

DISSERTATION

ECOLOGICAL ASPECTS OF PLANT SELENIUM HYPERACCUMULATION:
EFFECTS OF SELENIUM HYPERACCUMULATION ON PLANT-PLANT INTERACTIONS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

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Fall 2016

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ABSTRACT

ECOLOGICAL ASPECTS OF PLANT SELENIUM HYPERACCUMULATION: EFFECTS OF SELENIUM HYPERACCUMULATION ON PLANT-PLANT INTERACTIONS

Hyperaccumulators are plants that accumulate toxic elements to extraordinary levels. Selenium (Se) hyperaccumulators such as *Astragalus bisulcatus* and *Stanleya pinnata* can contain 0.1-1.5% of their dry weight in Se (1,000 - 15,000 mg Se kg⁻¹ DW), levels toxic to most other organisms. Selenium promotes hyperaccumulator growth and also offers the plant several ecological advantages through negative effects on Se-sensitive partners. Previous work has shown that high tissue Se levels reduce herbivory and pathogen infection. On the other hand, hyperaccumulators may offer an exclusive niche for Se-tolerant ecological partners. The focus of this dissertation study was on the effects of Se hyperaccumulation on plant-plant interactions.

The first Chapter presents a literature review of the phenomenon of Se hyperaccumulation, how Se hyperaccumulators are different from other plants, and an overview of previous studies on the effects of hyperaccumulated Se on ecological processes related to herbivore-plant interactions, microbe-plant interactions and pollinator-plant interactions. In addition, evolutionary aspects of Se hyperaccumulation are discussed, and their implications for their ecological partners. The findings presented in this overview formed the platform for the experiments carried out in this dissertation research, on the topic of plant-plant interactions.

In Chapter 2, experiments are described to address the question whether Se hyperaccumulation can negatively affect neighboring plants. Soil collected around hyperaccumulators on a seleniferous field site was measured and shown to contain more Se (up to 266 mg Se kg⁻¹) than soil around non-hyperaccumulators. Vegetative ground cover was somewhat lower around Se hyperaccumulators compared to non-hyperaccumulators. Thus, Se hyperaccumulators may increase surrounding soil Se concentration (phytoenrichment). The enhanced soil Se levels around hyperaccumulators were shown to

impair growth of a Se-sensitive plant species, *Arabidopsis thaliana*, pointing to a possible role of Se hyperaccumulation in elemental allelopathy.

In Chapter 3, potential positive effects of hyperaccumulator Se on neighboring plants are explored. It was found for two plant species, *Artemisia ludoviciana* and *Symphytotrichum ericoides*, that growing next to Se hyperaccumulators increased their Se content 10-20 fold (up to 800-2,000 mg Se kg⁻¹ DW) compared to when they were growing next to non-accumulators. Moreover, these neighbors of hyperaccumulators were 2-fold bigger, showed 2-fold less herbivory damage and harbored 3-4 fold fewer arthropods than when growing next to non-hyperaccumulators. When used in laboratory choice and non-choice grasshopper herbivory experiments, Se-rich neighbors of hyperaccumulators experienced less herbivory and caused higher grasshopper Se accumulation (10-fold) and mortality (4-fold). These results suggest that Se hyperaccumulators can facilitate the growth of Se-tolerant neighboring plants.

The fourth Chapter describes a more controlled greenhouse pot cocultivation study that investigated how Se affects relationships between Se hyperaccumulators (*A. bisulcatus* and *S. pinnata*) and related non-accumulator species (*A. drummondii* and *S. elata*), in terms of how these plants influence their neighbor's Se accumulation and growth. Selenium affected growth differently in hyperaccumulators and nonaccumulators: The hyperaccumulators performed 2.5-fold better on seleniferous than non-seleniferous soil, and grew up to 4-fold better with increasing Se supply, while the non-accumulators showed opposite results. Both hyperaccumulators and non-accumulators could affect growth (up to 3-fold) and Se accumulation (up to 6-fold) of neighboring plants. The mechanisms for these effects are largely unknown but may involve concentration of soil Se via exudation, root turnover and litter deposition. Exudate of selenate-supplied *A. bisulcatus* was shown by x-ray absorption spectroscopy to contain mainly C-Se-C.

In conclusion, Se hyperaccumulators may enhance the soil Se levels under their canopy, and also convert inorganic Se to organic Se. The Se-enriched soil around hyperaccumulators enhances Se levels in neighboring plants, which may negatively affect Se-sensitive neighboring plants via toxicity, but facilitate

Se-tolerant neighbors through reduced herbivory. The latter is an interesting finding, as it constitutes facilitation via enrichment with a non-essential element. It is also interesting that Se enrichment of neighbors by hyperaccumulators can result in competition when neighbors are Se-sensitive and in facilitation when neighbors are Se-tolerant. Via these competitive and facilitating effects, Se hyperaccumulators may affect plant species composition and, consequently, higher trophic levels. Hyperaccumulators may favor Se resistant species at different trophic levels, while selecting against Se sensitive species. If indeed Se hyperaccumulators affect soil Se distribution and speciation and local species composition and Se tolerance, Se hyperaccumulators may play an important role in Se entry into and Se cycling through their seleniferous ecosystems.

ACKNOWLEDGEMENTS

I thank Allah for helping me to finish this study. I would like to thank my parents and my family for supporting me. Special thanks to my advisor Lianne Pilon-Smits for all of her guidance on research and writing; I would also like to thank for her advice and guidance. I would also like to thank my committee members, Marinus Pilon, Mark Paschke and Jorge Vivanco. I would also like to thank my colleagues in the lab for helpful discussions, and specifically Colin Quinn for helping with plant-insect interaction studies and Stormy Dawn Lindblom and Jennifer Cappa for technical assistance with XAS studies. Finally, I would like to thank the Ministry of Higher Education in the Libyan government and the National Science Foundation for funding and the Advanced Light Source for beam time.

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CHAPTER 1:

ECOLOGICAL ASPECTS OF PLANT SELENIUM HYPERACCUMULATION¹:

INTRODUCTION

Hyperaccumulation is the intriguing phenomenon that some plant species accumulate one or more toxic elements to extraordinary high concentrations, typically 100-fold higher than other vegetation on the same site (Baker *et al.* 2000). The criterion used to distinguish a hyperaccumulator ranges from 0.01-1% of leaf dry matter, depending on the element. Elements that can be hyperaccumulated include arsenic (As, >0.1%), cadmium (Cd, >0.01%), cobalt (Co, >0.1%), copper (Cu, >0.1%), lead (Pb, >0.1%), manganese (Mn, >1%), nickel (Ni, >0.1%), selenium (Se, >0.1%) and zinc (Zn, >1%). These elemental levels would be lethal to other organisms, yet cause no toxicity in hyperaccumulators. About 450 plant species from over 40 families have been reported to hyperaccumulate, but hyperaccumulation is most prevalent in the Brassicaceae (Baker *et al.* 2000). Typically, hyperaccumulators are found only on soils that contain elevated levels of the toxic element in question, suggesting they have a physiological or ecological requirement for the element. Boyd and Martens (1992) proposed several possible physiological or ecological functions of hyperaccumulation, including roles in herbivore or pathogen defense, elemental allelopathy, drought resistance, or metal tolerance. Several of these hypotheses have been tested using different hyperaccumulator models. In this review we will give an overview of our current knowledge regarding the ecological effects of plant elemental hyperaccumulation, using Se hyperaccumulation as a model system.

Selenium naturally occurs as a trace element in most soils. Soil Se levels are typically below 1 mg kg⁻¹, but may be up to 100 mg kg⁻¹ in seleniferous soils. The main forms of bioavailable Se in oxidizing and reducing environments are selenate and selenite, respectively (White *et al.* 2007).

¹EI Mehdawi AF and Pilon-Smits EAH (2012) Plant Biology 14: 1-10.

Selenium has not been shown to be an essential element for higher plants, although it can have a growth-promoting effect for some species (Pilon-Smits *et al.* 2009). Since Se is chemically similar to sulfur (S), it is taken up and metabolized via the same mechanisms by organisms. Selenate and selenite can be taken up inadvertently by plants, via transporters for sulfate or other anions. Selenium uptake is dependent on Se concentration and speciation, the concentration of competing anions, rhizosphere pH and redox conditions (Mikkelsen *et al.* 1989). Roots take up selenate faster than selenite (Zhao *et al.* 2005; Pilon-Smits *et al.* 1999). Selenate and selenite can be further reduced to selenide and assimilated into the aminoacids selenocysteine (SeCys) and selenomethionine (SeMet) via the S assimilation pathway (White *et al.* 2007). Both SeCys and SeMet can be incorporated into proteins. Non-specific incorporation of SeCys into proteins, in the place of cysteine, is toxic. However, organisms that require Se as a micronutrient, including mammals, can also specifically incorporate SeCys into a small set of selenoproteins, which contain SeCys in their active site and perform redox functions. Some selenoproteins play a role in scavenging free radicals. In humans, Se deficiency has therefore been associated with enhanced risk of cancer (White *et al.* 2007). The window between Se deficiency and toxicity in animals is extremely narrow, and both are problems worldwide. Selenium-containing plants may be used both as a dietary source of Se (biofortification), and as a means to remove excess Se from the environment (phytoremediation).

What can plants do with Se and how are Se hyperaccumulators different from other plants?

Most plant species growing on seleniferous soils contain <10 mg Se kg⁻¹ DW, and experience toxicity at levels above ~100 mg Se kg⁻¹ DW (Rosenfeld and Beath 1964; Brown and Shrift 1982; White *et al.* 2004). Plants that can accumulate and tolerate moderately elevated Se levels (up to 1,000 mg Se kg⁻¹ DW) are called Se accumulators. True Se hyperaccumulator species, from the genera *Stanleya* and *Astragalus* can accumulate 1,000-15,000 mg Se kg⁻¹ DW (0.1-1.5% Se) without any toxicity symptoms. Hyperaccumulators differ from non-hyperaccumulators in several ways. Selenate uptake by hyperaccumulators is not inhibited by high sulfate concentration. Hyperaccumulators tend to enrich themselves with Se relative to S: they have a higher Se/S ratio in their tissues compared to their growth

medium; this is not found in non-hyperaccumulators. Hyperaccumulators of Se often also contain elevated S levels, compared to non-hyperaccumulators (El Mehdawi *et al.* 2011a).

Once Se is inside the plant, hyperaccumulators show a relatively high degree of root-to-shoot Se translocation compared to other plants. The form of Se accumulated by hyperaccumulator plants (Se speciation) is mainly organic methyl-SeCys (Freeman *et al.* 2006a), while in non-hyperaccumulators the majority of Se remains selenate. Perhaps because of this difference in speciation, the tissue Se sequestration pattern is different for hyperaccumulators: they store Se mainly in the leaf epidermis (sometimes in leaf hairs) and in reproductive tissues, particularly pollen, ovules and seeds (Freeman *et al.* 2006b; Quinn *et al.* 2011b). Non-hyperaccumulators mainly store Se in vascular tissues in leaves, and have higher levels in leaves than flowers. Plant Se levels also show a different seasonal fluctuation in hyperaccumulators and non-accumulators: the leaf Se concentration was highest in the early spring for hyperaccumulators, but peaked in summer for non-hyperaccumulators (Galeas *et al.*, 2007). The seasonal fluctuations in Se levels were correlated with S levels for non-hyperaccumulators, but not for hyperaccumulators. Table 1 summarizes these physiological differences between hyperaccumulators and non-hyperaccumulators.

Evolutionary aspects of Se hyperaccumulation

Selenium hyperaccumulation occurs in relatively few genera and species (~30 taxa in total), making it likely to be a derived trait: hyperaccumulators probably evolved from non-hyperaccumulators. Alternatively, it is possible that Se hyperaccumulation is an ancient trait that was more prevalent in times when seleniferous soils were more widespread, but that is increasingly being lost as soil Se concentrations decrease (Brown and Shrift 1982). Assuming Se hyperaccumulation is a derived trait, one question that arises is: which mutations gave rise to hyperaccumulation? The observed physiological differences in Se uptake, translocation and sequestration between hyperaccumulators and non-hyperaccumulators may be used to develop hypotheses about the underlying molecular mechanisms that have evolved to create hyperaccumulators. The observation that hyperaccumulators take up selenate independent from sulfate may indicate hyperaccumulators have evolved a selenate-specific transporter while non-hyperaccumulators

use the same transporters for sulfate and selenate. Such a specialization in function may e.g. happen after a polyploidy event: after doubling of a gene encoding a sulfate transporter, one may retain its original function while the duplicate evolves into a selenate transporter.

The hyperaccumulators' enhanced Se translocation rates and sequestration in reproductive organs could be due to different expression levels of particular transporters e.g. for selenate/sulfate or of organic selenocompounds. This may for instance involve upregulation of the transporter that exports sulfate from root cells into the root xylem, and from shoot cells into the shoot phloem. There may also be methyl-SeCys transporters in hyperaccumulators that mediate leaf phloem loading and sequestration in specialized epidermal cell types. If Se is (re)mobilized in different forms in hyperaccumulators and non-hyperaccumulators this may also explain the observed differences in sequestration patterns, both temporal and spatial. The molecular mechanism for the capacity of hyperaccumulators to accumulate Se as methyl-SeCys is a SeCys methyltransferase (SMT) (Neuhierl and Böck 1996). Methyl-SeCys can be safely accumulated because it does not get incorporated into proteins, and therefore does not disrupt protein function. This explains the extreme Se tolerance of hyperaccumulators. Methyl-SeCys can be converted to dimethyldiselenide, the main form of volatile Se produced by hyperaccumulators. Non-hyperaccumulators, on the other hand, produce volatile dimethylselenide, using selenomethionine (SeMet) as a starting point.

Assuming that Se hyperaccumulation is a derived trait, all hyperaccumulators are not likely to have evolved from a single ancestor, since they occur in several unrelated genera and are often considered derived taxa (Brown and Shrift 1982; White *et al.* 2004). It is therefore more likely that Se hyperaccumulation and hypertolerance arose independently in different genera via convergent evolution (Brown and Shrift 1982). This raises the question: what has/have been the selection pressure(s) for the evolution of Se hyperaccumulation? More than one selection pressure may act simultaneously. Since hyperaccumulators of Se are rarely observed in non-seleniferous areas (Brown and Shrift 1982), it has been hypothesized that Se may play an essential physiological role in hyperaccumulators. Although Se clearly is a beneficial nutrient for hyperaccumulators and many other plant species, owing to improved protection

against oxidative stress (Cartes *et al.* 2005; Djanaguiraman *et al.* 2005; Hartikainen 2005; Kong *et al.* 2005), to date there is no convincing evidence that supports an essential role for Se in any higher plant species. Alternatively, hyperaccumulators may have an ecological requirement for Se. As mentioned above, Boyd and Martens (1992) proposed several possible functions of hyperaccumulation, including roles in herbivore or pathogen defense, elemental allelopathy, drought resistance, or metal tolerance. So far, there is supporting evidence for both the elemental defense hypothesis and the elemental allelopathy hypothesis, as described in the next sections.

Effects of Se on ecological processes in seleniferous areas

Since Se is a toxic element, Se hyperaccumulation in plants can be envisioned to have ecological implications at many levels (Fig. 1). Below-ground, Se hyperaccumulator plants may affect the local soil Se distribution and speciation via litter deposition and root turnover and exudation. This may affect microbial composition and abundance, as well as neighboring vegetation. Selenium accumulation in plant tissues (root and shoot) as well as Se volatilization may affect plant-pathogen, plant-herbivore and plant-pollinator interactions. Below we summarize what is known about these ecological implications of Se (hyper) accumulation.

