DISSERTATION

# PLANT-MICROBE INTERACTIONS OF SELENIUM HYPERACCUMULATORS: EFFECTS ON PLANT GROWTH AND SELENIUM METABOLISM

Submitted by

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ABSTRACT

PLANT-MICROBE INTERACTIONS OF SELENIUM HYPERACCUMULATORS: EFFECTS ON PLANT GROWTH AND SELENIUM METABOLISM

Selenium (Se) hyperaccumulator (HA) plants can accumulate up to 1% of their dry weight as Se without any signs of toxicity. Since Se is not essential for plants, it is intriguing why these plants accumulate it to such high levels, and it is also interesting how they manage to accumulate and tolerate so much Se. Plants have been shown to take up and metabolize Se via the sulfur (S) assimilation pathway, due to chemical similarity between Se and S. The replacement of S by Se in proteins is toxic. The mechanism of hyper-tolerance in Se HA species is that they metabolize Se to methylselenocysteine (MeSeCys), a non-protein amino acid that can be stored safely. The mechanism of hyperaccumulation remains to be elucidated. As for the functional significance of hyperaccumulation, the concentration of the toxic element Se likely serves ecological functions i.e. protection from Se-sensitive herbivores and pathogens (elemental defense), and in competition with Se-sensitive neighboring plants (allelopathy). Interestingly, there is also mounting evidence of Se-resistant herbivores and plants that benefit from their association with Se HA plants. Thus, the high levels of Se in and around Se HA plants likely affect local ecology. Through negative effects on Se-sensitive ecological partners and positive effects on Se-resistant partners, HA species likely affect species composition at multiple trophic levels, as well as Se distribution and cycling.

Plant-animal and plant-plant interactions have received considerable attention in the last decade, still relatively little is known about the interactions of Se HA plants with microbes, the topic of this dissertation. From previous ecological studies there is some evidence of Se-tolerant microbes that live in association with HAs. Many of these microbes live in the root rhizosphere, and Se-resistant microbes have also been found on decomposing high-Se leaf litter. The specific nature of the Se HA-microbe

relationship is not known. It is reasonable to assume there are successful pathogenic microbes of Se HAs that have evolved Se resistance. There may also be mutualistic microbes that stimulate HA growth, aid in nutrient and water acquisition, or fight off pathogens. Microbes may even affect the acquisition, speciation and accumulation of Se.

As a first step in elucidating the specific interactions of HA plants and microbes, I characterized the Se localization and chemical speciation in roots of the HA *Astragalus bisulcatus*, commonly known as the two-grooved milk vetch (Fabaceae) and *Stanleya pinnata*, or Prince's plume (Brassicaceae), collected from a seleniferous area in Fort Collins, CO. The focus of this study was on the root since similar studies had already been done on all above-ground organs, and the root is of particular interest for rhizospheric plant-microbe interactions. I conducted X-ray microprobe analysis at the Advanced Light Source in Berkeley, CA to obtain a map of Se and other elements in taproot cross sections of the two HA species. Micro-X-ray Fluorescence (XRF) mapping revealed that Se was present throughout the roots, with the highest levels in the periphery (cortex) and lowest in the vascular tissue (stele). Further investigation with μ-X-ray near edge spectroscopy (XANES) revealed that the main form of root Se in both species was an organic C-Se-C compound (48-95%), likely MeSeCys. In addition, a surprisingly high percentage of Se was present as elemental Se ($Se^0$). Up to 35% of the Se was found to be $Se^0$, a form so far not reported in plants, but that has been reported to be produced by Se-resistant bacteria and fungi. Four fungi collected previously from HA roots were characterized with respect to their Se tolerance and ability to produce $Se^0$, and then used to inoculate HA plants in a controlled greenhouse study. X-ray microprobe analysis showed that three of fungi could produce $Se^0$. The roots of the greenhouse-grown HAs *Astragalus racemosus* and *S. pinnata* showed similar Se distribution patterns regardless of inoculation treatment, and contained almost exclusively C-Se-C. In fact, there was not even a minor fraction of $Se^0$ detected in the greenhouse-grown plants, with the exception of areas associated with microbial activity. A substantial fraction of $Se^0$ was found inside *A. racemosus* root nodules (that contain nitrogen-fixing

bacteria), and some $Se^0$ was observed on the surface of *A. racemosus* roots inoculated with *Alternaria astragali*. Thus, root Se speciation was strikingly different in HA plants collected from their natural habitat as compared to when grown from surface-sterilized seeds in the greenhouse. It appears that $Se^0$- producing endosymbionts, including nitrogen-fixing bacteria and to some extent the rhizosphere fungus *A. astragali* can affect Se speciation in the HA root, in the peripheral areas. Inoculation with rhizosphere fungi did not alter internal root Se speciation to the extent as was observed in field-collected HA taproots.

In the greenhouse inoculation studies I also investigated the effect of the rhizosphere fungi on plant growth and Se accumulation. Two *Astragalus* species, HA *A. racemosus* and the non-HA *Astragalus convallarius*, were inoculated with *A. astragali* (A3) and *Fusarium acuminatum* (F30), which were originally isolated from *Astragalus* HA species. Inoculation did affect growth in both species; A3 enhanced growth of *A. racemosus* yet inhibited growth of *A. convallarius*. Selenium treatment negated these differences in growth, perhaps because the A3 fungus is inhibited by Se. F30 reduced shoot-to-root Se translocation in *A. racemosus*, perhaps because of fungal trapping of Se on the root surface. X-ray microprobe analysis revealed no apparent differences between the inoculation treatments, but showed that the two *Astragalus* species differed in Se localization as well as chemical Se speciation. In the HA, root Se was distributed throughout the taproot as well as the lateral root, and relatively less Se was present as organic Se in the lateral root (90%) compared to the taproot (100%). In the non-HA root, Se was found to be concentrated in the extreme periphery of the taproot and in the vascular core in the lateral root. The taproot of the non-HA also contained mainly C-Se-C, yet there was a significantly lower percentage of organic Se in the lateral root of the non-HA (49%) as compared to the HA. Conclusions from this study were that the two *Astragalus* species differ in Se metabolism, and that rhizosphere fungi can affect translocation of Se.

In a similar experimental approach, HA *S. pinnata* and related non-HA *Stanleya elata* were inoculated with Alternaria *seleniiphila* (A1) or *Aspergillus leporis* (AS117), two rhizoplane fungi isolated from *S. pinnata*. Growth of *S. pinnata* was not affected by inoculation or by Se. *Stanleya elata* growth was inhibited by the presence of AS117 and by Se, but the combination of both treatments did not reduce growth of this non-HA. Selenium translocation was reduced in inoculated *S. pinnata*, and inoculation reduced S translocation in both the HA and non-HA species. X-ray microprobe analysis did not reveal inoculation-related differences in root Se distribution and speciation in either species; both species accumulated mainly (90%) organic Se. Sulfur, interestingly, was present equally in organic and inorganic forms in *S. pinnata* roots, suggesting Se-specific assimilation in this species. In conclusion, these rhizosphere fungi can affect growth and Se and/or S accumulation in *Stanleya*, and the effects were dependent on host species. The fungi generally enhanced root accumulation and reduced translocation. These effects could not be attributed to altered plant Se speciation but may involve altered rhizosphere speciation, as these fungi were shown to produce $Se^0$.

In the earlier studies, it was hypothesized that the root $Se^0$ was microbe-derived, yet none of the four rhizosphere fungi selected significantly affected plant Se speciation. Therefore, my attention shifted to another possible candidate. A fungus, shown to be an abundant seed endophyte in HA *A. bisulcatus* was identified as *Alternaria tenuissima* (A2) and was shown by X-ray microprobe analysis to be capable of producing $Se^0$ when supplied with selenite as well as when growing on *A. bisulcatus seed*. Uninfected *A. bisulcatus* seeds contained 100% organic Se (MeSeCys), which was located in the seed embryo but not the seed coat. Seeds harboring A2 contained up to 22% $Se^0$, as did the A2 mycelium growing on the seed. The production of $Se^0$ by A2 likely serves as a fungal Se tolerance mechanism. Surprisingly, while A2 successfully colonized seeds containing 10,000 mg $kg^{-1}$ MeSeCys, it was sensitive to 25 mg $kg^{-1}$ Se from flower extract or when supplied in the form of pure MeSeCys. Thus, this fungus likely occupies low-Se areas of the plant, such as in the seed coat and the apoplast. There is evidence that A2 may be

present throughout the plant, since $Se^0$ was also found in the root of a 5d old seedling as well as in the stem of a field-collected plant. Moreover, an endophytic fungus morphologically indistinguishable from A2 could be cultured from surface-sterilized stems. Although *A. tenuissima* is a known plant pathogen, it stimulated growth of *A. bisulcatus* up to 3-fold. A2-containing seedlings had lower shoot Se and S levels than seedlings from uninfected seeds. Perhaps less Se was available for translocation due to the formation of insoluble $Se^0$ in the root. It was concluded from this study that this fungal endophyte, *A. tenuissima*, can alter plant Se speciation and may contribute to the $Se^0$ observed in HA roots.

The studies described in this dissertation have shown for the first time Se distribution and speciation in roots of Se HA plants. The main form of Se was C-Se-C, likely MeSeCys, which has also been found in other plant parts. The most interesting finding was that roots from HA plants growing in the field contained a substantial fraction of $Se^0$, which has not been shown before in plants, and which was also not found in greenhouse-grown counterparts of these HA species. Among the four rhizosphere fungi and one endophytic fungus tested, all but one demonstrated the ability to produce $Se^0$ in pure culture. Furthermore, rhizospheric *A. astragali* and particularly endophytic *A. tenuissima* appeared to have a capacity to bring about the production of $Se^0$ in plants. Many of the fungi also affected plant growth as well as Se and S accumulation. The *Alternaria* species stimulated growth in HA hosts, and fungal inoculation was often associated with reduced Se and S translocation. This may be caused by the trapping of Se in the form of insoluble $Se^0$ in the root zone.These results shed light on plant-microbe interactions of Se HA species and suggest that microbes affect Se speciation and accumulation in these species as well as in related non-HAs. These findings may be useful for applications in Se phytoremediation or biofortification. Microbes identified to alter plant Se speciation to inert $Se^0$, and to reduce Se translocation may be useful in applications where toxicity to herbivores and movement of Se into the food chain is a concern.

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# Chapter 1

## Introduction

A brief history of selenium toxicity

Selenium (Se) is a naturally occurring element in most soils and can be found at very high levels in alkaline soils where Cretaceous shale or other seleniferous rocks are the soil parent material (Byers 1935, 1936; Rosenfeld and Beath, 1964; Beath 1982). Seleniferous soils range between 2-100 mg Se $kg^{-1}$, whereas non-seleniferous soils contain less than 1 mg Se $kg^{-1}$, (Mikkelsen et al., 1989; Beath et al., 1939). Selenium was discovered by Jons Jacob Berzelius in 1817 as a red deposit on the walls of a lead chamber used to make sulfuric acid; he was investigating the illnesses of several workers at the plant. He named the element after the Greek goddess of the moon Selene, perhaps because it drove the sick workers to crave eternal sleep. Selenium is chemically similar to sulfur (S), and therefore Se toxicity is thought to occur if Se levels are high enough to replace the essential macronutrient S in proteins, leading to misfolding and loss of function (Stadman 1996). Symptoms of Se poisoning include, "garlic breath", from dimethylselenide, gastro-intestinal problems, fatigue, transverse lines on finger nails, alopecia (loss of hair and nails) and peripheral neuropathy (Robinson 1989). The earliest known recordings of Se toxicity were by Marco Polo in 1295 during his travels along China's silk-road. The beasts of burden (pack animals) were reported to be very ill after eating some local vegetation (Ihnat 1989). This ailment was also recognized very early in the American West. In 1860, T. C. Madison reported sick cavalry horses that, after they lost hooves, could not get food or water, and eventually died (Ullrey et al. 1983). General Custer may have survived his infamous last stand if the animals of the cavalry had not been foraging on the native vegetation. The plants these animals were consuming are the so-called Se hyperaccumulators (HAs) and can concentrate Se up to 1.5% of their dry weight. Hyperaccumulation of Se has been reported for around 30 plant species, most are in the genus *Astragalus*; examples are *A. bisulcatus* and *A. racemosus* (Beath et al. 1939). The genus *Stanleya* also contains at least one Se

hyperaccumulating species, *S. pinnata* (Feist and Parker, 2001). Selenium HAs are found exclusively in high Se soils and typically have Se levels 100-fold greater than the surrounding vegetation (Beath et al. 1939). Selenium toxicity remains a problem in the American West as well as in other areas worldwide. Animal grazing on Se HAs is one source; another is the use of seleniferous soil for irrigated agriculture, or the use of seleniferous fossil fuels (Terry et al. 2000). Agricultural or industrial wastewaters from these applications end up in surface waters where the Se can get concentrated to levels that cause toxicity to fish and wildlife, and their consumers. A notorious example is the environmental disaster that happened in the early 1980s at the Kesterson reservoir in California (Ohlendorf et al. 1986).

Se as a beneficial element

Selenium was first recognized as a micro-nutrient for lab rats in 1957, and in 1973 it was discovered that Se is an essential component of the enzyme glutathione reductase in humans (for a review see Zhang and Gladyshev, 2009). Selenocysteine (SeCys) is now recognized as the 21$^{st}$ aminoacid in mammals, bacteria and algae (Stadtman, 1990). There are 25 recognized selenoproteins in humans, all of which have redox functions (Zhang and Gladyshev, 2009). Dietary Se supplementation has been shown to enhance overall antioxidant capacity, and associated with that reduce susceptibility to certain cancers as well as to infectious diseases (Sors et al., 2005). The chemical form of Se is important for the level of protection as well as for the toxicity of Se (Ip et al., 2000). Selenate and inorganic forms of Se may cause oxidative stress, whereas organic forms are less likely to produce free radicals when consumed in the correct dosages (Grant et al., 2011).

Plant Se metabolism

Selenium has not been shown to be essential for higher plants. During evolution onto dry land, higher plants are thought to have lost the SeCys insertion machinery essential for making selenoproteins (Hartikainen, 2005; Zhang and Gladyshev, 2009). However, many plant species produce organic seleno-

compounds and derive a physiological benefit from Se supplementation (Pilon-Smits et al., 2009). Lettuce, soybean, and ryegrass biofortified with Se had greater glutathione peroxidase and superoxide dismutase activity, and reduced lipid peroxidation (Hartikainen et al., 2000; Xue et al., 2001; Djanaguiraman et al., 2005). Plants take up Se inadvertently via S transporters, due to the chemical similarity between Se and S. Please see figure 1.1 for a diagram of plant Se metabolism.

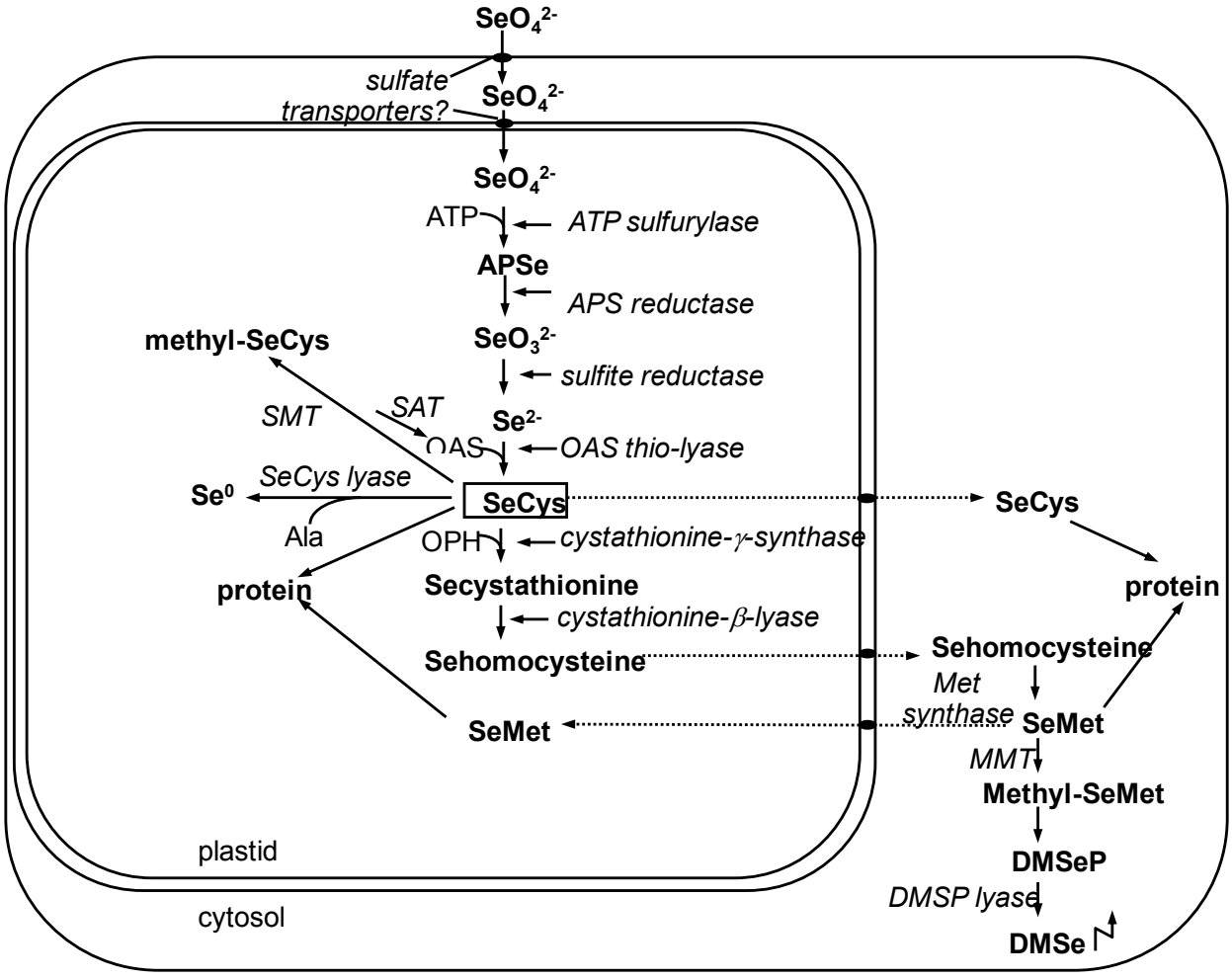


Figure 1.1 Selenium metabolism in plants

Selenate is taken up by plant sulfate transporters, and assimilated via the sulfate assimilation pathway into amino acids and other organic compounds including dimethylselenide (DMSe), which is volatile (Terry et al., 2000). In In HA plants, Se metabolism is markedly different compared to other plant species. Se HAs detoxify Se by adding a methyl group to selenocysteine (SeCys) via the enzyme SeCys

methyltransferase (SMT); the resulting methyl-SeCys (MeSeCys) can be accumulated safely, since it is a non-protein amino acid (Neuhierl and Bock, 1996). HAs can also convert MeSeCys to volatile dimethyldiselenide (DMDSe). Both DMSe and DMDSe are unstable and return to the soil as organic Se several days after volatilization (Martens and Suarez 1999; Kubachka et al. 2007).

X-ray microprobe analysis at a synchrotron particle accelerator can be used to obtain information about the chemical form (speciation) and tissue distribution (localization) of elements in intact biological material. Electrons are loaded into an accelerator ring; energy is emitted in the form of X-ray photons as the electrons travel the loop. Photons are focused into a narrow (16μm x 5μm) X-ray microbeam and directed

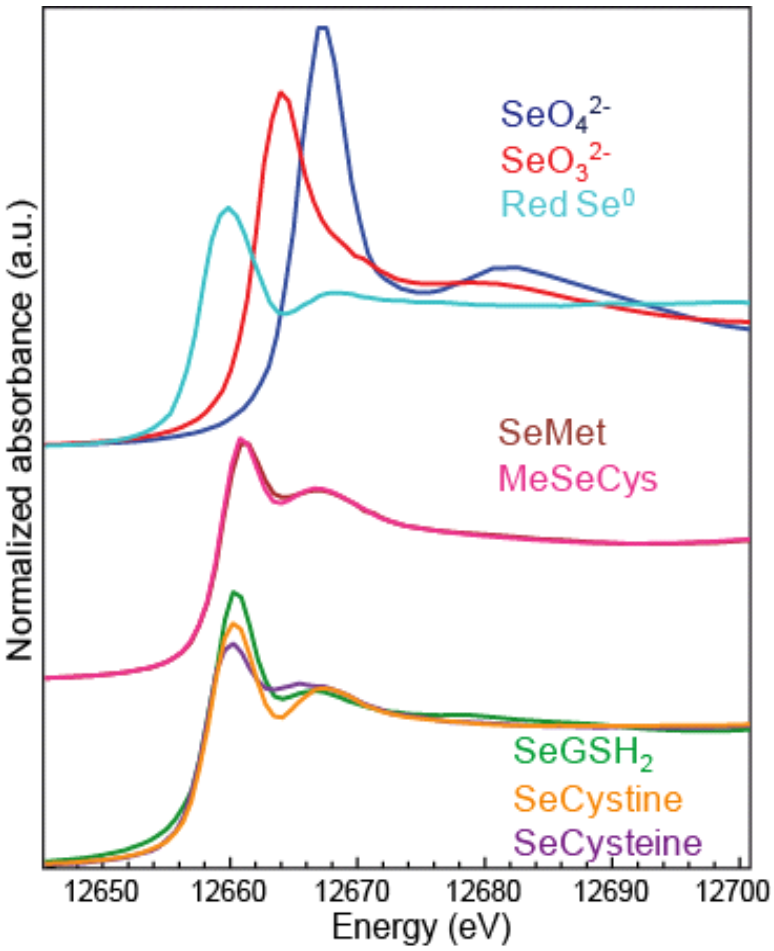


Figure 1.2 XANES spectra of different selenium compouds

Absorption of X-ray energy, or fluorescence due to X-ray induced excitation of atoms in the sample is read by a detector attached to the beam. Mapping the location of certain elements is achieved by X-ray Fluorescence (XRF); chemical speciation for a specific element is determined by μ-X-ray Absorption Near Edge Spectroscopy (XANES, see Fig. 1.2 for Se-spectra from standard compounds). Analyses by XANES have confirmed that MeSeCys is the primary form of Se found in young and old leaves of *A. bisulcatus* as well as in young leaves and

flowers of *S. pinnata* (Pickering et al., 2003; Freeman et al., 2006a; Quinn et al., 2011a). HAs also appear to have specialized storage areas for Se, as determined by XRF. In leaves of HAs, Se was found predominantly in peripherial areas: leaf margins, epidermis, and trichomes (Freeman et al., 2006a). Non-HA species were found to accumulate Se mainly in the vascular tissues, in the form of selenate (de Souza et al., 1998a; Van Hoewyk et al., 2005; Freeman et al., 2006a). Furthermore, HAs accumulate Se particularly in flowers and seeds, while non-HAs show the highest Se levels in leaves (Quinn et al., 2011a). It therefore appears that Se is sequestered by HAs in specialized areas in the periphery of the plant, as well as in the most precious structures, the reproductive organs. These patterns may be associated with plant defense, as discussed below.

Ecology of Se HAs

The ecological implications of Se accumulation are many-faceted, and impact all trophic levels (see figure 1.3). Of the hypotheses generated to explain why plants hyperaccumulate toxic elements like Se (Boyd and Martens, 1992), most evidence supports the elemental defense

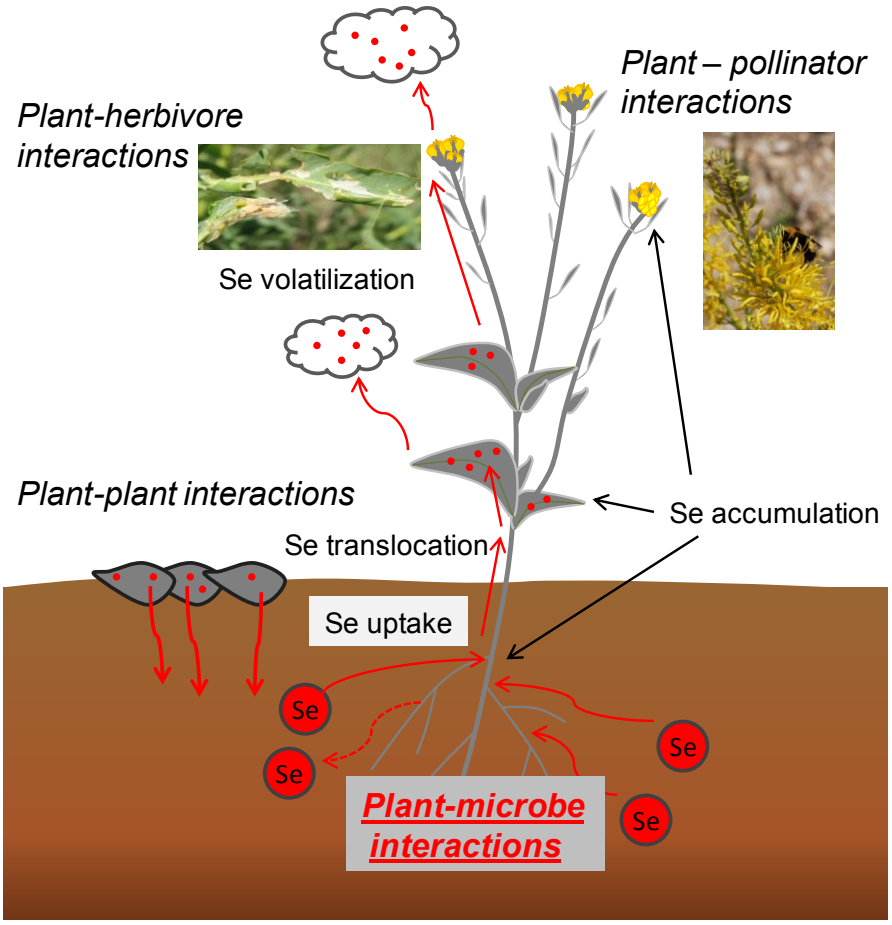


Figure 1.3 Selenium movement in and around plants, and ecological interactions it may affect, or that may affect it.

hypothesis. This states that the hyperaccumulated toxic element protects plants from pathogens or herbivores. Selenium accumulation was shown to protect both HAs and non-HAs from a variety of herbivores, both due to toxicity and deterrence (for a review see El Mehdawi and Pilon-Smits, 2012). HAs *A. bisulcatus* and *S. pinnata* were protected by Se from grasshoppers, moth larvae, thrips and spider mites, as well as from prairie dogs (Freeman et al. 2006b, 2009). Also, a field study comparing HA plants with non-HAs found more arthropods and arthropod species living on non-HAs than on HAs (Galeas et al. 2008). Similarly, the non-HA and crop species *Brassica juncea* (Indian mustard) was protected by Se from several important arthropod pests (aphids, butterfly/moth caterpillars, grasshoppers) as well as prairie dog herbivory (Bañuelos et al. 2002; Hanson et al. 2003, 2004; Freeman et al. 2009). Thus, even at lower levels Se can effectively protect plants from herbivores. This was also shown in studies where Se was added to an artificial diet, to levels equivalent to those found in *B. juncea*, which was confirmed to be toxic to *Spodoptera exigua* (beet armyworm) and the fly detrivore *Megaselia scalaris* (Vickerman and Trumble 1999). Indian mustard is popular as a Se-accumulating crop because it accumulates Se to 0.1% of its dry weight without significant toxicity, and is fast growing and high-biomass producing as compared to Se HA species. Indian mustard may be used to clean up areas with excess Se (phytoremediation), while producing Se-enriched material that may be used to alleviate Se deficiency in livestock or humans (biofortification).

However, as is commonly the case in biological "arms races", over time ecological partners have evolved resistance to the Se elemental defense (El Mehdawi and Pilon-Smits, 2012). Several Se-resistant symbionts of HAs have been reported. For instance, a Se-tolerant diamond back moth was found to feed on *S. pinnata* containing 0.2% Se of dry weight without ill effects; the moth accumulated MeSeCys, like its host, which can safely be accumulated and thus appears to be the basis of its Se tolerance (Freeman et al., 2006b). Similar associations were found between *A. bisulcatus* and three species of seed herbivores as well as two leaf herbivores (Valdez et al., 2012). Since Se is toxic to invertebrate

herbivores, and deters them, it may be hypothesized that plant Se accumulation affects plant-pollinator interactions as well. Pollinators may be deterred by, or suffer toxicity from the high Se found in flowers of HAs. Alternatively, pollinators may receive a benefit from Se in flowers such as increased antioxidant capacity, or protection from predators by elevated Se. Pollinator studies conducted at a seleniferous site found that honeybee and other pollinator visits were the same on Indian mustard flowers with less than 10 mg Se $kg^{-1}$ as compared to Indian mustard flowers with 230 mg Se $kg^{-1}$. The effect on pollinator health of foraging on high Se nectar/pollen is not known, but it is clear that Se-rich nectar and pollen is foraged by pollinators at high-Se sites. Honeybees and bumble bees collected while visiting a *S. pinnata* flower were shown to contain high levels of Se in pollen sacs on theirlegs, and lower levels were also present in their tissues (Quinn et al. 2011a). Future studies exploring how Se affects reproductive properties, pollination and pollinator health will be important to assess the potential ecological implications of Se phytoremediation.

Plant-plant interactions have also been shown to be impacted by Se hyperaccumulation. *Arabidopsis thaliana*, a Se-sensitive species, was reported to have poor germination and growth on high Se-soil collected from beneath Se HAs in the field, versus seeds that were germinated on low Se-soil from under the canopy of non-HA plants at the same site (El Mehdawi et al. 2011a). In contrast, plants found growing next to HAs in the field accumulated more Se, were larger and were more protected from arthropod herbivores compared to the same plant species growing far away (El Mehdawi et al. 2011b).

