter 4, the mechanism by which this was achieved was not determined. Without this determination I cannot ascribe a conclusive reason for the lack of inoculation effect reported here. It may be that the amount of inoculum was a factor, as much less was used here in the phytoremediation trial than in the soil origin experiment. In addition, existing microorganisms in the municipal biosolids

may have competed with the microorganisms from the inoculum and precluded their establishment or effectiveness.

B. napus coupled moderate shoot [Se] (17.4 *μ*g Se g^{-1} DW) with high shoot biomass to have the maximum phytoextraction potential in the inoculation trial. The next two highest performers, *M. officinalis* and *H. annuus,* shared that similar strategy of high biomass coupled with moderate shoot [Se]. *Astragalus racemosus* had the highest concentration of shoot [Se], but had a relatively low biomass, which resulted in that species having the fourth highest phytoextraction potential. *Amaranthus retroflexus* and *Carex pendula* appear to have concentrated more Se in their shoots than *Astragalus racemosus*, but this result is likely to be somewhat erroneous because of the small amounts of biomass used in the ICP-AES digestion process. Regardless, their small biomass production precluded those two species from being effective at phytoextraction. Similarly, *R. neomexicana* produced little biomass, but it also had low shoot [Se]. This species is a perennial tree that would likely require a longer growing period than two months to more accurately gauge its phytoextraction potential. *S. sudanense* produced a large amount of biomass, but the shoot [Se] was too low for it to be very effective at removing Se from the biosolids.

In all three trials the maximum Se phytoextraction potential occurred with *Astragalus crotalariae,* where it removed 113.8 *μ*g Se per plant growing in 50% biosolids. The next highest performing species was *B. napus* with 98.0 *μ*g Se per plant removed in the 75% biosolids dilution trial. To estimate the amount of harvests (x) it would take to remove enough Se to reach the Se ceiling

concentration allowable for land application, 100 μg g^{-1}, I used the following calculation:

$$x = \frac{(V_{Biosolids} \times BD_{Biosolids})([Se]_{Biosolids} - 100)}{\text{Total Se}_{Plant}}$$

The parameters are defined as the volume of biosolids in the pot ($V_{Biosolids}$), the bulk density of the biosolids ($BD_{Biosolids}$), the [Se] in the biosolids ($[Se]_{Biosolids}$), and the total amount of Se removed by a shoot (Total Se_{Plant}). The constant, 100, refers to the ceiling [Se] for land application of biosolids determined by the US-EPA. Pot volumes of 131 cm^3 and a biosolids bulk density of 0.56 g cm^{-3} were used in the calculation. I estimated that it would take 18 crops of *A. crotalariae* or 31 crops of *B. napus* to reduce the Se to ceiling concentration levels. It must be noted that this calculation ignores the sand portion in the pots, which effectively dilutes the [Se] of the substrate within the pot. Further analyses on the Se amount and concentration within the pots are required to fully assess the phytoextraction potential of these species. Notably, Se volatilization could have contributed additional Se removal from the biosolids that was not measured in this experimental procedure.

These trials were conducted under controlled greenhouse conditions which have been shown to have higher Se phytoextraction results than plants growing under field conditions (Bañuelos et al. 1998). Although phytoextraction requires considerable time to achieve, Bañuelos (2006) suggested that the

development of products with beneficial uses could make phytoremediation a more financially attractive option. An alternative use that may be explored for this substrate could be as a Se-rich fertilizer for Se-deficient soils. Selenate application has been practiced on soils in Finland since the recommendation to include Se in fertilizer in 1983 (Venäläinen et al. 1997). Selcote Ultra ® (Nufarm New Zealand, Auckland, New Zealand) and Top Stock ® (Frontier Agriculture Ltd., Witham St. Hughs, United Kingdom) are selenium fertilizers for use in agriculture. Perhaps the use of Se-enriched green manures like the non-accumulator legumes *Astragalus cicer* or *M. officinalis* could be investigated. In addition, biofortification of crops with Se has been gaining interest in recent years (Lyons et al. 2003; Broadley et al. 2006; Zhu et al. 2009; Broadley et al. 2010). Appropriate precautions would need to be taken to ensure that any beneficial uses of these biosolids or Se-enriched plants derived from the biosolids are safe.

Conclusions

Developing successful phytoextraction technologies for these municipal biosolids would require either development of agronomic techniques and seed sources for *Astragalus* Se-hyperaccumulators, or a market for Se-enriched crops that may include canola or green manures. I estimated that a best case scenario for phytoextraction would require 18 crops of *A. crotalariae,* but further examination of the remaining amount of Se in the biosolids must be conducted before final conclusions can be drawn.