I. Plant-herbivore interactions

Since Se is toxic to animals at high levels, ingestion of Se-rich plant material may be expected to have a toxic effect on herbivores. As a result, herbivores may learn to avoid high-Se plant material. Through such toxicity and/or deterrence Se accumulation may serve to defend the plants against herbivory. To investigate this so-called elemental defense hypothesis (Boyd and Martens 1992) different approaches have been used. In one approach, plants were pretreated under controlled conditions with different Se concentrations, and offered to herbivores in choice or non-choice experiments, to test for deterrence and toxicity, respectively. In another approach, herbivores or herbivory were surveyed in the field as a function of natural plant Se concentration. Already since the 1930s Se has been known to be the toxic component

of so-called “locoweeds”, responsible for substantial livestock losses in the Western U.S.A. (Beath *et al.* 1939). Six decades later we know Se in plants is toxic to a variety of insect and vertebrate herbivores, and also deters most herbivores. Varieties of *Atriplex* that accumulated more Se supported lower insect growth and survival of *Spodoptera exigua* (Vickerman *et al.* 2002). Caterpillars of the cabbage looper (*Trichoplusia ni*) preferred to feed on low-Se rather than high-Se *Brassica juncea* (Bañuelos *et al.* 2002). Selenium also protected *B. juncea* from the caterpillar of the cabbage white butterfly (*Pieris rapae*), both due to deterrence and toxicity (Hanson *et al.*, 2003). Selenium also protected plants against the other leaf chewing herbivores crickets and grasshoppers via deterrence and toxicity, both in *B. juncea* and in hyperaccumulator *S. pinnata* (Freeman *et al.*, 2007). In laboratory studies *B. juncea* plants were protected by Se at levels of 230 and 447 mg/kg DW, and in a subsequent manipulative field study levels as low as 50 mg/kg DW already protected *S. pinnata* plants. As described above, a difference between the two plant species is that the Se is sequestered specifically in the epidermis of *S. pinnata*, along the leaf edges, while *B. juncea* contains most Se in its vasculature. Sequestration in the periphery of the leaves, as found for *S. pinnata*, may offer additional protection from herbivores. Moreover, the main forms of Se in *B. juncea* and *S. pinnata* are selenate and methyl-SeCys, respectively. Since Se accumulation protected both plants, both forms of Se appear to be toxic to herbivores. The mechanism of methyl-SeCys toxicity was revealed in a comparative study using two populations of the diamondback moth (*Plutella xylostella*). In a lab study a Se-sensitive variety from a non-seleniferous area was shown to accumulate SeCys after feeding on Se-rich *S. pinnata* plants, while a Se-tolerant variety from a seleniferous area accumulated unaltered methyl-SeCys (Freeman *et al.* 2006b). Thus, the demethylation of methyl-SeCys likely causes toxicity via non-specific incorporation of the resulting SeCys into proteins. The Se-tolerant diamondback moth population may have lost this capacity to demethylate methyl-(Se)Cys. It appears to also have lost its aversion to feed and oviposit on high-Se plants: while the population from the non-seleniferous area preferred to oviposit and feed on low-Se plants, the population from the seleniferous area had no preference. Together these adaptations enable this herbivore to overcome the elemental plant defense and occupy the niche provided by Se hyperaccumulator plants. It is even feasible that the moth, which contained around 250 mg Se/kg

DW uses the Se for its own defense, against predators and parasites. If so, this defense likely will be overcome by Se-tolerant predators and parasites. Indeed, a parasitic wasp was found to complete its life cycle in the larvae of the Se-tolerant diamondback moth, and to contain similar Se levels as the moth, also in the form of methyl-SeCys (Freeman *et al.* 2006b).

In addition to leaf chewers, Se also was shown to protect plants from invertebrates with other feeding modes. Phloem-feeding aphids were shown to be deterred by high-Se *B. juncea* plants, and suffered toxicity already at plant Se levels as low as 10 mg/kg DW (Hanson *et al.*, 2004). This indicates that Se is present in the phloem of this plant, which is in agreement with the XAS data which showed predominant accumulation of Se in the vasculature. Selenium also protected the hyperaccumulators *S. pinnata* and *A. bisulcatus* from two herbivores with a cell-disrupting feeding mode, the two-spotted spider mite and the thrips (Quinn *et al.* 2010). Furthermore, in a preliminary study addressing the effect of root-accumulated Se on nematode colonization, high-Se *S. pinnata* plants harbored fewer nematodes than low-Se plants (Prins and Pilon-Smits, unpublished results). Besides protecting plants from a variety of invertebrate herbivores, Se was also shown to protect *B. juncea* and *S. pinnata* from a vertebrate herbivore: the black-tailed prairie dog (Quinn *et al.* 2008; Freeman *et al.* 2009). When high- and low-Se plants were offered to prairie dogs in the field they preferentially fed on the low-Se plants, and when high- and low-Se plants were followed over a 2-year period the low-Se plants survived significantly better and suffered less herbivory.

In a different approach to address the elemental defense hypothesis for Se, a field survey was done comparing arthropod load on hyperaccumulator and non-hyperaccumulator species in a seleniferous habitat. The two Se hyperaccumulator species *A. bisulcatus* and *S. pinnata* harbored significantly fewer arthropods (both in terms of animals per plant and number of species) compared to similar-sized non-Se hyperaccumulators *Medicago sativa* and *Helianthus pumilus* (Galeas *et al.* 2008). In another field survey it was found that hyperaccumulator *A. bisulcatus* containing 120-600 mg Se/kg DW was relatively abundant on prairie dog towns compared to outside these towns in seleniferous areas, and when growing on prairie dog towns it was hardly eaten (Quinn *et al.* 2008).

In summary, there is ample support for the Se elemental defense hypothesis, which states that Se hyperaccumulation may have evolved as a defense against herbivory. Even at low levels (10-50 mg/kg DW), Se can already protect plants against a variety of vertebrate and invertebrate herbivory, due to a combination of deterrence and toxicity. Herbivory may have served as an evolutionary selection pressure, leading to ever increasing Se accumulation in plants. Specialized metabolism, leading to storage of Se in the form of methyl-SeCys made it possible to accumulate even higher Se levels that would otherwise be toxic (>1,000 mg Se/kg DW), leading to true hyperaccumulation. Specific sequestration of Se in the peripheral plant tissues may have further optimized the protective effect of the Se, as well as plant Se tolerance. As with any plant defense, over time it will be overcome by some herbivores, and this appears to be the case as well for Se hyperaccumulation, as described for the case study of the diamondback moth.

II. Plant-pollinator interactions

High floral Se may be hypothesized to have a negative effect on plant reproduction if the Se in flowers impairs plant reproductive functions or plant-pollinator interactions. High Se levels may for instance impair pollen germination, pollen tube growth, number and size of seeds or seed germination. Selenium in flowers may deter pollinators or be toxic to them when ingested. Since Se has been shown to be toxic to many insect herbivores, and to deter them (Quinn *et al.* 2007), it may be expected to have similar effects on insect pollinators. Before addressing these questions it is helpful to know the distribution and chemical speciation of Se in reproductive tissues. In Se hyperaccumulator *S. pinnata*, flowers and seeds were found to have the highest Se levels of the entire plant, reaching levels upward of 4,000 mg kg⁻¹ DW (Quinn *et al.* 2011b). Within flowers, the pistil and anthers contained the highest Se levels, particularly the pollen and ovules. The nectar of hyperaccumulator *S. pinnata* also contained significant Se levels. The predominant form of Se in the *S. pinnata* flowers was a C-Se-C (organic) compound, presumably MeSeCys since the same compound was found earlier in its leaves (Freeman *et al.* 2006a). In contrast, the related non-hyperaccumulator *B. juncea* did not show particularly high floral Se levels compared to other organs (Quinn *et al.* 2011b). The majority (67%) of the Se in *B. juncea* flowers was also MeSeCys, with relatively

minor fractions of selenocystine (16%), selenate (11%) and selenite (6%). This Se speciation in *B. juncea* flowers is different from the predominant form in leaves, selenate (Pilon-Smits *et al.* 1999). Within *B. juncea* flowers the Se was evenly distributed (Quinn *et al.* 2011b). Thus, tissue-specific Se sequestration appears to be a trait that is unique for the hyperaccumulator. It is intriguing why the hyperaccumulator preferentially stores its Se in its pollen, ovules and seeds; perhaps the Se functions to protect these valuable reproductive structures from herbivores and pathogens.

Overall, the high Se levels in flowers, particularly in hyperaccumulators, may well have physiological and ecological implications. Prins *et al.* (2011) tested the effect of floral Se accumulation on reproductive functions in *B. juncea* and *S. pinnata*. At levels above ~ 500 - $1,000$ mg kg⁻¹ DW, pollen germination was significantly affected in *B. juncea* but not in *S. pinnata*. In addition, the number, size and germination rate of seeds were affected in *B. juncea* around the same concentration. Thus, in non-hyperaccumulators Se accumulation may negatively affect reproductive functions. It is worth noting, however, that in the field *B. juncea* typically does not accumulate more than 100 mg kg⁻¹ DW, and this level does not impair reproduction. There was no evidence that the high Se levels impaired reproductive functions in the hyperaccumulator, perhaps because it stored the Se in a less toxic form.

In another study, the ecological impacts of floral Se accumulation were investigated. *B. juncea* and *S. pinnata* plants containing high or low Se levels were monitored for floral visitation by honey bees and other potential pollinators (Quinn *et al.* 2011b). No differences in floral visitation were observed for either plant species, even at extremely high floral Se concentrations. Thus, results so far do not show evidence for a reproductive cost for Se hyperaccumulators due to deterrence of pollinators. It cannot be excluded at this point that the high-Se nectar and pollen ingested by the pollinators has a health effect on the pollinator. More studies are needed to address this question. If there is a health impact of the ingested Se on the bee, it could be positive or negative. Selenium is an essential trace element for insects, and therefore a Se-enriched diet may promote bee health. On the other hand, if ingested in excess, the Se will have a toxic effect on the bee. In preliminary studies with 2-5 animals per species, (non-native) honey bees were found to contain around 20 mg Se kg⁻¹ DW while (native) bumble bees contained around 250 mg Se kg⁻¹ DW.

While the bumble bees contained predominantly C-Se-C (presumably the non-toxic MeSeCys), the honey bee also contained more toxic forms of Se (Quinn *et al.* 2011b). Based on these limited data it is hard to predict whether these Se levels are likely to be beneficial or toxic. In Se toxicity studies on other – herbivorous- insects, animal Se levels of 10-90 mg Se kg⁻¹ DW were found to be lethal to Se-sensitive Lepidoptera larvae of *P. rapae* and *P. xylostella* (Hanson *et al.*, 2003; Freeman *et al.*, 2006b). The Se-tolerant *P. xylostella* population, on the other hand, accumulated 250 mg Se/kg DW without ill effects, as nontoxic MeSeCys (Freeman *et al.*, 2006b). It is possible that the bumble bee native to the seleniferous area has specialized to forage on Se hyperaccumulator and has evolved Se tolerance, as suggested by the observation that it contained the same high Se level and the same non-toxic form of Se as the Se-tolerant *P. xylostella*. The lower Se levels and presence of different forms of Se in the honey bee may reflect different foraging behavior (visiting both hyper- and non-hyperaccumulators), or may be an indication that it has reduced Se tolerance. More studies are needed to address this question and to assess the potential health effects of high-Se plants on local bee populations.

Honey from seleniferous areas contained around 1 mg Se/g FW (~1.25 mg Se/kg DW, Quinn *et al.*, 2011b), which is higher than the Se levels reported for honey from Turkey (Tuzen *et al.* 2010). At 1 mg Se/g FW, honey may be considered nutritionally enhanced. The recommended daily intake of Se is 50-75 microgram, corresponding with about three tablespoons of this Se-fortified honey. Thus, Se in flowers and the resulting Se-enriched honey may benefit human health via the reported capacity of Se to inhibit several types of cancers (Rayman 2005). Since not all forms of Se are equally potent in this regard, future studies investigating the chemical form of Se in honey could be helpful to better determine the health benefits of Se in honey.

III. Plant-microbe interactions

Plants live in close association with bacterial and fungal microbes. Some live inside the plant (endophytes), some on the plant surface and some in the sphere of influence of the plant, such as the rhizosphere. From the plant's perspective, the nature of the plant-microbe interaction may be positive

(mutualistic), negative (parasitic), or neutral (commensalistic). In mutualistic relationships the plant typically provides the microbe with organic carbon, and in return gets better access to mineral nutrients or protection from abiotic or biotic stresses. In parasitic relationships the microbe exploits the plant and may cause reduced growth, disease or death. Selenium accumulation in plant tissues may affect all of these plant-microbe interactions. Compared to other ecological interactions, the effects of Se on plant-microbe interactions are relatively understudied. In one study by Hanson *et al.*, (2003) it was found that Se accumulation in *B. juncea* at 300-750 mg/kg DW reduced fungal infection by two pathogens, one a leaf pathogen (*Alternaria alternata*) and one a stem-root pathogen (*Fusarium sp.*). Both pathogens are fairly Se-sensitive, showing 50% growth inhibition at 40-60 mg selenate/L when grown on petridishes. Thus, plant Se accumulation may protect plants from Se-sensitive fungal pathogens. It will be interesting in future studies to test whether Se also protects plants from bacterial and viral pathogens.

High-Se plant material may also have a profound effect on mutualistic relations with microbes, selecting for more Se tolerant species. The most extreme Se levels are likely experienced by hyperaccumulator endophytes and litter decomposers, which may experience levels >1,000 mg/kg DW. However, also in the hyperaccumulator rhizosphere Se levels can be quite elevated, up to 600 mg/kg soil (El Mehdawi *et al.* 2011a). This is likely to have a profound effect on microbial species composition and perhaps density. Indeed, in a survey of rhizosphere fungi collected from seleniferous and non-seleniferous habitats the isolates from seleniferous habitats were significantly more Se tolerant than those from a non-seleniferous habitat (Wangelin *et al.* 2011). The rhizosphere of hyperaccumulator plants harbored many highly Se tolerant fungi, some of which appear to benefit from Se in terms of overall growth and stress tolerance (Wangelin and Pilon-Smits, unpublished results). The high Se levels associated with hyperaccumulator habitats may lead to the evolution of specialized microbes that have evolved Se tolerance and therefore can occupy the niches provided by hyperaccumulator plants. In future studies it will be interesting to test the nature of these plant-fungus interactions, and the potential effects of associated microbes on plant Se accumulation and speciation. In the next section we will discuss another class of microbe that interacts with high-Se plant material: the litter decomposer.

IV. Litter decomposition and Se cycling

Decomposition of leaf litter in soil around plants is a significant ecosystem process that plays an important role in chemical cycling of elements (Aerts 2006). Many factors affect leaf litter decomposition rate, including physical, chemical, and biological factors. Physical factors include geography and climate (temperature, precipitation, evapotranspiration) (Aerts 2006; O'Neill *et al.* 2003; Silver and Miya 2001), and chemical factors include the elemental composition and pH of the soil. Biological factors include the quality of the litter and the local decomposer community (Swift *et al.* 1979, Smith and Bradford 2003). Among these factors, litter quality is considered one of the most important ones (Swift *et al.* 1979). The high Se levels observed in hyperaccumulator litter ($>1,000 \text{ mg kg}^{-1} \text{ DW}$) may be hypothesized to slow down decomposition, due to toxicity to the microbial and microarthropod decomposer communities. Surprisingly, the opposite was found when plant material with varying Se concentrations were left to decompose in a seleniferous area. High-Se *A. bisulcatus* material (around $600 \text{ mg Se kg}^{-1} \text{ DW}$) decomposed faster than low-Se *M. sativa* material and contained more microarthropods and culturable microbes (Quinn *et al.* 2011a). The *A. bisulcatus* material contained a higher N/C ratio than the *M. sativa* litter, potentially making it a higher quality litter for decomposers. The fast decomposition of the high-Se litter in seleniferous habitat suggests that the local microbial and microarthropod decomposer community has enhanced Se tolerance. Perhaps there even are specialist decomposers that specifically target hyperaccumulator litter. These Se tolerant decomposers may contribute to the local Se cycling, by recycling hyperaccumulator-bound Se and moving it into the food chain. Some may also volatilize Se, or promote Se leaching into groundwater. The presence of Se-tolerant decomposers enable hyperaccumulators to have a profound effect on the Se cycling in the local ecosystem. The plants concentrate Se many fold in their tissues, change its speciation from inorganic to organic, redeposit the Se to the soil, and through their tissues and their litter offer a significant portal for organic Se into the food chain. Organic Se is often taken up more readily by organisms, and affects organisms differently than inorganic Se; hyperaccumulators may

therefore mediate not only a quantitative but also a qualitative effect on Se fluxes through the local ecosystem.