Plant Se accumulation has also been shown to offer protection from microbial plant pathogens. Indian mustard plants treated with Se were less susceptible to a fungal leaf pathogen (*Alternaria brassicicola*) and a fungal root/stem pathogen (*Fusarium sp.*), compared to control plants not supplemented with Se (Hanson *et al*., 2003). Thus, Se hyperaccumulation now is considered a form of elemental defense (Boyd and Martens, 1992; Boyd, 2007, 2010). This may have important implications for Se phytoremediation

or biofortification: when growing Se-accumulating plants there may be less need for chemical antimicrobial agents and pesticides, and less biomass loss due to these pests.

Following a similar pattern to that observed for herbivores and plant neighbors, there are also many microbial symbionts of HAs that have evolved Se- resistance. For instance, Se-tolerant litter-decomposing microbes appear to be present in seleniferous habitat, and play an important role in breaking down HA plant material, and recycling Se in the local ecosystem (Quinn et al., 2011b). A litter decomposition study on seleniferous soil revealed that high-Se litter from HA *A. bisulcatus* decomposed faster and harbored significantly more microbes than low-Se litter of *Medicago sativa* (Quinn et al., 2011b). In another study, rhizosphere fungi from Se HAs were found to be significantly more Se-resistant than comparable fungi from a non-seleniferous area (Wangeline et al., 2011). Several Se-tolerant fungi cultured from the roots of HAs *A. bisulcatus* and *S. pinnata* were found to accumulate substantial Se levels and were not inhibited by Se (Wangeline and Reeves, 2007). One example is a *Fusarium species* (F30) in which growth and UV recovery were stimulated by Se (Wangeline, 2007). There also is a pathogenic oomycete, possibly *Albuga*, which grows successfully on high-Se *S. pinnata* leaves in the field, (Ami Wangeline, personal communication). These microbes may use a variety of Se resistance mechanisms, including Se reduction to insoluble, non-toxic elemental Se ($Se^0$), volatilization, and conversion to MeSeCys (Frankenberger and Karlson, 1994; Losi and Frankenberger, 1997; Hunter and Manter, 2009).

The effect of microbes on plant Se (hyper)accumulation

While the presence of Se-resistant microbial symbionts of HAs has been documented, as described above, there is still very little known about the importance of Se for the plant-microbe interactions of Se HA plants or, conversely, the potential role of microbes in plant Se hyperaccumulation. In general, plant-microbe interactions can be mutualistic, pathogenic, and/or saprophytic. The nature of this interaction

may vary, depending on the conditions. Plant-fungal mutualisms can become pathogenic if the plant becomes stressed, and/or saprophytic after the plant dies. The tendency for the nature of plant-microbe interactions to change depending on the environmental conditions or the health or nutritional status of the plant has been well documented for many grass symbionts in the *Alternaria* genus (Dugan and Lupien 2004). Some of the known beneficial effects of microbes on plants include stimulation of plant root growth and changes in root morphology (de Souza et al. 1998b), protection from pathogens and herbivores, as well as from abiotic stresses such as drought, salt, heat, and toxic elements (for a review see Harmon 2011). Many plants cannot survive without their fungal symbiont(s), indicating an essential role of plant-fungal mutualism in the evolution of plant stress tolerance (Rodriguez and Redman 2008). A similar dependence on microbial symbionts may have played a role in the evolution of hyperaccumulation.

Even less is known about whether and how Se HA-associated microbes influence plant Se uptake, metabolism and tolerance. Rhizospheric microbes may make Se more or less bioavailable to plants. Microbes may stimulate plant Se uptake and assimilation, and stimulate plant root growth leading to a larger Se uptake capacity. Or, microbes may reduce plant Se uptake and assimilation. It has been shown that the presence of rhizosphere bacteria enhances Se accumulation and volatilization in Indian mustard, as well as certain wetland species (de Souza et al., 1999).The mechanism proposed for how Se levels were enhanced in these plants is an increase in root hair density and higher rhizosphere levels of the aminoacid (O-acytyl)serine, which has been shown to stimulate S uptake and metabolism in plants.

In addition to free-living microbes in the soil surrounding Se HAs, there are microbes that live inside of the plant tissue; these are called endophytes. Most plants tested so far contain multiple bacterial and fungal endophyte species, which can colonize all plant tissues and be transmitted horizontally (to neighboring plants) or vertically (via the seeds, to the next generation) (for reviews see Saikkonen 1998;

Sturz 2000). Endophytes have been successfully cultured from Se HA stems and roots (Jose Rodolfo Valdez, personal communication). It is feasible that endophytes with high Se tolerance, accumulation or volatilization facilitate plant Se accumulation, volatilization and/or tolerance. Increased understanding of the role microbes play in Se accumulation and hyperaccumulation will prove to be valuable when designing phytoremediation and biofortification projects.

In the studies described here, Se speciation and distribution were characterized in roots of *Astragalus* and *Stanleya* HAs with the goal to obtain better insight into below-ground Se metabolism and the potential of microbial symbionts to affect Se speciation and distribution in host tissues. In chapter 2, XRF and XANES were used to compare the distribution and speciation of Se in taproots of *S. pinnata* and *A. bisulcatus* collected from the field with those collected from surface-sterilized, greenhouse-grown plants of the same species. In Chapters 3 and 4, *Astragalus* and *Stanleya* HAs and non-accumulator species were inoculated with rhizosphere fungi collected from HAs, to test whether these fungi can alter root Se speciation and/or plant Se accumulation and tolerance. The rationale was that inoculation with these fungi may result production of $Se^0$ in the roots, since fungi have been shown to be able to reduce Se to the elemental form, a form of Se that has so far not been reported in plants. In addition, if these fungi convert more toxic forms of Se to inert, insoluble $Se^0$, this may affect their host plant's Se uptake, accumulation and tolerance. Many fungi are known to be promiscuous with respect to their host range and so root symbionts of HAs may also be able to infect non-HAs, and their Se-related properties. In the study described in chapter 3, two *Astragalus* species, HA *A. bisulcatus* and non-accumulator *A. convallarius*, were inoculated with Se-HA rhizoplane fungi isolated from *Astragalus* HAs growing in the field. These fungi are *Alternaria astragali* (A3) and *Fusarium acuminatum* (F30). Chapter 4 describes a similar study using two *Stanleya* species. *Stanleya pinnata* and *Stanleya elata* were inoculated with rhizosphere fungi collected from *S. pinnata*, *Alternaria seleniiphila* and *Aspergillus leporis*. To determine the levels of Se and other elements in plant roots and shoots, inductively coupled plasma atomic

emissions spectroscopy (ICP-AES) was used as described by Zarcinas et al. 1987). To determine the potential positive and/or negative effects of these fungi on plant growth and the potential effect of the fungi on plant Se tolerance, plant biomass was also monitored. X-ray fluorescence mapping and XANES were used to compare the distribution and speciation of Se in roots of inoculated and uninoculated plants.

In Chapter 5 of this dissertation, a very close association of *A. bisulcatus* with a seed endophyte is investigated. This fungus had been observed earlier to be a significant pathogen to *A. bisulcatus* seeds when germinated on agar, or other artificial media. However, much less pathogenicity was observed when *A. bisulcatus* seeds were germinated on sand or soil. The question addressed in this chapter is: what is the effect of this fungus on seedling growth and Se accumulation? An *in vitro*, fungal inoculation experiment with *A. bisulcatus* was conducted to gain insight into the potential positive or negative effects of this fungus on plant growth and Se accumulation. Biomass was measured as an indicator of plant tolerance (to the fungus, and to Se), and ICP-AES was used to determine levels of Se and other elements. These studies are of particular interest in understanding the potential roles of microbes in Se hyperaccumulation.

As described in the following chapters, it appears that microbial symbionts can indeed affect plant Se speciation, as well as plant growth and Se accumulation. These findings shed new light on the ecology of hyperaccumulation. Moreover, such environmental components that contribute to plant Se accumulation and tolerance may be exploited in phytotechnological applications, to prevent both Se deficiency (via biofortification) and toxicity (via phytoremediation) in animals and humans.

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## Chapter 2

## Influence of Microbial Associations on Selenium Localization and Speciation in Roots of *Astragalus* and *Stanleya* Hyperaccumulators


**Summary**

Selenium (Se) hyperaccumulator plants can accumulate and tolerate Se up to 1% of their dry weight. Since little is known about below-ground processes of Se uptake and metabolism in hyperaccumulators, X-ray absorption spectromicroscopy was used to characterize the chemical composition and spatial distribution of Se in roots of *Astragalus* and *Stanleya* hyperaccumulators. Selenium was present throughout the roots, with the highest levels in the cortex. The main form of Se (48-95%) in both species collected from naturally seleniferous soil was an organic C-Se-C compound, likely methyl-selenocysteine. In addition, surprisingly high fractions (up to 35%) of elemental Se ($Se^0$) were found, a form so far not reported in plants but commonly produced by Se-tolerant bacteria and fungi. Four fungi collected from hyperaccumulator roots were characterized with respect to their Se tolerance and ability to produce $Se^0$, and then used to inoculate hyperaccumulators in a controlled greenhouse study. The roots of the greenhouse-grown *Astragalus* and *Stanleya* contained mainly C-Se-C; in most plants no $Se^0$ was detected, with the exception of *Astragalus* nodules and roots of *Astragalus* inoculated with *Alternaria astragali*, an $Se^0$-producing fungus. Apparently, $Se^0$- producing endosymbionts including nitrogen-fixing bacteria and endophytic fungi or bacteria in the root can affect Se speciation in hyperaccumulator roots. Microbes that affect plant Se speciation may be applicable in phytoremediation and biofortification, especially if they are promiscuous and affect Se tolerance in crop species.

## Introduction

Selenium (Se) is a naturally occurring element in most soils, and can be found at very high levels in alkaline soils where Cretaceous shale or other seleniferous rocks are the soil parent material (Byers 1935, 1936; Rosenfeld and Beath, 1964; Beath 1982). Non-seleniferous soils contain less than 1 mg Se $kg^{-1}$, whereas seleniferous soils can range between 2-100 mg Se $kg^{-1}$ (Mikkelsen et al., 1989; Beath *et al.*, 1939). Selenate is thought to be the predominant bioavailable form of Se in seleniferous soil (Rosenfeld and Beath, 1962; Zhao et al., 2005).

Selenium is an essential nutrient for many animals including mammals, yet is also toxic at higher levels, leaving a narrow margin between deficiency and toxicity (Chen et al., 1980; Hoffmann and Berry 2008; Li et al., 2009). Selenium toxicity is thought to occur because of the similarity of Se to sulfur (S) (Brown and Shrift, 1982; Stadtman, 1990; Smith et al., 1995; Terry et al., 2000). Selenium is metabolized into selenocysteine (SeCys), or selenomethionine (SeMet); these Se-amino acids can be non-specifically incorporated into proteins instead of cysteine or methionine, causing the proteins to lose function (Brown and Shrift, 1982; Stadtman, 1990). Plants that accumulate Se may be used to both clean up excess Se from the environment (phytoremediation) and to prevent Se deficiency in consumers (biofortification). Crop species that are particularly good Se accumulators and that may be used for biofortification are garlic, onion, and broccoli (Fairweather-Tait et al., 2011).

Selenium enters the food chain primarily through plants that inadvertently take up and assimilate selenate via S transporters and enzymes (Terry et al., 2000; Sors et al., 2005). Plant species differ in their capacity to accumulate and tolerate Se. The so-called Se hyperaccumulator (HA) plants can accumulate more than 0.1% of their dry weight as Se, without showing any symptoms of toxicity (Beath et al., 1939; White et al., 2007). Most plant species that are Se HAs occur within the genus *Astragalus*; examples are *A. bisulcatus* and *A. racemosus* (Beath et al., 1939). The genus *Stanleya* also contains at least one Se

hyperaccumulating species, *S. pinnata* (Feist and Parker, 2001). HAs detoxify Se by adding a methyl group to SeCys via the enzyme SeCys methyltransferase (SMT); the resulting methyl-SeCys (MeSeCys) can be accumulated safely since it is a non-protein amino acid (Neuhierl and Bock, 1996). Methyl-SeCys is the primary form of Se found in young and old leaves of *A. bisulcatus* as well as in young leaves and flowers of *S. pinnata* (Pickering et al., 2003; Freeman et al., 2006a; Quinn et al., 2011). Within HA leaves the Se was found predominantly in the periphery, such as the margins, epidermis, and trichomes (Freeman et al., 2006a). Non-HA plant species accumulate Se mainly in the vascular tissues, in the form of selenate (de Souza et al., 1998a; Van Hoewyk et al., 2005; Freeman et al., 2006a); since this form of Se is more toxic, these species are less Se tolerant. Selenium speciation in plant roots has so far not been reported for any species, including HAs.

In contrast to many other organisms, Se has not been shown to be essential for higher plants (Brown and Shrift, 1982; Zhang and Gladyshev, 2009). Selenium does appear to be a beneficial nutrient for many plants, especially HAs, which can reach two-fold higher biomass in the presence of Se (Pilon-Smits et al. 2009). Thus, the functional significance of Se hyperaccumulation may be to offer better growth, perhaps due to better oxidative stress resistance (Cartes et al., 2005; Hartikainen, 2005). An additional benefit of Se hyperaccumulation is enhanced resistance to Se-sensitive herbivores and pathogens (Vickerman et al., 2002; Hanson et al., 2003, 2004; Freeman et al., 2006b, 2007; 2009; Quinn et al., 2007, 2008, 2010). Thus, Se hyperaccumulation may be considered a form of elemental defense (Boyd and Martens, 1992; Boyd, 2007, 2010). As with any plant defense, herbivores and pathogens are likely to overcome it over time. There is indeed evidence of Se-tolerant herbivores and rhizosphere microbes (Freeman et al., 2006b; Wangeline et al., 2007, 2011). A Se-tolerant diamond back moth was found to feed on *S. pinnata* containing 0.2% Se dry weight without ill effects. It accumulated MeSeCys, like its host, explaining its tolerance. Se-tolerant microbes can use different Se tolerance mechanisms, including Se reduction to insoluble, non-toxic elemental Se ($Se^0$), volatilization, or conversion to

MeSeCys (Frankenberger and Karlson, 1994; Losi and Frankenberger, 1997; de Souza et al., 2001; Hunter and Manter, 2009). Some plant-associated microbes have been shown to affect plant Se accumulation and volatilization (de Souza et al., 1998b). The capacity of microbes to affect plant Se speciation has not been investigated.

In the study described here, Se speciation and distribution were characterized in roots of *Astragalus* and *Stanleya* HAs with the goal to obtain better insight into below-ground Se metabolism and the potential of microbial symbionts to affect Se speciation and distribution in host tissues.

## Material and Methods

Biological material

*Astragalus racemosus* seeds were obtained from Western Native Seed, Coaldale, CO, USA. *Stanleya pinnata* seeds were collected in the summer (June-July) of 2009 at Pine Ridge Natural Area, a seleniferous site in Fort Collins, CO, USA. *Astragalus bisulcatus* and *S. pinnata* tap roots were collected from Pine Ridge Natural Area in June of 2009.

The four fungal isolates were obtained and described previously by Wangeline et al. (2007, 2011) from HAs growing on naturally seleniferous soil. They are (i) *Fusarium acuminatum* (F30), collected from *A. racemosus* in Lysite, WY, (ii) *Alternaria astragali* (A3) collected from *A. bisulcatus* in Laramie, WY, (iii) *Aspergillus leporis* (AS117), isolated from *S. pinnata* at Pine Ridge in Fort Collins, CO

and (iv) *Alternaria seleniiphila* (A1), isolated from *S. pinnata* at Pine Ridge in Fort Collins, CO.

Field root preparation for XAS

Plants roots with rhizosphere soil attached were collected from Pine Ridge Natural Area in Fort Collins, CO. Live, intact roots were cut approximately 10 cm below the crown (top of the tap root). The tap root was inserted into flexible plastic tubing with a diameter of 2 cm. A two-component silicon epoxy was

inserted into the tubing to fill up the space around the root, and to fix soil particles in place. After the epoxy was set, the tubing containing the root plus rhizosphere soil was cut into 0.5-1 mm thick cross-sections that were then frozen and stored at -80$^{o}$C for XAS analysis.

Fungal cultivation

All fungi were cultivated under continuous fluorescent light at 22°C in sealed Petri dishes containing 0.5 strength malt extract agar (0.5 MEA, Difco, Detroit, MI) supplemented with different Se concentrations as specified below.

For determination of Se tolerance, the fungal isolates were supplied with 0, 30, 300 or 600 mg $L^{-1}$ Se as $Na_2SeO_4$. After five days of growth, colony diameter measurements were taken to quantify Se tolerance; at this point none of the colonies had reached the edge of the petri dish.

Fungi for X-ray absorption spectroscopy (XAS) were grown for 5d on 0.5 MEA with either $Na_2SeO_3$ or $Na_2SeO_4$ at 30 μM unless otherwise indicated. Mycelium was carefully collected, avoiding agar medium, rinsed with sterile distilled water, frozen in liquid nitrogen and stored at -80$^{o}$C for XAS.

Fungi to be used for plant inoculation were initially cultivated on 0.5 MEA medium with 30 μM $Na_2SeO_4$.

Plant preparation for fungal co-cultivation studies

*Stanleya pinnata* seeds were surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10 minute rinses in sterilized water. The *A. racemosus* seeds were first scarified with sandpaper and then surface-sterilized. Seeds were germinated on half-strength Murashige and Skoog medium with 10 g $L^{-1}$ sucrose (Murashige and Skoog, 1962) under continuous light at 23$^{o}$C in a plant growth cabinet. After 14 days the seedlings were carefully transferred to 1 L cones filled with steam-sterilized gravel (in the bottom) and coarse sand (on top). The plants were watered bi-weekly with ¼ Hoagland's solution (Hoagland and Arnon, 1938) for two weeks and then inoculated with fungi as described below.

Co-cultivation and Harvest

*Stanleya pinnata* was inoculated with either fungal isolate A1 or AS117; a control treatment received no inoculum. The *Astragalus racemosus* inoculation treatments consisted of fungal isolates A3, F30, or no inoculum. Plants were inoculated with a standard quantity of fungal hyphae and/or spores. The fungi were grown on 0.5 strength MEA plates for 5d, and fungal materials were collected at the perimeter of the fungal colony. In preparation for plant inoculation, mycelium from F30 and mycelium and spores of A1 and A3 were macerated in sterile water in 1.5 mL tubes using sterile glass beads and a micro pestle. Spores from AS117 were placed in a tube with sterile water and 0.05% Tween-20 to break the water surface tension. Fragments of hyphae from F30, as well as spores and spore chains of A1 and A3 were quantified using a hemocytometer to estimate mm hyphae and spores and spore chains $mL^{-1}$ water. Spores from an AS117 colony were quantified in the same way and diluted to 2,000 spores $mL^{-1}$ water. F30, A1, and A3 were diluted to a final concentration of 2,000 mm $mL^{-1}$ water. The inocula were delivered via peat moss to the plants in the greenhouse. Each plant received 1.5 mL of peat moss saturated with 1 mL of inoculum, or peat moss with sterile water for the control treatments. The Se treatment was delivered to the plants via watering with 12.5 $\mu$M $Na_2SeO_4$ twice weekly beginning at the time of inoculation. After inoculation, plants and fungi were co-cultivated for 12 weeks before harvesting. On the day of harvest, the plants were gently washed to remove adhering sand from the roots, and the roots were frozen in liquid nitrogen and stored at -20$^{o}$ C for XAS. Root cross sections were made on frozen roots using a chilled razor blade, a Petri dish, and a block of dry ice to maintain the integrity of the sample.

X-ray absorption spectroscopy

Selenium distribution and speciation were determined using micro-focused X-ray fluorescence (XRF) mapping and X-ray absorption near edge structure (XANES) spectroscopy, respectively. Intact biological samples were flash-frozen in liquid nitrogen and shipped on dry ice for microprobe analyses at the

Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Lab (Marcus *et al.,* 2004). Frozen samples (plant roots and fungal mycelia) were transferred onto a Peltier stage kept at -33$^{o}$ C to reduce potential beam radiation damage. XRF elemental maps were recorded at 13 keV, using a 15 µm (H) x 6 µm (V) beam, 15 µm x 15 µm pixel size, 50 ms dwell time per pixel. The chemical forms of Se in particular areas of interest were further investigated using Se K-edge XANES. XANES provides information about the oxidation state and, when compared to well-characterized Se standard compounds, information about its chemical speciation (Pickering *et al.,* 1999). XRF maps and XANES spectra were recorded with a 7 element Ge solid state detector (Canberra, ON, Canada). Spectra were deadtime corrected, pre-edge background subtracted, and post-edge normalized using standard procedures (Kelly *et al.,* 2008). Red selenium (white line position set at 12660 eV) was used to calibrate the spectra. Least square linear combination (LSQ) fitting of Se XANES spectra was performed in the 12630-12850 eV range, using a library of standard seleno-compounds. Se standards used include: $Na_2SeO_4$, $Na_2SeO_3$, SeCystine, SeMet purchased from Sigma-Aldrich (St Louis, MO, USA), MeSeCys, γ-GMeSeCys, SeCysth, and $SeGSH_2$ purchased from PharmaSe (Austin, TX, USA). SeCys was obtained by reducing SeCystine overnight at 25$^{o}$C in 100 mM sodium borohydride at a 1:1 molar ratio; reduction was verified enzymatically using SeCys lyase as described by Pilon-Smits et al. (2002). Gray and red elemental Se were provided by Amy Ryser and Dan Strawn. All data processing and analyses were performed with a suite of custom LabVIEW (National Instruments, Austin, TX, USA) programs available at the beamline.

**Results**

In field-collected plants from two Se hyperaccumulating species, *A. bisulcatus* and *S. pinnata*, Se was distributed throughout the roots, as judged from XRF mapping (Fig. 2.1). In both plant species the highest Se signal intensity was found in the peripheral (cortex) region, with relatively lower signal in the center. Soil particles attached to the roots are visible in panels A and C, tri-color coded elemental maps showing Ca (in green), Fe (in blue) and Se (in red). There is not a clear Se signal in the isolated soil particles, but at the root-soil interface both plant species show a ring of Se that overlaps with Ca- and Fe-rich soil particles. It cannot be distinguished whether this Se-rich area corresponds with the epidermis, the rhizosphere, or both.

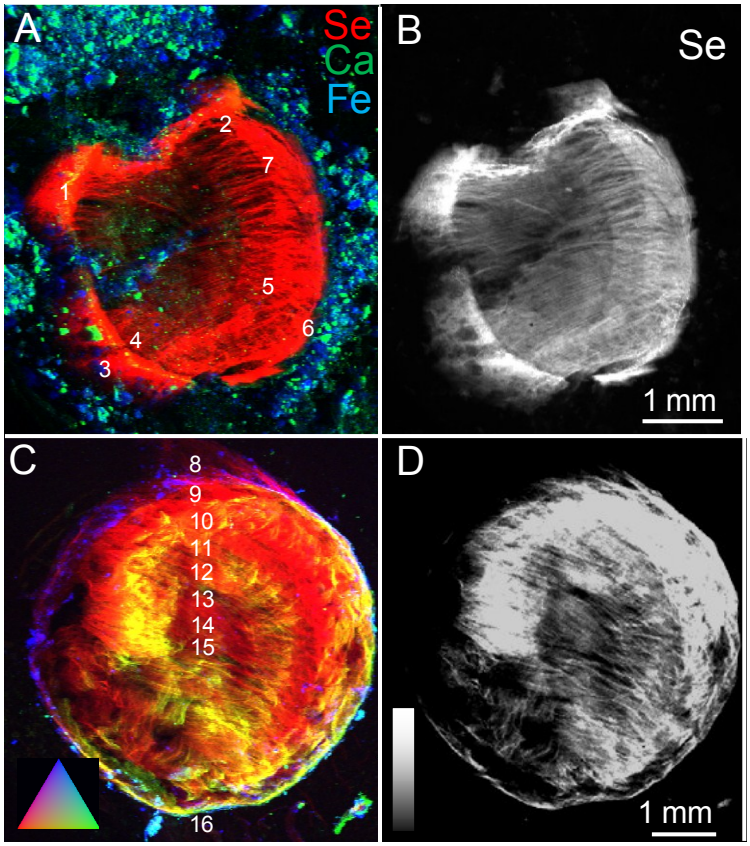


Figure 2.1 X-rayfluorescence elemental mapping of *Astragalus bisulcatus* and *Stanleya pinnata* roots with soil particles attached, collected from the field. A, B) crown root of an *A. bisulcatus* plant. B, D) root of *S. pinnata*. A, C) tricolor-coded maps, with Se in red, Ca in green, and Fe in blue. B, D) black and white Se distribution map (high Se = white). Numbers indicate locations of XANES analyses.

The speciation of Se in the roots and in the Se-rich ring at the root-soil interface was determined by XANES (Table 2.1; the numbers in figure 2.1 correspond to the locations where spectra were collected).

Table 2.1. Results from least squares linear combination fitting of experimental XANES spectra with sta seleno-compounds. The points where the spectra were collected are indicated in Figure 2.1.
C-Se-C: methyl-selenocysteine, Se-Methionine or Se-Cystathionine. $Se^0$: red or gray elemental Se.
SS: normal sum of squares (quality of fit; 0 = perfect fit); nd: compound not detectable.

| | SS ($x10^{-4}$) | $SeO_4^{2-}$ | $SeO_3^{2-}$ | $SeGSH_2$ | SeCysteine | SeCystine | C-Se-C | $Se^0$ |
|---|---|---|---|---|---|---|---|---|
| *A. bisulcatus* | | | | | | | | |
| 1 | 9.30 | nd | 5% | nd | nd | nd | 95% | nd |
| 2 | 4.30 | nd | 16% | nd | nd | nd | 50% | 35% |
| 3 | 1.76 | nd | 11% | nd | nd | nd | 64% | 26% |
| 4 | 1.77 | nd | 5% | nd | nd | nd | 88% | 8% |
| 5 | 3.43 | nd | 6% | nd | nd | nd | 89% | 6% |
| 6 | 2.11 | nd | 11% | nd | nd | nd | 66% | 24% |
| 7 | 1.86 | nd | 7% | nd | nd | nd | 80% | 14% |
| *S. pinnata* | | | | | | | | |
| 8 | 4.78 | nd | 4% | nd | nd | nd | 84% | 13% |
| 9 | 2.96 | nd | nd | nd | nd | nd | 93% | 7% |
| 10 | 2.67 | nd | 9% | nd | nd | nd | 67% | 24% |
| 11 | 3.88 | nd | 4% | nd | 70% | nd | nd | 22% |
| 12 | 3.57 | nd | 15% | nd | nd | nd | 50% | 34% |
| 13 | 2.30 | nd | 13% | nd | nd | nd | 56% | 31% |
| 14 | 2.41 | nd | 11% | nd | nd | nd | 59% | 31% |
| 15 | 5.05 | nd | 18% | nd | nd | nd | 48% | 34% |
| 16 | 2.00 | nd | 5% | nd | nd | nd | 82% | 13% |

In all of the locations analyzed for *A. bisulcatus* and all but one of the *S. pinnata* XANES locations the predominant form of Se (49-95%) is an organic form of Se with a C-Se-C configuration (SeMet, MeSeCys, or SeCysth). The one location of XANES analysis in *S. pinnata* (#11) that showed a different speciation was most similar to SeCys. Interestingly, all but one of the XANES spectra from the *A. bisulcatus* root and all of the spectra from the *S. pinnata* root indicate a high amount of elemental selenium ($Se^0$), which has not been previously reported in plants. $Se^0$ was the second most abundant form of the total Se in these field-collected roots (5-35%). The one location that did not contain $Se^0$ (#1) was on the periphery of the root. Selenite was the third most abundant form of Se (4-18%), found in all XANES spots on the *A. bisulcatus* roots and all spots on *S. pinnata* except peripheral spot #9. On

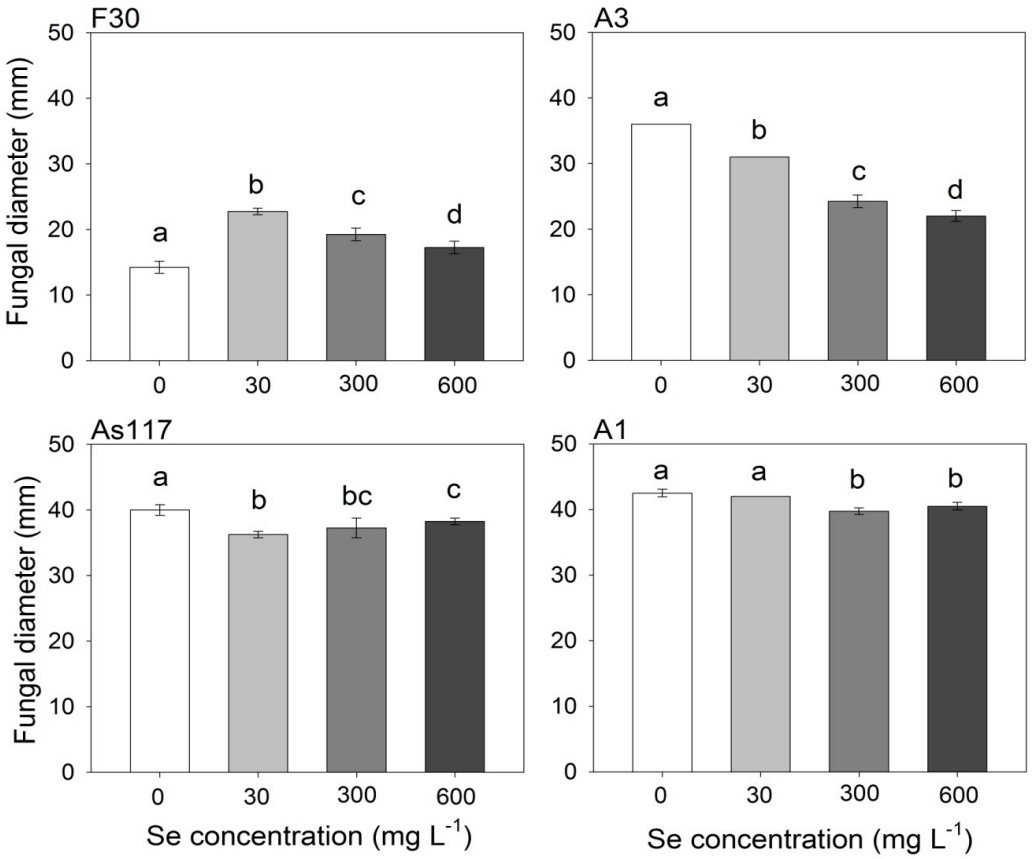


Figure 2.2 Selenium tolerance of the four fungi used in this study, F30 (*Fusarium acuminatum*), A3 (*Alternaria seleniiphila*), AS117 (*Aspergillus leporis*), A1 (*Alternaria astragali*), quantified as colony diameter after 5d of growth on 0.5-strength malt extract agar supplied with either 0, 30, 300 or 600 mg $L^{-1}$ sodium selenate. Values shown represent the means, and the error bars are the standard error of the mean. Lower case letters above bars indicate statistically different means (ANOVA, $P<0.05$).

average, the speciation in the *A. bisulcatus* root was 9% $SeO_3^{2-}$, 76% C-Se-C, and 16% $Se^0$. The average Se composition across all the *S. pinnata* locations analyzed was 9% $SeO_3^{2-}$, 68% C-Se-C, and 23% $Se^0$. The Se signal was high enough throughout the *S. pinnata* root that a transect of XANES locations could be analyzed, which revealed interesting differences in Se speciation between the cortex (spots 8-10,16) and the stele (spots 12-15) (Fig. 2.1, Table 2.1). The stele contained a significantly higher percentage of $Se^0$ than the cortex (32±1 vs 14±4; p=0.0026), and relatively more $SeO_3^{2-}$ (14±1 vs 4±2, p=0.005), while containing a lower percentage of C-Se-C than the cortex (53±2 vs 81±5; p=0.0032). The Se signal in the stele of *A. bisulcatus* was too low to obtain a good XANES spectrum, so speciation cannot be compared with the cortex. However, the cortex showed a similar relative distribution of organic vs inorganic Se as the cortex of *S. pinnata*. Since $Se^0$ has not been reported to be produced or found in plants before, but is well-known to be produced by many microbes, it is possible that the $Se^0$ in the roots of both Se HAs is produced by microbial endophytes. To investigate this possibility, several fungi were selected and tested for their effects on root Se speciation. These fungi come from a collection isolated from the rhizoplane of *Astragalus* and *Stanleya* HAs as described by Wangeline et al. (2011). Four isolates were selected for controlled inoculation studies with *Astragalus* and *Stanleya*. Two of these fungi were isolated from *S. pinnata*: *Alternaria seleniiphila* (A1) a newly described small-spored species (Wangeline et al., 2007), and a Se-tolerant *Aspergillus leporis* (AS117). The other two fungi were isolated from *Astragalus* species: *Alternaria astragali* (A3) was isolated from *Astragalus bisulcatus* and newly described by Wangeline et al. (2007). *Fusarium acuminatum* (F30) was isolated from *Astragalus racemosus*.