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Chapter 6

A Synthesis: Soil Microorganisms Contribute to Se-hyperaccumulation in *Astragalus*

Science is a process of learning. It starts with observing things in nature and wondering how and why what you observe works the way it does. Then you develop an idea or hypothesis about why and how these things work. Development of ideas comes with investigation of what others already know about the system. Once ideas that have been constructed are recorded the process of testing those ideas by experimentation ensues. By analyzing the results of the experiment you are able to refine your initial ideas about how the system you are investigating truly works.

In my case I had happened upon the distinctive plant *Astragalus bisulcatus* by chance one day while I was engaged in other work. I found the plant notable because of its large size and the garlicky smell emanating from magenta and purple flowers, rather than a light and sweet floral scent. Since I am a legumeophile and *Astragalus* is the largest constituent in the Fabaceae I became instantly intrigued. Through discussion I learned that the garlicky odor comes from methylated Se and *A. bisulcatus* has a somewhat unique trait of Se hyperaccumulation.

From there I became interested in the root nodule symbiosis in this species and the other Se-hyperaccumulators in the genus. I read about hyperaccumulation as a unique plant trait and found that little research had been conducted on these hyperaccumulators in the long history of plant science. In fact, an explosion of interest in the entire group of trace element hyperaccumulators had only very recently begun. Most of the information on hyperaccumulators focused on above-ground organs and interactions, while it

seemed obvious to me that belowground organs and interactions must be crucial for hyperaccumulation. With the help of my committee members I formalized this into a review paper that was published in Plant and Soil and also serves as the first chapter in this work.

From this viewpoint of realizing the importance of rhizosphere processes and the stark lack of investigation by others I developed the theme for my dissertation. I was lucky that my personal interest intersected with a knowledge gap. In addition, this knowledge gap has practical applications for biologically based pollutant remediation strategies.

I decided to investigate if and how soil microorganisms affect Se-hyperaccumulation. My hypotheses were that (i) soil microorganisms affect Se accumulation in plants; (ii) specifically the root-nodule symbiosis has a role in Se-hyperaccumulation; (iii) the mechanism for the role of root-nodule symbiosis in hyperaccumulation is through nitrogen allocation into selenoamino acids.

In the preceding four chapters I described experiments that I have conducted to establish if there is a role for soil microorganisms in Se-hyperaccumulation in *Astragalus.* At all times I have conducted experiments with a consortium of soil microorganisms that served as inoculants for plants. In Chapter 2 I found that Se occurs throughout nodules from Se-hyperaccumulators. I did not find evidence for a cost of tolerance where Se-hyperaccumulators exhibited diminished nodulation ability compared to non-accumulators. Results from chapter 3 indicated that amidst the consortia used as an inoculant a role for symbiotic organisms could be detected. Nodulation

increased shoot [Se] in Se-hyperaccumulators through increased levels of the selenoamino acid γ-glutamyl-methylselenocysteine. In chapter 4 I found that the source of the soil inoculant mattered for plant [Se] in both Se-hyperaccumulators and non-accumulators, where inoculum originating from Se-hyperaccumulators produced higher [Se] in both plant types. The experiments in Chapter 5 were an attempt to utilize this knowledge of inoculant effects on altering shoot [Se] to extract more Se from polluted substrates. Although I did not observe an inoculation effect in my attempt, it shows the importance of knowing the mechanism of these effects, and that there is still much more to learn. So, as is the way of science, it is back to the drawing board – time to refine the hypothesis and conduct more experiments so that the effect of inoculation can be effectively applied in other situations.

Now that I have some information about the mechanism that causes nodulation to increase [Se] in hyperaccumulators, I am still left wondering: how does this work? What is it about the metabolism of this symbiotic interaction that affects γGMSC? There was no similar effect in MeSeCys, a molecule widely regarded as a significant detoxification mechanism in *A. bisulcatus*. Is there an advantage for producing γGMSC? And thus the scientific process continues, there is the wonder - how does this work?

From this experience, I found that science requires a bunch of patience and persistence. As I have told my students, “if you like being wrong, you might like being a scientist.” I think this idea sums up much of the well-trodden path of scientific discovery. The really intriguing part of science that I have discovered is

that often it is being wrong (in terms of your research hypothesis) that is more interesting and rewarding than the being “right”.