V. Plant-Plant Interactions: Scope of this Dissertation Research

From the various ecological studies summarized above it is clear that the accumulated Se in hyperaccumulators has a profound effect on its ecological interactions with animals and microbes. An area that was unstudied at the start of this dissertation research was that of plant-plant interactions: does the Se in hyperaccumulators have any effects on neighboring plants? If so, are these effects negative (competitive) or positive (facilitation) and what could be the underlying mechanisms? Plants are known to be able to affect neighboring plants negatively or positively via various mechanisms. Negative effects may include the production or concentration of toxic chemicals (allelopathy). It may be envisioned that decomposition of Se hyperaccumulator leaf litter or exudation of selenocompounds can increase soil Se concentration, which may be toxic to neighboring plants. Another potential negative effect on neighbors may be enhanced herbivore loads as these herbivores avoid Se hyperaccumulators. On the other hand, it can also be envisioned that deterrence of herbivores by Se in hyperaccumulators via volatiles would extend to its neighbors, which would constitute facilitation. The objectives of these studies were to determine how Se hyperaccumulators affect their surrounding soil Se concentration, local plant community composition, and the germination, growth and Se accumulation of surrounding plant species. The findings were expected to give better insight into the importance of Se hyperaccumulators for the ecology of seleniferous ecosystems, and also into the ecological implications of growing high-Se plants in agricultural settings, e.g. for phytoremediation or biofortification.

Table 1: Overview of Physiological differences between Se hyperaccumulators and non-hyperaccumulators.

<u>Property</u>	<u>Se hyperaccumulators</u>	<u>Non-hyperaccumulators</u>
Se uptake	Sulfur-independent	Inhibited by sulfur
Root-to-shoot Se translocation	Higher	Lower
Se accumulation and tolerance	1,000-15,000 mg Se kg ⁻¹ DW	< 1,000 mg Se kg ⁻¹ DW
Se volatilization	Higher, as dimethyldiselenide	Lower, as dimethylselenide
Sequestration (organ level)	Highest in reproductive organs	Highest in leaves
Sequestration (tissue level)	Highest in epidermis, pollen, ovules	Highest in vascular tissues
Main Se form in tissues	Methyl-SeCys	Selenate
Seasonal fluctuations of Se, S	Highest in spring for Se, summer for S	Highest in summer for both

FIGURES

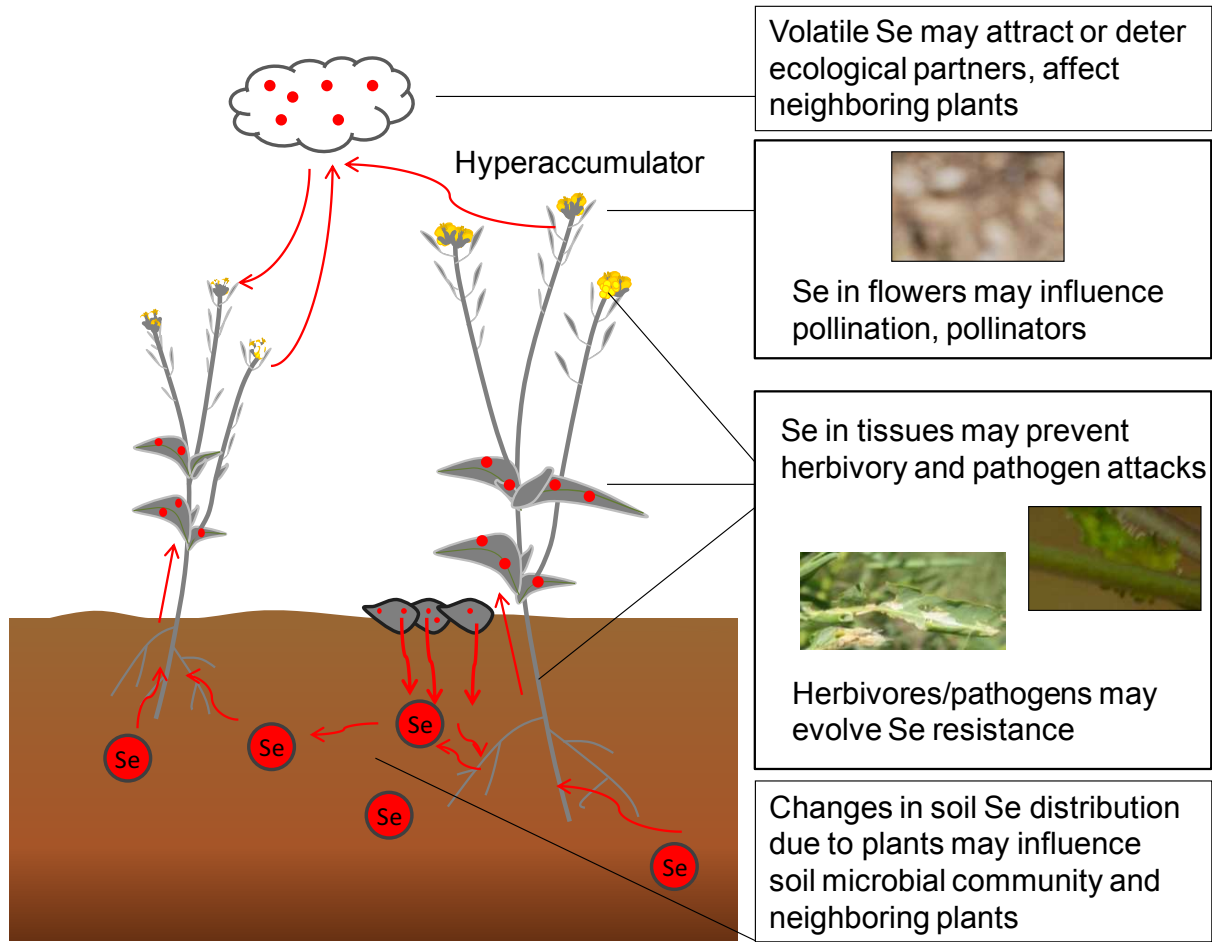


Figure 1. Ecological aspects of Se hyperaccumulation.

Figure1. Ecological aspects of Se hyperaccumulation.

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CHAPTER 2:

EFFECTS OF SELENIUM HYPERACCUMULATION ON PLANT-PLANT INTERACTIONS²: EVIDENCE FOR ELEMENTAL ALLELOPATHY?

Few studies have investigated plant-plant interactions involving hyperaccumulator plants. Here we investigate the effect of selenium (Se) hyperaccumulation on neighboring plants. Soil and litter Se concentration were determined around hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* and non-hyperaccumulators *Medicago sativa* and *Helianthus pumilus*. We also compared surrounding vegetative cover, species composition, and Se concentration in two plant species (*Artemisia ludoviciana*, *Symphytotrichum ericoides*) growing either close to or far from Se hyperaccumulators. Then, *Arabidopsis thaliana* germination and growth were compared on soils collected next to the hyperaccumulators and non-hyperaccumulators.

Soil collected around hyperaccumulators contained more Se (up to 266 mg Se kg⁻¹) than soil around non-hyperaccumulators. Vegetative ground cover was somewhat lower around Se hyperaccumulators compared to non-hyperaccumulators. Selenium concentration was higher in neighboring species *A. ludoviciana* and *S. ericoides* when growing close to, compared to far from Se hyperaccumulators. *A. thaliana* showed reduced germination and growth, and higher Se accumulation when grown on soil collected around Se hyperaccumulators compared to non-accumulators.

In conclusion, Se hyperaccumulators may increase surrounding soil Se concentration (phytoenrichment). The enhanced soil Se levels around hyperaccumulators can impair growth of Se-sensitive plant species, pointing to a possible role of Se hyperaccumulation in elemental allelopathy.

Elemental allelopathy influences on plant distribution and abundans around hyperaccumulators

²El-Mehdawi AF, Quinn CF, Pilon-Smits EAH (2011) New Phytologist 191: 120-131. Co-author Colin Quinn contributed Figures 2 and 3 and Table 3.

INTRODUCTION

The element selenium (Se) is an essential micronutrient for many organisms including mammals. In these organisms Se is incorporated into essential selenoproteins, some of which have antioxidant functions and may help prevent a variety of cancers (Burke 2002; Smits and LeDuc. 2009; Zhang *et al.* 2006). Although Se is essential for some algae and has been shown to promote growth for many higher plant species, there is no evidence it is essential for higher plants (Novoselev *et al.*, 2002; Zhang and Gladyshev, 2010). Selenium is toxic to most organisms at higher concentrations, due in large part to its similarity to sulfur (S) which leads to non-specific replacement of S by Se in proteins (Stadtman 1990).

Bioavailable Se in soil occurs primarily in the form of selenate (SeO_4^{2-}) or selenite (SeO_3^{2-}) (Kocot and Kita, 2003). Soil Se concentrations vary, and most soils contain between 0.01 and 2.0 mg kg⁻¹; some seleniferous soils can have Se concentrations above 10 mg kg⁻¹ (Zhu *et al.*, 2009). Despite their apparent lack of a physiological requirement for Se, higher plants readily take up selenate or selenite and convert it into organic forms via S assimilation mechanisms. Plants can also volatilize Se, in the forms of dimethylselenide or dimethyldiselenide, which have a pungent odor that helps to identify Se-rich plants (Terry *et al.* 2000). Some plants even actively accumulate Se to levels between 0.1 and 1.5% of dry weight (DW), typically 100-fold higher than other species growing on the same site (Beath *et al.*, 1939). Such species are called Se hyperaccumulators and are found exclusively on seleniferous soils. Species with intermediate Se levels, between 100 and 1,000 mg Se kg⁻¹ DW, are called Se accumulators, and non-Se accumulators only have trace concentrations of Se when grown in seleniferous habitats (Hawrylak-Nowak, 2008, Terry *et al.* 2000). Selenium hyperaccumulating species such as *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae) have the ability to store and tolerate extremely high concentrations of Se because they sequester Se in specialized tissues in the form of methylselenocysteine (MeSeCys) (Neuhierl and Böck, 2009; Freeman *et al.*, 2009). This form of Se does not get incorporated into proteins and thus is relatively non-toxic compared to selenate, the primary form of Se found in soils and in non-hyperaccumulating species (Neuhierl and Böck, 1996; de Souza *et al.*, 1998).

Why do some plants hyperaccumulate the toxic and non-essential element Se? There is substantial evidence for the elemental defense hypothesis: Se can protect plants from a variety of herbivores and pathogens (Hanson *et al.*, 2003, 2004; Freeman *et al.*, 2007, 2009; Galeas *et al.*, 2008). While this sheds some light on the possible functional significance of Se hyperaccumulation, alternative hypotheses have been proposed and may be explored (Boyd and Martens 1992). One alternative hypothesis is that elemental hyperaccumulation may serve an allelopathic function to keep neighboring plants at a distance, if hyperaccumulators concentrate the element in their surrounding soil, a phenomenon called phytoenrichment (Morris *et al.*, 2006, 2009). Past studies investigating the role of hyperaccumulation serving an allelopathic function have shown mixed results. Nickel hyperaccumulators were shown to increase surrounding soil Ni concentration, but did not decrease neighboring plant germination (Zhang *et al.* 2005, 2007). Increased Zn concentration in media was shown to reduce germination rates of a variety of species (Bottoms 2001). However, the Zn concentrations in the media were much higher than what is found in the field around Zn accumulating plants, and therefore this cannot be considered representative for elemental allelopathy. Morris *et al.* (2006) found that soil with elevated Zn concentrations collected from around Zn-rich *Acroptilon repens* did not decrease the germination rate of several species.

The effect of Se hyperaccumulator plants on germination, growth and Se accumulation in neighboring plants has yet to be reported. It has been shown that the decomposition of Se hyperaccumulator leaf litter can increase soil Se concentration (Quinn *et al.* 2010). Furthermore, it can be envisioned that Se is exuded from hyperaccumulator plant roots or from germinating seeds, further contributing to elevated Se levels around hyperaccumulator plants, which may have an effect on neighboring plants. The objectives of this study were to determine how Se hyperaccumulators affect their surrounding soil Se concentration, local plant community composition, and the germination, growth and Se accumulation of surrounding plant species.

MATERIALS AND METHODS

Study Site

The field site for this study, Pine Ridge Natural Area, is located in South West Fort Collins, CO, USA (40°32.70N, 105°07.87W). Pine Ridge Natural Area is a seleniferous habitat with sandy loam of Cretaceous shale origin, dominated by forb and grass species. The soil has a pH of 7.6, and 11% soil organic matter. Some DTPA-extractable nutrient levels were (in mg kg⁻¹): nitrate 5.1, sulfate 18.6, Ca 291, Fe 12, and K 425 (Quinn *et al.* 2010). At least two species of Se hyperaccumulating plants, *A. bisulcatus* (two-grooved milkvetch) and *S. pinnata* (prince's plume), thrive at Pine Ridge Natural Area (Galeas *et al.* 2008). The populations of *A. bisulcatus* and *S. pinnata* at Pine Ridge Natural Area are known to accumulate high concentrations of Se, up to 10,000 mg Se kg⁻¹ for *A. bisulcatus* and over 6,000 mg Se kg⁻¹ for *S. pinnata* (Freeman *et al.* 2006b; Galeas *et al.* 2007).

Effect of Se hyperaccumulation on soil Se distribution

To investigate the effect of Se hyperaccumulation by plants on the distribution of soil Se concentration, soil samples were collected from around the Se hyperaccumulators *A. bisulcatus* (Fabaceae) and *S. pinnata* (Brassicaceae) and the non-hyperaccumulators *Medicago sativa* (alfalfa, Fabaceae) and *Helianthus pumilus* (little sunflower, Asteraceae) at Pine Ridge Natural Area. These control species have been used before in earlier studies and were chosen because among the species on the site these showed the most similar growth habit and (in the case of the Fabaceae) relatedness. A soil sample was collected from the top 2 cm of topsoil from around 7 individuals of each plant species directly next to the stem and at 10 cm, 20 cm and 50 cm from the stem. In addition, soil samples were collected 0-5 cm from the root at depths of 0 cm, 5cm, 10 cm and 50 cm. Soil samples were dried for 72 hours as described below and were then sieved using mesh with 1 mm² holes, which removed leaf litter material and larger arthropods. Soil samples were analyzed for metal concentration as described below. In addition, young mature leaves and lateral roots were collected from each of the seven individuals of each of the four plant species, and analyzed for Se concentration as described below.

Effect of Se hyperaccumulation on plant community

To investigate the effect of Se hyperaccumulation on the surrounding plant community, the percent ground cover and plant species composition was determined around the same *A. bisulcatus*, *S. pinnata*, *M. sativa* and *H. pumilus* individuals described above (n = 7). Ground cover was determined by placing two 0.1 m² Daubenmire plots directly East and West of the plant, with the edge of the plot touching the stem of the plant. The percent ground cover for each plot was estimated as described by Daubenmire (1959) and the number of individuals from each plant species within each plot was counted. The percent ground cover and species composition was then averaged between the two plots.