The four fungal isolates were tested for their Se tolerance by quantifying their growth on agar media supplied with 0-600 mg $L^{-1}$ $Na_2SeO_4$. Judged from this test F30 is the most Se tolerant, growing better in the presence of Se than without Se (Fig. 2.2). A1 and AS117 are also highly Se tolerant, showing only a marginal growth reduction up to 600 mg Se $L^{-1}$ (Fig. 2.2). Among the four fungi, A3 was the least

tolerant, with ~40% growth reduction at 600 mg Se $L^{-1}$. This is still ten-fold more Se-tolerant than many other fungi tested in earlier studies (Hanson et al., 2003; Wangeline et al., 2011). Preliminary measurements of Se accumulation in the selenate-supplied fungi indicate that A1 is a particularly good Se accumulator, and A3 and F30 are moderate Se accumulators. AS117 is a low Se accumulator; this may be related to the observation that AS117 appears to volatilize Se at high levels, based on qualitative assessment of the strength of the distinct volatile Se smell (Wangeline, unpublished results).

To test whether these four fungi can produce $Se^0$ when supplied with selenate or selenite, the fungi were grown on media containing one of these compounds, and their speciation determined by XANES. Indeed, with the exception of F30, all fungi accumulated $Se^0$, which comprised 28-98% of their total Se; F30 accumulated organic forms of Se (Table 2.1). Based on these characteristics with respect to Se tolerance and metabolism, it was decided to use all four fungal species for HA inoculation studies.

To test whether production of $Se^0$ in HA roots is associated with the presence of microbial symbionts, HAs *A. racemosus* and *S. pinnata* were grown from surface-sterilized seeds in sterilized growth medium, with or without the fungi described above. Each plant species was inoculated with two different fungi that were isolated previously from plants in the same genus. The reason why *A. racemosus* was chosen over *A. bisulcatus* was that 50-80% of the *A. bisulcatus* seeds contained an endophytic fungus, which might confound the results; *A. racemosus* seeds did not show any evidence of endophytes. In the *A. racemosus* roots from all three treatments (no inoculum, A3 inoculum and F30 inoculum) Se was found throughout the roots, with a higher signal coming from the cortex compared to the stele (Fig. 2.3), similar to what was observed in field-collected *A. bisulcatus* roots. The majority of *A. racemosus* plants from all greenhouse treatments had root nodules at the time of harvest. Nodules can be seen on the lateral root of *A. racemosus* inoculated with F30 (Fig. 2.3D), and in the cross-section of *A. racemosus*

inoculated with A3 (Fig. 2.3F). The Se signal is very high in the nodules of these roots compared to the rest of the root. There are no obvious differences in Se distribution between the three treatments.

Table 2.2. Results from least squares linear combination fitting of experimental XANES spectra with standard seleno-compounds. XANES fits were obtained from fungi originally isolated from HA roots in the field, grown on plates with Se. C-Se-C: Methyl-selenocysteine, Se-Methionine or Se-Cystathionine. $Se^0$: red or gray elemental Se. SS: normal sum of squares (quality of fit); nd: compound not detectable.

| | SS ($x10^{-4}$) | $SeO_4^{2-}$ | $SeO_3^{2-}$ | $SeGSH_2$ | SeCys | SeCystine | C-Se-C | $Se^0$ |
|---|---|---|---|---|---|---|---|---|
| F30 on 30μM $SeO_3^{2-}$ | | | | | | | | |
| 1 | 12.1 | nd | 10% | 38% | nd | nd | 56% | nd |
| 2 | 16.2 | nd | 6% | 35% | nd | nd | 63% | nd |
| 3 | 5.47 | nd | 6% | 40% | nd | nd | 55% | nd |
| A3 on 30μM $SeO_3^{2-}$ | | | | | | | | |
| 1 | 3.12 | 1% | nd | 16% | 43% | nd | nd | 38% |
| 2 | 2.56 | nd | 4% | 11% | nd | nd | 58% | 29% |
| 3 | 3.73 | nd | 5% | 12% | nd | 23% | 21% | 40% |

| | SS ($x10^{-4}$) | $SeO_4^{2-}$ | $SeO_3^{2-}$ | $SeGSH_2$ | SeCys | SeCystine | C-Se-C | $Se^0$ |
|---|---|---|---|---|---|---|---|---|
| AS117 on 30μM $SeO_4^{2-}$ | | | | | | | | |
| 1 | 6.57 | nd | nd | nd | nd | 37% | nd | 61% |
| 2 | 12.9 | nd | nd | nd | nd | 37% | nd | 60% |
| AS117 on 360μM $SeO_3^{2-}$ | | | | | | | | |
| 1 | 8.89 | nd | nd | nd | nd | nd | nd | 98% |
| A1 on 30μM $SeO_3^{2-}$ | | | | | | | | |
| 1 | 2.53 | nd | nd | 13% | nd | nd | nd | 87% |
| 2 | 2.01 | nd | 4% | 17% | nd | 22% | nd | 57% |
| 3 | 1.25 | nd | nd | 11% | nd | nd | nd | 90% |

Selenium speciation in the roots and root nodules was determined using Se K-edge XANES. The numbers in Figure 2.3 indicate where XANES spectra were collected; Se speciation results can be found in Table 2.3. The most abundant form of Se in the *A. racemosus* roots from the greenhouse had a C-Se-C configuration. A small fraction of Se was $SeO_3^{2-}$ in most of the XANES spots. Spots 2-8 and 12-20 all gave similar Se spectra with on average 95% C-Se-C and 5% $SeO_3^{2-}$. Interestingly, in the majority of root spectra collected from the greenhouse plants there is a noted absence of $Se^0$ (which was a significant Se species in the field-collected *A. bisulcatus* roots). The only spots from the greenhouse roots that contained $Se^0$ (Table 2.3) were located in the root nodule of *A. racemosus* inoculated with F30

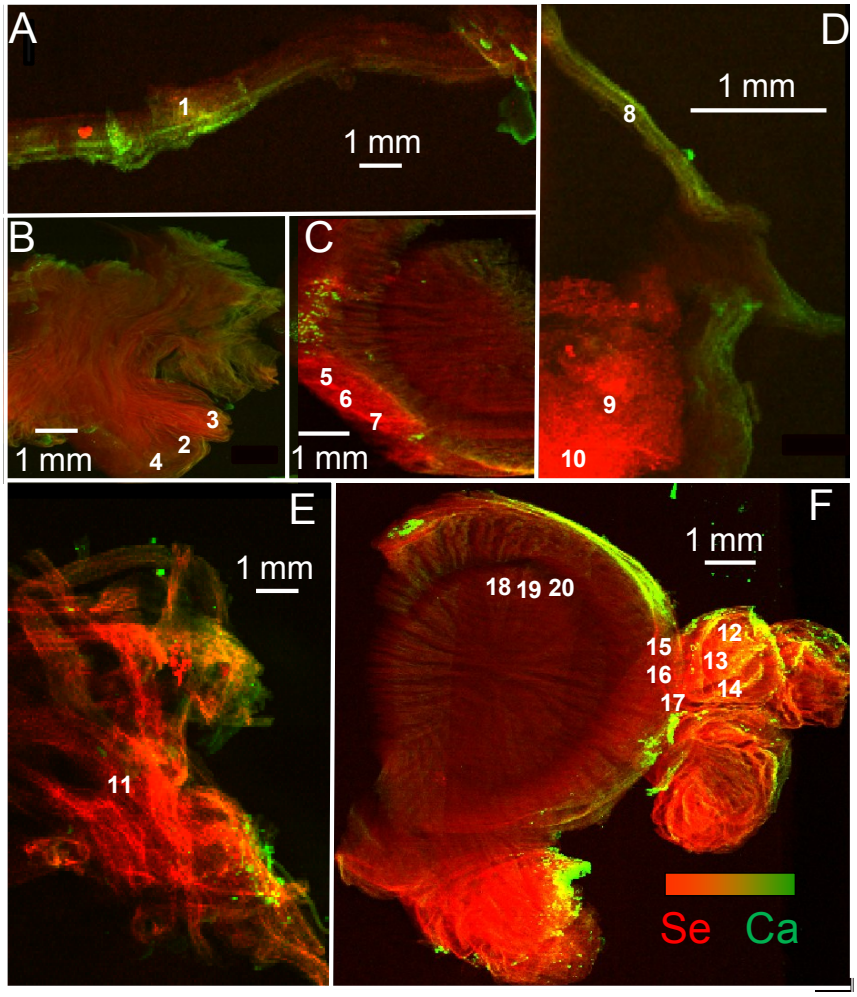


Figure 2.3 X-rayfluorescence elemental mapping of *Astragalus racemosus* roots grown in greenhouse conditions. A, B) lateral root and root thin section respectively from an un-inoculated plant. C, D) thin section and lateral root with nodule, respectively, from plants inoculated with *Fusarium acuminatum* (F30). E, F) from plants inoculated with *Alternaria astragali* (A3). All panels show bicolor –coded maps with Se in red and Ca in green. Numbers indicate locations of XANES analyses.

Table 2.3. Results from least squares linear combination fitting of experimental XANES spectra with standard seleno-compounds. XANES fits were obtained at the points indicated in Figure 2.3 on the roots of greenhouse-grown *A. racemosus* plants. C-Se-C: Methyl-selenocysteine, Se-Methionine or Se-Cystathionine. $Se^0$: red or gray elemental Se.

SS: normal sum of squares (quality of fit); nd: compound not detectable.

| | SS ($x10^{-4}$) | $SeO_4^{2-}$ | $SeO_3^{2-}$ | $SeGSH_2$ | SeCysteine | SeCystine | C-Se-C | $Se^0$ |
|---|---|---|---|---|---|---|---|---|
| *No inoculum* | | | | | | | | |
| 1 | 91.1 | Nd | nd | 63% | nd | nd | 8% | nd |
| 2 | 10.3 | Nd | 3% | nd | nd | nd | 97% | nd |
| 3 | 4.25 | Nd | 3% | nd | nd | nd | 98% | nd |
| 4 | 4.47 | Nd | 4% | nd | nd | nd | 95% | nd |
| *F30 inoculum* | | | | | | | | |
| 5 | 6.71 | nd | 5% | nd | nd | nd | 95% | nd |
| 6 | 9.57 | nd | 6% | nd | nd | nd | 96% | nd |
| 7 | 6.98 | nd | 8% | nd | nd | nd | 93% | nd |
| 8 | 10.9 | nd | 4% | nd | nd | nd | 100% | nd |
| 9 | 2.92 | 1% | nd | 15% | nd | 38% | nd | 45% |
| 10 | 5.06 | nd | 8% | 23% | nd | nd | 41% | 28% |
| *A3 inoculum* | | | | | | | | |
| 11 | 9.42 | nd | 18% | 18% | nd | nd | 46% | 22% |
| 12 | 5.76 | nd | 9% | nd | nd | nd | 91% | nd |
| 13 | 3.61 | nd | 9% | nd | nd | nd | 85% | nd |
| 14 | 3.68 | nd | 6% | nd | nd | nd | 95% | nd |
| 15 | 6.43 | nd | 7% | nd | nd | nd | 91% | 2% |
| 16 | 5.02 | nd | 7% | nd | nd | nd | 90% | 3% |
| 17 | 5.77 | nd | 7% | nd | nd | nd | 89% | 4% |
| 18 | 6.21 | nd | nd | nd | nd | nd | 103% | nd |
| 19 | 3.94 | nd | 3% | nd | nd | nd | 99% | 1% |
| 20 | 4.89 | nd | nd | nd | nd | nd | 103% | nd |

(Fig. 2.3D, spots 9 and 10, 28-45% $Se^0$), on the lateral root mass of *A. racemosus* inoculated with A3 (Fig. 2.3E, spot 11, 22% $Se^0$), and on the root-nodule interface of *A. racemosus* inoculated with A3 (Fig. 2.3F, spots 15-17, <5% $Se^0$). The XANES spectra from location 1 (Fig. 2.3A) was notably different from the others, and is likely unreliable based on the poor quality of the fit (sum of squares value of 91.1 x $10^{-4}$).

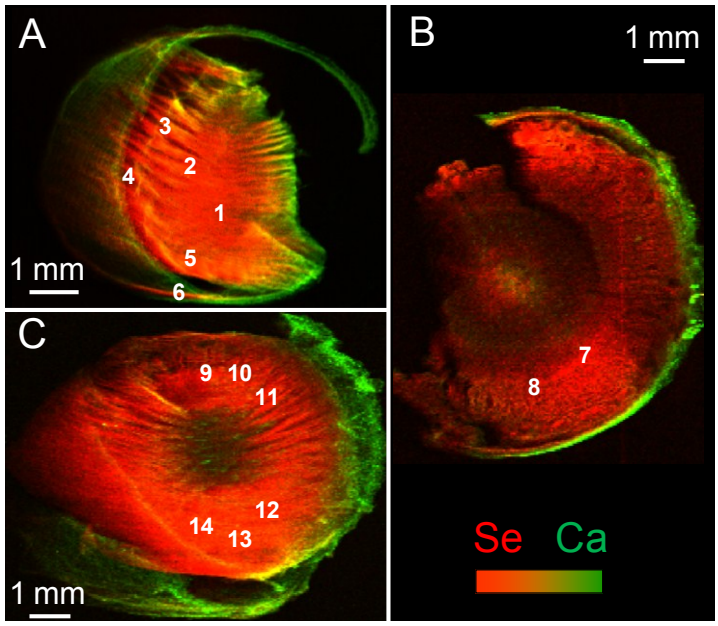


Figure 2.4 X-ravfluorescence elemental mapping of *Stanleya pinnata* roots grown in greenhouse conditions. A) Un-inoculated control, B) plant inoculated with *Aspergillus leporis* (AS117), C) plant inoculated with *Alternaria seleniiphila* (A1). All panels show bicolor –coded maps with Se in red and Ca in green. Numbers indicate locations of XANES analyses.

Figure 2.4 shows the XRF elemental distribution maps of the root sections of the greenhouse-grown *S. pinnata*. Selenium was predominantly localized in the cortex, and lower or absent in the center of *S. pinnata* plants inoculated with A3 or F30 (Fig. 2.4B, C). The same may be true for the uninoculated root (Fig. 2.4A), but this cannot be determined from this sample due to the angle of the cross-section. XANES spectra from the un-inoculated root (Fig.2. 4A) reveal that all of the Se is present in a C-Se-C configuration (Table 2.4). The XANES spectra obtained from the root inoculated with AS117 indicated that the Se was also almost exclusively present as C-Se-C, with a small fraction of selenite (Fig. 2.4B, Table2. 4). The spot on the cortex showed relatively more inorganic Se compare to the spot on the cortex-stele interface, but the fit of the cortex spot was less reliable. The Se present in the cortex of the root inoculated with A1 was also almost completely C-Se-C, with small fractions (up to 4%) of $Se^0$ and

selenite (Fig. 2.4C, Table2. 4). Thus, similar to *Astragalus*, *S. pinnata* roots contained a substantial $Se^0$ fraction in the field, but not in the greenhouse.

Table 2.4. Results from least squares linear combination fitting of experimental XANES spectra with standard seleno-compounds. XANES fits were obtained at the points indicated in Figure 2.4 on the roots of greenhouse-grown *S. pinnata* plants. C-Se-C: Methyl-selenocysteine, Se-Methionine or Se-Cystathionine. $Se^0$: red or gray elemental Se.

SS: normal sum of squares(quality of fit); nd: compound not detectable.

| | SS ($x10^{-4}$) | $SeO_4^{2-}$ | $SeO_3^{2-}$ | $SeGSH_2$ | SeCysteine | SeCystine | C-Se-C | $Se^0$ |
|---|---|---|---|---|---|---|---|---|
| *No inoculum* | | | | | | | | |
| 1 | 6.47 | nd | nd | nd | Nd | nd | 100% | nd |
| 2 | 7.58 | nd | nd | nd | Nd | nd | 100% | nd |
| 3 | 7.07 | nd | nd | nd | Nd | nd | 100% | nd |
| 4 | 6.46 | nd | nd | nd | Nd | nd | 100% | nd |
| 5 | 3.91 | nd | nd | nd | Nd | nd | 100% | nd |
| 6 | 6.08 | nd | nd | nd | Nd | nd | 100% | nd |
| *AS117 inoculum* | | | | | | | | |
| 7 | 9.02 | nd | 6% | nd | Nd | nd | 101% | nd |
| 8 | 38.9 | nd | 16% | nd | Nd | nd | 86% | nd |
| *A1 inoculum* | | | | | | | | |
| 9 | 4.16 | nd | 2% | nd | Nd | nd | 93% | 7% |
| 10 | 4.18 | nd | nd | nd | Nd | nd | 96% | 4% |
| 11 | 3.51 | nd | nd | nd | Nd | nd | 98% | nd |
| 12 | 3.64 | nd | 1% | nd | Nd | nd | 100% | nd |
| 13 | 3.26 | nd | nd | nd | Nd | nd | 99% | 2% |
| 14 | 11.5 | 1% | 6% | nd | Nd | nd | 91% | nd |

## Discussion

This study describes Se distribution and speciation in roots of Se HAs, both from the field and from a greenhouse experiment. The main form of root Se was a C-Se-C compound in both *Astragalus* and *Stanleya* HA species, either growing in the field or the greenhouse. There was an interesting difference in Se speciation between plants collected from naturally seleniferous soil and plants germinated from surface-sterilized seeds and grown on sterilized medium in the greenhouse. The plants in the field contained a substantial fraction (up to 35%) of elemental Se ($Se^0$), a form usually associated with microbial activity. This form was in most cases not observed in greenhouse plants. When observed in greenhouse-grown plants, the $Se^0$ was found particularly in root nodules. In the field it was found throughout the roots: in the cortex, stele, and at the root-soil interface. There were no nodules on these particular field-collected roots, but other *A. bisulcatus* nodules were shown to contain around 30% $Se^0$ (Valdez-Barillas, Lindblom, Fakra, Marcus and Pilon-Smits, unpublished results).

Since the nodules of *A. racemosus* contained relatively high levels of $Se^0$ compared to the rest of the root, an as yet unidentified nitrogen fixing bacterium in the nodules appears to significantly affect Se speciation. It is interesting that the roots of the greenhouse-grown plants were in most cases nodulated, even though no attempt was made to inoculate with *Rhizobium*, and the seeds and growth medium were sterilized at the beginning of the experiment. Apparently, there were bacterial strains in the vicinity that could colonize this HA species and were not affected by its elevated Se levels. Selenium-hyperaccumulating *Astragalus* species have been reported before to have root nodules (Defaria et al., 1989) and to host bacteria that could nodulate other, non-HA *Astragalus* species (Wilson and Chin 1947). So far these HA-associated Rhizobiaceae have not been characterized, and nothing is known about their relative Se tolerance. In earlier studies Rhizobiaceae associated with other plant species have been shown to differ in Se sensitivity. While 1 mg $L^{-1}$ selenate impaired nodulation and $N_2$ fixation in *Melilotus indica*, a non-HA (Wu et al. 1994), the symbionts *Rhizobium selenireducens, Sinorhizobium fredii,* and *S. meliloti* were Se-tolerant (Kinkle et al. 1994; Hunter et al. 2007). Several Se-tolerant fungi

were tested for the ability to reduce Se to $Se^0$; among isolates shown to have this capacity were an *Aspergillus* and a *Fusarium* species (Gharieb et al., 1995).

The high levels of $Se^0$ in the root proper (cortex and stele) that was found only in the plants growing in the field may be due to endophytic fungi or bacteria, since these are known to colonize these tissues (Wyens et al., 2009a,b). Inoculation of greenhouse-grown plants with four rhizoplane fungi isolated from HAs in seleniferous areas did not significantly affect root Se speciation, although one fungus gave rise to some (2-22%) $Se^0$ accumulation. When this fungus (A3) was grown on plates and supplied with selenite, it accumulated predominantly $Se^0$, it has the potential to affect root Se speciation. It is interesting to note that the XANES spectrum indicating the highest $Se^0$ fraction in the greenhouse roots was on a lateral root where the x-ray beam was hitting the outside of the root; the area of initial contact with the fungus. In the field the $Se^0$ fractions in the roots were much higher, however, than those observed in the greenhouse. Perhaps additional microbes are present in the field that further influence root Se speciation, or perhaps root colonization by the fungus requires more time. The plants in the greenhouse experiment were only co-cultivated with the fungi for 3 months, while the field plants were several years old. The finding that most of the greenhouse-grown *A. bisulcatus* and *S. pinnata* plants did not accumulate $Se^0$ indicates that this form of Se is not commonly made in these HAs, and makes it more likely that it is the result of microbial activity. Since $Se^0$ is insoluble and therefore not mobile, the compound is likely produced in the root itself, perhaps by an endophyte as a detoxification mechanism. Microbial conversion of Se to $Se^0$ could also protect the plant from Se toxicity. Furthermore, trapping of insoluble Se in the roots by microbes may reduce shoot Se concentration. Higher plant tolerance and lower shoot Se levels may benefit the practice of phytostabilization- where plants are used to prevent the movement of pollutants and prevent toxic levels from entering the food chain. In future studies it will be interesting to study the microbiome of HAs, and to characterize the properties of the endophytes and their effects on plant metabolism.

The predominant form of Se in roots of all of the HAs tested was C-Se-C. This may either be SeCysth, SeMet, MeSeCys, or γ-glutamyl-MeSeCys. The XANES spectra for these Se species are practically indistinguishable (Pickering et al., 1999). In young leaves the predominant form of Se (~90%) was also shown to be a C-Se-C compound (Pickering et al., 2003; Freeman et al., 2006a). Liquid chromatography mass spectrometry showed this compound to be 50% MeSeCys and 50% γ-glutamyl-MeSeCys (Freeman et al., 2006a). Pickering *et al.* (2000) reported that in roots of *A. bisulcatus* Se was mainly present in organic form, which is in agreement to our study. This appears to be different from non-accumulators: the non-HA *Triticum aestivum* (wheat) accumulated predominantly selenate in its roots when supplied with selenate, and mainly selenite and SeMet when applied with selenite (Li *et al*., 2008).

The roots of greenhouse- and field-grown HAs contained a small fraction of selenite. In earlier studies, leaves of the same HA species showed minor fractions of selenate (Freeman et al. 2006a, 2006b). The finding that the minor fraction of inorganic Se in roots (apart from $Se^0$) is selenite rather than selenate may reflect the more reducing (anoxic) conditions in the root tissues as compared to the leaves with their large intracellular spaces. It is thought that the main bioavailable form of Se in soil is selenate, and that selenate assimilation takes place predominantly in the leaf chloroplasts (Pilon-Smits et al., 1999). It is interesting that organic Se is the main form of Se in the root, because this either suggests that selenate is quickly translocated to the shoot, transformed to organic Se and remobilized back to the root, or that the roots are capable of assimilating selenate into organic Se directly. Yet another explanation is that the form of Se in seleniferous soil in the vicinity of HAs is already to a large extent organic, due to litter deposition and root turnover and/or exudation. It will be interesting in future studies to investigate Se speciation in soil around HAs. In this context it is interesting to note that in an earlier XRF study rice roots were found to influence soil Fe, Mn and As distribution and speciation in their rhizosphere (Frommer et al., 2011).

In both plants from the field and plants from the greenhouse the highest Se signal intensity was found in the peripheral (cortex) region, with relatively lower signal in the center (stele). This may reflect a difference in physiology between the two tissue types, with more metabolic activity and better storage capacity in the cortex (ground tissue) than in the stele (vascular tissue). Since Se may have a defensive function, the plant may also accumulate more Se in its periphery where it can better defend the plant from attacks initiated from outside. It will be interesting in future studies to compare Se and S distribution to see if the two chemically similar elements co-localize, particularly because in earlier studies Se and S distribution and seasonal fluctuations were often shown to be correlated for non-HAs, but not correlated in HAs (Galeas et al., 2007; El Mehdawi et al. 2011).

In the plants from the field there was a high-Se ring at the root-soil interface of both HA species, which may either correspond with the epidermis or rhizosphere. Earlier, leaf Se levels were found to be especially high in the epidermis (Freeman et al., 2006a), and it is possible that the root, too, sequesters Se preferentially in the epidermis. Concentration in the periphery of organs, away from vital metabolic processes may contribute to Se tolerance, and may also offer the plant better protection from pathogens and herbivores. It is also possible that Se levels are elevated in the rhizosphere. Recently it has been reported that Se levels in rhizosphere soil of HAs *A. bisulcatus* and *S. pinnata* were significantly higher (7-13 fold) compared to bulk soil (El Mehdawi et al. 2011). The form of Se in the Se-enriched area at the root-soil interface was mainly C-Se-C in all cases. Interestingly, in *S. pinnata* the fraction of C-Se-C was relatively higher on the root-soil interface than inside the root, while in *A. bisulcatus* the fraction of C-Se-C was relatively lower there (Fig. 1, Table 2). The *A. bisulcatus* root periphery contained a large fraction of $Se^0$, indicative of microbial activity. Perhaps the high-Se peripheral ring of *A. bisulcatus* reflects the rhizosphere and is highly colonized by microbes that can reduce Se into insoluble $Se^0$. In the *S. pinnata* field root, on the other hand, the stele had a higher $Se^0$ fraction than the cortex, possibly indicating colonization by endophytic Se reducing microbes in the xylem apoplast. In either case, the

degree of microbial colonization may affect plant Se accumulation; this will be interesting to further investigate in future studies.

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# Chapter 3

## Co-cultivation of *Astragalus racemosus* and *Astragalus convallarius* with selenium-hyperaccumulator rhizosphere fungi: Effects on plant growth and accumulation of selenium and other elements


**Summary**

Little is known about how fungi affect selenium (Se) accumulation. Here we investigate the effects of two fungi on Se accumulation, translocation, and chemical speciation in hyperaccumulator *Astragalus racemosus* and non-accumulator *Astragalus convallarius*. The fungi, *Alternaria astragali* (A3) and *Fusarium acuminatum* (F30), were isolated from *Astragalus* HA rhizosphere. A3- inoculation enhanced growth of *A. racemosus* yet inhibited growth of *A. convallarius*. Selenium treatment negated these growth effects. F30 reduced shoot-to-root Se translocation in *A. racemosus*. X-ray microprobe analysis showed no differences in Se speciation between inoculation groups. The *Astragalus* species differed in Se localization and speciation. *Astragalus racemosus* root-Se was distributed throughout the taproot and lateral root, and was 90% organic in the lateral root. Interestingly, the related element sulfur (S) was present in about equal fractions of organic and inorganic forms in the hyperaccumulator. *Astragalus convallarius* root-Se was concentrated in the extreme periphery of the taproot. In the lateral root, Se was exclusively in the vascular core, and was only 49% organic. These findings indicate differences in Se assimilation between the two species, and differences between Se and S speciation in the hyperaccumulator. The finding that fungi can affect translocation may have applications in phytoremediation and biofortification.