In my work over the past 5 years I have significantly increased the amount of information regarding belowground interactions of Se-hyperaccumulators. No one has previously shown an effect of nodulation on Se-hyperaccumulation. This opens up new opportunities for further exploration of this root nodule symbiosis. In addition my findings may help shed light on the evolution and ecology of root nodule symbioses in general. Perhaps this will help to eventually explain why there are so many species of *Astragalus*. Is it related to their ability to specialize and adapt their symbiotic interactions to suit their lifestyle?

I have also conducted one of the few projects that have investigated pollutant removal from biosolids. Often researchers have investigated the effects of land applied biosolids or how plants extract trace elements from the substrate once it is applied to land. My phytoextraction trials showed that upland plants can be successfully grown in 100% biosolids without any amendments. In addition, I have shown that some species of *Astragalus* Se-hyperaccumulators have potential for use in phytoremediation settings. My greatest joy in this work has been the ability to conduct basic ecological science that has informed the design and implementation of a project geared to help resolve a real-life issue.

Appendices

Appendix 1. Mean [Se] (μg Se g^{-1} DW) with standard error in different legume organs collected from the field. Some organs were not collected and elemental concentrations were not determined (--). The asterisk (*) indicates that n = 1 for that sample.

Species	Leaf	Flower	Pod	Root	Nodule
Non-accumulators					
A. argophyllus	28 ± 6	31 ± 9	--	19 ± 9	0*
A. convallarius	50 ± 14	--	--	21 ± 11	--
A. missouriensis	52 ± 18	32 ± 16	26 ± 14	32 ± 8	--
*M. alba**	39	40	28	17	26
M. officinalis	35 ± 14	--	--	35 ± 8	--
O. sericea	8 ± 2	16 ± 4	5*	18 ± 4	7 ± 7
Hyperaccumulators					
A. bisulcatus	148 ± 35	252 ± 73	131 ± 80	13 ± 5	26 ± 6
A. praelongus	600 ± 153	959 ± 340	1,557 ± 349	171 ± 59	40 ± 8
A. racemosus	89 ± 36	57*	31 ± 12	31 ± 7	40 ± 14
A. rafaelensis	487 ± 101	1,261± 890	975 ± 288	--	--

Appendix 2. Mean values for nodulation index and dry weight per nodule (mg) are shown with standard error for each of the *Astragalus* species.

Species	Nodulation Index	Nodule dry weight (mg)
A. bisulcatus	66.7 ± 7.6	0.822 ± 0.145
A. convallarius	26.0 ± 3.6	1.642 ± 0.335
A. drummondii	51.9 ± 6.2	0.972 ± 0.178
A. praelongus	39.3 ± 6.4	1.852 ± 0.348
A. racemosus	58.8 ± 5.8	1.292 ± 0.141
A. shortianus	67.2 ± 9.2	0.920 ± 0.167

Appendix 3. Mean shoot dry weights (mg) with standard error in different *Astragalus* species. Soil origin (home or foreign) and Se treatment (0 µM or 100 µM) are shown separately (n = 8).

	0 µM		100 µM	
Species	**Home**	**Foreign**	**Home**	**Foreign**
A. bisulcatus	124 ± 25	167 ± 25	115 ± 21	164 ± 19
A. convallarius	196 ± 38	583 ± 132	302 ± 60	388 ± 57
A. drummondii	189 ± 14	222 ± 39	213 ± 29	132 ± 30
A. praelongus	636 ± 133	387 ± 123	427 ± 71	735 ± 143
A. racemosus	1,081 ± 114	716 ± 38	964 ± 84	825 ± 70
A. shortianus	122 ± 14	81 ± 9	100 ± 21	69 ± 11

Appendix 4. Mean root dry weights (mg) with standard error in different *Astragalus* species. Soil origin (home or foreign) and Se treatment (0 µM or 100 µM) are shown separately (n = 8).

	0 µM		100 µM	
Species	**Home**	**Foreign**	**Home**	**Foreign**
A. bisulcatus	144 ± 34	177 ± 36	120 ± 24	106 ± 19
A. convallarius	88 ± 25	221 ± 37	165 ± 24	257 ± 45
A. drummondii	159 ± 15	218 ± 38	142 ± 19	106 ± 28
A. praelongus	408 ± 116	326 ± 130	285 ± 85	592 ± 164
A. racemosus	699 ± 120	474 ± 29	703 ± 86	623 ± 73
A. shortianus	250 ± 35	122 ± 24	188 ± 44	89 ± 19