Effect of proximity to Se hyperaccumulators on neighboring plant Se concentration

Studies were conducted to determine if proximity to Se hyperaccumulating plants affects Se concentration in non-hyperaccumulating plant species. Young mature leaves were sampled for Se concentration from the species *Artemisia ludoviciana* (white sage; Asteraceae) and *Symphotrichum ericoides* (white heath aster; Asteraceae), either growing in close proximity (< 1 m) to the hyperaccumulator species *A. bisulcatus* or *S. pinnata* or far away (> 4 m) from any hyperaccumulator (n=3). Young mature leaves from *A. bisulcatus*, *S. pinnata*, *M. sativa* and *H. pumilus* were also collected for elemental analysis. In addition, litter was collected under the canopy of *A. bisulcatus*, *S. pinnata*, *M. sativa*, and *H. pumilus*, as well as soil from 0-2 cm and from 2-5 cm depth.

Effect of soil Se concentration on *Arabidopsis thaliana* germination and growth

Litter and soil were collected at Pine Ridge Natural Area from around hyperaccumulators *A. bisulcatus* and *S. pinnata* (n=10), and from around non-hyperaccumulators *M. sativa* and *H. pumilus* (n=5), and analyzed for Se concentration as described below. For each plant sampled (30 plants total) topsoil (0-2 cm) with an equal volume of leaf litter on top was placed in petri dishes and 50 *Arabidopsis thaliana* seeds were sown in each petri dish (n = 3 per plant sampled). Germination rates for *A. thaliana* were recorded after 14 days. In a second experiment, 25 *A. thaliana* seeds were sown on soil taken from each

plant at 2-5 cm depth, using 10 x 10 cm pots (n=3 for each plant sampled). Three weeks after germination plants were analyzed for growth by determining biomass dry weight (DW) by harvesting and washing the whole plant including roots and drying at 50° C for 72 hours. Shoot material from the dried *A. thaliana* plants collected from each pot was then analyzed for elemental concentrations as described below.

Selenium-dependent *Arabidopsis thaliana* germination was investigated using half-strength Murashige and Skoog (MS) basal salts agar medium (Murashige and Skoog, 1962) spiked with different concentrations of Se as sodium selenate. Germination rates were compared 6 days after sowing seeds on media with 0, 2.5, 5, 10, 25, 100, 250, and 1000 ppm Se, using 3 replicates of 25 seeds each per concentration.

Elemental analysis

Leaves, seeds, litter and soil collected as described above were analyzed for elemental composition as follows. All samples were dried at 50°C for 72 hours and 100 mg DW of each sample was digested in nitric acid as described by Zarcinas *et al.* (1987). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used as described by Fassel (1978) to determine each digest's elemental composition.

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 (ANOVA) followed by a post-hoc Tukey Kramer test was used when comparing multiple means. Correlation analysis and linear regression were used to correlate *A. thaliana* germination rate with substrate Se concentration.

RESULTS

Effect of Se hyperaccumulators on soil Se distribution

To investigate if Se hyperaccumulators change soil Se distribution we determined soil Se concentration at different distances from the Se hyperaccumulators *A. bisulcatus* and *S. pinnata* and the non-Se hyperaccumulators *M. sativa* and *H. pumilus* growing at Pine Ridge Natural Area. In addition, we

measured soil Se concentration at different depths next to the tap root of the same plants. Plant Se concentration in leaves and roots were also determined. *A. bisulcatus* and *S. pinnata* had higher concentrations of Se in leaves and roots than the non-hyperaccumulators, reaching levels exceeding 1,000 mg kg⁻¹ DW in both roots and shoots (Fig. 2A). Shoots and roots of the non-hyperaccumulator species all contained less than 120 mg Se kg⁻¹ (Fig. 2A). Sulfur (S), an element that is chemically similar to Se, was also present at higher levels in both Se hyperaccumulator species than in non-hyperaccumulators (Table 2A, B). Among the other nutrients tested, magnesium (Mg) and manganese (Mn) were present at higher levels, and copper (Cu) and iron (Fe) at lower levels, in leaves of hyperaccumulators compared to non-hyperaccumulators (Table 2).

Surface soil collected from beneath the canopy of hyperaccumulator species (0-20 cm) generally showed higher Se levels than soil from around non-accumulators (Fig. 2B). The most pronounced difference was found between soil collected 20 cm from *A. bisulcatus*, the species with the highest Se concentration, and soil collected 20 cm from *M. sativa*, a non-hyperaccumulator in the same family as *A. bisulcatus* (Fig. 2B). For *A. bisulcatus* the soil Se concentration next to the tap root was even higher below the soil surface. Soil collected right next to *A. bisulcatus* roots at 5 cm, 10 cm and 30 cm depth contained 71-103 mg Se kg⁻¹, which was significantly more Se than in soil collected next to any of the other plant species, and 3-5 fold higher than on the *A. bisulcatus* soil surface (Fig. 2C).

Effect of Se hyperaccumulation on the neighboring plant community

To determine if the plant communities around Se hyperaccumulators were different from those around non-hyperaccumulators on the same site we measured vegetative ground cover and species composition around Se hyperaccumulators *A. bisulcatus* and *S. pinnata* and non-hyperaccumulators *M. sativa* and *H. pumilus* at Pine Ridge Natural Area. The percentage ground cover was slightly lower around hyperaccumulators than non-accumulators (Fig. 3A). When single species were compared, only *S. pinnata* and *M. sativa* differed significantly, but when combined the two hyperaccumulators had significantly lower average surrounding vegetative cover than the two non-hyperaccumulators (Fig. 3A).

Average species richness was also slightly lower around the two hyperaccumulators compared to the non-accumulators, but not significantly (Fig. 4B). Table 3 lists the plant species found in the neighboring vegetation. There were no significant differences between the four species with respect to neighboring species composition, with the exception that *Bromus japonicus* (field brome) occurred more frequently around *H. pumilus* than around the other three species, and *Agropyron repens* (quackgrass) occurred more frequently around *A. bisulcatus* than *M. sativa* (Table 3). Other species that showed an interesting though non-significant trend were *Chenopodium berlandieri* which was found more frequently around hyperaccumulators than non-accumulators, and *Descurainia sp.* which was found less frequently around hyperaccumulators than non-accumulators.

Two species, *Artemisia ludoviciana* (white sagebrush) and *Symphyotrichum ericoides* (white heath aster), that were found around hyperaccumulator species and also away from hyperaccumulators throughout the site were further analyzed for any effect of the presence of the hyperaccumulator neighbor on leaf Se concentration. Their leaf Se levels were higher when the plants were growing next to a hyperaccumulator species (*A. bisulcatus* or *S. pinnata*) than when they were growing away from hyperaccumulators; the difference was 6-7 fold for *S. ericoides* and 2-6 fold for *A. ludoviciana* (Fig. 4). Interestingly, the Se concentration in *A. ludoviciana* actually reached hyperaccumulator level ($> 1,000 \text{ mg kg}^{-1} \text{ DW}$) when it was growing next to a hyperaccumulator species, but not when it was growing away from them. To our knowledge neither of these two species has been reported to be a hyperaccumulator before. Despite their elevated tissue Se levels, neither of the two neighboring species showed any signs of Se toxicity such as chlorosis or stunted growth (results not shown).

Effects of Se hyperaccumulators on germination and growth of Se-sensitive neighbors

In a first experiment toward determining whether Se hyperaccumulator plants may have a negative effect on Se-sensitive neighbors due to their apparent ability to concentrate Se in their surrounding soil, litter and soil were collected from around the two hyperaccumulators *A. bisulcatus* and *S. pinnata* as well as from two non-accumulator control species, *M. sativa* and *H. pumilus*, to be used in controlled growth

experiments. Before use, the litter and soil were analyzed for elemental composition, as were the corresponding leaves and seeds of the plants around which the litter and soil were collected.

The Se levels in leaves and seeds of *A. bisulcatus* and *S. pinnata* were higher than those in *M. sativa* and *H. pumilus*, as expected (Fig. 5A). Hyperaccumulators had a higher Se concentration in seeds compared to leaves, while non-hyperaccumulators did not show such a pattern. Similar to Se, tissue sulfur (S) levels were higher in seeds and leaves of hyperaccumulators than in non-accumulators (Fig. 5B). The hyperaccumulators had a higher S concentration in leaves compared to seeds, and thus showed an opposite partitioning for S compared to Se; the non-hyperaccumulators did not show a difference in S level between these organs. Leaf Se and S concentrations were not significantly correlated in hyperaccumulators, but were correlated in non-hyperaccumulators. Table 4 shows the concentrations of some other nutrients in the leaves and seeds. Leaf Mg and Mn levels were again higher in Se hyperaccumulators than in non-accumulators (Table 4A). As for seeds, Fe levels were 2-5 times lower in hyperaccumulators compared to non-accumulators (Table 4B).

Selenium was present at similar levels in fresh leaves and decaying plant litter (Fig. 6A, B), with hyperaccumulators -particularly *A. bisulcatus*- having higher Se concentrations than non-accumulators. The same trend was found for soil taken from 0-2 cm and 2-5 cm depth: soil from around hyperaccumulators, particularly *A. bisulcatus*, generally had a higher Se concentration than soil around non-hyperaccumulator species. As shown in Table 5, the hyperaccumulator species showed positive and highly significant correlations between their Se concentration in fresh leaves, leaf litter, and surrounding soil. For non-hyperaccumulator species the only significant correlation was found between leaf litter and soil at 2-5cm depth.

To test the potential negative effect of the elevated litter and soil Se levels around hyperaccumulator species on Se-sensitive plant species, the litter and soil take from around the two hyperaccumulators (*A. bisulcatus* and *S. pinnata*) and the two non-accumulators (*M. sativa* and *H. pumilus*) was used for a comparative germination and growth study using *Arabidopsis thaliana* accession Landsberg erecta (Ler). This accession was shown earlier to be particularly sensitive to selenate (Zhang *et al.*, 2006a,b). In a first

test, the soil from 0-2 cm depth was placed in petri dishes and covered with litter collected around the same plant. *A. thaliana* seeds were sown on this re-created topsoil-litter medium and the plates were cultivated in the lab. The germination rates of *A. thaliana* were significantly higher on soil and litter collected around non-hyperaccumulator species as compared to hyperaccumulator species (Fig. 7A). There was a significant negative relationship between average Se concentration in soil and litter and the germination rate of *A. thaliana* across the four species (Fig. 7B).

A second experiment was done to determine plant growth as well as Se accumulation in *A. thaliana* plants sown on soil collected from around hyperaccumulators or non-accumulators. The *A. thaliana* seeds were sown in pots on soil collected at 2-5 cm depth around each of the four species. Fewer *A. thaliana* seeds germinated when sown on soil from around hyperaccumulator species compared to non-hyperaccumulator soil (Fig. 8A); there was a negative correlation between soil Se concentration and *A. thaliana* germination (Fig. 8B). From visual observation the plants that germinated on soil from around hyperaccumulators appeared substantially smaller compared to those growing on soil collected around non-hyperaccumulators (Fig. 8C). While the average dry weight per plant was 2-fold lower for plants growing on *A. bisulcatus* soil and 30-fold lower for plants growing on *S. pinnata* soil as compared to non-accumulator soils (Fig. 8D), these averages were not significantly different due to the high standard deviation. Selenium concentrations were higher in *A. thaliana* growing on soil collected from around hyperaccumulator species *A. bisulcatus* compared to plants growing on soil taken next to non-hyperaccumulator species *M. sativa* and *H. pumilus* (Fig. 9A). The elemental concentrations could not be determined for *A. thaliana* growing on *S. pinnata* soil, since none of the sown plants survived beyond the seedling stage. The levels of S and to a lesser extent Mg and Mn were also elevated in *A. thaliana* growing on *A. bisulcatus* soil compared to soil taken next to non-hyperaccumulator species (Fig. 9).

To further investigate whether the Se concentration in the litter and soil used in these studies (100 - 2000 mg kg⁻¹ DW) was sufficient to inhibit *A. thaliana* germination, a controlled experiment was carried out in which seed germination was determined as a function of Se (selenate) concentration in agar medium. The germination rate decreased above a Se concentration of around 5 mg kg⁻¹ DW (Fig. 10). The 50%

germination inhibition point was around 10 mg kg⁻¹ DW (125 μM sodium selenate), an order of magnitude lower than the Se levels in the soil and litter collected around hyperaccumulators.

DISCUSSION

The main finding of this study is that soil Se levels around hyperaccumulators in the field are enriched with Se, resulting in enhanced Se accumulation in neighboring plants, to levels that are potentially phytotoxic. This may suggest a role for Se in elemental allelopathy: hyperaccumulators may use Se to keep Se-sensitive neighbors at a distance. This finding is of significance since it sheds new light on the functional significance of elemental hyperaccumulation. Thus, in addition to the previously reported benefits of elemental hyperaccumulation as a protectant against herbivores and pathogens, it may reduce competition with neighboring plants. All of these benefits may have contributed as selection pressures during the evolution of hyperaccumulation.

Soil and litter around Se hyperaccumulators was enriched in Se by on average 2-3 fold for soil and 6-7 fold for litter, as compared to soil around non-hyperaccumulators growing on the same site. The hyperaccumulator plants themselves contained on average 20-fold higher Se concentrations than the non-accumulators. The higher Se levels in the soil around hyperaccumulators may be due to phytoenrichment (deposition over time by the hyperaccumulator), or simply because soil Se distribution is heterogeneous and the hyperaccumulators are indicators of the high-Se patches. Although based on our sampling we cannot distinguish between the two, there is some circumstantial evidence that hyperaccumulator plants concentrate certain elements in their surrounding soil. The hyperaccumulators were shown to have higher tissue levels than non-accumulators of not only Se but also S, Mg and Mn, and in *A. thaliana* grown on soil collected around hyperaccumulators the levels of Se, S, Mn and Mg all tended to be elevated compared to *A. thaliana* growing on non-accumulator soil. Moreover, the decomposing hyperaccumulator litter collected on top of the surrounding soil was very high in Se (600-2000 mg kg⁻¹ DW), and its decomposition is likely to enrich the soil underneath over time, as was found recently in a litter decomposition study (Quinn

et al., 2010). Both *S. pinnata* and *A. bisulcatus* are perennial species, so the level of phytoenrichment may increase with time.

If hyperaccumulator plants indeed phytoenrich their surrounding soil with Se, this may be due not only to litter deposition but also root turnover and exudation. In each of these processes the Se deposition likely is in the form of organic Se, since hyperaccumulators accumulate Se as methyl-selenocysteine (Freeman *et al.*, 2006a, 2010). Based on earlier uptake studies with different Se species (Zayed *et al.*, 1998), methyl-SeCys likely is more readily taken up by neighboring plants than selenate, the predominant form of bioavailable Se in bulk (oxic) soil. In addition to enriching total Se in their surrounding soil via deposition of litter and root-released compounds, it is feasible that hyperaccumulators mobilize non-labile pools of soil Se via special exudates, further increasing the level of bioavailable Se for neighboring plants. In this respect it will be interesting for future studies to compare total and bioavailable Se levels in rhizosphere soil of hyperaccumulators and bulk soil, and the forms of Se in neighboring plants with those in the same species growing far away from hyperaccumulators.