## Introduction

The element selenium (Se) is chemically similar to sulfur (S). Selenium in the form of selenocysteine (SeCys) occurs in selenoproteins, which perform essential functions in many animals, bacteria and algae (Stadtman, 1990; Zhang and Gladyshev, 2009). Many selenoproteins have antioxidant activity, and dietary Se supplementation can enhance antioxidant capacity which may in part explain reduced susceptibility to cancer. Studies also suggest that the chemical speciation of Se is important in determining the level of protection (Ip et al., 2000). Higher plants are thought to have lost the machinery to make selenoproteins, and are therefore not known to require Se (Lobanov et al., 2009; Zhang and Gladyshev, 2009). Still, many plant species have been shown to produce organic seleno-compounds, and derive a physiological benefit from Se supplementation leading to increased antioxidant capacity (Hartikainen, 2005; Pilon-Smits et al., 2009). Lettuce, soybean, and ryegrass biofortified with Se had greater glutathione peroxidase and superoxide dismutase activity, and reduced lipid peroxidation (Hartikainen et al., 2000; Xue et al., 2001; Djanaguiraman et al., 2005).

At elevated levels Se is toxic to most organisms. Selenium toxicity is thought to occur when SeCys is incorporated into protein in the place of cysteine, leading to misfolding and loss of function (Stadtman, 1996). In addition, inorganic forms of Se may cause oxidative stress (Grant et al., 2011). Another aspect of Se toxicity may be S starvation. Because of the similarity between S and Se, they can use the same transporters and metabolic pathways. In plants, selenate is taken up via sulfate transporters and assimilated via the sulfate assimilation pathway into aminoacids and other organic compounds (Terry et al., 2000).

Some plant species stand out as having the ability to accumulate levels of Se that are 100-fold higher than surrounding vegetation, levels toxic to most organisms. These so-called hyperaccumulator (HA) plants can tolerate and actively accumulate Se up to 1.5% of their dry weight (Beath et al., 1939; Galeas

et al., 2007). Hyperaccumulation of Se has been reported for around 30 plant species, most of which are in the *Astragalus* genus (Beath, 1982). One thing that sets HAs apart is their ability to methylate SeCys via SeCys-methyltransferase (SMT) (Neuhierl and Bock, 1996). Methyl-SeCys is the predominant form of Se in leaves of many HAs (Pickering et al., 2003; Freeman et al., 2006). Roots of two HA species were shown recently by μ- X-ray Absorption Near Edge Spectroscopy (XANES) to contain most of their Se in the form of C-Se-C compounds (Lindblom et al., 2012); most likely this was methyl-SeCys, but could also have included selenomethionine (SeMet) or selenocystathionine (SeCyst) since the spectra from these three compounds are virtually indistinguishable. The capacity of HAs to store Se as methyl-SeCys may explain their extreme Se tolerance, since this is a non-protein aminoacid and therefore does not disrupt protein function.

Several hypotheses have been proposed to explain why plants hyperaccumulate (Boyd and Martens, 1992). Most evidence supports the elemental defense hypothesis which states that hyperaccumulation protects plants from pathogens and herbivores. Indeed, Se accumulation has been shown to protect plants from a variety of herbivores (El Mehdawi and Pilon-Smits, 2012). Plant Se accumulation has also been shown to offer protection from Se-sensitive microbial plant pathogens. *Brassica juncea* (Indian mustard) plants treated with Se were less susceptible to a fungal leaf pathogen (*Alternaria brassicicola*) and a fungal root/stem pathogen (*Fusarium sp.*), compared to control plants not supplemented with Se (Hanson *et al*., 2003). HAs may also use Se as a form of elemental allelopathy, inhibiting Se-sensitive neighboring plants via Se phytoenrichment of surrounding soil (El Mehdawi et al., 2011). In addition to these ecological benefits, HAs derive a significant physiological benefit from Se, producing 2-3 fold more biomass (El Mehdawi et al., 2012).

While HAs negatively affect Se-sensitive ecological partners, they offer a niche for Se-resistant ecological partners, including rhizosphere and endophytic microbes, litter decomposers, herbivores, and

neighboring plants (El Mehdawi and Pilon-Smits, 2011). Many microbes can live in association with HAs. For instance, there is evidence of Se-tolerant litter-decomposing microbes in seleniferous areas. In a litter decomposition study on seleniferous soil the high-Se litter from HA *Astragalus bisulcatus* lost weight faster and harbored significantly more microbes than low-Se litter of *Medicago sativa* (Quinn et al., 2011). In addition, several fungi have been successfully cultured from the roots of HAs *A. bisulcatus* and *Stanleya pinnata* (Wangeline and Reeves, 2007). When these rhizoplane fungi were grown in the presence of Se, they were significantly more tolerant to Se than fungi from non-seleniferous areas (Wangeline et al., 2011).

Relatively little is known about whether and how HA-associated microbes influence plant Se uptake, metabolism and tolerance. In one study, inoculation with rhizosphere bacteria collected from a high-Se area enhanced Se accumulation in the non-HA *Brassica juncea* (de Souza et al., 1998a). Other results suggestive of a potential influence of microbes on plant Se metabolism were obtained in a recent XANES study, where field-collected roots of Se HAs were shown to contain a relatively high percentage of elemental Se ($Se^0$), compared to greenhouse-grown HA roots, where $Se^0$ was found only in areas associated with microbial activity, particularly in nodules (Lindblom et al., 2012). Since $Se^0$ has not been reported in plants before, but has been found to be produced by many microbes, the $Se^0$ found inside field roots may be due to the presence of microbial endophytes. If Se HA-associated microbes indeed affect Se speciation and accumulation, they may also have a profound effect on plant growth and Se tolerance.

In the greenhouse study reported here, two *Astragalus* species, one HA and one non-HA, were inoculated with Se-HA rhizoplane fungi isolated from *Astragalus* HAs in the field, and the effect on plant growth and the accumulation of Se and other elements was investigated. Moreover, XRF and XANES were used to investigate localization and speciation of Se and other elements in both species.

## Materials and Methods

### Biological Material

*Astragalus racemosus* and *Astragalus convallarius* seeds were obtained from Western Native Seed, Coaldale, CO, USA. The two fungal isolates were obtained and described previously by Wangeline et al. (2007, 2011) from HAs growing on naturally seleniferous soil. They are (i) *Fusarium acuminatum* (F30), collected from *A. racemosus* in Lysite, WY, and (ii) *Alternaria astragali* (A3) collected from *A. bisulcatus* in Laramie, WY.

### Fungal growth

Fungi were cultivated under continuous fluorescent light at 22°C in sealed Petri dishes containing 0.5 strength malt extract agar (0.5 MEA, Difco, Detroit, MI) supplemented with 30μm sodium selenate.

### Plant growth

The *A. racemosus* and *A. convallarius* seeds were first scarified with sandpaper and then surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10 minute rinses in sterile water. Seeds were germinated on half-strength Murashige and Skoog medium with 10 g $L^{-1}$ sucrose (Murashige and Skoog, 1962) under continuous light at 23$^{o}$C in a plant growth cabinet. After 14 days the seedlings were carefully transferred to 1 L cones filled with steam-sterilized gravel (in the bottom) and coarse sand (on top). The plants were watered bi-weekly with ¼ Hoagland's solution (Hoagland and Arnon, 1938) for two weeks and then inoculated with fungi as described below.

### Co-cultivation

The *A. racemosus* and *A. convallarius* inoculation treatments consisted of fungal isolates A3, F30, or no inoculum. Plants were inoculated with a standard quantity of fungal hyphae and/or spores. The fungi

were grown on 0.5 strength MEA plates for 5d, and fungal materials were collected at the perimeter of the fungal colony. In preparation for plant inoculation, mycelia from F30 and mycelia and spores of A3 were macerated in sterile water in 1.5 mL tubes using sterile glass beads and a micropestle. Fragments of hyphae from F30, as well as spores and spore chains of A3 were quantified using a hemocytometer to estimate mm hyphae, spores and spore chains $mL^{-1}$ water. F30 and A3 were diluted to a final concentration of 2,000 mm $mL^{-1}$ water. The inocula were delivered via peat moss to the plants in the greenhouse. Each plant received 1.5 mL of peat moss saturated with 1 mL of inoculum, or peat moss with sterile water for the control treatments. The Se treatment was delivered to the plants via watering with 12.5 μM $Na_2SeO_4$ twice weekly beginning at the time of inoculation. This Se concentration was chosen so as to not induce toxicity in the non-HA. After inoculation, plants and fungi were co-cultivated for 10-13 weeks before harvest.

Elemental analysis

At harvest the plant roots were washed of sand that was bound in the root mass, and then dried for 48h at 45°C. Samples were digested in nitric acid as described by Zarcinas *et al.* (1987). Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used to determine Cu, Fe, Mg, Se and S concentrations in the acid digest (Fassel 1978).

Elemental distribution and speciation

X-ray microprobe analysis was performed on intact frozen root material from *A. racemosus* and *A. convallarius* supplied with 12.5 μM $Na_2SeO_4$. Elemental tissue distribution and chemical speciation were determined using XRF mapping and XANES spectroscopy, respectively, both as described by Lindblom et al. (2012).

Statistical Analysis

The software JMP-IN (3.2.6, SAS Institute, Cary, NC) was used for statistical data analysis. A student's t-test was used to compare differences between two means. Analysis of variance followed by a post-hoc Tukey Kramer test was used when comparing multiple means. It was verified that the assumptions underlying these tests (normal distribution, equal variance) were met.

## Results

Plant growth of Se HA *A. racemosus* and non-accumulator *A. convallarius* was affected by co-cultivation with HA-derived rhizosphere fungi. When grown in the absence of Se, *A. racemosus* shoots grew significantly larger in the presence of the A3 fungus; this positive effect was not observed in the presence of Se (Fig. 3.1A). The roots of A3-inoculated *A. racemosus* plants showed a similar trend (Fig. 3.1B): shoots and roots of F30-treated *A. racemosus* were similar in biomass to un-inoculated plants, and were only different in that the Se-treated, F30-inoculated plant roots were significantly smaller than A3-treated roots grown without added Se (Fig. 3.1A and 3.1B). *A. convallarius* plants were more affected by the fungal co-cultivation than by the addition of Se. Inoculation with A3 had a negative effect on shoot biomass, but when Se was added, *A. convallarius* plant shoots grew to a similar size as those of uninoculated plants (Fig. 3.1C). The same trend was observed for the roots of *A. convallarius*: A3 appeared to negatively affect root growth when Se was absent from the system, as judged from the observation that the A3-inoculated roots were smaller compared to the un-inoculated roots, although not quite significant at the 0.05 level ($p=.075$) (Fig. 3.1D).

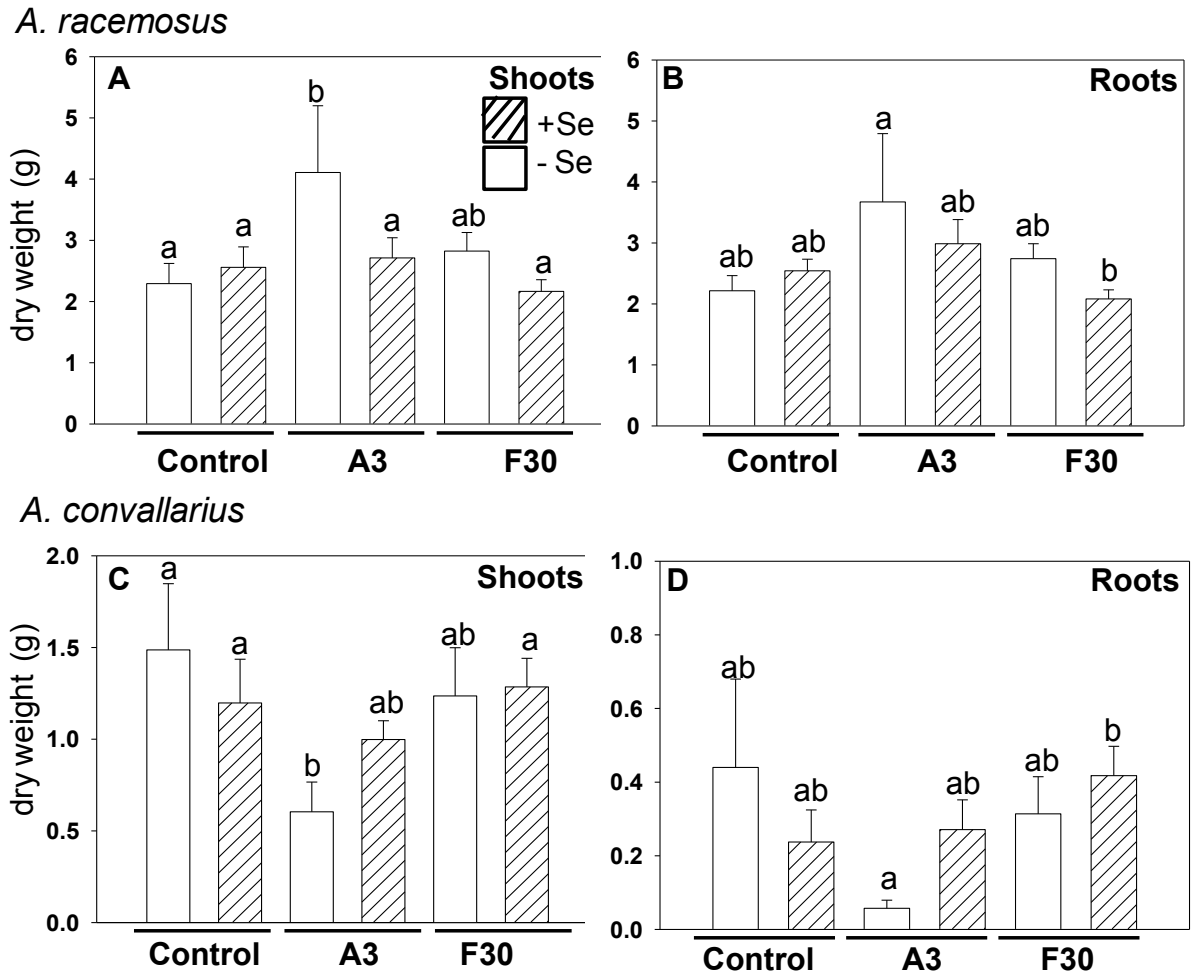


Figure 3.1 Biomass of *A. racemosus* shoots (A) and roots (B) and *A. convallarius* shoots (C) and roots (D) that were either uninoculated, or inoculated with rhizosphere fungi A3 or F30. Shown values are the mean ± standard error of the mean (SEM). Lower case letters above bars indicate statistically significant differences ($p < 0.05$).

Selenium and sulfur concentrations in *A. racemosus* shoots and roots, and the ratio of shoot concentration divided by root concentration differed with fungal co-cultivation treatment. Shoot Se levels in *A. racemosus* inoculated with either of the two fungi were lower than those left uninoculated (Fig. 3.2A). The average root Se was somewhat higher in the inoculated plants (Fig. 3.2B), but this was not significant. Due to the combined effects of lower shoot Se and higher root Se levels, however, the ratio of shoot to root Se was significantly reduced in the F30-inoculated *A. racemosus* plants (Fig. 3.2C).

Sulfur levels in the shoots of *A. racemosus* were lower in the Se-treated plants compared to plants grown without Se for the uninoculated group and the A3-inoculated group, but were unaffected by Se in the F30-treated group (Fig. 3.). In the presence of Se, plants co-cultivated with F30 contained less S in the shoots compared to control plants. Sulfur in the shoots of A3 plants treated with Se was similar to that in the control plants (Fig. 3.2D). Sulfur concentration in the roots of plants inoculated with A3 or F30 was higher than in the control plants within the Se-treated plant groups. In the absence of Se, A3 did not appear to affect S levels in the roots, but F30-treated plants showed significantly reduced root S levels (Fig. 3.2E). The fraction of S that was moved to the shoot, as determined by the concentration ratio of shoot S and root S was significantly lower in the fungus-

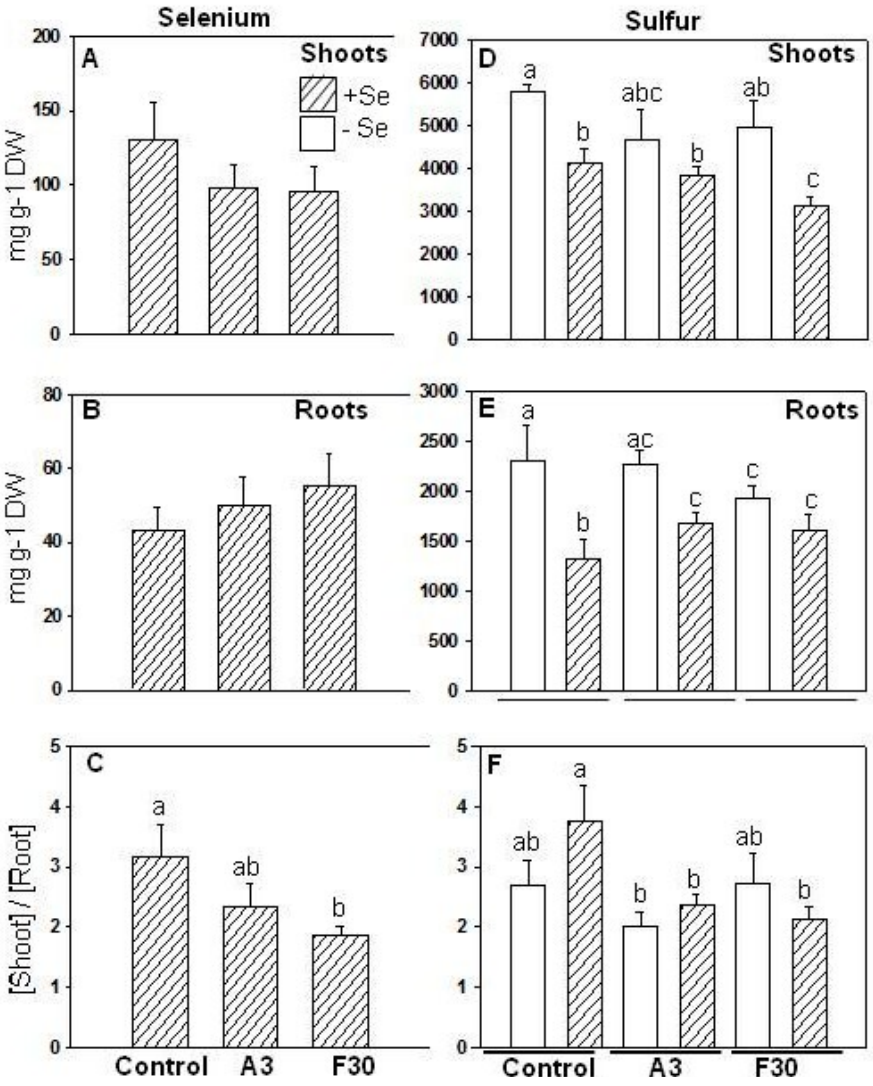


Figure 3.2 Selenium and sulfur concentration in the shoots (A, D) and roots (B, E) of *A. racemosus* plants, either uninoculated or inoculated with rhizosphere fungi A3 or F30. Panels C and F show the shoot/root ratio of the Se and S concentration, respectively. Shown values are the mean ± SEM. Lower case

inoculated plants than in the uninoculated plants in the presence of Se; in the absence of Se there was no effect of fungal co-cultivation on the S root-to-shoot ratio (Fig. 3.2F).

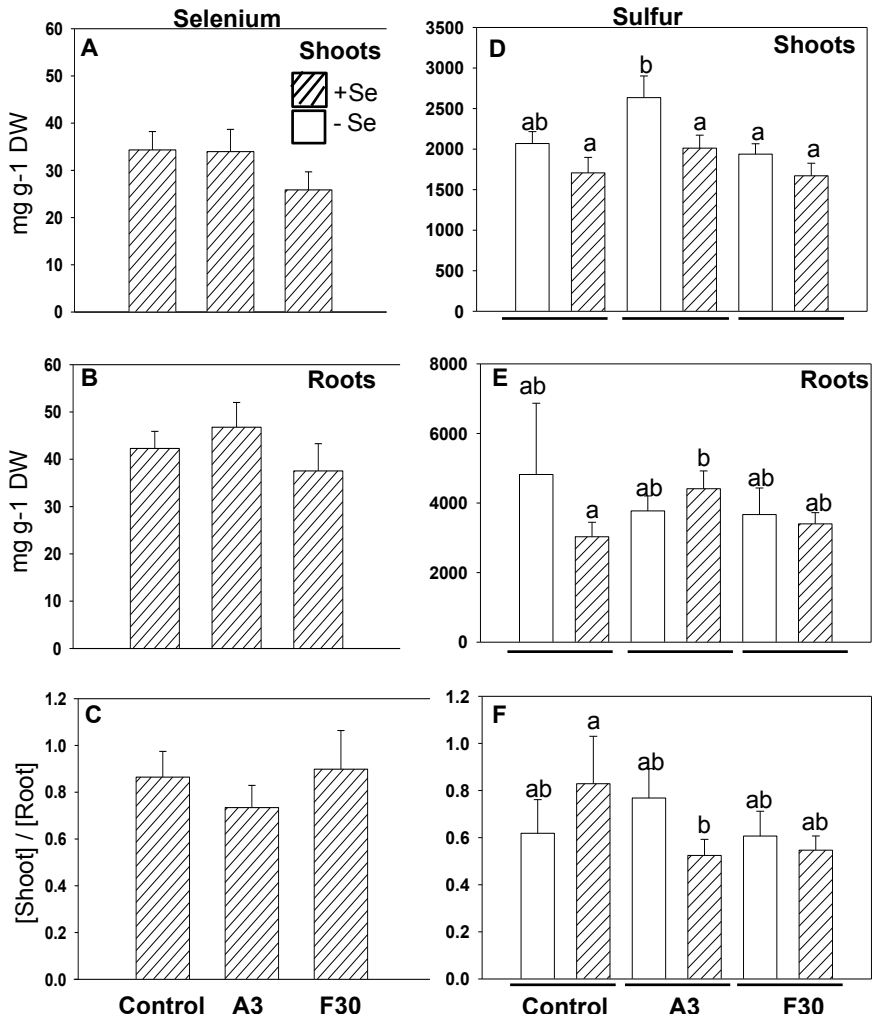


Figure 3.3 Selenium and sulfur concentration in the shoots (A, D) and roots (B, E) of *A. convallarius* plants, either uninoculated or inoculated with rhizosphere fungi A3 or F30. Panels C and F show the shoot/root ratio of the Se and S concentration, respectively. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

There were no inoculation-related differences in Se concentration in the shoots (Fig. 3.3A) or roots (Fig. 3.3B) of *A. convallarius*, nor were there differences in the ratio of shoot : root Se concentration (Fig. 3.3C). There were, however, differences in shoot S levels in the absence of Se. Shoots of *A. convallarius* co-cultivated with the A3 fungus contained higher S levels than those within the F30 group (Fig. 3.3D). The shoots of *A. convallarius* that were given Se showed no differences in shoot S levels between the inoculated and uninoculated plants (Fig. 3.3D). The roots of *A. convallarius* co-cultivated with the A3 fungus and supplied with Se, contained a higher S level in the roots compared to uninoculated plants (Fig. 3.3E). There were no other differences observed for S levels in *A. convallarius* roots due to either the presence of Se, or fungal treatment (Fig. 3.3E). The shoot : root S

concentration ratio was lower in the A3-inoculated plants compared to uninoculated *A.convallarius* when grown in the presence of Se (Fig. 3.3F). As for the F30-inoculated plants, there were no differences in shoot : root S compared to the uninoculated plants (Fig. 3.3F).

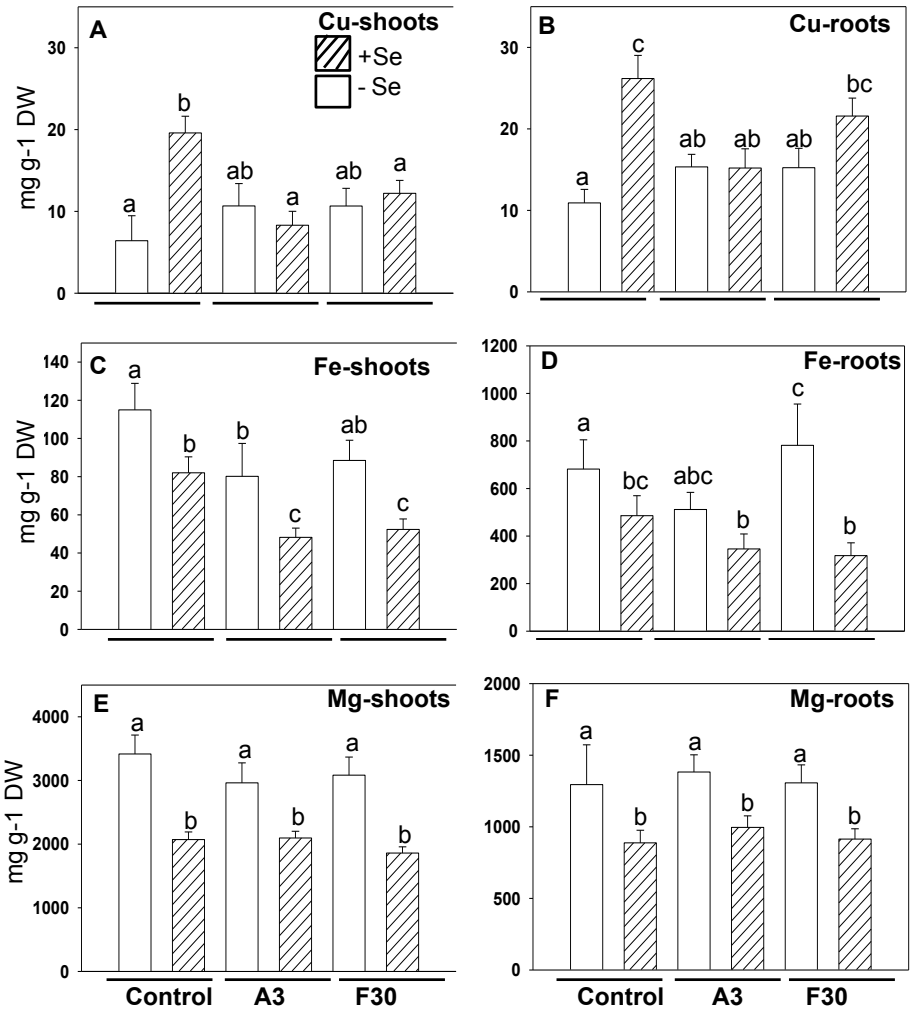


Figure 3.4 Copper (A, B), iron (C, D), and magnesium (E, F) concentration in the shoots (A, C, E) and roots (B,D, F) of *A. racemosus* either uninoculated or inoculated with rhizosphere fungi A3 or F30. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

Both the fungal and Se treatments also affected the levels of other nutrients in *A. racemosus*. The shoot and root Cu levels were higher in the Se–treated plants than in plants grown without Se, for the uninoculated group (Fig. 3.4A, B). Furthermore, inoculation with A3 or with F30 was associated with a decreased shoot Cu concentration in the presence of Se (Fig. 3.4A). There was also a lower root Cu level in A3-inoculated plants compared to uninoculated plants in the presence of Se (Fig. 3.4B). Shoot Fe levels were lower in Se–treated plants compared to plants grown without Se, and lower in A3- or F30- inoculated plants than in uninoculated plants (Fig.

3.4C). Root Fe levels were also lower in Se-treated plants compared to plants grown without Se for the uninoculated and F30-inoculated groups; no such difference was observed for the A3-inoculated group. Finally, the levels of Mg in both the shoots (Fig. 3.4E) and roots (Fig. 3.4F) were significantly lower in the Se-treated plants than in plants grown without Se; Mg levels were not affected by fungal inoculation.