If hyperaccumulators affect the Se concentration and/or form of Se in their surrounding soil, and consequently in their neighbors, this may have a positive or negative effect on those neighbors. Higher or lower Se levels may affect plant physiology as well as the plant's ecological interactions. Our findings indicate that in the field there may be some negative effects on neighboring species because the percentage ground cover was slightly lower around hyperaccumulators. The lower percentage ground cover around hyperaccumulators may mean reduced competition for hyperaccumulators as well as for Se-tolerant neighboring species, and selection against Se-sensitive neighbors. As a model Se-sensitive plant, the *A. thaliana* accession Ler indeed showed significant reduction in germination and growth on soil collected next to hyperaccumulators. In the field we did not see any evidence of toxicity such as chlorosis or stunted growth, even though the two plant species tested showed 4-7 fold higher Se levels when growing next to hyperaccumulators compared to when growing away from hyperaccumulators in the field. Since the neighboring species composition in the field was different around hyperaccumulators and nonaccumulators, and some neighboring species appeared to thrive next to hyperaccumulators it will be interesting for future

studies to study the individual responses of different neighboring species. Particularly interesting for further studies are the potential beneficial ecological effects of Se accumulation in neighbors, such as protection from herbivores or pathogens.

It cannot be excluded that a factor other than Se was responsible for the apparent toxicity of the soil and litter collected around hyperaccumulator plants. However, based on the agar experiment the concentrations found in soil and litter around hyperaccumulators is high enough to impair *A. thaliana* seed germination for this accession. Even if only 10% of the Se in the litter and soil would be bioavailable, this would still be expected to affect germination. Also, the toxic effect was found for soil collected from two hyperaccumulator species from different families, and not for soil collected from two non-accumulators from two different families, making it less likely that the two hyperaccumulators both exuded a toxic compound other than Se while the other two species did not. Also, the levels of Se in plants growing on hyperaccumulator soil was elevated, both in the field and in the pot experiment, making it more feasible that Se was indeed the toxic compound.

Hyperaccumulators showed preferential Se accumulation in seeds versus leaves, which was opposite to their S partitioning pattern. Non-hyperaccumulators, on the other hand, showed similar Se and S partitioning patterns, and accumulated both elements to similar levels in leaves and seeds. This may suggest hyperaccumulators have different transporters for Se and S that are regulated differently, while non-accumulators cannot distinguish between Se and S. Furthermore, hyperaccumulators generally had higher S levels in roots, leaves and seeds than non-accumulators, suggesting they have upregulated levels of S uptake and translocation. This was indeed recently found in a transcriptomic and biochemical study comparing hyperaccumulator *S. pinnata* with non-hyperaccumulator *Stanleya albescens* (Freeman *et al.*, 2010). Another interesting finding was that hyperaccumulators tended to have higher levels of Mn and Mg. At this point we do not have an explanation for this phenomenon, but it is feasible that Mn and Mg uptake or translocations are facilitated by elevated plant S levels.

This study is the first to provide insight into the effects of Se hyperaccumulators on soil Se distribution and plant-plant ecological interactions. This information is interesting not only from a

fundamental scientific perspective, but also because Se is both a micronutrient and an environmental pollutant, and plants are increasingly used both as Se-fortified foods and for phytoremediation of excess Se (Banuelos and Bradley, 2010). Better insight into the effects of hyperaccumulator plants on Se accumulation and speciation in their neighbors may be useful for the further development of these applications. For instance, if Se hyperaccumulators enhance Se accumulation in neighboring crop species and perhaps also facilitate accumulation of more organic, anticarcinogenic Se in these neighbors, this would be very applicable for the development of efficient co-cropping practices.

Table 2: Leaf (A) and root(B) tissue concentration (mg kg⁻¹) of various nutrients in hyperaccumulator (*A. bisulcatus* and *S. pinnata*) and non-hyperaccumulator (*H. pumilus* and *M. sativa*) species growing in the same seleniferous habitat. Shown are means ± standard error. Superscript letters denote statistically different means (ANOVA, α = 0.05)

A. leaf				
	<i>A. bisulcatus</i>	<i>S. pinnata</i>	<i>M. sativa</i>	<i>H. pumilus</i>
Cu	1.7 ± 0.3 ^a	1.9 ± 0.1 ^{ac}	5.1 ± 0.5 ^b	4.0 ± 0.4 ^c
Fe	50 ± 2 ^{ab}	44 ± 4 ^a	62 ± 2 ^b	76 ± 8 ^c
Mg	1997 ± 107 ^a	2017 ± 126 ^a	1062 ± 33 ^b	1679 ± 106 ^c
Mn	23 ± 2 ^a	22 ± 3 ^a	15 ± 2 ^b	10 ± 1 ^b
S	9781 ± 775 ^a	7756 ± 816 ^b	2132 ± 816 ^c	2876 ± 461 ^c
B. root				
	<i>A. bisulcatus</i>	<i>S. pinnata</i>	<i>M. sativa</i>	<i>H. pumilus</i>
Cu	2.9 ± 0.4 ^a	2.0 ± 0.1 ^a	3.6 ± 0.4 ^{ab}	4.8 ± 0.9 ^b
Fe	393 ± 102	502 ± 90	393 ± 127	825 ± 614
Mg	631 ± 101 ^a	921 ± 80 ^{bc}	680 ± 63 ^{ac}	899 ± 97 ^{bc}
Mn	14 ± 3	9 ± 1	12 ± 3	21 ± 12
S	1877 ± 451 ^a	6569 ± 917 ^b	777 ± 91 ^a	816 ± 87 ^a

Table 3: Plant species surrounding Se hyperaccumulating and non-Se hyperaccumulating plants.

Values show average number of plants per m² ± standard error.

Family	Common name	Species	Se hyperaccumulators		Non-hyperaccumulators	
			<i>Astragalus bisulcatus</i>	<i>Stanleya pinnata</i>	<i>Medicago sativa</i>	<i>Helianthus pumilus</i>
Poaceae	Quackgrass	<i>Agropyron repens</i>	90.0 ± 18.6 ^a	52.1 ± 12.5 ^{ab}	32.8 ± 17.8 ^b	48.6 ± 15.8 ^{ab}
Liliaceae	textile onion	<i>Allium textile</i>	1.4 ± 1.4	0	0	0
Brassicaceae	Alyssum	<i>Alyssum minus</i>	0.7 ± 0.7	0	2.1 ± 1.5	0
Asteraceae	white sagebrush	<i>Artemisia ludoviciana</i>	8.6 ± 7.0	2.1 ± 1.5	3.6 ± 3.6	8.6 ± 5.0
Poaceae	field brome	<i>Bromus japonicus</i>	11.4 ± 4.5 ^a	24.3 ± 6.7 ^a	20.0 ± 5.5 ^a	56.4 ± 12.2 ^b
Chenopodiaceae	pitseed goosefoot	<i>Chenopodium berlandieri</i>	3.6 ± 2.4	7.1 ± 7.1	0	1.4 ± 1.4
Santalaceae	bastard toadflax	<i>Comandra umbellata</i>	0	0	2.9 ± 2.9	0
Brassicaceae	mustard sp	<i>Descurainia sp</i>	0	1.4 ± 1.4	10.0 ± 5.0	7.1 ± 2.6
Poaceae	slender wheatgrass	<i>Elymus trachycaulus</i>	4.3 ± 3.3	2.9 ± 2.9	0	4.3 ± 2.0
Poaceae	Indian ricegrass	<i>Oryzopsis hymenoides</i>	3.6 ± 2.1	3.6 ± 3.6	2.8 ± 2.1	0
Brassicaceae	tall tumbled mustard	<i>Sisymbrium altissimum</i>	0	0	0	0.7 ± 0.7
Poaceae	needle and thread grass	<i>Stipa comata</i>	2.1 ± 2.1	6.4 ± 3.7	6.4 ± 3.6	14.3 ± 4.7
Poaceae	green needlegrass	<i>Stipa viridula</i>	0.7 ± 0.7	2.9 ± 2.1	0	1.4 ± 0.9
Asteraceae	yellow salsify	<i>Tragopogon dubius</i>	0.7 ± 0.7	2.1 ± 1.5	3.6 ± 0.9	1.4 ± 0.9

Table 4: Leaf (A) and seed (B) tissue concentrations (mg kg⁻¹DW) of various plant nutrients in hyperaccumulator (*A. bisulcatus* and *S. pinnata*) and non-hyperaccumulator (*H. pumilus* and *M. sativa*) species growing side by side in seleniferous habitat (Fort Collins, CO). Shown are mean and standard error of the mean. Superscript letters denote statistically different means (ANOVA, $\alpha = 0.05$)

A. leaf				
	<i>A. bisulcatus</i>	<i>S. pinnata</i>	<i>M. sativa</i>	<i>H. pumilus</i>
Cu	1.5 ± 0.27 ^a	2.6 ± 0.22 ^a	6.7 ± 4.9 ^b	0.9 ± 0.25 ^a
Fe	26.3 ± 3.2 ^{ab}	21.9 ± 3.3 ^b	40.3 ± 10.2 ^a	11.2 ± 6.2 ^b
Mg	1670 ± 213 ^a	1329 ± 177 ^a	520 ± 124 ^b	166 ± 23 ^b
Mn	20.1 ± 2.9 ^a	10.3 ± 1.7 ^b	4.5 ± 1.7 ^{bc}	0.6 ± 0.13 ^c
Mo	0.4 ± 0.2 ^a	2.7 ± 0.7 ^b	0.8 ± 0.2 ^a	3.1 ± 0.7 ^b
Zn	0.7 ± 0.15 ^a	3.9 ± 1.1 ^{ab}	7.9 ± 2.5 ^b	5.2 ± 1.6 ^b

B. Seed				
	<i>A. bisulcatus</i>	<i>S. pinnata</i>	<i>M. sativa</i>	<i>H. pumilus</i>
Cu	1.5 ± 0.27 ^a	0.7 ± 0.13 ^a	1.6 ± 0.42 ^a	1.6 ± 0.37 ^a
Fe	14.6 ± 3.5 ^a	12.4 ± 2.7 ^a	61.4 ± 14.2 ^b	23.9 ± 4.7 ^a
Mg	460 ± 100 ^a	220 ± 95 ^a	332 ± 22 ^a	382 ± 89 ^a
Mn	5.2 ± 1.6 ^a	2.6 ± 1.8 ^a	2.2 ± 0.2 ^a	2.5 ± 0.9 ^a
Mo	0.9 ± 0.2 ^a	0.7 ± 0.2 ^a	0.8 ± 0.2 ^a	0.9 ± 0.3 ^a
Zn	7.4 ± 1.9 ^a	2.2 ± 0.4 ^b	8.7 ± 0.9 ^a	5.3 ± 2.2 ^{ab}

Table 5: Correlation coefficient (R) and statistical significance (P) of a correlation analysis between Se concentration in leaf, litter, soil 0-2 cm and soil 2-5 cm depth. Statistically significant correlations ($P < 0.05$) are shown in bold. All correlations are positive.

	Leaf	Litter	Soil 0-2cm
Hyperaccumulator species (<i>A. bisulcatus</i> & <i>S. pinnata</i>)			
Litter	R=0.61/ P<0.01		
Soil 0-2cm	R=0.39/ P<0.01	R=0.64/ P<0.01	
Soil 2-5cm	R=0.56/ P<0.01	R=0.39/ P<0.01	R=0.20/ P=0.39
Non-hyperaccumulator species (<i>M. sativa</i> & <i>H. pumilus</i>)			
Litter	R=0.11/ P=0.76		
Soil 0-2cm	R=0.50/ P=0.14	R=0.05/ P=0.99	
Soil 2-5cm	R=0.07/ P=0.87	R=0.60/ P<0.01	R=0.39/ P=0.86
All 4 species			
Litter	R=0.70/ P<0.0001		
Soil 0-2cm	R=0.48/ P<0.01	R=0.66/ P<0.0001	
Soil 2-5cm	R=0.65/ P<0.0001	R=0.50/ P<0.0001	R=0.31/ P=0.09

FIGURES

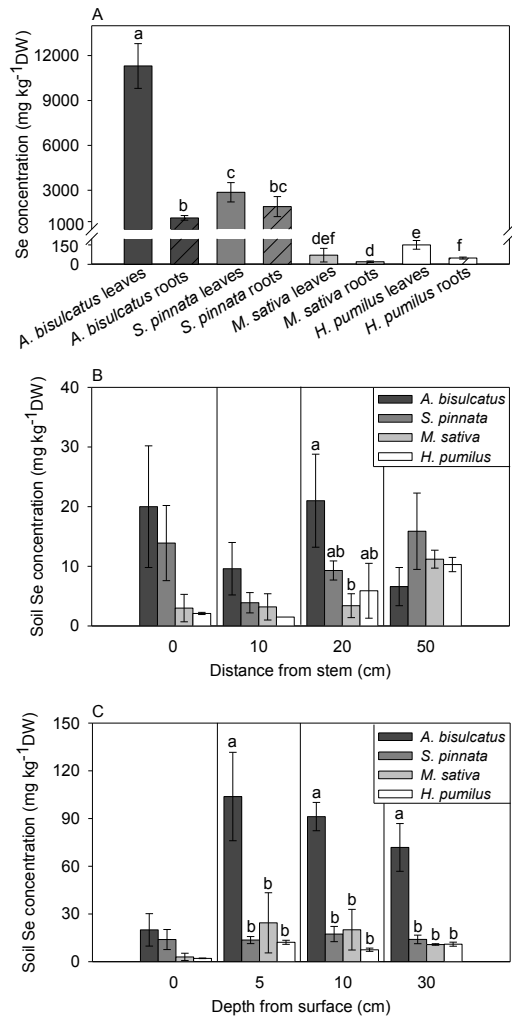


Figure 2: (A) Selenium concentration in roots and leaves in Se hyperaccumulators (*A. bisulcatus* and *S. pinnata*) and non-hyperaccumulators (*M. sativa* and *H. pumilus*). (B) Soil Se concentrations from the top 2 cm of soil at different distances from the base of Se hyperaccumulators and non-hyperaccumulators and (C) soil Se concentration from rhizospheric soil (0-3 cm from root) at different depths next to hyperaccumulator and non-hyperaccumulator roots. Values are means +/- SE; different letters above bars represent a significant difference ($\alpha = 0.05$).

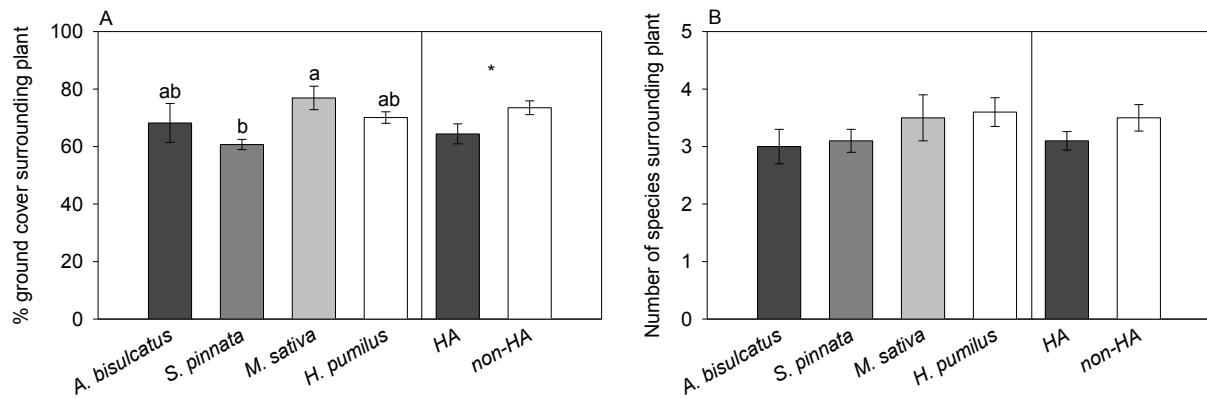


Figure 2

Figure 3: (A) Percentage ground cover and (B) number of species around Se hyperaccumulators (HA, *A. bisulcatus* and *S. pinnata*) and non-hyperaccumulators (non-HA, *M. sativa* and *H. pumilus*). Values are means \pm SE; different letters above bars or an asterisk between bars represents a significant difference ($\alpha = 0.05$).