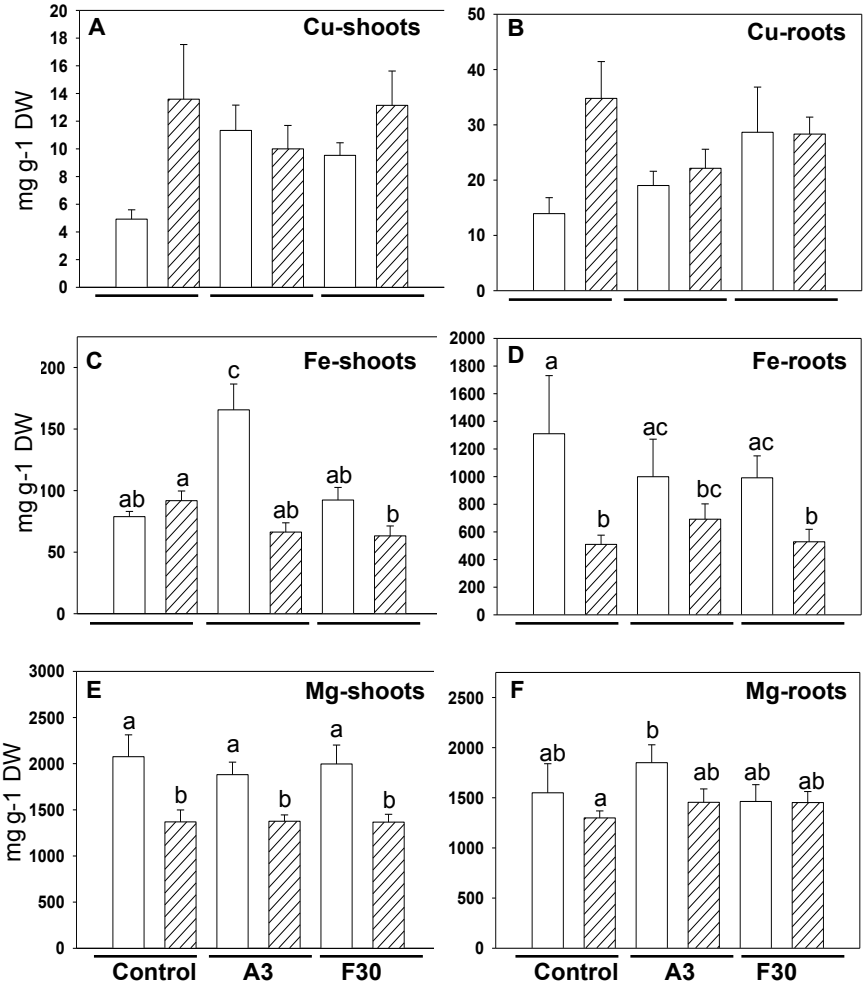


Figure 3.5 Copper (A, B), iron (C, D), and magnesium (E, F) concentration in the shoots (A, C, E) and roots (B,D, F) of *A. convallarius* either uninoculated or inoculated with rhizosphere fungi A3 or F30. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

There were no significant differences in *A. convallarius* shoot (Fig. 3.5A) or root (Fig. 3.5B) Cu levels between any of the treatment groups. Shoot Fe levels were 2-fold higher in the A3–inoculated plants compared to the uninoculated plants, but only when grown in the absence of Se (Fig. 3.5C). Root Fe levels were lower in plants treated with Se than in plants grown without Se, independent of inoculation treatment (Fig. 3.5D). Similarly, shoot Mg concentration was lower in the presence of Se than in its

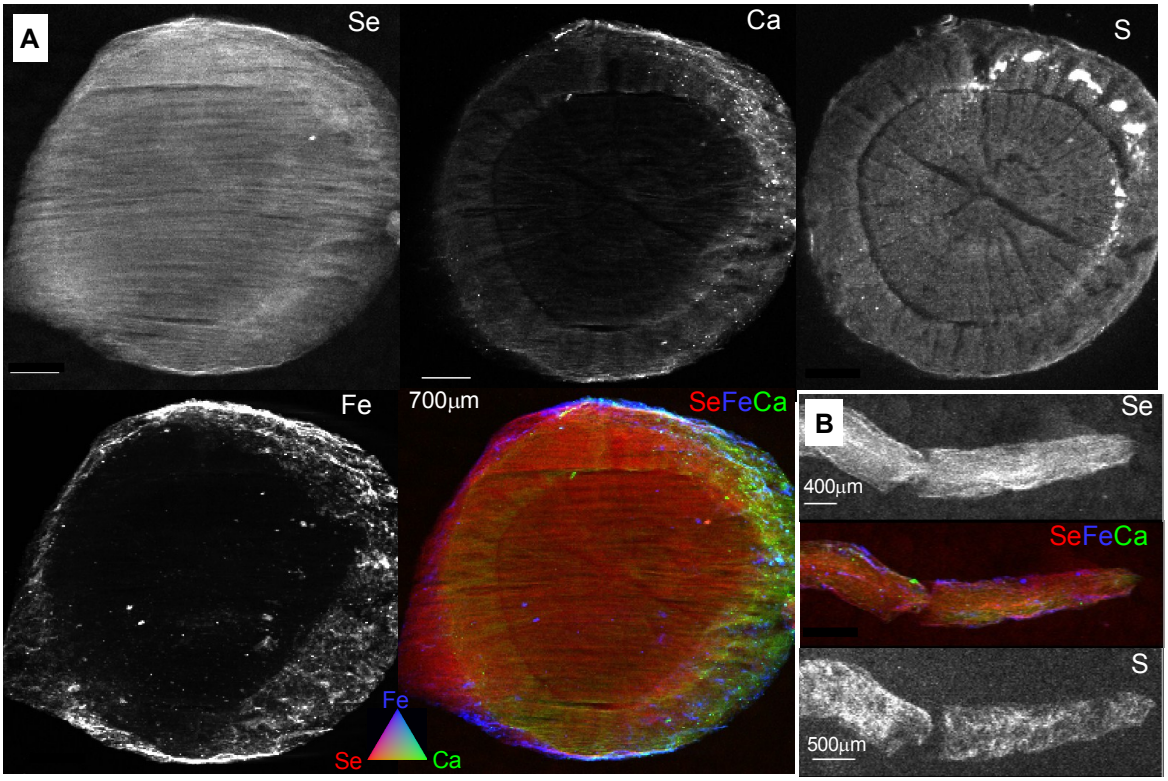


Figure 3.6 XRF maps showing the distribution of Se, Ca, and Fe in root cross-sections (A) and lateral root (B) ofuninoculated *A. racemosus* plants.

absence, independent of inoculation treatment (Fig. 3.5E) There was no effect of Se treatment or fungal inoculation on Mg levels in the roots of *A. convallarius* (Fig. 3.5F).

The Se distribution in *A. racemosus* roots was not affected by the fungal treatments. Representative roots are shown in Figure 3.6. In the cross-sectioned tap root (Fig. 3.6A) Se is shown to be distributed throughout the root, with a somewhat higher concentration in the peripheral tissues (cortex and periderm) relative to the central stele. Calcium showed a similar distribution as Se. Sulfur, on the other hand, appeared to be present at equal levels in stele, cortex and periderm. Iron was concentrated greatly in the cortex and periderm, with negligible levels in the central stele. In the lateral root (Fig. 3.6B) both Se and S were distributed throughout the root. Calcium was also found throughout the lateral root as well, while Fe was present in specks along the outside of the root.

In *A. convallarius* the different fungal treatments also did not appear to affect the root Se distribution pattern. As can be seen in the representative cross-section of a tap root, Se was localized to a great extent in the extreme periphery, in what appears to be the periderm (Fig.3.7A). Also, the Se appeared to be present at somewhat higher level in the cortex than the stele. Calcium and S followed a similar distribution pattern. Iron was also concentrated in the extreme periphery, and within the root appears to be present in two concentric rings, one in the cortex and one in the stele. In the lateral root (Fig 3.7B) Se was less concentrated in the periphery of the root, but rather was concentrated in what appears to be vascular tissue. Calcium and Fe, on the other hand, appear to be present to a large extent in the periphery of the lateral root. Sulfur does not show a particular localization pattern.

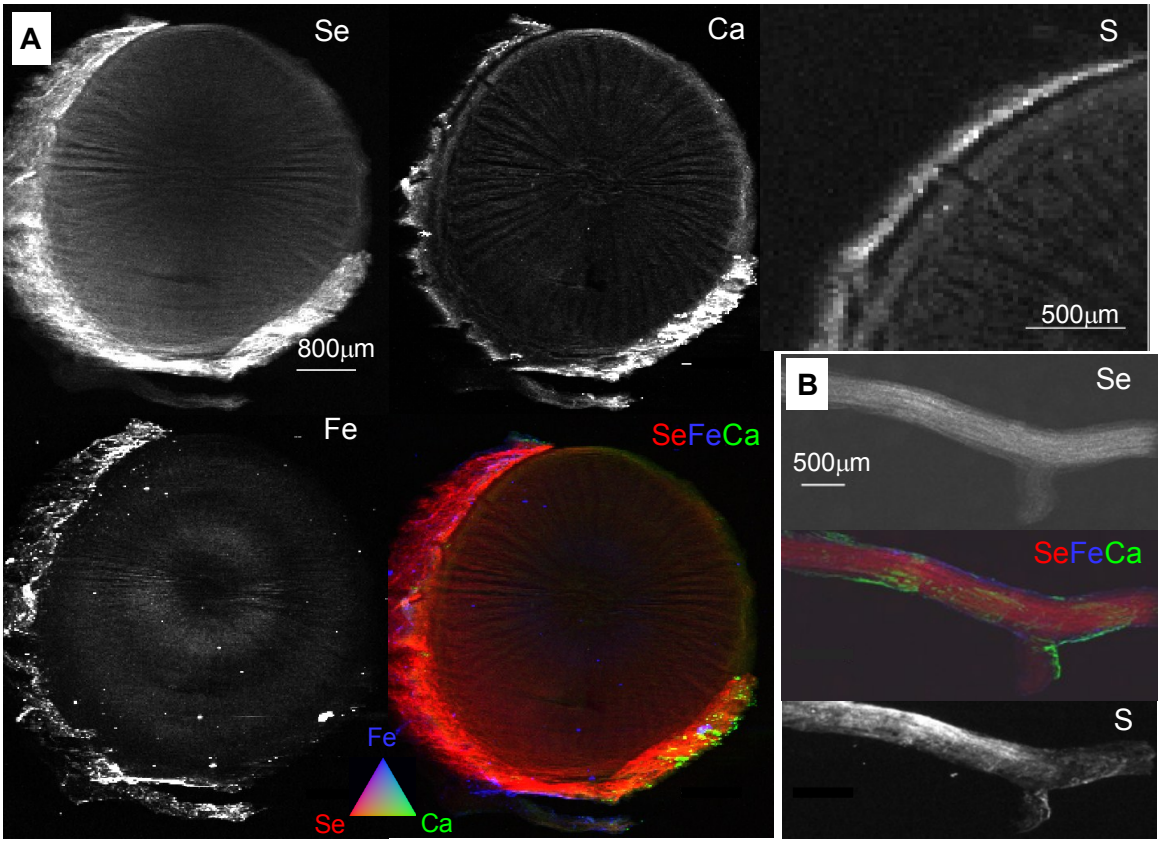


Figure 3.7 XRF maps showing the distribution of Se, Ca, and Fe in root cross-sections (A) and lateral root (B) ofuninoculated *A. convallarius* plants.

Like Se distribution, the Se speciation in roots of *A. racemosus* and *A. convallarius* did not appear to be affected by the fungal treatments. Typical K-edge Se XANES spectra are shown in Figure 3.8A. The spectrum from the *A. racemosus* root showed a high degree of similarity to the SeMet standard. The spectrum of the *A. convallarius* root, on the other hand, showed similarity to a combination of the spectra from selenate, selenite and SeMet. Least square linear combination (LSQ) analysis showed that lateral roots of *A. racemosus* contained predominantly organic Se (91% of total Se), mainly in the C-Se-C configuration (Fig. 3.8B). *A. convallarius* contained 49% organic Se (mainly C-Se-C); its large inorganic Se fraction consisted roughly equally of selenite and selenate (Fig 3.8C). Sulfur speciation was also analyzed using S XANES on the *A. racemosus* tap root shown in Figure 3.6. Unlike Se, S was present mostly as inorganic sulfate in the stele and the cortex (Fig. 3.9). In the periphery of the root (periderm) there was a mixture of sulfate and organic S (($S^0$) oxidation state, Fig. 3.9).

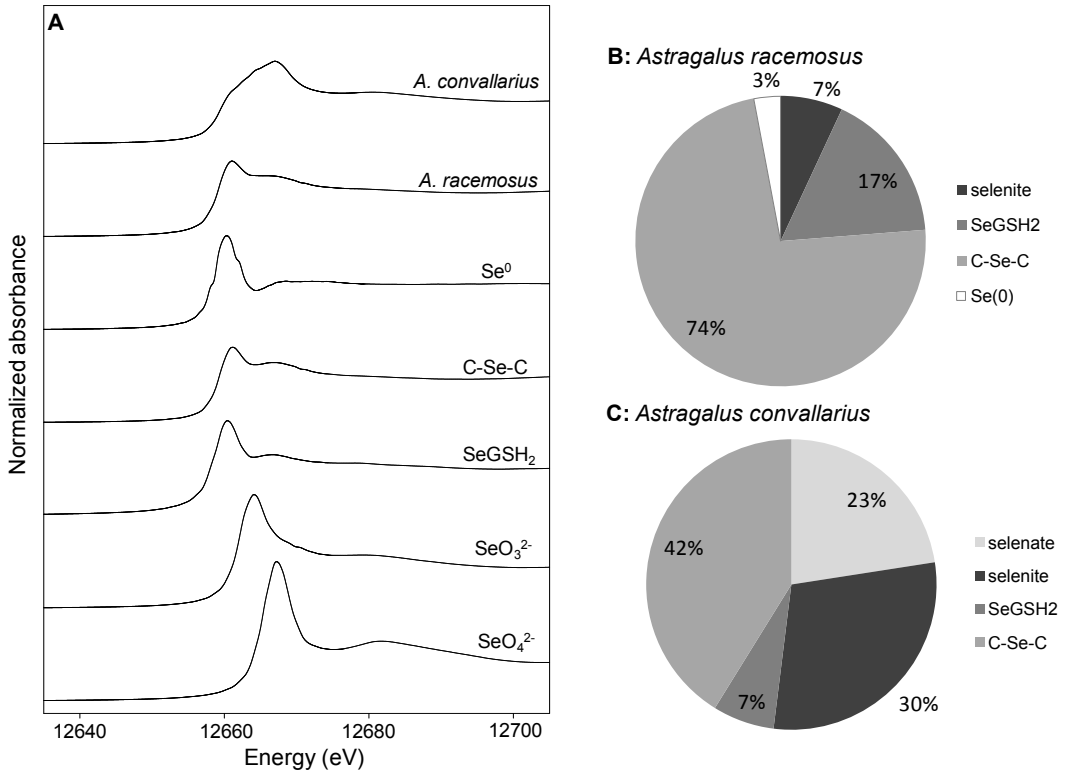


Figure 3.8 XANES K-edge Se spectra from representative lateral roots of A. racemosus and (A) and lateral root (B) ofuninoculated *A. convallarius* as well as from the isolated F30 and A3 fungi grown on selenite, in comparison with spectra from five standard seleno-compounds: gray elemental Se, selenite, selenite, seleno-glutathione, and sleno-methionine

## Discussion

The inoculation of Se HA *A. racemosus* and non-accumulator *A. convallarius* with rhizoplane fungi isolated from HAs significantly affected plant growth and accumulation of Se and S.

The effects of fungal inoculation on plant growth were generally positive for *A. racemosus* and negative for *A. convallarius*, and were Se-dependent. The fungi reduced shoot/root Se ratio in *A. racemosus* and shoot/root S ratio in both species. The observed effects of fungal inoculation on Se accumulation could not be explained by differences in Se speciation and localization, as judged from XANES and XRF analysis. There were, however, differences in Se localization and speciation between the two *Astragalus* species; furthermore, Se and S showed different localization patterns in both species.

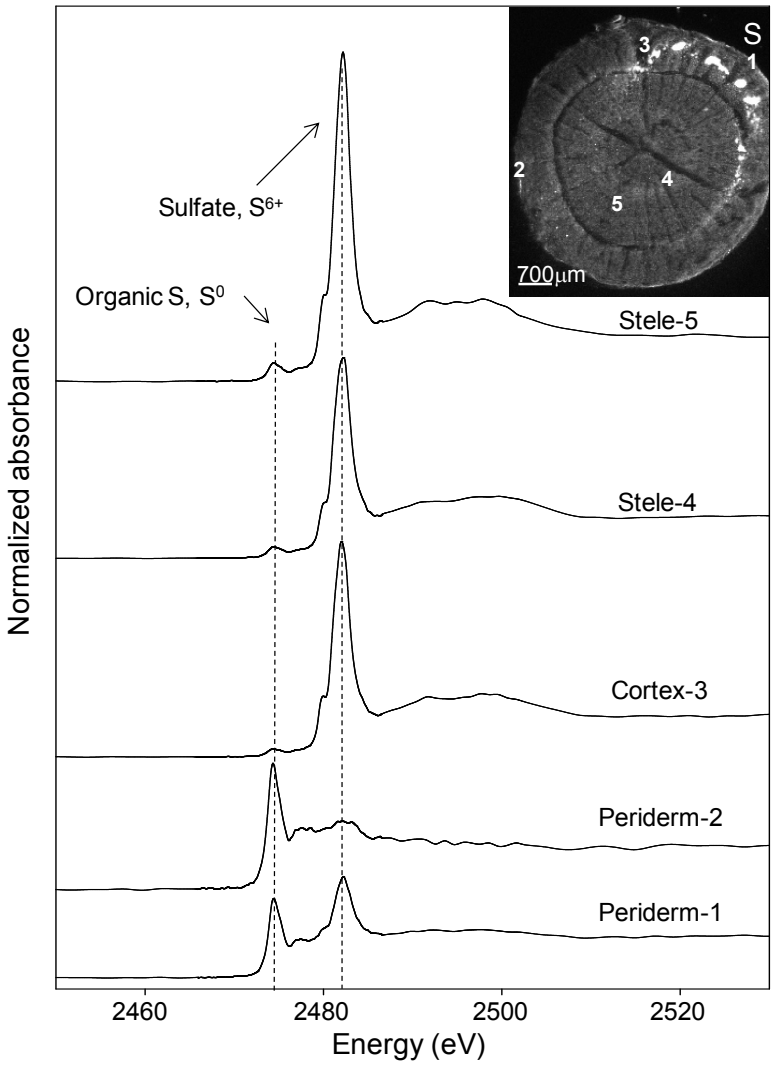


Figure 3.9 XANES K-edge S spectra from the tap root of *A. racemosus*. Spectra were obtained inside the central vascular cylinder (stele); in the surrounding cortex, and in the extreme periphery (periderm).

The effect of A3, *Alternaria astragali*, on plant growth was Se-dependent as well as species-dependent. When no Se was added, A3 had a positive effect on *A. racemosus* but a negative effect on *A.*

*convallarius*. Thus, A3, originally isolated from HA *A. bisulcatus*, promoted growth of a related HA, but appeared to be pathogenic to the related nonaccumulator. In the presence of Se these growth effects were not observed. The positive and negative growth effects did not correlate with root or shoot Cu, Fe, Mg or S levels; the underlying mechanisms are not clear but may involve fungal production of growth-affecting hormones.

The observation that there was a negative growth effect of A3 on *A. convallarius* in the absence but not in the presence of Se, suggests that Se accumulation may protect *A. convallarius* from the pathogenicity of this fungus. Selenium accumulation was shown earlier to be able to protect *B. juncea* from two Se-sensitive pathogenic fungi, one *Alternaria* and one *Fusarium* species (Hanson et al., 2003). A3 was shown earlier (Lindblom et al., 2012) to be inhibited by Se at the lowest concentration tested, 30 mg/L. For comparison, the tissue Se concentration in *A. convallarius* root and shoot was 30-40 mg Se/kg DW, or ~3-4 mg Se/kg FW. In *A. racemosus* the tissue Se levels were 50-100 mg Se/kg DW. The positive effect of A3 on the growth of this species was also only observed in the absence of Se, again indicating that this fungus may be inhibited by Se. Of course the form of Se was not the same in the two plant species and the medium, making comparison not so straightforward.

Inoculation with either of the two fungi was associated with a reduced increased root surface area or upregulation of sulfate/selenate transporters. Such effects were reported for rhizosphere bacteria (de Souza et al., 1998a). It is also feasible that the fungi affected root Se and S levels by altering Se and S speciation in the rhizosphere or root, either into a form more readily taken up by the root or a form less readily translocated to the shoot. XANES did not show a significant difference in speciation between the inoculated and uninoculated roots, but it cannot be excluded that the XANES locations examined were not colonized by fungal hyphae. Another reason the fungi affected root Se and S levels may have been that the fungal hyphae were particularly rich in Se and S and were so tightly bound to the roots that they

were collected together with the root for elemental analysis. This is perhaps less likely, in view of the finding that Se accumulation was only affected by the fungi in *A. racemosus* and not in *A. convallarius*. Some other differences between the two plant species worth mentioning are that the HA generally showed a much higher Se and S translocation ratio compared to the non-accumulator, and that S uptake and translocation were negatively affected by Se in the HA but not the nonaccumulator. HAs of Se have been reported before to have a higher Se translocation factor (El Mehdawi et al., 2012); this may be caused by different expression levels of selenate transporters, or to differential speciation of Se in roots of HAs and non-HAs. The finding that Se impeded S uptake and translocation more in the HA may suggest that the HA sulfate/selenate transporters that mediate uptake and translocation have a higher affinity for selenate than sulfate.

The two plant species differed markedly with respect to root Se localization and speciation. HA *A. racemosus* contained Se throughout its taproot and lateral root, with a slightly higher level in the cortex. Nonaccumulator *A. convallarius*, on the other hand, showed a strong Se localization in the extreme periphery of the taproot (periderm and/or rhizosphere), and in the lateral root Se was strongly concentrated in the vascular core. Concentration of Se in the root cortex has been observed earlier in another *A. racemosus* plant as well as in *Astragalus bisulcatus* (Lindblom et al., 2012). The Se in *A. racemosus* lateral roots was 90% organic while *A. convallarius* contained only 49% organic Se; the other shoot/root Se ratio in *A. racemosus* as well as a lower shoot/root S ratio in both *Astragalus* species. The effect of the fungi on S translocation was only observed in the presence of Se. The lower shoot/root Se/S ratios were mainly the result of elevated root Se or S levels, although the shoot Se or S levels were also somewhat lower. Thus, the fungi appeared to promote Se and S uptake into the root, and perhaps also root-to-shoot translocation. Enhanced root Se and S uptake under influence of the fungi may have been caused by

half of the Se in *A. convallarius* was selenite and selenate. The organic Se in both species consisted mainly of C-Se-C compounds. This may have been SeMet, methyl-SeCys or selenocystathionine, as their XANES spectra are indistinguishable. The other non-accumulator species, *Astragalus drummondii* was shown recently to contain predominantly C-Se-C in its leaves (El Mehdawi et al., 2012). It is interesting that these non-accumulator *Astragalus* species apparently are capable of producing substantial levels of organic Se from selenate, since non-accumulators from other genera were found to predominantly accumulate selenate when supplied with selenate (de Souza et al., 1998b; Van Hoewyk et al., 2005). The speciation in this lateral root of *A. racemosus* was similar to that found earlier in the taproot of another plant of the same species growing under the same conditions, but the lateral root contained a somewhat smaller fraction of C-Se-C (90%) compared to the taproot, which contained exclusively C-Se-C (Lindblom et al. 2012).

The root distribution of S was somewhat different from that of Se for HA *A. racemosus*: S was evenly dispersed throughout the taproot, while Se was most concentrated in the cortex. No such difference was observed in the taproot of *A. convallarius* where S, like Se, was strongly concentrated in the extreme periphery. The different tissue distribution patterns for Se and S in the HA can perhaps be explained by differences in Se and S speciation. Indeed, while the two elements were supplied in analogous forms, as selenate and sulfate, respectively, they were apparently metabolized differently in the HA. Most of the Se was accumulated in organic form throughout the root; S, however, was present to a large extent as inorganic S (sulfate) in the stele and cortex, with only the periderm showing a higher fraction of organic S (as S(0), Fig. 9). This suggests that the HA *A. racemosus* has the ability to discriminate between Se and S analogs, and that it assimilates much of its Se in its root while S assimilation happens more in the shoot. Indeed, S assimilation is thought to happen mostly in mesophyll chloroplasts (Pilon-Smits and Pilon, 2006).

Our finding that fungal inoculation may affect Se and S uptake and translocation may have ecological implications and potential applications. It appears that the capacity of a plant to (hyper)accumulate Se is determined not only by the attributes of the plant, but also by its microbial partners. If fungi can affect Se uptake and translocation, this could influence the above- and below-ground interactions with different ecological partners, including herbivores, pollinators and other microbes. The finding that one fungus had an opposite effect on growth in the HA and the related non-accumulator, and that this effect was Se-specific, may suggest that fungi can affect competition between plant species in the field and that this effect will be different on different soil types. In a phytoremediation setting, fungus-associated enhanced uptake and root accumulation may enhance phytostabilization efficiency. Moreover, reduced translocation of Se to the shoot may reduce movement of Se into the food chain, and associated toxicity to wildlife and livestock. In future investigations it will be interesting to further explore the impact of microbial symbionts on plant Se (hyper)accumulation, for better insight into the ecology of seleniferous areas and potential application in phytoremediation or biofortification.

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# Chapter 4

## Inoculation of selenium hyperaccumulator *Stanleya pinnata* and related non-accumulator *Stanleya elata* with hyperaccumulator rhizosphere fungi - Investigation of effects on Se accumulation and speciation


**Summary**

Little is known about how fungi affect elemental accumulation in hyperaccumulators. Here, two rhizosphere fungi from selenium (Se) hyperaccumulator *Stanleya pinnata*, *Alternaria seleniiphila* (A1) and *Aspergillus leporis* (AS117) were used to inoculate *S. pinnata* and related nonhyperaccumulator *Stanleya elata*. Growth and Se and sulfur (S) accumulation were analyzed. Furthermore, X-ray microprobe analysis was used to investigate elemental distribution and speciation. Growth of *S. pinnata* was not affected by inoculation or by Se. *Stanleya elata* growth was negatively affected by AS117 and by Se, but combination of both did not reduce growth. Selenium translocation was reduced in inoculated *S. pinnata*, and inoculation reduced S translocation in both species. Root Se distribution and speciation were not affected by inoculation in either species; both species accumulated mainly (90%) organic Se. Sulfur, in contrast, was present equally in organic and inorganic forms in *S. pinnata* roots.

Thus, these rhizosphere fungi can affect growth and Se and/or S accumulation, depending on host species. They generally enhanced root accumulation and reduced translocation. These effects cannot be attributed to altered plant Se speciation but may involve altered rhizosphere speciation, as these fungi are known to produce elemental Se. Reduced Se translocation may be useful in applications where toxicity to herbivores and movement of Se into the food chain is a concern. The finding that fungal inoculation can enhance root Se accumulation may be useful in Se biofortification or phytoremediation using root crop species.

## Introduction

The element selenium (Se) naturally occurs in the earth's crust. Selenium is an essential element for many species including humans, but can be toxic at higher levels. In areas where soil Se levels or Se bioavailability are low, Se deficiency in livestock is a serious and costly problem (Maas et al. 2011). In high-Se areas, Se toxicity can occur and cause health- or behavioral problems, and even death in animals and humans (Aldosary et al. 2012). Although Se has not been shown to be essential to plants, it has been reported to be beneficial at low levels, increasing antioxidant activity and promoting growth (for a review see Pilon-Smits et al. 2009). Selenium is chemically similar to sulfur (S) and readily taken up and assimilated by plants via the S assimilation pathway (for a review see Terry et al. 2000). The capacity of plants to accumulate Se from soil is limited by the concentration and bioavailability of Se, as well as the physiological properties of the plant. Genetic and environmental components that contribute to plant Se accumulation and tolerance may be exploited to prevent both deficiency (via biofortification) and toxicity (via phytoremediation).

Plant species differ in their capacity to accumulate Se. Most plants are sensitive to Se at high levels and accumulate no more than 100 mg $kg^{-1}$ DW under natural conditions (Beath et al. 1982). However, a small number of plant species endemic to seleniferous soils can accumulate Se to levels typically 100-fold higher than other local vegetation, reaching 1,000-15,000 mg $kg^{-1}$ DW (Beath et al. 1939). These so-called hyperaccumulators hyperaccumulators (HAs) differed from non-HAs with respect to their Se biochemistry and physiology. Micro-focused X-ray fluorescence (XRF) mapping and Se K-edge X-ray absorption near-edge structure (XANES) have shown that non-HA plants sequester Se primarily in the leaf vasculature as selenate (de Souza et al. 1998a; Freeman et al. 2006), but Se HAs accumulate Se predominantly in the leaf epidermis in organic forms with a C-Se-C configuration, which contain Se coupled to two organic groups (Freeman et al. 2006). Hyperaccumulator *Astragalus bisulcatus*

(Fabaceae) showed specific Se sequestration in the leaf trichomes, in the form of C-Se-C compounds that were identified as methyl-selenocysteine (MeSeCys) and □-glutamyl-MeSeCys using liquid chromatography – mass spectroscopy (Pickering et al. 2003, Freeman et al. 2006). Hyperaccumulator *Stanleya pinnata* (Brassicaceae) sequestered Se in its leaf epidermis, in specialized cells along the leaf margin in the form of C-Se-C compounds that were identified as 80% MeSeCys and 20% selenocystathionine (SeCysth), while non-HA *Stanleya albescens* showed a diffuse Se distribution throughout the leaf and accumulated 100% SeCysth (Freeman et al. 2006, 2010). The amino acid MeSeCys does not get incorporated into proteins, and can therefore be safely accumulated. This is thought to be the main mechanism of the Se hypertolerance of HAs (Neuhierl and Böck, 1996).

While the biochemistry and physiology of Se hyperaccumulation have received a lot of attention, there is still very little known about the plant-microbe interactions of Se HA plants, or of any elemental HA (for a review see Alford et al. 2010). In general, plant-microbe interactions can be mutualistic, pathogenic, and/or saprophytic. Sometimes a plant-fungal mutualism can turn pathogenic when the plant becomes stressed, and/or saprophytic after the plant dies, as has been shown for many grass symbionts in the *Alternaria* genus (Dugan and Lupien 2004). Some of the known beneficial effects of microbes on plants include: stimulation of plant root growth and changes in root morphology (de Souza et al. 1998b), protection from pathogens and herbivores, as well as from abiotic stresses such as drought, salt, heat, and toxic elements (for a review see Harmon 2011). Many plants cannot survive without their fungal symbiont(s), indicating an essential role of plant-fungal mutualism in the evolution of plant stress tolerance (Rodriguez and Redman 2008). A similar dependence on microbial symbionts may have played a role in the evolution of hyperaccumulation.

Selenium accumulation has likely also played a role in the evolution of microbial partners. There is evidence of a wide range in Se tolerance in fungi. In a decomposition study conducted on naturally

seleniferous soil, high-Se leaf litter contained more culturable microbes than low-Se leaf litter (Quinn et al. 2011). Of the hypotheses presented as to why elemental accumulation evolved in plants, the elemental defense theory, stating that plants are protected by the accumulated element from pathogens and herbivores, has been well-supported (for a review see El Mehdawi and Pilon-Smits, 2012). Most elemental defense studies have focused on plant-herbivore interactions, but one study using *Brassica juncea* demonstrated that plants treated with Se were better protected against two fungal pathogens, an *Alternaria* and a *Fusarium* species (Hanson et al. 2003).

The objective of this study was to investigate whether two rhizosphere fungi from Se HA *S. pinnata* can affect Se uptake, accumulation, speciation, and/or growth of *S. pinnata* as well as of a related non-HA, the Se-sensitive species *Stanleya elata* (El Mehdawi et al. 2012). The two fungi used in this study were described earlier by Wangeline and Reeves (2007) and further characterized by Lindblom et al. (2012). *Alternaria seleniiphila* (A1) and *Aspergillus leporis* (AS117), isolated from the rhizoplane of *S. pinnata*, are both highly Se-tolerant and accumulate high levels of Se, mainly in elemental form ($Se^0$). The capacity of these fungi to convert other, toxic forms of Se to inert, insoluble $Se^0$ may not only confer Se tolerance to the fungus, but also affect their host plant's Se uptake, accumulation and tolerance. Since many fungi are promiscuous with respect to their host range, root symbionts of HAs may have similar effects on non-HAs. If fungi can indeed affect Se accumulation and tolerance in a range of plants, they may be useful for biofortification and phytoremediation technologies.