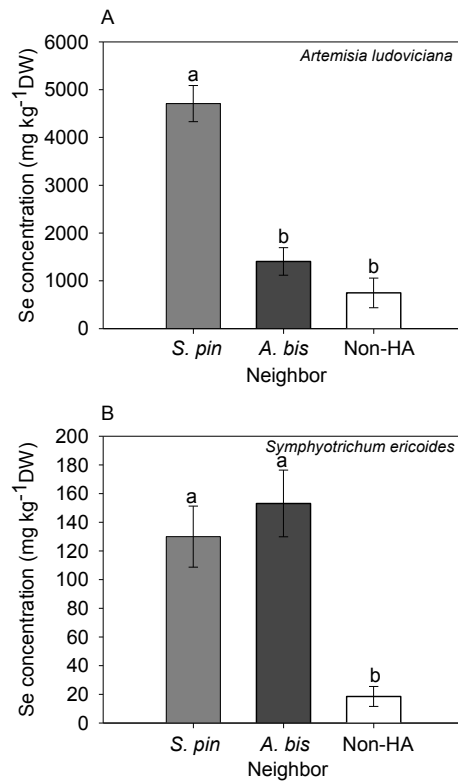


Figure 4: Leaf Se levels in the plant species *Artemisia ludoviciana* and *Symphyotrichum ericoides* when growing next to hyperaccumulator species *A. bisulcatus* or *S. pinnata* or >4 m away from hyperaccumulators.

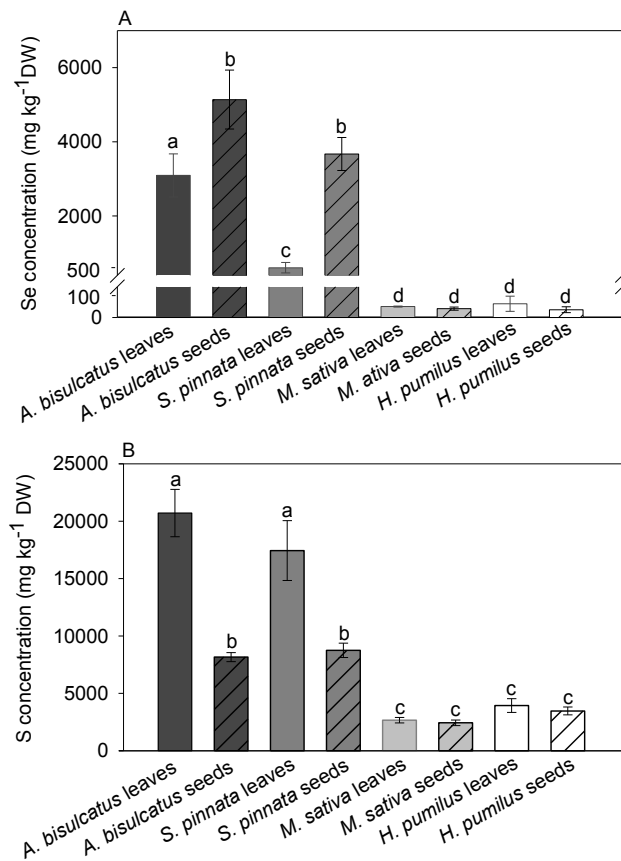


Figure 5: Selenium (A) and S (B) levels in leaves and seeds of Se hyperaccumulator species *A. bisulcatus* and *S. pinnata* and non-hyperaccumulator species *M. sativa* and *H. pumilus* plants growing in the field at Pine Ridge Natural Area. Leaf litter and soil taken from these same plants were used for subsequent experiments.

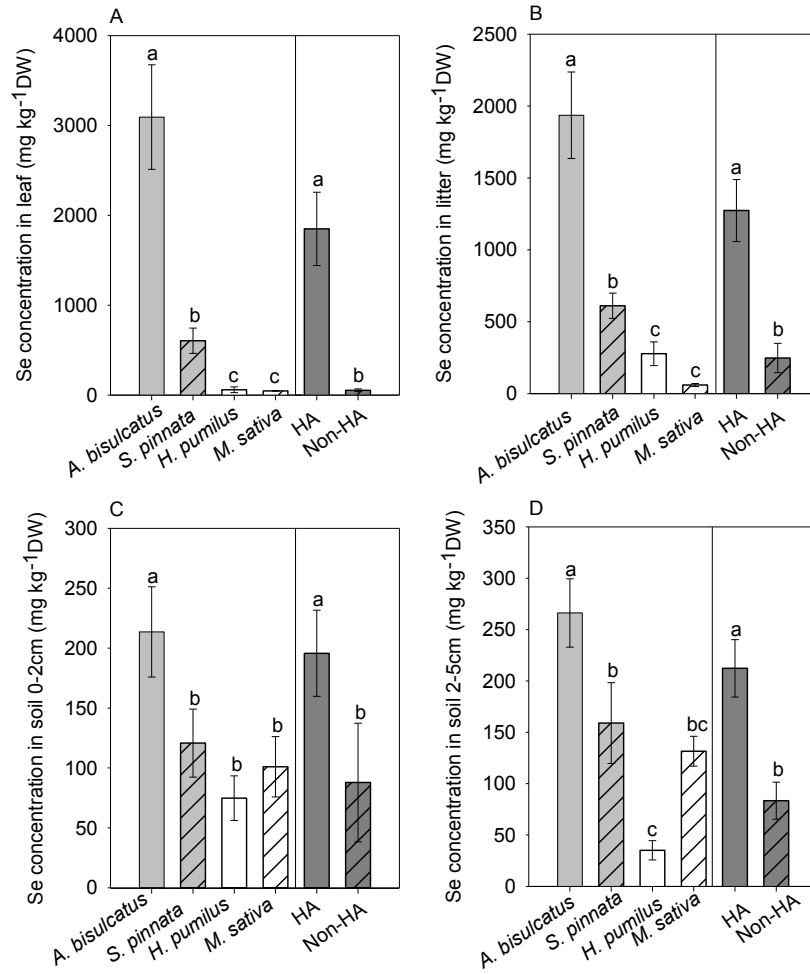


Figure 6: Selenium levels in leaf (A), litter (B), soil from 0-2cm depth (C) and soil from 2-5cm depth (D) around hyperaccumulator species *A. bisulcatus* and *S. pinnata* and non-hyperaccumulator species *M. sativa* and *H. pumilus* growing in the field at Pine Ridge Natural Area.

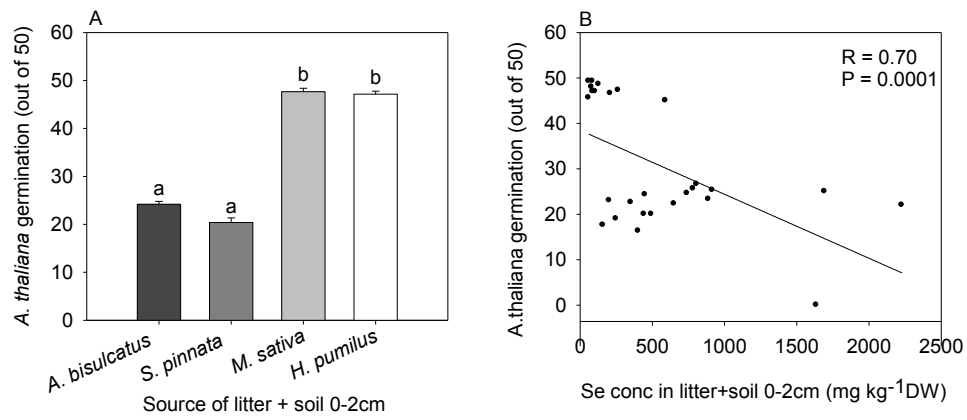


Figure 7: (A) Germination rate of *A. thaliana* seeds on litter + soil collected around hyperaccumulator species *A. bisulcatus* and *S. pinnata* compared to material collected around non-hyperaccumulator species *M. sativa* and *H. pumilus*. (B) Correlation between Se concentration in litter + soil and the germination rate of *A. thaliana*.

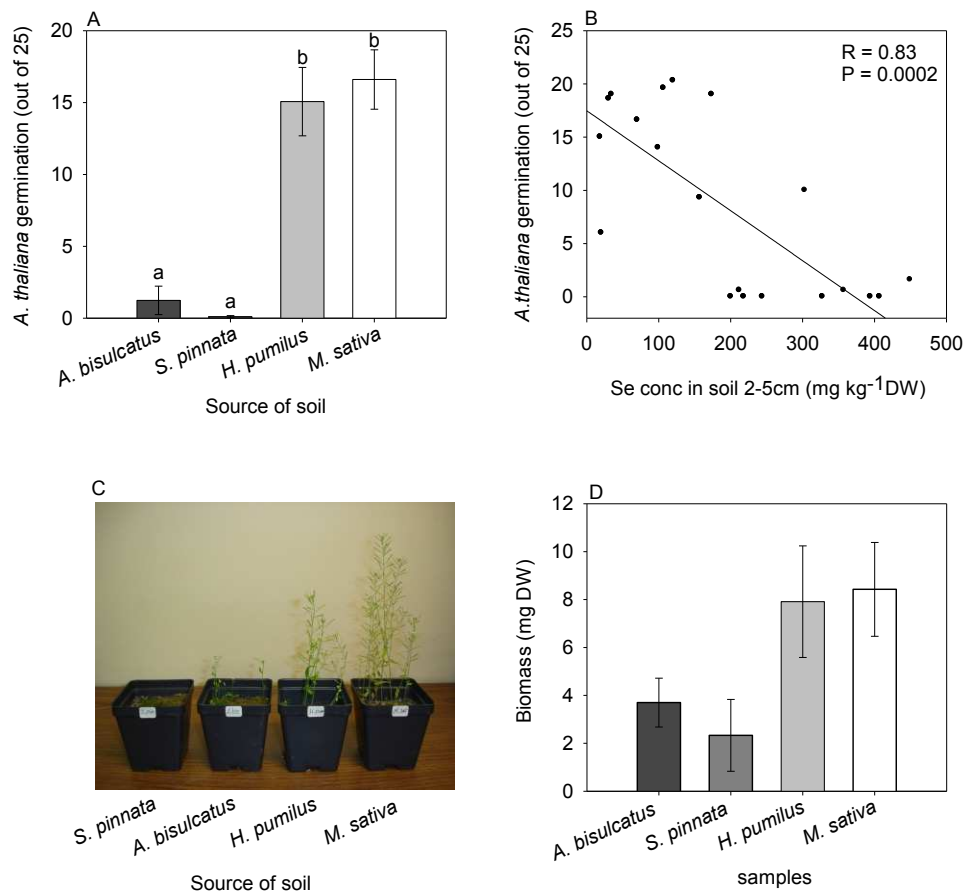


Figure 8: (A) Germination of *A. thaliana* on soil taken at 2-5cm depth around hyperaccumulator species *A. bisulcatus* and *S. pinnata* compared to non-hyperaccumulator species *M. sativa* and *H. pumilus*. (B) Correlation between Se concentration in soil and the germination rate of *A. thaliana*. (C) *A. thaliana* plants growing on soils collected around (from left to right) *S. pinnata*, *A. bisulcatus*, *H. pumilus* and *M. sativa*. (D) Dry weight per *A. thaliana* plant when grown on soil collected from *S. pinnata*, *A. bisulcatus*, *H. pumilus* and *M. sativa*.

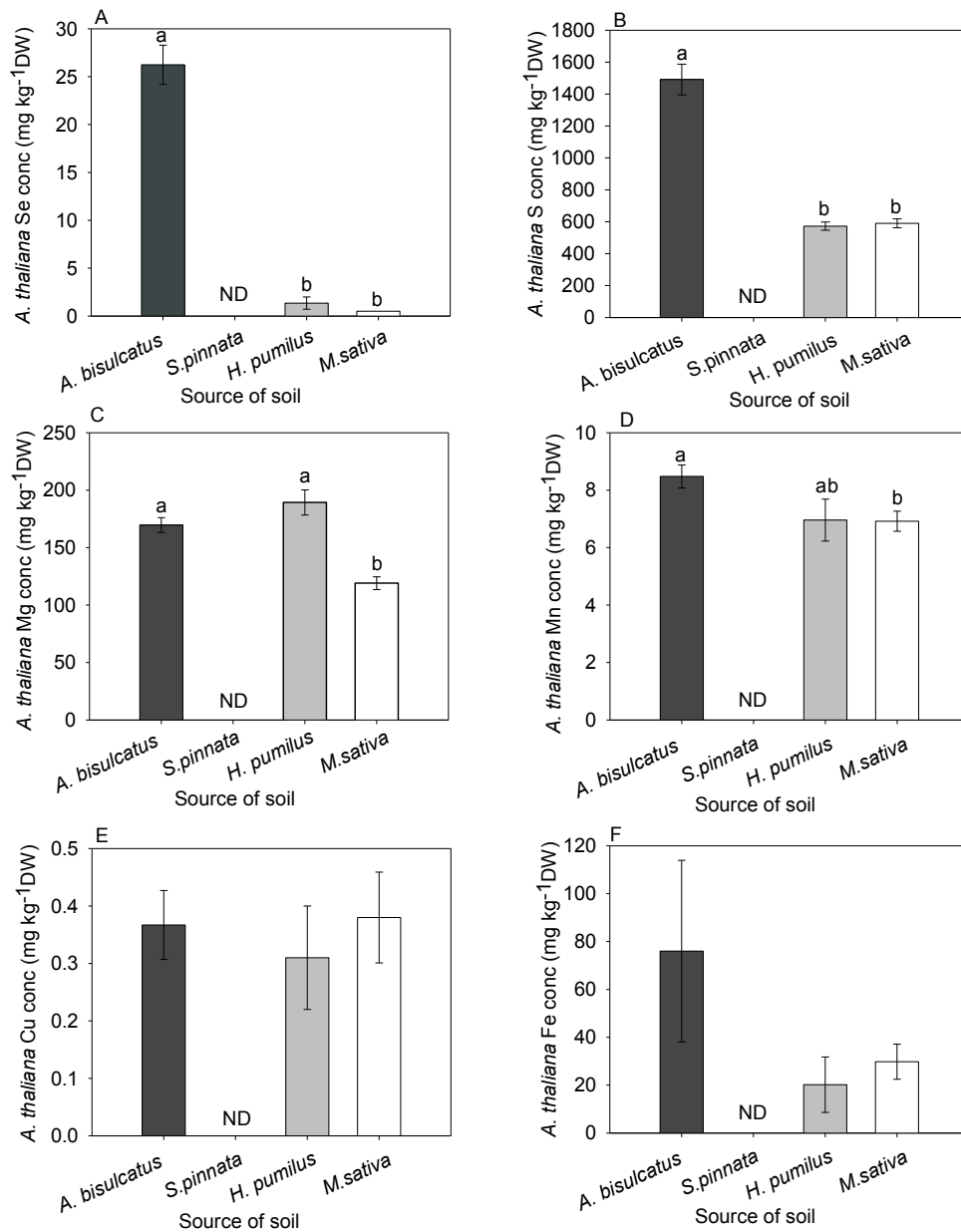


Figure 9: Leaf elemental concentrations (A-F: Se, S, Mg, Mn, Cu, Fe) in *A. thaliana* grown on soil from around hyperaccumulator *A. bisulcatus* or non-hyperaccumulator species *M. sativa* and *H. pumilus*. ND: not determined since not enough biomass could be collected for *A. thaliana* germinated on soil collected around *S. pinnata*.