## Materials and Methods

Biological Material

*Stanleya pinnata* seeds were obtained from Western Native Seed, Coaldale, CO, USA. *Stanleya elata* seeds were collected in Southern Nevada by Jennifer Cappa (Colorado State University). The two fungal isolates were obtained as described previously by Wangeline et al. (2007, 2011) from *S. pinnata* growing

on naturally seleniferous soil around Fort Collins, CO, USA. They are (i) *Alternaria seleniiphila* (A1), and (ii) *Aspergillus leporis* (AS117).

Fungal cultivation

Fungi were cultivated under continuous fluorescent light at 22°C in sealed Petri dishes containing half-strength malt extract agar (0.5 MEA, Difco, Detroit, MI) supplemented with 30 m sodium selenate.

Plant cultivation

The *S. pinnata* and *S. elata* seeds were surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10 minute rinses in sterile water. Seeds were germinated on half-strength Murashige and Skoog medium with 10 g $L^{-1}$ sucrose (Murashige and Skoog, 1962) under continuous light at 23$^{o}$C in a plant growth cabinet. After 14 days the seedlings were carefully transferred to 1 L cones filled with steam-sterilized gravel (in the bottom) and coarse sand (on top). The plants were watered bi-weekly with quarter-strength Hoagland's solution (Hoagland and Arnon, 1938) for two weeks and then inoculated with fungi as described below.

Inoculation

The *S. pinnata* and *S. elata* inoculation treatments consisted of fungal isolates A1, AS117, or no inoculum. Plants were inoculated with a standard quantity of fungal hyphae and/or spores. The fungi were grown on 0.5 strength MEA plates for 5d, and fungal materials were collected at the perimeter of the fungal colony. In preparation for plant inoculation, mycelia and spores or spore chains of A1 were macerated in sterile water in 1.5 mL tubes using sterile glass beads and a micropestle. Fragments of hyphae, spores and spore chains of A1 were quantified using a hemocytometer to estimate mm hyphae, spores and spore chains $mL^{-1}$ water. Spores from AS117 were collected in sterile water with 1% tween.

A1 and AS117 were diluted to a final concentration of 2,000 mm hyphae $mL^{-1}$ water and 2,000 mm spores $mL^{-1}$ water, respectively. The inocula were delivered via peat moss to the plants in the greenhouse. Each plant received 1.5 mL of peat moss saturated with 1 mL of inoculum, or peat moss with sterile water for the control treatments. The Se treatment was delivered to the plants via watering with 12.5 μM $Na_2SeO_4$ twice weekly beginning at the time of inoculation. This Se concentration was chosen so as to not induce toxicity in the non-HA. After inoculation, plants and fungi were co-cultivated for 13 weeks before harvest.

Elemental analysis

At harvest the plant roots were washed of sand that was bound in the root mass, and then dried for 48h at 45°C. Samples were digested in nitric acid as described by Zarcinas *et al.* (1987). Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used to determine elemental concentrations in the acid digest (Fassel 1978).

Elemental distribution and speciation

X-ray microprobe analysis was performed on intact frozen root material from *S. pinnata* and *S. elata* that had been supplied with 12.5 μM $Na_2SeO_4$. Elemental tissue distribution and chemical speciation were determined using XRF mapping and XANES spectroscopy, respectively, both as described by Lindblom et al. (2012).

Statistical Analysis

The software JMP-IN (3.2.6, SAS Institute, Cary, NC) was used for statistical data analysis. A student's t-test was used to compare differences between two means. Analysis of variance followed by a post-hoc Tukey Kramer test was used when comparing multiple means. It was verified that the assumptions underlying these tests (normal distribution, equal variance) were met.

**Results**

The dry weight biomass of Se hyperaccumulator (HA) *S. pinnata* was not affected by fungal-inoculation, Se treatment, or the combination of both (Fig. 4.1 top). The non-accumulator *S. elata* was negatively affected by Se treatment, and by *Aspergillus leporis* (AS117) inoculation (Fig. 1 bottom). Selenium toxicity, particularly in the late stages of the experiment presented itself as die-back from the tips of *S. elata* leaves and eventually, death of 5 of the original 12 un-inoculated plants within the experimental group. *Stanleya elata* inoculated with (AS117) did not have inhibited growth compared to un-inoculated, minus Se, control plants (Fig. 4.1 bottom).

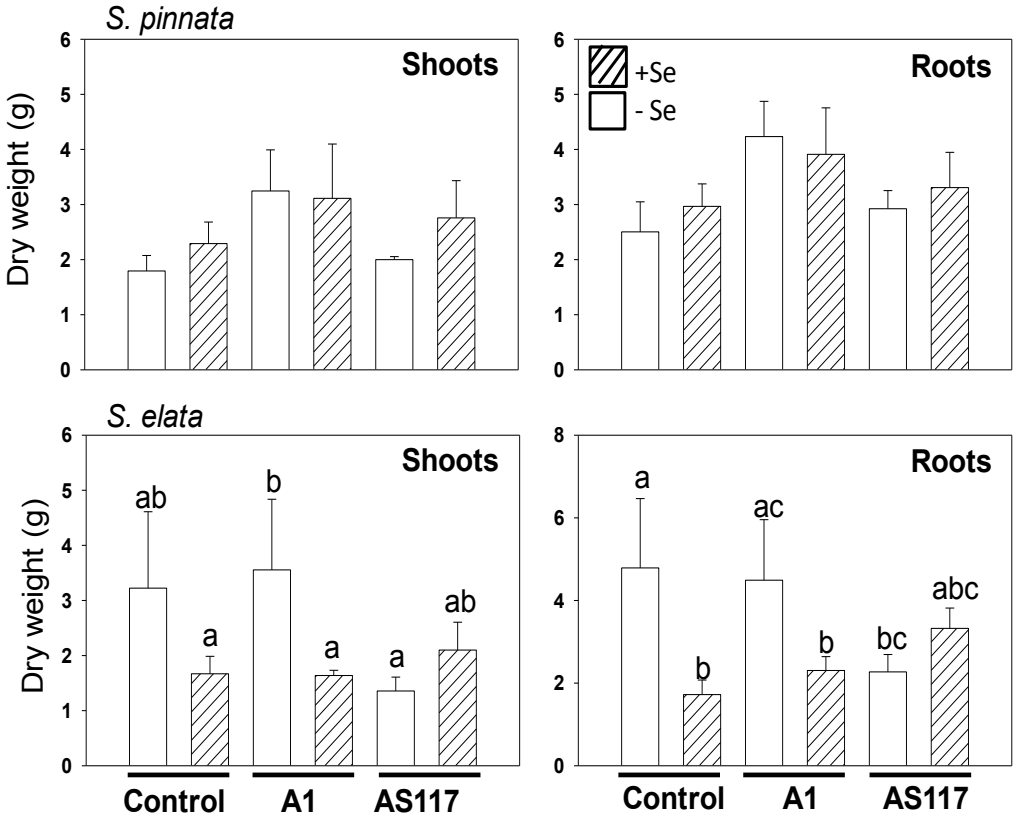


Figure 4.1 Biomass of *S. pinnata* shoots (top) and roots (bottom) that were either un-inoculated or inoculated with rhizosphere fungi A1 or AS117. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

Selenium levels in the roots of A1-inoculated *S. pinnata* were 30% higher compared to the un-inoculated plants, or to plants inoculated with AS117. In shoots of inoculated plants, there were two-fold lower Se

levels compared to un-inoculated plants (Fig. 4.2, left). The ratio of shoot and root Se concentration was two- to three-fold lower in inoculated *S. pinnata*. Since S is chemically similar to Se, S tissue concentration was also determined in the shoots and roots. The effect of fungal inoculation on tissue S levels was similar -in the presence of Se- to that observed for Se. Shoot S concentration was lower in inoculated plants, as was the shoot-to-root S concentration ratio in A1-inoculated plants compared to un-inoculated plants (Fig. 4.2, right). In Se-treated plants, root S concentration was significantly lower in all cases, whereas in the shoots, S levels were only reduced in fungus-inoculated *S. pinnata*.

The total amount of Se accumulated, calculated as the product of Se concentration and biomass, was 2-fold higher in the roots of A1-inoculated *S. pinnata* compared to AS117-inoculated or un-inoculated plants (Fig. 4.3, left).

Total shoot Se accumulation was 1.5- to 2-fold lower in plants inoculated with either fungus. The translocation factor (total shoot accumulation / total root accumulation), was on average 5 in un-inoculated *S. pinnata*, i.e. there

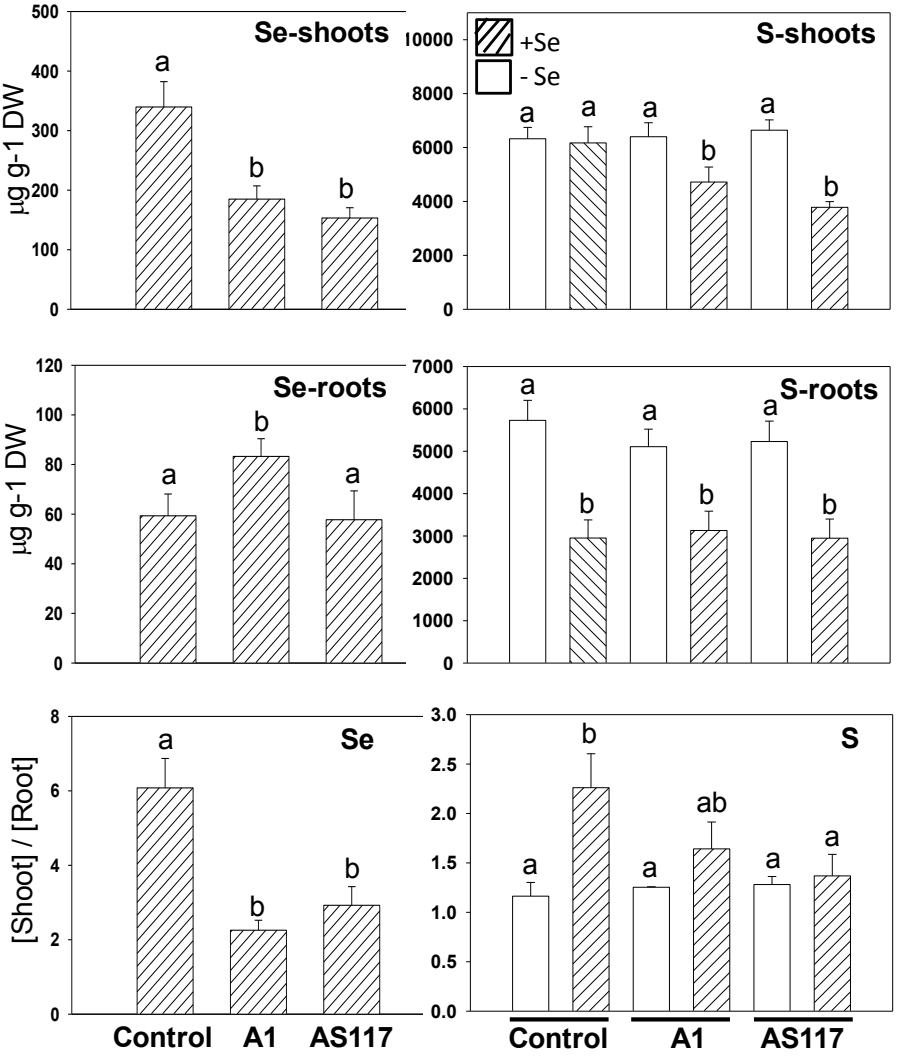


Figure 4.2 Selenium (left) and S (right) concentration in shoot and roots, as well as the shoot-to-root concentration ratio in un-inoculated or inoculated with rhizosphere fungi A1 or AS117. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

was five-fold more Se in shoots than roots, compared to only 2-fold more Se in shoots than roots in inoculated plants. The total Se accumulated per plant (root and shoot) was 1.5 times lower in AS117-inoculated plants than in un-inoculated plants ($p < .05$); AS117-inoculated plants showed intermediate results between A1- and un-inoculated plants, and this treatment was not significantly different from either other. Tissue Se levels were not affected by fungal inoculation in *S. elata* (Fig. 4.4, left). There were also no differences in S tissue levels in the presence of Se (Fig. 4.4, right). However, in the absence of Se, root S levels were higher in A1-inoculated plants compared to AS117- and un-inoculated plants, and shoot S levels were elevated in inoculated plants compared to un-inoculated plants. Selenium-

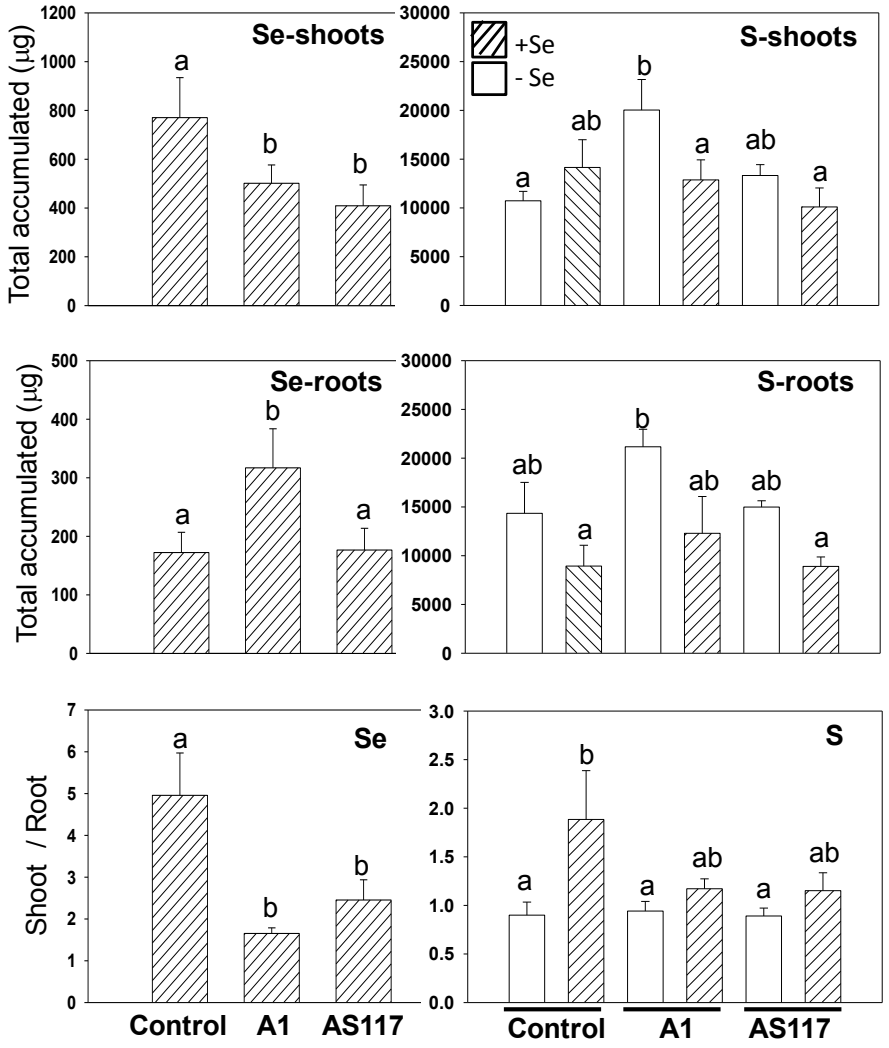


Figure 4.3 Total Se (left) and S (right) accumulated in the shoot and roots, as well as the shoot/root ratio of shoot and rootaccumulation in un-inoculated or inoculated with rhizosphere fungi A1 or AS117. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

treated *S. elata* plants had lower S in roots and shoots in all cases. The Se-related reduction in S accumulation in the shoots was more pronounced in the inoculated plants (-50%) than in un-inoculated plants (-20%). Total root Se accumulation was two-fold higher in AS117-inoculated plants

compared to un-inoculated plants ($p < .05$); A1-inoculated plants showed intermediate Se root accumulation which was not significantly different from the other two treatments (Fig 4.5, left).

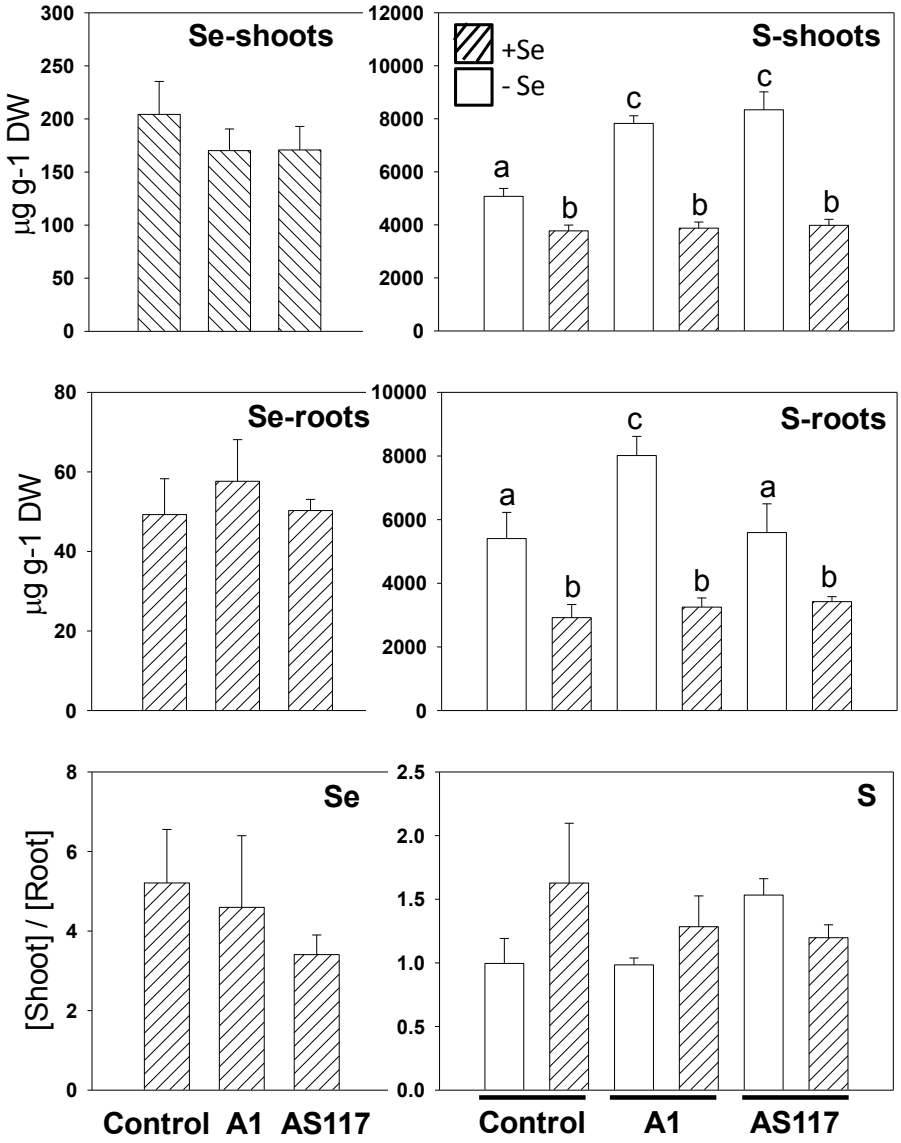


Figure 4.4 Selenium (left) and S (right) concentration in shoot and roots, as well as the shoot-to-root concentration ratio in *S. elata* un-inoculated or inoculated with rhizosphere fungi A1 or AS117. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

Total shoot Se accumulation was not affected by the fungal inoculations. In the presence of Se, there were no differences in shoot or root S accumulation (Fig. 4.5, right). However, there was an effect on S partitioning between the roots and shoots of *S. elata*. There was relatively less S in the shoots of inoculated plants.

In the absence of Se, total S accumulation in shoots and roots was highest in A1-inoculated plants and lowest in AS117-inoculated plants ($p < .05$); un-inoculated plants showed intermediate S accumulation.

Total shoot and root S accumulation were three to four times higher in the absence of Se than in the presence of Se for A1-inoculated and un-inoculated plants; there was no such effect on S accumulation in AS117-inoculated *S. elata*, where S accumulation was independent of the Se treatment.

XRF mapping was used to determine the localization of Se and other elements in inoculated and un-inoculated plants.

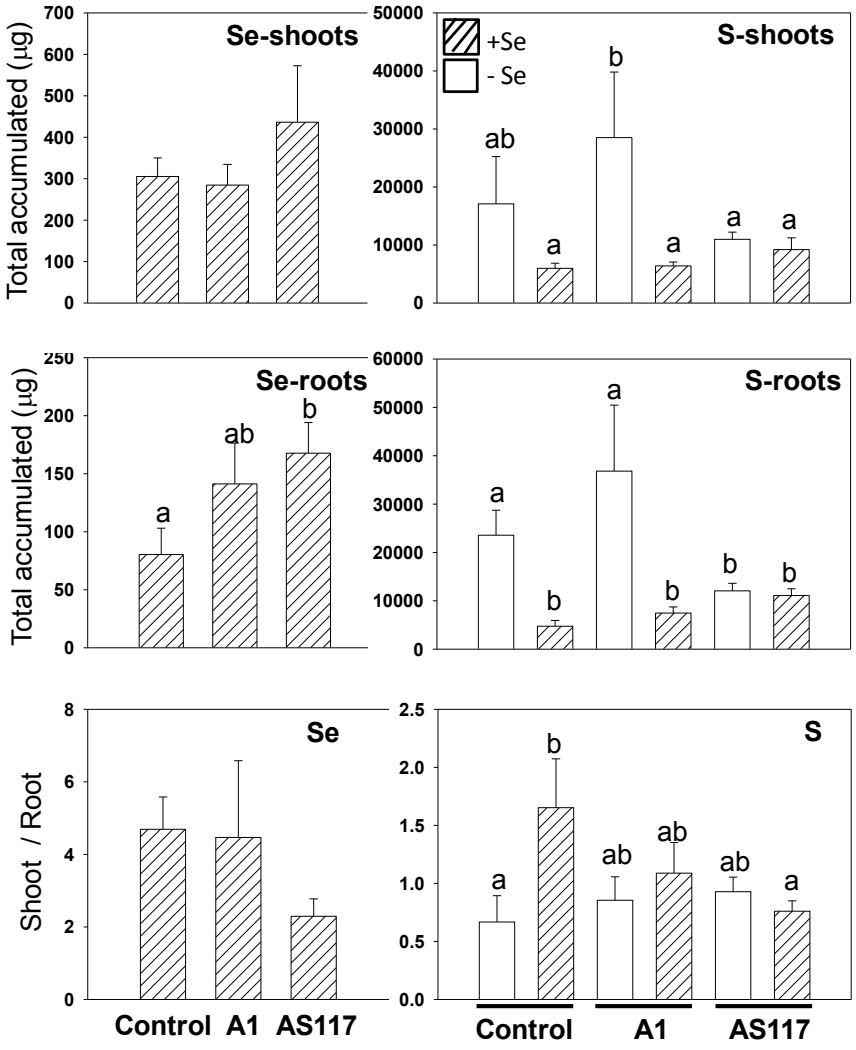


Figure 4.5 Total Se (left) and S (right) accumulated in the shoot and roots of *S. elata*, as well as the shoot/root ratio of shoot and root accumulation in un-inoculated or inoculated with rhizosphere fungi A1 or AS117. Shown values are the mean ± SEM. Lower case letters abouve bars indicate statistically significant differences ($p < 0.05$).

Selenium distribution and speciation were not affected by the fungal inoculations for either of the two *Stanleya* species. Representative roots of *S. pinnata* are shown in Figure 6. In the tap root cross-section (left six panels), Se was found throughout but was more concentrated in the cortex than the stele (particularly low Se signal was detected from the xylem). There was one particularly Se-rich area in the cortex where a lateral root appears to be emerging from the pericycle. Selenium did not appear to be very abundant in the periderm, where calcium (Ca) and iron (Fe) were most concentrated (see overlay of

Se, Ca and Fe in Fig. 4.6). The distribution of zinc (Zn) and Se were strongly correlated (Pearson r= 0.87) as is also evident from the purple color in the overlay of Se, Ca and Zn (Fig. 4.6). From the side view of the lateral root of *S. pinnata* (Fig. 4.6, right six panels), Se was present throughout the root and not concentrated in any particular area. Calcium and Fe were concentrated in the periphery and did not correlate strongly with Se localization (see overlay of Se, Ca and Fe, Fig. 4.6). Similar to the tap root, Zn in the lateral root co-localized with Se (Pearson r= 0.88) (see overlay of Se, Ca and Zn, Fig. 4.6).

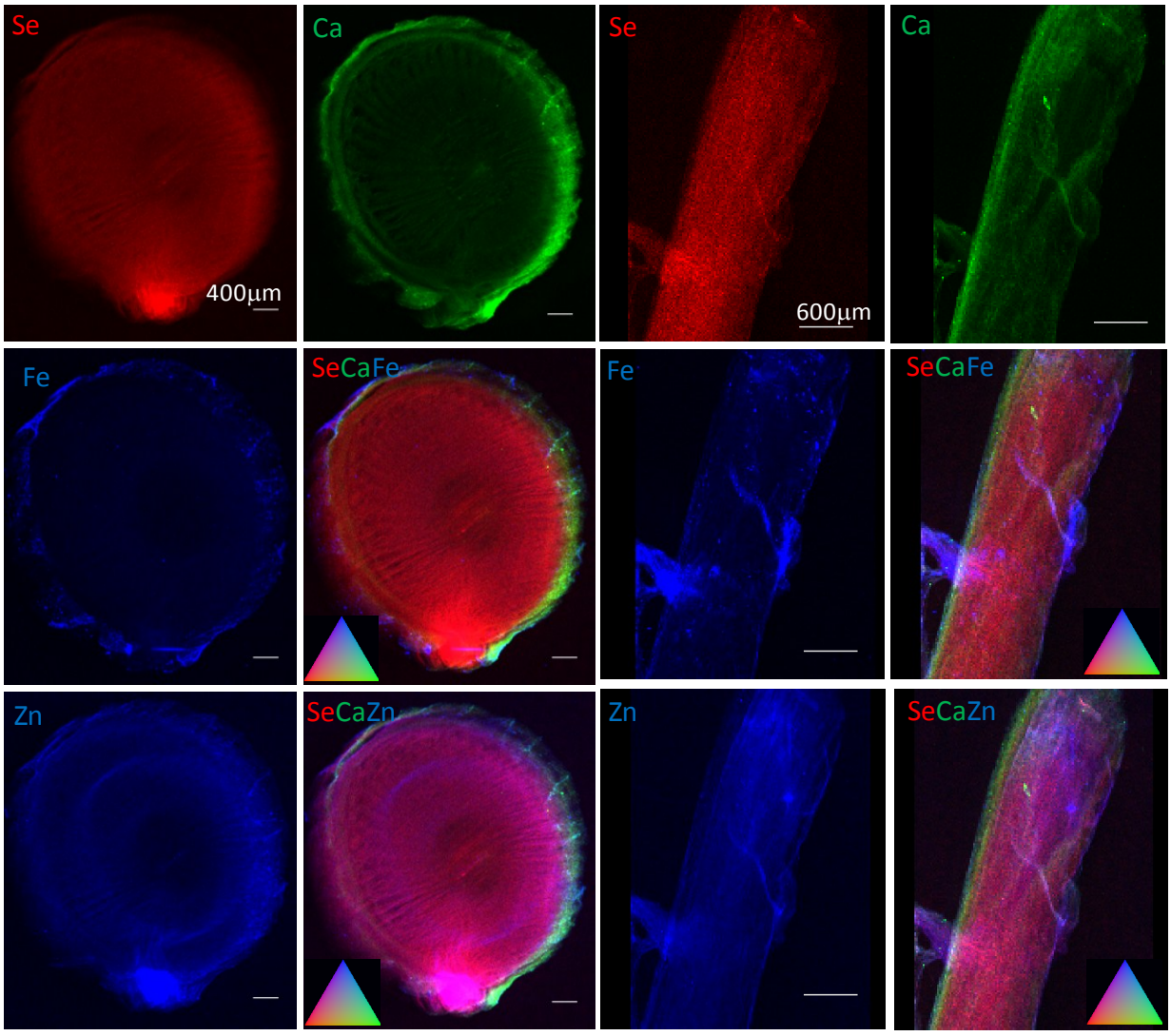


Figure 4.6 Micro-XRF maps showing distribution of Se, Ca, Fe, and Zn in a root cross-section (left six panels) and lateral root (right six panels) of *S. pinnata*.

Elemental distribution in a tap root cross-section of *S. elata* is shown in the left six panels of Figure 7. Selenium was present mainly in the cortex. Calcium and Fe, on the other hand, were present mostly in the periderm. Zinc showed co-localization with Se (Pearson r= 0.78). Elemental distribution in a lateral root of *S. elata* is shown in the right six panels of Figure 7. Selenium was concentrated in discrete structures that ran longitudinally through the lateral root and may represent vascular tissues. Calcium and Fe also showed a non-homogeneous distribution, while Zn was more homogeneously distributed and was co-localized with Se (Pearson r= 0.85). Selenium also co-localized with Fe in the lateral root of *S. elata* (Pearson r= 0.90).