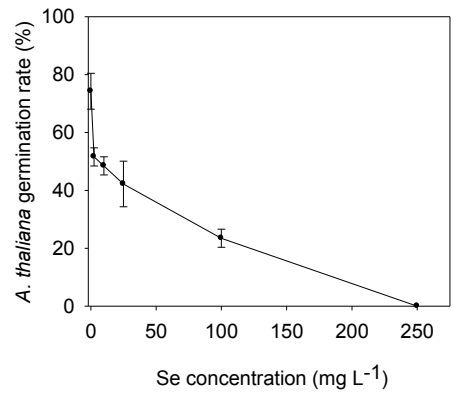


Figure 10: Germination rate of *A. thaliana* seeds as a function of Se concentration when sown on half-strength Murashige and Skoog (MS) medium supplemented with sodium selenate.

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CHAPTER 3:

SELENIUM HYPERACCUMULATORS FACILITATE SELENIUM-TOLERANT NEIGHBORS VIA PHYTOENRICHMENT AND REDUCED HERBIVORY³:

Soil surrounding selenium (Se) hyperaccumulator plants was shown earlier to be enriched in Se, impairing the growth of Se-sensitive plant species. Since Se levels in neighbors of hyperaccumulators were higher, and Se has been shown to protect plants from herbivory, we investigate here the potential facilitating effect of Se hyperaccumulators on Se-tolerant neighboring species in the field.

We measured growth and herbivory of *Artemisia ludoviciana* and *Symphytichum ericoides* as a function of their Se concentration and proximity to hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata*. When growing next to hyperaccumulators, *A. ludoviciana* and *S. ericoides* contained 10-20 fold higher Se levels (800-2,000 mg kg⁻¹ DW) than when growing next to non-accumulators. The roots of both species were predominantly (70-90%) directed toward hyperaccumulator neighbors, not toward other neighbors. Moreover, neighbors of hyperaccumulators were 2-fold bigger, showed 2-fold less herbivory damage and harbored 3-4 fold fewer arthropods. When used in laboratory choice and non-choice grasshopper herbivory experiments, Se-rich neighbors of hyperaccumulators experienced less herbivory and caused higher grasshopper Se accumulation (10-fold) and mortality (4-fold).

Enhanced soil Se levels around hyperaccumulators can facilitate growth of Se-tolerant plant species through reduced herbivory and enhanced growth.

This study is the first to show facilitation via enrichment with a non-essential element. It is interesting that and in facilitation when neighbors are Se-tolerant. Via these competitive and facilitating effects, Se hyperaccumulators may affect plant species composition and, consequently, higher trophic levels.

³El-Mehdawi AF, Quinn CF, Pilon-Smits EAH (2011) *Current Biology* 21: 1440-1449.

The data for Figures 17-19 were collected together with co-author Colin Quinn.

Se enrichment of neighbors by hyperaccumulators can result in competition when neighbors are Se sensitive

and in facilitation when neighbors are Se-tolerant. Via these competitive and facilitating effects, Se hyperaccumulators may affect plant species composition and, consequently, higher trophic levels.

INTRODUCTION

The element selenium (Se) is a trace element for many animals as a component of selenoproteins, which are redox-active and have a variety of essential functions (Zhang and Gladyshev, 2009). Although Se has not been shown to be essential for higher plants it is a beneficial nutrient for many species (Pilon-Smits *et al.*, 2009). Most plants take up selenate inadvertently because of its similarity to sulfate, and readily metabolize it via the sulfur (S) assimilation pathway (Terry *et al.*, 2000). At higher levels Se becomes toxic due to its chemical similarity to S. Non-specific replacement of cysteine by selenocysteine (SeCys) in proteins disrupts protein function, leading to toxicity and death (Stadtman 1996).

While Se is present at low levels in most soils, it is particularly abundant in seleniferous soils such as Cretaceous shale, which typically contains 1-10 mg Se kg⁻¹ and may reach 100 mg Se kg⁻¹ (Beath *et al.*, 1939). Some plants native to such seleniferous soils hyperaccumulate Se to levels >1,000 mg kg⁻¹ dry weight (DW), and can even reach levels up to 15,000 mg kg⁻¹ DW (1.5%) (Galeas *et al.*, 2007). While most plants cannot distinguish between Se and S, hyperaccumulators preferentially take up Se over S, and store Se in all plant parts. Most of the Se in hyperaccumulators is stored in the form of methyl-SeCys. This amino acid does not get incorporated into protein and can therefore be safely accumulated, explaining the extreme Se tolerance of hyperaccumulators (Neuhierl & Böck, 1996). Non-hyperaccumulator plants store more toxic forms of Se such as inorganic selenate (de Souza *et al.*, 1998; Zayed *et al.*, 1998; Van Hoewyk *et al.*, 2005).

The functional significance of Se hyperaccumulation has been a topic of recent study. Selenium accumulation has been shown to protect plants from a wide variety of herbivores, including vertebrates and invertebrates with different feeding modes (Hanson *et al.*, 2003, 2004; Freeman *et al.*, 2007, 2009; Quinn *et al.*, 2008, 2010). This protection was based on both deterrence and toxicity. Selenium-based deterrence might be due to the highly odoriferous forms of volatile Se that are emitted by Se-rich plants (Terry *et al.*,

2000); additionally, it is possible that Se-rich plant material has an unattractive taste. Besides Se, other hyperaccumulated elements (As, Cd, Ni, Zn) have been shown to protect plants from herbivory (Boyd 2010). In addition to protecting plants from herbivores, Se accumulation has been shown to reduce infection by two pathogenic fungi (Van Hoewyk *et al.*, 2005).

More recently, evidence was found that Se hyperaccumulators phytoenrich their surrounding soil with Se, and that this may serve as a form of elemental allelopathy against Se-sensitive neighboring plants (El Mehdawi *et al.*, 2011a). Soil collected around Se hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* was toxic to the Se-sensitive species *Arabidopsis thaliana* and resulted in enhanced Se accumulation as compared to soil collected around non-hyperaccumulators in the same seleniferous area. The Se concentration in hyperaccumulator soil appeared to be high enough to be responsible for the observed toxicity, as judged from agar experiments with similar Se concentrations. In the field, neighboring plants of hyperaccumulators also showed enhanced Se levels (El Mehdawi *et al.*, 2011a). Although the degree of ground cover was slightly lower around hyperaccumulator species in the field (El Mehdawi *et al.*, 2011a), there was no apparent toxicity in neighboring plants of hyperaccumulators, in contrast to the laboratory experiments using *A. thaliana*. This observation prompted us to further study two neighboring species that contained particularly elevated Se levels without ill effects: *Artemisia ludoviciana* and *Symphyotrichum ericoides*. Since enhanced plant Se accumulation has been shown to provide ecological benefits, particularly protection from herbivores, we hypothesized that the enhanced Se levels in Se-tolerant neighbors of hyperaccumulators may be facilitative for these species.

In facilitation, benefactors (also called nurse plants) can benefit neighboring plants (beneficiaries) in several ways. Direct facilitative effects may involve giving protection from sun, wind, extreme temperatures or herbivores, better access to nutrients, or protection from toxins. Facilitated nutrient access may be achieved via nutrient pumping, N₂ fixation, or excretion of metal chelators. Indirect facilitative effects may involve a negative effect of the benefactor on competitors or herbivores of the beneficiary (Callaway 1995; Brooker *et al.*, 2008). Facilitation is especially important during the most sensitive seedling stage of the beneficiary. It is most prevalent in areas where the beneficiary is at the edge of its

geographical range, and more generally in harsh environments with respect to water supply, temperature, exposure, soil quality and herbivory pressure. Under such adverse conditions, competition is thought to become a less important and facilitation a more important ecosystem process (Brooker *et al.*, 1998). The benefactor plant may experience the relationship with its beneficiary as neutral (commensalism), or may experience competition, particularly when the beneficiary increases in size.

So far there is little information on the possible effects of plant elemental accumulation on plant-plant interactions, particularly with respect to facilitation. In co-cropping experiments on metal-polluted soil, in the context of phytoremediation, metal accumulating species were found to facilitate the growth of less metal-tolerant neighbors by removing the toxic metal. This was found for *Salix caprea*, which improved the growth of *Carex flava* (Koelbener *et al.*, 2008), and for the Zn/Cd hyperaccumulator *Sedum alfredii* which improved the growth of *Zea mays* (Wu *et al.*, 2007). In this study we investigated the facilitative effects of two Se hyperaccumulators, *A. bisulcatus* and *S. pinnata*, on the neighboring species *A. ludoviciana* and *S. ericoides*, all growing in their natural seleniferous habitat. We measured the neighbors' Se concentration and size, as well as their susceptibility to herbivory, as a function of their proximity to hyperaccumulators.

RESULTS

Se hyperaccumulators *A. bisulcatus* and *S. pinnata* positively affect growth and Se accumulation in neighbors

In their natural seleniferous habitat the species *A. ludoviciana* and *S. ericoides* were 2-3 fold taller and had more leaves when growing next to the hyperaccumulators *A. bisulcatus* and *S. pinnata* than when growing next to non-hyperaccumulators (Fig. 11). There was also a pronounced difference in leaf Se concentration in *A. ludoviciana* and *S. ericoides* plants depending on their proximity to hyperaccumulators: leaf Se levels were 10-20 fold higher when they were growing next to hyperaccumulators as compared to when they were growing away from them (Fig. 12A,B). As a result, overall Se accumulation per plant (concentration x biomass) was 20-40 fold higher for *A. ludoviciana* and *S. ericoides* growing next to

hyperaccumulators. Since Se hyperaccumulators are known to contain not only higher Se levels but also higher S levels than other vegetation on seleniferous soils (Galeas *et al.*, 2007), we also compared the S levels of the *A. ludoviciana* and *S. ericoides* plants under study. Leaf S levels in *A. ludoviciana* were significantly higher (by 40-50%) when growing next to hyperaccumulators (Fig. 12C); in *S. ericoides* the S level was also somewhat elevated (25%) when growing next to *A. bisulcatus*, but not next to *S. pinnata* (Fig. 12D). The soil Se levels around *A. bisulcatus* and *S. pinnata* were 7-13 fold higher compared to those in soil collected around non-hyperaccumulators (Fig. 12E). The soil S levels were 3-5 fold higher around the hyperaccumulators than around non-accumulators (Fig. 12F), but this difference was only significant for *A. bisulcatus*.

A. ludoviciana and *S. ericoides* roots grow toward hyperaccumulator neighbors

The finding that *A. ludoviciana* and *S. ericoides* appear to benefit from their elevated Se levels when growing next to hyperaccumulators in terms of above-ground biomass led us to investigate below-ground root-root interactions. The taproots of both *A. ludoviciana* and *S. ericoides* were directed predominantly (70-90%) toward their hyperaccumulator neighbor when growing next to *A. bisulcatus* (Fig. 13A, B) or *S. pinnata* (Fig. 13C, D). In contrast, roots of both species did not grow in any particular horizontal direction when the plants were situated next to the non-accumulator legume *Medicago sativa* (Fig. 13E, F). While the angle of root growth was horizontal in almost all cases when the plants were growing next to a hyperaccumulator, it was vertical in about a third of the plants growing next to *M. sativa*.

High-Se neighbors of hyperaccumulators are protected from herbivory

Since Se accumulation has been found to protect other plant species from herbivory, the number of herbivores and degree of herbivore damage were surveyed on the high- and low-Se *A. ludoviciana* and *S. ericoides* plants growing next to or far away from hyperaccumulators. The number of arthropods per plant was 3-4 fold lower on plants growing next to hyperaccumulators than on plants growing away from hyperaccumulators (Fig. 14A, B). Similarly, the number of damaged leaves per plant was ~2-fold lower

for plants growing next to hyperaccumulators (Fig. 14A, D). This difference is all the more impressive considering the fact that the plants growing next to hyperaccumulators were twice as big (Fig. 11).

S. ericoides plants growing next to hyperaccumulators had two distinct leaf types, prompting us to further investigate herbivory and Se concentration in the small versus big leaves. Even though the plants had many more small leaves than big leaves (~17-fold, Fig. 15A), herbivory damage was 3-fold more prevalent on big than small leaves (Fig. 15B). Overall, ~75% of the big leaves showed herbivory, versus only 2.5% of the small leaves (Fig. 15C). Interestingly, the Se concentration was 10-25 fold higher in the small leaves than in the big leaves (Fig. 15D).

To compare herbivory on *A. ludoviciana* and *S. ericoides* as a function of their proximity to hyperaccumulators under more controlled conditions, plants from both species were collected next to *A. bisulcatus* or away from hyperaccumulators, transferred to pots and taken to the laboratory. Grasshoppers were also collected, from the same field site. Before being offered to grasshoppers in choice and non-choice experiments, the plants were characterized in terms of height, number of leaves and Se concentration. Similar to our earlier survey, plants of both species were taller, had more leaves and a higher Se concentration (16-22 fold) when growing next to the hyperaccumulator than when growing next to non-hyperaccumulators (Fig. 16).

In the choice experiment, the grasshoppers preferentially targeted the low-Se plants collected next to non-hyperaccumulators rather than high-Se plants of the same species collected next to hyperaccumulators (Fig. 17A-C). *Symphytichum ericoides* showed significantly less stem height loss and less leaf loss for the high-Se plants. For *A. ludoviciana* this preference was only significant for leaf loss but not for stem height loss, but we noticed that its stems were in several cases clipped by the grasshoppers but the clippings left uneaten. Despite the apparent avoidance of high-Se plants by the grasshoppers, their mortality over the course of the experiment was substantial: only 5-20% survived (Fig. 17D, E). This mortality may have been due to grasshopper Se accumulation, since the animals that had fed on *A. ludoviciana* and *S. ericoides* for 6 days contained 10- and 20-fold higher Se levels, respectively, than

grasshoppers from the field (Fig. 17F). The grasshoppers that fed on *S. ericoides* reached higher tissue Se levels, showed lower survival and died more rapidly than those that fed on *A. ludoviciana* (Fig. 17D-F).

In the non-choice experiment, the high-Se *A. ludoviciana* and *S. ericoides* plants originating from around hyperaccumulators lost less stem height and fewer leaves than their low-Se counterparts collected next to non-hyperaccumulators (Fig. 18A-C, Fig. 19). The animals that had fed on high-Se plants showed 20% survival after 6 days, while the animals that had fed on low-Se plants showed 50-80% survival over the same time period (Fig. 18D). The animals may have died of Se toxicity, since the tissue Se concentration in grasshoppers that had fed on plants collected next to hyperaccumulators was 15- (*A. ludoviciana*) and 40-fold (*S. ericoides*) higher than in animals collected in the field (Fig. 18F). Animals that fed on plants collected from around hyperaccumulators also contained on average 2-10 fold higher Se levels than animals that fed on plants from the same species collected from around non-hyperaccumulators; these levels were not significantly higher though (Fig. 18F).

DISCUSSION

In this study we present evidence that Se hyperaccumulators can act as benefactor/nurse plants, facilitating the growth of Se-tolerant neighboring plants. When growing next to hyperaccumulators *A. bisulcatus* and *S. pinnata*, as compared to non-hyperaccumulator neighbors, *A. ludoviciana* and *S. ericoides* were bigger and showed reduced herbivory damage and arthropod load. These neighbors of hyperaccumulators were also better protected from grasshopper herbivory in laboratory experiments, owing to both deterrence and toxicity. The herbivory protection was likely due to Se enrichment: *A. ludoviciana* and *S. ericoides* contained 10-20 fold elevated Se levels (800-2,000 mg kg⁻¹ DW) when growing next to a hyperaccumulator neighbor. These are similar to hyperaccumulator levels, and high enough to protect plants from a wide variety of herbivores (Hanson *et al.*, 2003, 2004; Freeman *et al.*, 2006b, 2009; Quinn *et al.*, 2010). Indeed, for *S. ericoides* the herbivory experienced by the high-Se neighbors of hyperaccumulators was almost exclusively on those leaves that had the lowest Se levels.