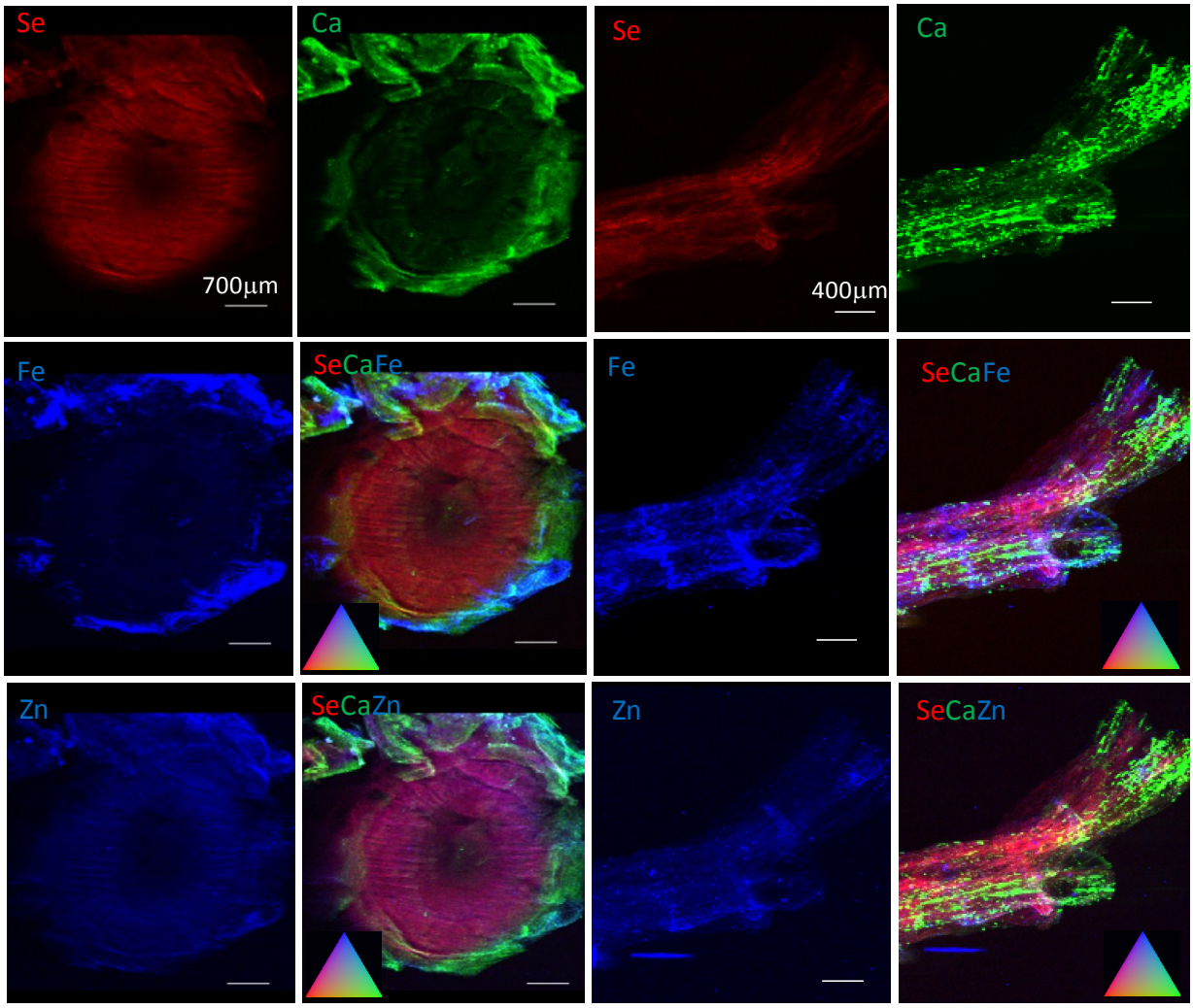


Figure 4.7 Micro-XRF maps showing distribution of Se, Ca, Fe, and Zn in a root cross-section (left six panels) and lateral root (right six panels) of *S. elata*.

XANES was used to determine the speciation of Se in the roots of *S. pinnata* and *S. elata* from the different treatments. There were no differences in speciation between the inoculation treatments (results not shown). The two plant species were similar in Se speciation (see Fig. 4.8a for representative spectra). In the tap root of *S. pinnata* as well as *S. elata* most Se (95%) was organic, with a C-Se-C configuration, i.e. MeSeCys, SeCysth or SeMet; the remainder of the Se was inorganic (selenate, selenite, or $Se^0$). It should be noted that the error margin of the linear least squares combination fitting is $\pm$10%, so the identification of the minor Se fractions is not certain. In the lateral roots of *S. pinnata* as well as *S. elata* there was relatively less C-Se-C (87%) compared to the tap roots; the remaining, minor

fractions of Se again were inorganic species (selenate or selenite).

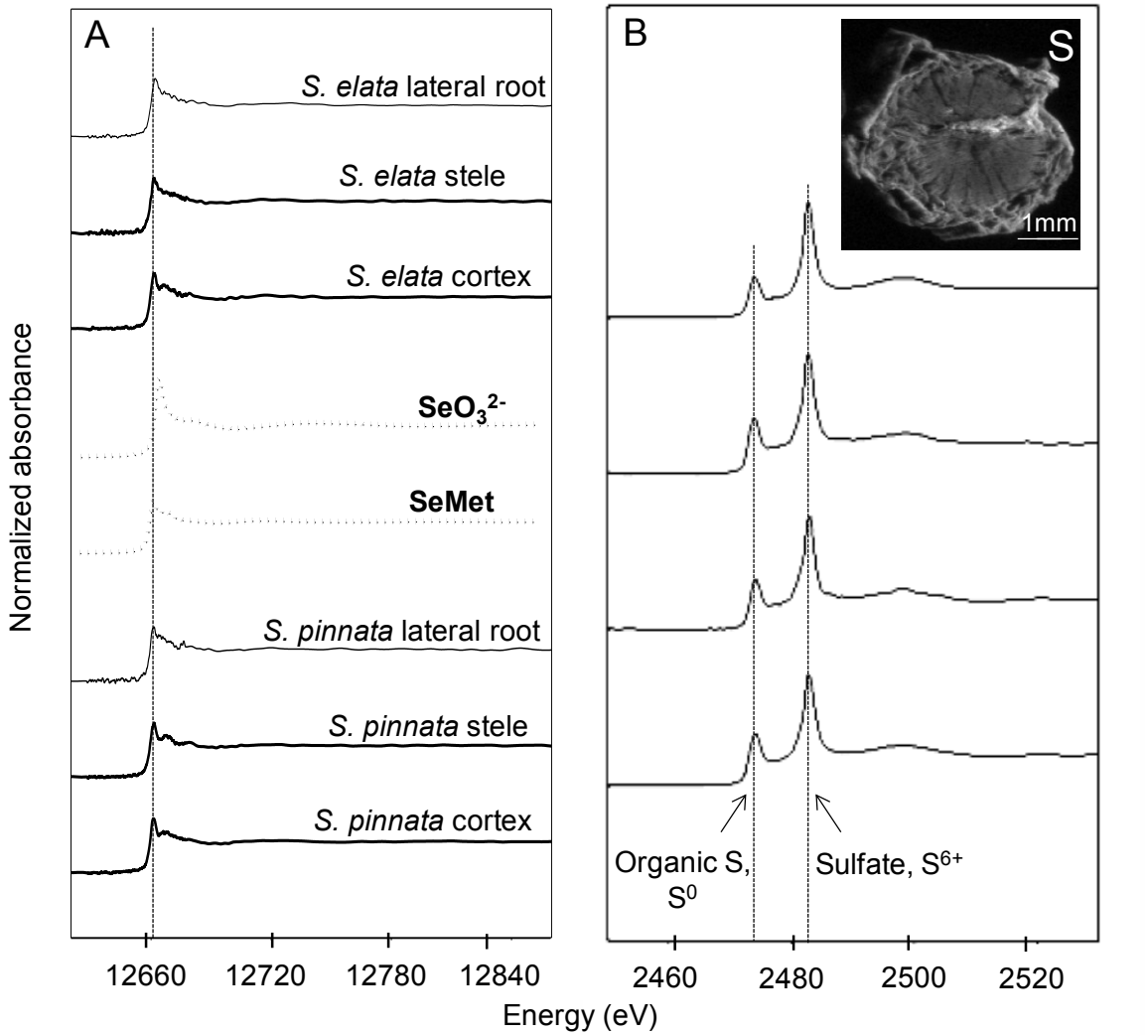


Figure 4.8 Selenium (A) and sulfur (B) speciation as determined by XANES. A. Se spectra obtained from taproot and lateral roots of *S. pinnata* and *S. elata* in comparison with two Se standards, selenomethionine (SeMet) and seleinte ($SeO_3^{2-}$). B. Sulfur spectra obtained fromfour locations in the cortex and periderm of the taproot of *S. pinnata*. Organic and inorganic S peaks were identified using sulfur standards (not shown). Inset shows X-ray fluorescence map showing S (in white) in the taproot from which the S XANES spectra were obtained.

Since S is chemically similar to Se and supposedly uses the same transporters and enzymes, S distribution and speciation were determined in a tap root of *S. pinnata* (Fig. 6.8b). Sulfur XRF mapping showed Se to be distributed fairly evenly throughout the root (Fig. 6.8b, inset). Sulfur speciation analysis

(XANES) in cortex and periderm showed a mixture of organic and inorganic S in roughly equal fractions (Fig. 6.8b).

## Discussion

The objective of this study was to investigate whether two rhizosphere fungi, *Alternaria seleniiphila* (A1) and *Aspergillus leporis* (AS117), isolated from the rhizosphere of Se HA *Stanleya pinnata* affect growth, Se accumulation, localization and/or speciation in host plant *S. pinnata*, as well as in the related Se-sensitive, non-HA species, *Stanleya elata*. Neither the fungal-inoculations, nor the addition of Se affected growth in *Stanleya pinnata*. There appeared to be an increase in biomass when inoculated with A1, but this was not significant; this effect may have become more pronounced if the fungus-plant co-cultivation had been allowed to proceed for longer than thirteen weeks. *Stanleya elata* growth was negatively affected by Se, as well as by AS117 inoculation; these effects were not additive but rather antagonistic. The *S. elata* AS117-inoculated plants showed the greatest survival rate in the presence of Se, and this was the only treatment group where the addition of Se did not result in reduction of biomass, but rather the biomass was slightly increased.

The greatest impact of fungal inoculation on *S. pinnata* Se accumulation was in A1- inoculated roots, where there was two-times more Se compared to un-inoculated roots. In contrast, AS117- inoculated *S. pinnata* contained 1.5-times less Se per plant. As to be expected, the two fungi affected the host plant properties differently; however, some trends held up for both fungi. Selenium translocation in *S. pinnata* was lower when inoculated with either fungus, judged from both Se tissue concentration and total Se accumulated. The most interesting result of inoculation in *S. elata* was up to 2-fold higher root Se accumulation in AS117-inoculated plants. This finding was somewhat surprising since it was A1-inoculation that led to enhance root Se accumulation in *S. pinnata*, again indicating that the functional significance of fungal-inoculation is host-dependent.

Analysis of S accumulation, chemically similar to Se, did show similarities with Se accumulation, but provided further information about the different interactions between host plant and fungus. In *S. pinnata*, translocation of S was somewhat reduced. In the non-HA *S. elata*, fungal inoculation had more of an impact on S than on Se accumulation. In the absence of Se, *S. elata* inoculated with either fungus had higher shoot S levels. In the presence of Se, S translocation was lower in AS117-inoculated plants, and to a lesser extent in A1-inoculated plants. Chemical speciation of Se was not altered by inoculation in roots of *S. pinnata* or *S. elata*, and was found in both species to be mainly organic Se with a C-Se-C configuration.

Thus, the overall effect of the fungi may be to enhance root Se accumulation, and to reduce Se (and S) translocation to the shoots. The effect was more pronounced for Se than for S in HA *S. pinnata*, and more pronounced for S than for Se in non-HA *S. elata*, although Se accumulation was enhanced in roots of *S. elata* inoculated with AS117. The mechanism by which these fungi affected Se translocation in the HA, and mainly S accumulation in the non-HA, may be that these two elements are metabolized differently in the two plant species. XANES analysis revealed that Se was present as C-Se-C compounds in the HA root as well as the non-HA root, whereas S was accumulated as a roughly equal mixture of organic and inorganic S compounds in *S. pinnata*. It is feasible that the fungi affect translocation of organic selenocompounds differently than the inorganic and /or organic S compounds. In addition, the non-HA may have accumulated a different form of C-Se-C than the HA. Earlier, C-Se-C was found to be the predominant form of Se in leaves of *S. pinnata* and this was identified as 80% MeSeCys and 20% SeCysth (Freeman et al., 2006) In leaves of non-HA *S. albescens* the Se was also 100% C-Se-C, but this was identified as 100% SeCysth (Freeman et al., 2010). Thus, it is possible that the form of Se in *S. pinnata* and *S. elata* roots is actually different, and if so, different C-Se-C compounds may be differently affected by the fungus. The finding that -although similar- root Se distribution was somewhat different between *S. pinnata* and *S.elata* may suggest that the Se was present in different forms in the two

species. While Se was present at the highest levels in the cortex in both species, in *S. elata* Se was present more exclusively in the cortex. Since the stele contains the vascular tissues, the low Se levels in the stele of *S. elata* may indicate that there is relatively less Se in the vascular core to be translocated. Thus, the somewhat different localization and, potentially, speciation of Se may have made the Se in *S. elata* less available for translocation.

The possible mechanism behind the observed reduction in Se and S translocation in the presence of the fungi may be reduction of Se and S to elemental, insoluble Se and S by the fungi in the rhizosphere, reducing bioavailability and therefore uptake and translocation. These fungi were isolated from the rhizoplane of *S. pinnata*, and thus were in close contact with the root surface, and both fungi have been shown to produce $Se^0$ (Lindblom et al. 2012). If these fungi chemically reduce Se (and S) on the root surface, it is feasible that reduced, insoluble $Se^0$ (and $S^0$) is trapped in fungal hyphae attached to roots, which may explain the observed enhanced root Se and S levels. The XANES analysis on inoculated roots did not find a substantial $Se^0$ fraction in taproot cross sections or lateral roots, but the microbeam (15 x 6 $\mu m^2$) may have missed fungal hyphae. In addition to possible reduction of Se or S, translocation of Se and S may have been affected by fungal volatilization in the rhizosphere, reducing Se uptake and translocation. AS117- inoculation in *S. elata* led to the lowest plant Se levels, and AS117 has been observed to strongly volatilize Se (Wangeline 2007).

The fungus AS117 had different interactions with the two plant species, and interaction with non-tolerant *S. elata* was Se-dependent. It appears that AS117 ameliorated the negative effect of Se on Se-sensitive *S. elata*. The mechanism, as described above, may involve reduction of Se in the root zone to inert $Se^0$, or volatilization of Se, both of which it has been demonstrated to be capable of (Lindblom et al. 2012). Alternatively, it may be hypothesized that Se ameliorated the pathogenicity of AS117. If so, the addition of Se may have triggered systemic defense mechanisms in the plant that made it more

resistant to the fungus. Indeed, Se has been reported to upregulate the production of the hormones salicylic acid and jasmonic acid in two *Stanleya* species (Freeman et al. 2010).

In plants treated with selenate, root S concentration was lower in both species. Shoot S concentrations were lower in Se-treated *S. elata*, but not *S. pinnata*. Since selenate and sulfate are analogous compounds, the Se-associated reduction in tissue S levels may be the result of competition for the same transporters. The degree of competition was different, however, for the two *Stanleya* species. In *S. pinnata*, no reduction in total plant S accumulation was observed in the presence of Se (Fig. 3), whereas the total S accumulation per *S. elata* plant was 4-fold reduced in the presence of Se (Fig. 5). Thus, the interaction of Se and S was different in the HA and the non-HA, perhaps due to differences in kinetic properties of the respective sulfate/selenate transporters. The HA bioconcentrated Se to higher levels than the non-HA, and in contrast to the non-HA it did not compromise S accumulation, perhaps indicating the presence of separate selenate and sulfate uptake systems in the HA. It is worth mentioning here that the selenate:sulfate ratio was 0.05 (12.5 □M selenate vs. 250 □M sulfate), yet addition of this relatively low concentration of Se led to a 4-fold reduction in S accumulation in *S. elata*. Either selenate strongly outcompeted sulfate for uptake, or S accumulation was affected by secondary toxic effects of the Se.

Selenium co-localizated with Zn in tap roots and lateral roots of both *Stanleya* species, and in lateral roots of *S. elata* Se also co-localized with Fe. The reason for this co-localization is not clear, but could reflect conjugation by, or incorporation of, these elements in the same molecules, such as the chelators glutathione and phytochelatins. The production of these non-protein thiols has been shown to be induced by a variety of cations and anions, and they can bind both anions (such as arsenite) and cations (e.g. $Cd^{2+}$) (Cobbett and Goldsbrough, 2002).

In conclusion, this study shows that rhizoplane fungi isolated from a Se HA can enhance root Se accumulation. The effect of inoculation on root Se accumulation was similar for the HA and non-HA, however, the specific fungal inoculant that resulted in enhanced root Se accumulation was different for each *Stanleya* species. A1 enhanced root Se accumulation in *S. pinnata* whereas AS117 enhanced root Se accumulation in *S. elata.* The mechanisms behind the observed effects of fungal inoculation on Se accumulation are not entirely clear, but do not appear to include alteration of plant Se speciation. It is possible that the fungi alter Se speciation and Se bioavailability in the rhizosphere. The apparent capacity of one fungus (AS117) to alleviate reduction in biomass from Se toxicity indicates that specific fungal inoculation may be useful in applications where plant Se toxicity is a concern. Furthermore, the observed effect of fungal-inoculation in reducing Se translocation to the shoot may be useful in applications where shoot Se accumulation is undesirable, e.g. in cases where there is a concern for toxicity to herbivores and for the movement of Se into the food chain. The finding that fungal inoculation can enhance Se accumulation in roots is also significant and may be useful in root crop Se biofortification practices.

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# Chapter 5

## *Astragalus bisulcatus* seed-coat fungus, friend or foe?


**Summary**

In earlier studies, roots of selenium (Se) hyperaccumulators were shown to contain up to 35% elemental Se ($Se^0$) when growing in seleniferous soil, while no $Se^0$ was found in a greenhouse setting. Since microbes, but not plants, are known to produce $Se^0$, it was hypothesized that the root $Se^0$ was microbe-derived. Here, a fungus identified as *Alternaria tenuissima* (A2), an abundant seed endophyte in Se hyperaccumulator *Astragalus bisulcatus*, is shown to produce $Se^0$. While uninfected seeds contained 100% organic Se (methylselenocysteine), seeds harboring A2 also contained up to 22% $Se^0$, as did the A2 mycelium growing on the seed. The production of $Se^0$ by A2 may serve as a fungal Se tolerance mechanism. A2 successfully colonized seeds containing 10,000 mg $kg^{-1}$ methylselenocysteine, but was sensitive to 25 mg $kg^{-1}$ Se from flower extract or supplied as pure methylselenocysteine. It likely occupies low-Se areas of the plant, in seed coat and apoplast. A2 may be present throughout the plant, since $Se^0$ was also found in the root of a 5d old seedling and in the stem of a field-collected plant. Although *A. tenuissima* is a known plant pathogen, it stimulated growth of *A. bisulcatus* up to 3 fold. A2-containing seedlings had lower shoot Se and sulfur levels than seedlings from uninfected seeds. It is possible that less Se was translocated due to the formation of insoluble $Se^0$ in the root. In conclusion, this fungus can alter plant Se speciation and may contribute to the $Se^0$ observed in hyperaccumulator roots.

## Introduction

Selenium is an essential micronutrient for many organisms including humans, but is toxic in high doses. The gap between Se deficiency and toxicity is narrow and both are problems worldwide. Selenium is toxic due to its similarity to sulfur (S). Selenium readily replaces S in essential S proteins, interfering with their function (Stadtman 1990). In the Western United States, where soils have elevated Se concentrations, chronic ingestion of high-Se plants by livestock has been reported to result in $330 million in losses annually (Rosenfield and Beath 1964; Wilbur 1980).

Selenium serves no known essential function in plants or fungi (Zhang and Gladyshev, 2009). However, Se is beneficial to many plants as it increases growth and antioxidant activity and offers protection against a wide variety of herbivores and pathogens (Hanson et al. 2003; Freeman et al. 2006a). All plants readily take up and assimilate Se into organic compounds, due to the similarities of Se and S. Hyperaccumulator (HA) plants are special in that they accumulate and tolerate up to 15,000 mg Se $kg^{-1}$ at seleniferous sites (Terry et al. 2000). These plants are also unique in that they preferentially allocate Se to the reproductive tissues, i.e. flowers and seeds (Quinn et al., 2011a). Selenium accumulation in plants can be used for phytoremediation i.e. the environmental cleanup of pollutants by plants. Selenium HAs are found in the families Asteraceae, Brassicaceae and Fabaceae.

There have been several hypotheses that have been tested for why plants hyperaccumulate; inadvertent uptake, drought tolerance, elemental tolerance, allelopathy, and elemental defense against herbivores and pathogens (Boyd and Martens, 1992). For Se hyperaccumulation, the evidence for the elemental defense hypothesis is well supported. Selenium has been show to protect plants from a variety of generalist herbivores, for a review see El Mehdawi and Pilon-Smits (2012). There is evidence that HAs may deposit Se in surrounding soil as a form of elemental allelopathy against Se-sensitive neighboring plants (El Mehdawi et al., 2011a).

While Se-sensitive ecological partners suffer in their interactions with Se HAs, Se-resistant partners may exploit the high Se niche offered by HA plants. Se-resistant herbivores have been found to feed on HA seeds and leaves. In some of these herbivores resistance is based on tolerance and in others it is based on exclusion (Freeman et al., 2006a (Valdez Barillas et al., 2012; Freeman et al., submitted). Furthermore, Se-tolerant neighboring plants of HAs in the field were shown to benefit from enhanced Se levels, in that they were less susceptible to herbivory (El Mehdawi et al. 2011b).

Relatively little is known about how Se affects the plant-microbe interactions of HAs. Depending on whether the associated microbe lives in the rhizosphere, phyllosphere, or as endophyte, it may experience different Se levels, and with that, Se toxicity. The microbe's relationship with the plant may involve pathogenicity, mutualism and commensalism. Some microbes may perform beneficial functions for the HA: stimulating growth, aiding in nutrient and water acquisition, or fighting off pathogens. In HAs, microbes may also affect the acquisition, speciation and accumulation of the hyperaccumulated element.

There is evidence that Se can protect plants from Se-sensitive pathogens. In a study with non-HA *Brassica juncea*, Se was shown to protect plants from two Se-sensitive fungal pathogens, *Alternaria brassicicola* and a *Fusarium* species (Hanson et al., 2003). There is also evidence for the presence of Se-resistant microbes that live in association with HAs. A litter decomposition experiment on seleniferous soil revealed that there were more microbes on high-Se leaf litter from HAs than on low-Se litter from related species collected from the same site, suggesting specialist Se-resistant decomposing microbes are present at seleniferous sites (Quinn et al. 2011b). Furthermore, a Se-resistant Rhizobacterium apparently lives in association with the HA *Astragalus bisulcatus* (Fabaceae), since this species produces high-Se nodules (Valdez Barillas et al., 2012). This bacterium may affect plant Se speciation, since the nodule produced a high fraction of elemental Se ($Se^0$) (Valdez Barillas et al., 2012). In addition, many Se-

tolerant fungi have been isolated from the rhizosphere of HAs (Wangeline et al., 2011). For most of these rhizosphere fungi it is not known what effect they may have on Se hyperacculators. A few were characterized in more detail and were found to produce $Se^0$ (Chapters 3 and 4 of this dissertation). Interesting in this context was the finding that HA roots collected from the field contained high fractions of $Se^0$ (up to 35%) while greenhouse-grown plants from the same species contained exclusively organic selenocompounds with a C-Se-C configuration (Se attached to two organic groups) (Chapter 2 of this dissertation: Lindblom et al. 2012). Based on these findings it was hypothesized that microbes are responsible for the production of $Se^0$ observed in HAs in their natural habitat. To test this hypothesis, HA plants were grown from surface-sterilized seeds and inoculated with several fungi shown earlier to be able to produce $Se^0$. However, no significant effect on plant Se speciation was observed, only one fungus led to a little $Se^0$ in the root (Lindblom et al., Chapters 3 and 4 of this dissertation). It was concluded that either the cocultivation time was not long enough, or these rhizosphere microbes do not significantly contribute to root $Se^0$ production.

In this study we test another HA-associated fungus that appears to be a seed endophyte. It was found to emerge regularly from surface-sterilized seeds of *A. bisulcatus*. As an endophyte, it has a very close association with the HA and maximal opportunity to impact plant Se speciation. In this study we identify this fungus, characterize its Se-related properties, and its impact on plant Se speciation, Se accumulation and overall growth.

## Materials and Methods

Biological material and selection of A2-containing *A. bisulcatus* seeds/seedlings

*Astragalus bisulcatus* seeds were obtained from Western Native Seed, Coaldale, CO, USA. The A2 fungus was isolated from these seeds for tests on fungal Se-tolerance, Se speciation studies, as well as for cultivation for species identification purposes. Seeds were first scarified for 10 min with

concentrated sulfuric acid and then washed with sterile water and germinated on sterile filter paper. As soon as the seeds germinated, it was apparent which seeds (nearly half) contained the A2 seed-coat fungus. The half that did not show evidence of the fungus, as well as those that did contain A2 were transferred to culture tubes with sterile peat moss and 0.5 strength Murashige and Skoog salts (Murashige and Skoog, 1962) plus 5 g $L^{-1}$ sucrose to encourage the growth of both plant and fungus.

Fungal growth for tolerance and species identification

For the analysis of A2 tolerance to different seleno-compounds, the fungus was cultivated under continuous fluorescent light at 22°C in sealed Petri dishes containing 0.5 strength malt extract agar (0.5 MEA, Difco, Detroit, MI) supplemented with sodium selenate or sodium selenite at 0, 10, 30, or 300 μg $ml^{-1}$. Fungal tolerance was also tested on extract made from the flowers of *A. bisulcatus* determined to contain 0, 10, 30, 60, and 150 μg $ml^{-1}$ Se added in the 0.5 MEA, as well as on different concentrations of MeSeCys (0, 10, 30, 60, 150 μg $ml^{-1}$), also added in MEA.

For identification, the A2 fungus was grown on V-8 colony conditions for conidium analysis with continuous light and desiccation. PCA slide culture conditions and PCA colony conditions – 8hr light-16hr dark, continuous desiccation.

Plant growth

The seeds of *A. bisulcatus* were first scarified for 10 min with concentrated sulfuric acid, and then further surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10 min rinses in sterile water. Seeds were then germinated on sterile filter paper under continuous light at 23°C in a plant growth cabinet. Half of the seedlings in each group, (A2-associated and seeds without A2), were watered with 80μM Se as selenate while the other half obtained water. The culture tubes were sealed with breathable tape and opened only to add water with nutrients, either with Se added or without Se,

depending on the treatment group. The experiment was terminated after four weeks. At that point many of the replicates in the control group had died (i.e. the group without the fungus and without Se added).

Elemental analysis

At harvest the plant roots were washed of sand that was bound in the root mass, and then dried for 48h at 45$^{o}$C. Samples were digested in nitric acid as described by Zarcinas *et al.* (1987). Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used to determine Se and S concentrations in the acid digest (Fassel 1978).

Elemental distribution and speciation

X-ray microprobe analysis was performed on intact frozen *A. bisulcatus* seeds, on *A. bisulcatus* seedlings that had been supplied with 20 µM $Na_2SeO_4$, and on A2 fungus material that had been grown on liquid malt extract medium containing 30 mg $L^{-1}$ $Na_2SeO_4$ or $Na_2SeO_3$ and washed briefly in 1 mM $Na_2SO_4$ to removed adsorbed Se. Elemental tissue distribution and chemical speciation were determined using XRF mapping and XANES spectroscopy, respectively, both as described by Lindblom et al. (2012).

Statistical Analysis

The software JMP-IN (3.2.6, SAS Institute, Cary, NC) was used for statistical data analysis. A student's t-test was used to compare differences between two means. Analysis of variance followed by a post-hoc Tukey Kramer test was used when comparing multiple means. It was verified that the assumptions underlying these tests (normal distribution, equal variance) were met.

## Results

X-ray microprobe analysis of seeds and seedlings

When seeds of *A. bisulcatus* were surface-sterilized and germinated on sterile filter paper, about half of them contained an endophytic fungus, which was designated A2. To characterize the effect of A2 on the location and chemical speciation of Se in *A. bisulcatus* seeds and seedlings, XRF and XANES analysis were performed on infected and uninfected individuals. In the seeds, Se was found in the embryo and was not detected in the seed coat (Fig. 5.1 A and B).

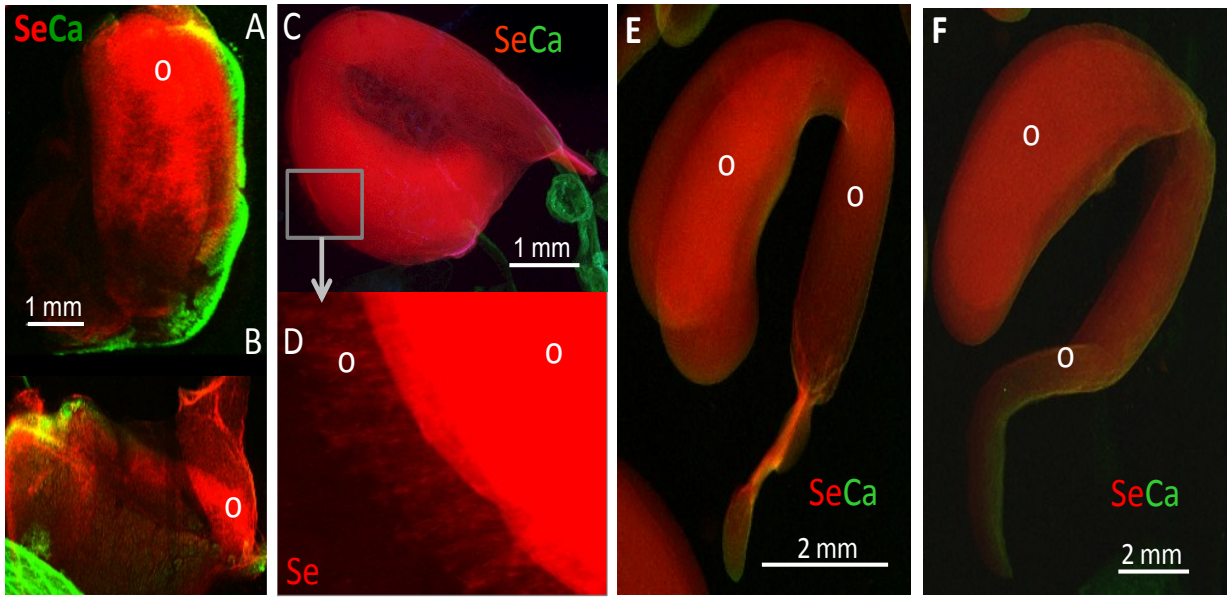


Figure 5.1 Micro-XRF maps showing distribution of Se, Ca, Fe, and Zn in (A) An uninfected seed. (B) A seed in the early stages of infestation. (C) A seed in late stage, terminal infestation and (D)magnified mycelia emerging from a terminally infested seed. (E) A 24h old seedling and (F) a 5d old seedling. White circiles indicate locations where XANES spectra were collected; the XANES shown in Table 5.1.

There were no apparent differences in the localization of Se that correlated with the presence of the A2 fungus. Selenium in the uninfected seed was found to be 100% C-Se-C (Table 5.1). An infected seed in the early stage of infection contained Se mainly as an organic compound with a C-Se-C configuration, but a seed in the late phase of infestation contained a large fraction (20%) of elemental Se ($Se^0$). The

remaining Se present was C-Se-C (74%), and seleniten (6%). The A2 mycelia outside of the seed also contained Se (Fig. 5.1 C and D), which consisted of 59% C-Se-C, 22% $Se^0$ and 19% selenite (Table 5.1).

Table 5.1 Results from least squares linear combination fitting of experimental XANES spectra with standard selno-compounds. XANES spectra were obtained from the *Astragalus bisulcatus* seeds, seedlings and funfal mycelium shown in Figure 5.1. C-Se-C: Methyl-selenocysteine, Se-Methionine or Se-Cystathionine. $Se^0$: red or gray elemental Se. SS: normal sum of squares; nd: compound not detected.

| | SS ($x10^{-4}$) | $SeO_4^{2-}$ | $SeO_3^{2-}$ | $SeGSH_2$ | C-Se-C | $Se^0$ |
|---|---|---|---|---|---|---|
| **Seeds** | | | | | | |
| Uninfected (3A) | 3.10 | nd | nd | nd | 100% | nd |
| Infected, early stage (3B) | 3.54 | 2% | 1% | nd | 97% | nd |
| Infected, late stage (3D) | 1.70 | nd | 6% | nd | 74% | 20% |
| Mycelia , late stage (3D) | 7.35 | nd | 19% | nd | 59% | 22% |
| **Seedlings** | | | | | | |
| 24 hrs old (3E, both) | 2.76 | nd | nd | nd | 100% | nd |
| 5d old-cotyledons (3F) | 3.03 | nd | 4% | nd | 96% | nd |
| 5d old-root (3F) | 5.71 | nd | 13% | nd | 70% | 17% |

*A. bisulcatus* seeds were germinated on selenate-containing growth medium and the resulting seedlings flash-frozen for XAS analysis at two different time points after germination. Micro XRF mapping demonstrated that *A. bisulcatus* had a fairly uniform distribution of Se in seedling cotyledons, hypocotyls and roots (Fig. 5.1 E and F). Twenty-four hours following germination a seedling contained 100% C-Se-C in cotyledons and root (Table 1). Five days following germination of another seedling, the cotyledon contained 95% C-Se-C and 5% selenite, while the root contained relatively less C-Se-C (70%) and a substantial fraction of inorganic Se: 18% $Se^0$ and 12% selenite (Table 5.1).

The finding that the A2 endophytic fungus may alter seed and seedling Se speciation, and may be responsible for the formation of $Se^0$ in plants, prompted us to further investigate the identity and properties of this fungus and its effects on plant growth and Se accumulation. The identity of A2 was initially investigated by DNA sequencing of the internal transcribed spacer (ITS) of the small ribosomal subunit.

As shown in Figure 2, the sequence from A2 showed 100% sequence similarity with the known plant pathogen *Alternaria tenuissima* and 99.6% similarity with *Alternaria astragali*, a

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A2                      ------------------CTGGACCTCTCGGGGTTACAGCCTTGCTGAAT 32
Alternaria_astragali    ------------------CTGGACCTCTCGGGGTT-CAGCCTTGCTGAAT 31
Alternaria_tenuissima   CATAAATATGAAGGCGGGCTGGACCTCTCGGGGTTACAGCCTTGCTGAAT 50
                                          ***************** **************

A2                      TATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCC 82
Alternaria_astragali    T-TTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCC 80
Alternaria tenuissima   TATTCACCCTTGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCC 100
                        * ********************************************

A2                      ACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC 132
Alternaria_astragali    ACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC 130
Alternaria_tenuissima   ACCACTAGGACAAACATAAACCTTTTGTAATTGCAATCAGCGTCAGTAAC 150
                        **************************************************

A2                      AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 182
Alternaria_astragali    AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 180
Alternaria_tenuissima   AAATTAATAATTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA 200
                        **************************************************

A2                      TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG 232
Alternaria astragali    TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG 230
Alternaria_tenuissima   TGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTG 250
                        **************************************************

A2                      AATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGC 282
Alternaria_astragali    AATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGC 280
Alternaria_tenuissima   AATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGC 300
                        **************************************************

A2                      ATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC 332
Alternaria astragali    ATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC 330
Alternaria_tenuissima   ATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGTTGGGC 350
                        **************************************************

A2                      GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCG 382
Alternaria_astragali    GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCG 380
Alternaria_tenuissima   GTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCCG 400
                        **************************************************

A2                      GCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGG 432
Alternaria_astragali    GCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGG 430
Alternaria tenuissima   GCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGG 450
                        **************************************************

A2                      TCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAG 482
Alternaria_astragali    TCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAG 480
Alternaria_tenuissima   TCTAGCATCCATTAAGCCTTTTTTTCAACTTTTGACCTCGGATCAGGTAG 500
                        **************************************************

A2                      GGATACCCGCTGAACTTAAGCATATCA-------------- 509
Alternaria_astragali    GGATACCCGCTGAACTTAAGCATATCA-------------- 507
Alternaria tenuissima   GGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA 540
                        **************************
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Figure 5.2 DNA sequence alignment of the internal transcribed spacer region (ITS) of the small ribosomal subunit, comparing the A2 fungus with *Alternaria astragali* (99% similarity) and *Alternaria tenuissima* (100% similarity).

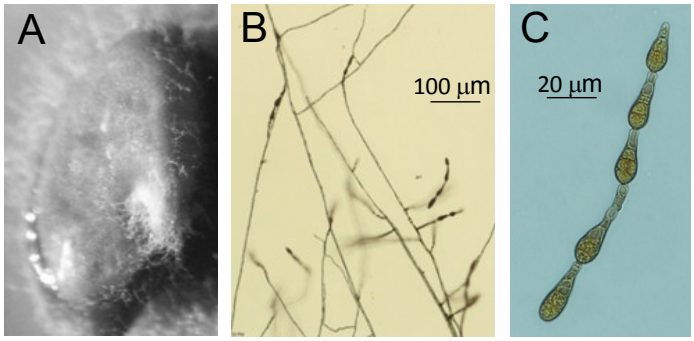


Figure 5.3 (A) A picture of a surface sterilized seed with A2 endophyte mycelia emerging from the seed coat. (B) Conidiaphores and conidia of A2, and (C) magnified conidia used to identify this fungus as *Alternaria tenuissima*.

rhizosphere fungus associated with *A. bisulcatus* (Wangeline and Reeves, 2007). Alternaria species are difficult to distinguish by commonly used molecular techniques, since they are genetically very similar. Therefore, morphological characteristics are more oftenly used to identify *Alternaria* species. Particularly, the conidiophores and conidia are structures useful for identification. The conidiophores and conidia of A2 are shown in Figure 5.3. Below is the complete description of the morphological properties of A2, which confirm its identity as *A. tenuissima*.

Fungal isolate taxonomic description

*Alternaria tenuissima* (Nees and T. Nees: Fr.) Wiltshire, anamorph (no known teleomorph), specific isolate designation Wangeline A2.

Host/substrate: seed pod of *Astragalus bisulcatus*

Locality: Pine Ridge Natural Area, Fort Collins, Colorado, U.S.A. 40°32.70N, 105°07.87W

Culture conditions: V-8 colony conditions (conidium analysis) – continuous light and desiccation. PCA slide culture conditions and PCA colony conditions – 8hr light- 16hr dark, continuous desiccation.

Colony at 7 to 8 d, PCA and V-8: Colony 5-6 cm diameter. On PCA is a surface network of interwoven radial hyphae, gray to buff with little sporulation and no discernible rings. Also present is a dense mat of reduced aerial hyphae. The reverse is dark green to black and uniform. The colony on V-8 is similar to PCA. Mycelium cottony, with an advancing colony edge of approximately 10 mm. Colony has dark

mycelial rings alternating with dense aerial rings when infrequently present. Mycelia grey, buff, and medium to dark brown. Mycelia layers upwards with increasing age. The reverse is dark green to black.

Conidiophores (40x) produced on V-8 range on average between 130-155 µm in length. Juvenile conidia are ovoid and have no definable beak, while most mature into a body narrowly ellipsoid with a long beak. The conidiophores are mostly simple but may have low amounts of branching (1-2), sometimes proliferating at 1-2 conidogenous sites. Conidia chains of 2-6 (8) are produced (most common 4). Older areas of mycelial growth can have a distinctive rope-like formation, commonly up to or less commonly surpassing 11 wide.

Conidium bodies (100x) are ovoid or long ellipsoid; commonly with a long beak or less commonly with no distinctive beak. Conidium length, with beak, ranging from 14 to 39µm with 2-5 transverse septa, width ranging from 6 to 11µm with 0-3 longitudinal septa, and beak length from 1.5 to 17µm. Note worthy that mature conidia have beaks >10 µm, and continue to divide becoming septate in both the beak (2-3 cells) and body gaining additional longitudinal septa. Also note worthy that roughly 50% of conidia have 0 longitudinal septa. Few conidium walls are rough or thickened.

Characterization of Se-related properties of the A2 seed coat fungus, *A. tenuissima*

X-ray microprobe analysis was carried out on mycelia of A2 that was grown on medium containing different forms of Se. The Se speciation data are listed in Table 5.2. When the fungus was supplied with selenite ($SeO_3^{2-}$), 76-100% of Se in the mycelia was present as $Se^0$; the remainder was seleno-glutathione ($Se\text{-}GSH_2$). When supplied with selenate ($SeO_4^{2-}$) the fungus accumulated a large fraction (40-54%) of Se as C-Se-C; other compounds detected were $Se\text{-}GSH_2$ (17-27%), selenate (19-24%) and selenite (8-12%).

Table 5.2 Results from least squares linear combination fitting of experimental XANES spectra with standard selno-compounds. XANES spectra were obtained from A. tenuissima A2, grown on MEA with 30 mg L-1 selenate ($SeO_4^{2-}$) or selenite ($SeO_3^{2-}$)C-Se-C: Methyl-selenocysteine, Se-Methionine or Se-Cystathionine. $Se^0$: red or gray elemental Se. SS: normal sum of squares; nd: compound not detected.

| | SS ($x10^{-4}$) | $SeO_4^{2-}$ | $SeO_3^{2-}$ | $SeGSH_2$ | C-Se-C | $Se^0$ |
|---|---|---|---|---|---|---|
| $SeO_3^{2-}$ supplied, 1 | 7.69 | nd | nd | nd | nd | 100% |
| $SeO_3^{2-}$ supplied, 2 | 7.65 | 3% | nd | 20% | nd | 76% |
| $SeO_4^{2-}$ supplied, 1 | 3.14 | 19% | 12% | 24% | 45% | nd |
| $SeO_4^{2-}$ supplied, 2 | 4.88 | 22% | 12% | 27% | 40% | nd |
| $SeO_4^{2-}$ supplied, 3 | 5.85 | 24% | 8% | 17% | 54% | nd |

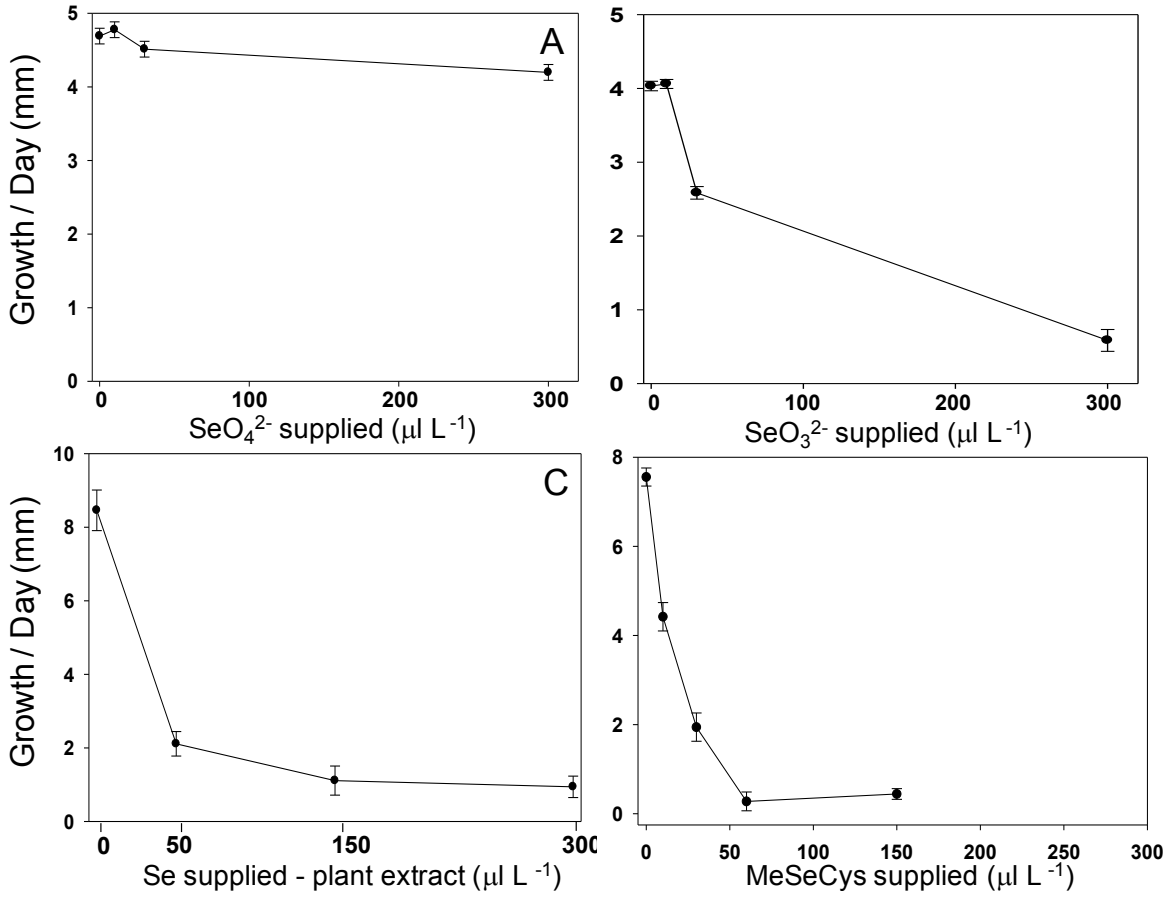


Figure 5.4 Alternaria tenuissima (A2) tolerance measured as colony growth per day on varying concentrations of (A) selenite, (B) selenite, (C) extract from the flowers of Astragalus bisulcatus, and (D) methy-selenocysteine.

The Se tolerance of the A2 fungus to different forms of Se is shown in Figure 4. The fungus was most tolerant to selenate: its growth was still 90% of the control when supplied with 300 mg Se $L^{-1}$ (Fig. 5.4A). The fungus was also fairly tolerant to selenite: it showed 50% inhibition around 100 mg Se $L^{-1}$ (Fig. 5.4B). To test A2 growth as a function of plant-derived Se, flower material was extracted in water and added to the growth medium at different dilutions; the Se concentration in the extract was determined using ICP-AES. The fungus was very inhibited by the plant extract, showing 50% inhibition around 25 mg Se $L^{-1}$ (Fig. 5.4C). To test whether this was likely due to the Se or (also) to other toxic compounds in the flowers, fungal growth was also determined as a function of MeSeCys concentration. When pure MeSeCys was added to the medium, the

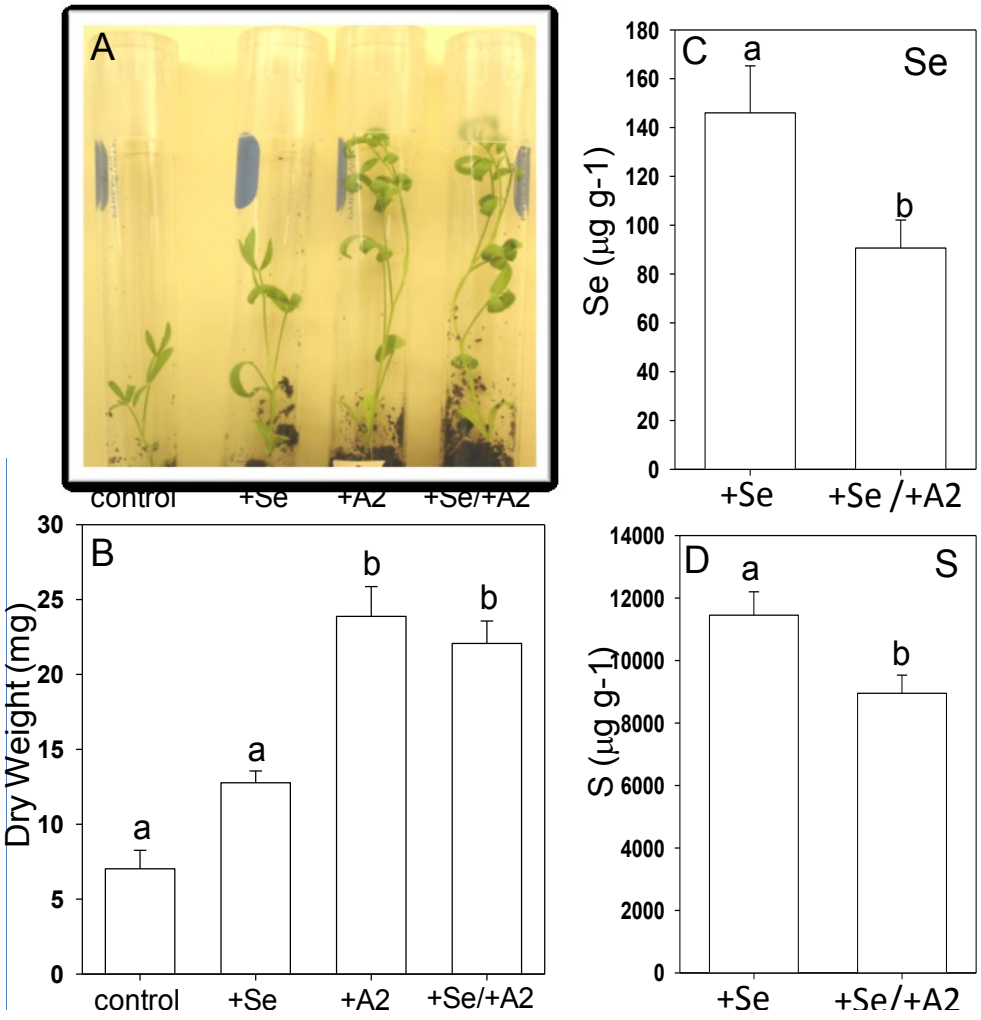


Figure 5.5 (A) Photograph of *A. bisulcatus* plants from the A2 co-cultivation experiment. (B) Dry weight of plants in milligrams. (C) Selenium concentration and, (D) sulfur concentration in shoots. The number of replicates for each group was 10, except for the control treatment which had only 5 living plants at the end. Different letters above bars indicate significant differences ($p < 0.05$) between means.

growth of A2 was 50% inhibited around 20 mg Se $L^{-1}$ (Fig. 5.4D), i.e. A2 growth was similarly inhibited by pure MeSeCys and by the Se in *A. bisulcatus* flowers.

Effect of *A. tenuissima* (A2) on *A. bisulcatus* growth and Se and S accumulation

When seeds that showed evidence of the presence of A2 upon germination were separated from those that did not show fungal hyphae, and the two groups were cultivated on sterile peat moss for 4 weeks with or without selenate, the A2-containing seedlings had reached a 2-3 fold greater dry weight compared to uninfected seedlings (Fig. 5A, B). The addition of Se did not significantly affect *A. bisulcatus* growth for either group (Fig. 5A, B). The presence of the A2 fungus was also associated with reduced shoot Se and S levels (Fig. 5C, D).

## Discussion

The A2 fungus, identified as *A. tenuissima*, appears to be an abundant endophyte in *A. bisulcatus*, since it was found in approximately half of field-collected surface-sterilized seeds. The A2 fungus may contribute to the fraction of $Se^0$ found earlier in field-collected HA roots, since its presence was associated with the presence of $Se^0$ in seeds. A2 may be present not only in the seed but also throughout the plant, since a 5d old seedling was shown here to also contain $Se^0$ in its root, as did root and stem of mature plants in the field (Lindblom et al. 2012; Valdez Barillas et al. 2012). While *A. tenuissima* is generally known as a plant pathogen with a wide host range (Mishra and Parakash, 1975), there is no evidence from this study that it acts as a pathogen on *A. bisulcatus*; it acted more like a growth-promoting microbe.

The form of Se in uninfected seeds was 100% C-Se-C; this was likely MeSeCys, as has been reported for seeds (Nigam and McConnell, 1969) as well as leaves (Freeman et al., 2006b) and flowers (Valdez Barillas

et al., 2012). The mycelium of the A2 fungus produced a mixture of C-Se-C and $Se^0$ when growing on seeds, and therefore apparently converted C-Se-C (MeSeCys) to $Se^0$, likely as a tolerance mechanism. Grown in pure culture, A2 also produced $Se^0$ when supplied with selenite. Conversion of more toxic forms of Se to insoluble, inert $Se^0$ is known to be a tolerance mechanism for many microbes (Gharieb et al. 1993 and citations within: Gadd 1993; and Lovely 1993).

Although A2 grew very well on seeds that contained in the order of 10,000 mg Se $kg^{-1}$ (Galeas et al., 2007; Quinn et al., 2011a), it was already 50% inhibited by 25 mg $kg^{-1}$ Se extracted from *A. bisulcatus* flowers, as well as by 20 mg $kg^{-1}$ MeSeCys. An explanation for the ability of A2 to successfully grow on these high-Se plants may be that A2 occupies areas of the plant where there is relatively less Se, i.e. the seed coat and the apoplast. As is shown here, the seed coat contains very little Se, and in earlier studies energy dispersive X-ray analysis of HA leaves revealed that Se was in the vacuole, and not in the apoplast (Freeman et al., 2006, 2010). Thus, A2 may not encounter toxic Se levels in the living plant, like it does when grown on pure selenocompounds or homogenized plant extract.

The ITS sequence alignment identification of the fungus revealed an interesting similarity to another fungal-symbiont of *A. bisulcatus*, *A. astragali* (A3), which was originally isolated from the rhizoplane of surface sterilized roots (Wangeline and Reeves 2007). An additional *Alternaria* species, *A. seleniiphila* (A1) was isolated from the rhizoplane of HA *Stanleya pinnata* (Wangeline and Reeves, 2007). Both A1 and A3 were characterized for Se tolerance and speciation by Lindblom et al. (2012). The Se-related characteristics of A2 are somewhat similar to A1 and A3. All are capable of reducing selenite to $Se^0$ and all are fairly tolerant to selenate. All three also stimulated the growth of their HA host. However, A2 is the only one among the three that may significantly affect Se speciation in its host.

Perhaps related to its effect on Se speciation toward more insoluble $Se^0$ in roots, the A2-containing *A. bisulcatus* seedlings showed significantly lower Se and S levels in their shoots. In the previous studies

where HAs were inoculated with related *Alternaria* species A1 and A3, there was a reduction in translocation, as the same may be the case here with A2; the root biomass was too small to determine root elemental concentrations. The possible mechanism for reduced translocation could be the production of $Se^0$ in the rhizosphere or inside the root apoplast, trapping Se in a non-translocatable form.

The implication of altered Se speciation in HAs is that it may affect the interactions of the plant and its ecological partners, as well as Se cycling in the ecosystem. Selenium that is in the elemental form is not bioavailable and therefore is less toxic and less likely to be recycled thereby also reducing toxicity to herbivores and other ecological partners. There may also be potential applications of microbes that affect plant Se speciation, for instance in phytoremediation. These fungi may also affect the speciation and translocation of Se in non-HA species, and thus may enhance Se tolerance and overall growth.

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## *Curriculum Vitae*


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**Education**

Jan 2009- present: PhD Botany. Projected graduation date: July 2012. Funded by Graduate Teaching Assistantship (GTA) Aug 2009 - graduation

Current GPA: 3.75

2008: B.S. Botany/Biosciences (double major), Colorado State University.

**Graduate research**

Spring 2009- present: Laboratory of Dr. E.A.H. Pilon-Smits, Department of Biology, Colorado State University.

Project: Evolutionary and Ecological Aspects of Selenium Hyperaccumulation; plant-microbe interactions in hyperaccumulator plants.

**Undergraduate research**

Sept. 2000 – Dec. 2008: Laboratory of Dr. E.A.H. Pilon-Smits, Department of Biology, Colorado State University.

Projects: 1. Genetic Engineering Approaches for the Study of Plant Selenium Metabolism: 2. Evolutionary and Ecological Aspects of Selenium Hyperaccumulation.

**Training**

May 2009 - Dec 2010: Training in the use of X-ray Absorption Spectroscopy and collaboration with the scientists at the Advanced Light Source, Berkeley, CA for graduate research (four trips).

**Teaching Experience**

Spring 2012: Graduate teaching assistant for Basic Concepts of Plant Life (BZ105), 3 lab sections

Fall 2011: Graduate teaching assistant for Attributes of Living Systems (Life102), 2 lab sections

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Spring 2003: Undergraduate teaching assistant ($TA^2$) for Plant Physiology (BZ440)

**Honors and Awards**

WINS travel grant (Women in Natural Sciences), Spring 2009

CSEM scholarship (women and minorities in Computer Science, Engineering and Mathematics), Fall 2008

**Services and Synergistic Activities**

2009-2010: Mentor of undergraduate research for three students registered for Independent Study (BZ495, 6-15h/wk for each student).

**Publications (*shared first authors)**

1. **Lindblom SD**, Fakra SC, Pilon-Smits EAH (**2012**) *Astragalus bisulcatus* seed-coat fungus- friend or foe? In preparation
2. **Lindblom SD**, Fakra SC, Tracy B, Landon JK, Schultz P, Pilon-Smits EAH (**2012**) Inoculation of selenium hyperaccumulator, *Stanleya pinnata* and related non-accumulator, *Stanleya elata* with selenium-tolerant rhizosplane fungi: effects on plant growth and accumulation of selenium and other elements. Submitted
3. El Mehdawi AF, **Lindblom SD**, Cappa JJ, Fakra SC, Pilon-Smits EAH (**2012**) Comparison of selenium accumulation, localization and speciation in *Artemisia ludoviciana*, *Symphyotrichum ericoides* and *Chenopodium album* growing next to hyperaccumulators and non-hyperaccumulators - Do hyperaccumulators affect selenium speciation in neighboring plants? Submitted
4. **Lindblom SD**, Fakra SC, Tracy B, Landon JK, Schultz P, Pilon-Smits EAH (**2012**) Co-cultivation of *Astragalus racemosus* and *Astragalus convallarius* with selenium-hyperaccumulator rhizosphere fungi: effects on plant growth and accumulation of selenium and other elements. Submitted
5. Alford ER, **Lindblom SD**, Pittarello M, Freeman JL, Fakra SC, Marcus MA, Broeckling C, Pilon-Smits EAH, Paschke MW (**2012**) Plant selenium hyperaccumulation - With a little help from rhizobial friends. Submitted

6. El Mehdawi AF, Reynolds RJB, Prins CN, **Lindblom SD**, Cappa JJ, Fakra SC, Pilon-Smits EAH (**2012**) Analysis of selenium accumulation, speciation and tolerance of potential selenium hyperaccumulator *Symphyotrichum ericoides*. Submitted
7. Valdez Barillas JR, Quinn CF, Freeman JL, **Lindblom SD**, Marcus MS, Fakra SC, Gilligan TM, Alford ER, Wangeline AL, Pilon-Smits EAH (**2012**) Selenium distribution and speciation in hyperaccumulator *Astragalus bisulcatus* and associated ecological partners. Submitted
8. **Lindblom SD**, Valdez-Barillas JR, Fakra SC, Marcus MA, Wangeline AL, Pilon-Smits EAH **(2011)** Influence of microbial associations on selenium localization and speciation in roots of *Astragalus* and *Stanleya* hyperaccumulators. Environmental and Experimental Botany, In press. doi:10.1016/j.envexpbot.2011.12.01*1*
9. Quinn QF, **Lindblom SD**, Pilon-Smits EAH (**2011**) Ecological aspects of selenium phytoremediation. In: "Phytoremediation: Processes, Characteristics, and Applications" (Columbus F, Ed.). Nova Publishers, 651-666
10. Quinn CF, Freeman JL, Reynolds RJB, Cappa JJ, Fakra SF, Marcus MA, **Lindblom SD**, Quinn EK, Bennett LE, Pilon-Smits EAH (**2010**) Selenium hyperaccumulating plants are protected from the cell disruptor herbivores. BMC Ecology 10: 1
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17. Garifullina GF, Owen JD, **Lindblom SD**, Tufan H, Pilon M, Pilon-Smits EAH (**2003**) Expression of a Mouse Selenocysteine Lyase in *Brassica juncea* Chloroplasts Affects Selenium Tolerance and Accumulation. Physiologia Plantarum 118: 538-544

**Presentations**

Spring 2001, 2003 - Biology Department student research symposium. Poster.

Spring 2001 - CSU undergraduate research symposium. Poster. Honorable mention.

Spring 2002, 2003, 2004 – CSU undergraduate research symposium. Poster.

Fall 2011 – Plant Super-Group- Colorado State University.