The Se enrichment of *A. ludoviciana* and *S. ericoides* may in part be explained by the finding that soil Se levels were 7-13 fold higher around hyperaccumulators. The beneficiary plants showed preferential root growth toward their hyperaccumulator neighbors, which was not observed toward non-hyperaccumulator *M. sativa*. Thus, it appears that when growing next to a Se hyperaccumulator, *A. ludoviciana* and *S. ericoides* actively tap into this source of Se, reaching hyperaccumulator Se levels themselves that they can tolerate and from which they derive ecological benefit. For further studies it will be interesting to investigate the Se tolerance mechanisms of *A. ludoviciana* and *S. ericoides*, e.g. whether they store Se mainly as methyl-SeCys like their hyperaccumulator neighbors. It will also be interesting to study the mechanisms responsible for their apparent preferential root growth toward high-Se areas.

In addition to the demonstrated ecological benefit, it is feasible that *A. ludoviciana* and *S. ericoides* enjoyed a physiological benefit from their enhanced Se levels, since they were so much taller (2-fold) next to hyperaccumulators. Selenium is a beneficial nutrient for many plants, particularly for hyperaccumulators, perhaps via protection from oxidative stresses (Pilon-Smits *et al.*, 2009; Hartikainen 2005). The hyperaccumulators may also provide other benefits like shelter from wind or extreme temperatures, or better access to other elements besides Se. In this context it is interesting to note that S levels were 3-5 fold elevated in hyperaccumulator soil and up to 1.5-fold elevated in the beneficiaries; S levels were shown earlier to be higher in Se hyperaccumulators than in non-hyperaccumulators on the same site (El Mehdawi *et al.*, 2011a). There may also be an indirect facilitating effect on *A. ludoviciana* and *S. ericoides* if hyperaccumulators use Se as a form of elemental allelopathy to reduce competition from Se-sensitive neighbors, as indicated by results from our earlier work (El Mehdawi *et al.*, 2011a). In addition, intraspecific competition within *A. ludoviciana* and *S. ericoides* may play a role: we observed that the density of *A. ludoviciana* and *S. ericoides* was lower around hyperaccumulators than away from hyperaccumulators (data not shown). Perhaps there is genetic variation with respect to Se tolerance within *A. ludoviciana* and *S. ericoides*. If so, the more sensitive individuals may be selected against around hyperaccumulators, leaving the tolerant individuals with less competition, resulting in better growth.

The *A. bisulcatus* and *S. pinnata* nurse plants did not show any obvious positive or negative effects when growing next to their beneficiaries. *A. bisulcatus* and *S. pinnata* are substantially bigger than *A. ludoviciana* and *S. ericoides*, so the relationship of the hyperaccumulators to their beneficiaries is likely neutral, unless the beneficiaries tap so much Se from the hyperaccumulators that it would compromise the physiological and ecological benefits the hyperaccumulators derive from the Se.

The Se levels in the beneficiary plants were increased 10-20 fold while the levels in the soil were only 7-13 fold elevated. As mentioned, roots of *A. ludoviciana* and *S. ericoides* grew preferentially toward their hyperaccumulator neighbors, which may have enabled them to maximize their access to Se. The preferential root growth of *A. ludoviciana* and *S. ericoides* toward Se hyperaccumulators may indicate they have positive chemitropism toward Se. Plant roots are well-known to respond positively or negatively to soil pockets with elevated levels of nutrients or toxins, as well as to the presence of roots from neighboring plants of the same or different species (Boyd 2010; de Kroon 2007; Hodge 2009). In several earlier reports hyperaccumulator roots were shown to preferentially proliferate in soil containing the hyperaccumulated element; this was found for Zn hyperaccumulator *Thlaspi caerulescens* (Schwartz *et al.*, 1999; Haines 2002), Cd/Zn hyperaccumulator *Sedum alfredii* (Liu *et al.*, 2009) and Se hyperaccumulator *S. pinnata* (Goodson *et al.*, 2003). Since the soil Se levels around hyperaccumulators were found to be elevated compared to soil around non-accumulators in the same area, it is possible that the neighboring *A. ludoviciana* and *S. ericoides* responded positively to this soil Se gradient. However, it is also possible that the hyperaccumulator plants provide some other positive stimulus that affects their neighbors' root growth, e.g. higher levels of the nutrient S. The stimulus does not appear to be nitrogen, since the related and similarly sized non-accumulator legume *M. sativa* did not influence the direction of root growth in *A. ludoviciana* and *S. ericoides*.

An additional explanation for the finding that the Se levels in the companion plants were increased by 10-20 fold and those in the soil only 7-13 fold may be that the soil Se around hyperaccumulators is particularly bioavailable. It is interesting to note in this respect that the Se/S ratio in *S. ericoides* and *A.*

ludoviciana was elevated when they were growing next to hyperaccumulators (0.5 next to *A. bisulcatus* and 1.2 next to *S. pinnata*, as compared to 0.07 when growing next to a non-hyperaccumulator). The soil Se/S ratio was not that different: 0.04 next to *A. bisulcatus*, 0.13 next to *S. pinnata* and 0.03 next to non-hyperaccumulators. The hyperaccumulators may affect bioavailability as well as the form of Se in their surrounding soil. For instance, since hyperaccumulators accumulate mainly methyl-SeCys (Freeman *et al.*, 2006b) litter deposition may over time change the predominant form of Se in soil surrounding hyperaccumulators from inorganic Se (e.g. selenate) to more organic Se such as methyl-SeCys, which may be more readily taken up by neighbors (Zayed *et al.*, 1998). Non-protein amino acids such as methyl-SeCys have been reported to commonly occur in soils, and their importance in ecological and physiological processes is becoming increasingly clear, e.g. via antiherbivory, antimicrobial and allelochemical activity, or protection from stress (Vranova *et al.*, 2011). It is also feasible that the bioavailability of Se around hyperaccumulators is affected via the excretion of chelators (Bais *et al.*, 2006). In future studies it will be interesting to analyze the composition and activity of hyperaccumulator exudates, particularly with respect to the presence of Se chelators and selenocompounds. Yet another possible explanation for the finding that *S. ericoides* and *A. ludoviciana* beneficiaries were particularly enriched in Se relative to their soil may be that these neighboring plants are connected directly with their hyperaccumulator neighbors via mycorrhizae, and derive selenocompounds via this access. It is known that mycorrhizal fungi are often not host-specific and one fungal individual can interconnect neighboring plants of different species, distributing resources and facilitating plant growth (Van der Heijden and Horton, 2009).

Facilitation is thought to be especially important in extreme, harsh environments (Callaway and Walker, 1997). The seleniferous site studied here fits that description well. Not only is the soil seleniferous shale rock with low soil depth, but the climate is very dry (average annual precipitation 374 mm per year), frequent high winds, cold winters (average -10°C minimum temperature in January) and hot summers (average 30°C maximum temperature in July). In that sense the results from this study fit the pattern observed for facilitation. The novelty of the study presented here is that it is the first to show how phytoenrichment with a non-essential element can facilitate growth in neighboring plants in an ecologically

relevant setting. Earlier studies with metal hyperaccumulators have only been carried out in phytoremediation settings, which are not very ecologically relevant. Moreover, in those studies neighbors of hyperaccumulators did show facilitated growth in several cases, but due to lower levels of the toxic metal, rather than higher levels as shown here for Se. Another very interesting aspect of our current study is that the same process, enrichment by hyperaccumulators of neighboring plants with Se, can at the same time have a competitive effect on one class of neighbors (Se-sensitive plants) and a facilitating effect on another class of neighbors (Se-tolerant plants).

The finding that hyperaccumulators have a negative effect on Se-sensitive ecological partners, but offer a niche that may benefit Se-tolerant ecological partners is a recurring theme in our studies of the ecology of Se hyperaccumulators. While Se-sensitive plants may suffer toxicity when growing on the high-Se soil next to hyperaccumulators, Se-tolerant plants benefit from the associated elevated Se levels because it protects them from herbivores. Earlier we found that while Se-sensitive herbivores are deterred by hyperaccumulators and suffer toxicity when forced to feed on them, a Se-tolerant diamondback moth thrives on hyperaccumulator *S. pinnata* (Freeman *et al.*, 2006b). Additional leaf and seed herbivores have been found to occupy this and other hyperaccumulator species (Quinn and Pilon-Smits, unpublished results). Similarly, while Se-sensitive fungal pathogens were less successful in colonizing high-Se than low-Se plants, Se-tolerant fungi were observed to thrive in hyperaccumulator rhizosphere and litter (Quinn *et al.*, 2011a; Wangeline *et al.*, 2011). Moreover, there are indications that native bumble bee pollinators of hyperaccumulators in seleniferous areas are Se tolerant (Quinn and Pilon-Smits, unpublished results). Taken together, Se appears to be very important for the ecological interactions of Se hyperaccumulator species, and hyperaccumulators may have a profound effect on the overall ecology of seleniferous habitats. The enhanced soil Se levels around hyperaccumulators may have a negative effect on Se-sensitive plant species, while facilitating Se-tolerant ones. Via these mechanisms, hyperaccumulators may affect plant species composition and, consequently, higher trophic levels. Could hyperaccumulators be ecosystem engineers? This will be an intriguing question to address in future studies.

CONCLUSIONS

This study is the first to show a facilitating effect by means of enrichment with a non-essential element. Selenium hyperaccumulators enrich their neighbors with Se, and when these neighboring plants are Se-tolerant they enjoy the ecological benefits associated with elevated Se, i.e. reduced herbivory. Some Se-tolerant neighbors appear to actively forage for Se, judged from preferential root growth toward the hyperaccumulator. Earlier, Se-sensitive plants were shown to be negatively impacted by their elevated Se when growing on soil collected around hyperaccumulators. Thus, the Se deposited by hyperaccumulators likely has both competitive and facilitating effects, which may together affect species composition in seleniferous areas. This study provides the framework for future studies investigating the facilitative effects of hyperaccumulating plants on their neighbors.

Experimental Procedures

Study Site

The field site for this study was Pine Ridge Natural Area in Fort Collins, CO, USA (40°32.70N, 105°07.87W). The soil and vegetation properties of this seleniferous area were described in detail before (El Mehdawi *et al.*, 2011a). For this study we made use of naturally occurring plant species on the site: the two Se hyperaccumulating species *A. bisulcatus* (two-grooved milkvetch, Fabaceae) and *S. pinnata* (prince's plume, Brassicaceae), as well as two species often found in the vicinity of these hyperaccumulators: *Artemisia ludoviciana* (white sage; Asteraceae) and *Symphyotrichum ericoides* (white heath aster; Asteraceae). Furthermore, in one study *Medicago sativa* (alfalfa; Fabaceae) was used as a control species.

Effect of proximity to Se hyperaccumulators on neighboring plant size and elemental concentration

Artemisia ludoviciana and *S. ericoides* plants were collected from three locations within the same area: (i) in close proximity (<1 m) to the hyperaccumulator *A. bisulcatus* (ii) in close proximity to hyperaccumulator *S. pinnata*, and (iii) next to non-hyperaccumulator species and >4 m from any

hyperaccumulator. The sampling was as follows: four plants from each hyperaccumulator species (*A. bisulcatus* or *S. pinnata*) were selected, and around each hyperaccumulator plant four *A. ludoviciana* and four *S. ericoides* plants were collected. The *A. ludoviciana* and *S. ericoides* were analyzed for total biomass (dry weight of root plus shoot), stem height and number of leaves. The shoot Se and S concentration was also determined, as described below. In addition, soil was collected from between the *A. ludoviciana* / *S. ericoides* plants and their neighbors (*A. bisulcatus*, *S. pinnata*, or non-hyperaccumulators). The soil samples were collected from the top 5 cm, after removal of any litter. The soil was sieved, acid-digested and analyzed for Se and S as described earlier (El Mehdawi *et al.*, 2011a).

Determination of root directional growth

Four plants from each of the hyperaccumulator species (*A. bisulcatus* or *S. pinnata*) were selected, and around each hyperaccumulator plant the direction of root growth was determined for ten *A. ludoviciana* and ten *S. ericoides* plants. The root direction was classified as toward the hyperaccumulator when the root was bent horizontally and grew in the direction of the hyperaccumulator neighbor (i.e. in the quarter section of the radius that was closest to the hyperaccumulator). Root direction was classified as neutral when the root grew vertically, or when it grew bent horizontally in a direction that was neither toward nor away from the hyperaccumulator neighbor (i.e. in the two quarter sections of the radius that were at intermediate distance from the hyperaccumulator neighbor). Finally, root growth was classified as away from the hyperaccumulator when the root grew bent horizontally, in a direction pointing away from the hyperaccumulator (i.e. in the quarter section of the radius that was furthest from the hyperaccumulator). The same experimental procedure was followed for the control species *M. sativa*, except that only four *A. ludoviciana* and four *S. ericoides* plants were analyzed around each of the four selected *M. sativa* individuals. The reason for this lower number was that there were fewer *A. ludoviciana* and *S. ericoides* plants around *M. sativa*.

Field arthropod and herbivory survey

The same sixteen *A. ludoviciana* and *S. ericoides* individuals, whose collection is described above under the heading “Effect of proximity to Se hyperaccumulators on neighboring plant size and elemental concentration” were surveyed for the number of arthropods they harbored in the field at the time of collection, as well as for their number of leaves with signs of herbivory. The arthropod collection was carried out as described earlier (Quinn *et al.*, 2011a). In short, this was done by shaking the plant vigorously inside a bucket and using an aspirator to collect the resulting animals. Leaves were classified as showing herbivory when part of the leaf or leaf margin was missing or when there was a hole in the leaf; necrotic spots were not counted as herbivory. Since *S. ericoides* showed two different types of leaves (small and large), herbivory was scored separately for both leaf types, and the Se levels in both leaf types were measured.

Laboratory herbivory experiments

Entire *A. ludoviciana* and *S. ericoides* plants were dug out in the field and placed in 10 cm diameter pots in their own field soil. For each species, two categories of plants were collected: (i) in close proximity to *A. bisulcatus*, (ii) next to a non-hyperaccumulator and >4 m away from any hyperaccumulator. Note: there were not enough *A. ludoviciana* and *S. ericoides* plants left in the field next to *S. pinnata* to look at the effect of that hyperaccumulator as well. The plants were taken to the lab, and in preparation for controlled herbivory experiments the stem height of each individual plant was measured and the number of leaves counted; in addition, a leaf sample was collected for Se analysis. Grasshoppers were collected in bulk from the same field site, using a sweep net. Earlier (Freeman *et al.*, 2009), a similar sweep on this site yielded the following genera: *Amphitornus*, *Arphia*, *Aulocara*, *Cordillacris*, *Dissosteira*, *Hesperotettix*, *Melanoplus*, *Mermiria*, *Spharagemon*, *Trachyrhachys* and *Trimerotropis*. This mixture of Orthoptera species collected from a Se

hyperaccumulator habitat was used to simulate Orthoptera herbivory experienced by these plants under natural conditions.

Choice feeding experiment – For each of the two species *A. ludoviciana* and *S. ericoides* three aquaria were prepared, each containing three (high-Se) plants collected next to *A. bisulcatus* and three (low-Se) plants collected next to non-hyperaccumulator neighbors. Each of these plants had been analyzed for height, number of leaves and Se concentration as described above. Eight grasshoppers were added to each aquarium, making sure that for each aquarium animals of similar size were used, and the aquaria were covered. Over the subsequent six days, the plants were watered every two days, and grasshopper survival was counted daily. At the end of the 6-day herbivory trial, the remaining stem height and number of leaves of each plant were measured, and plant height and leaf loss were calculated from the difference between the initial and final numbers. Furthermore, the live and dead grasshoppers were collected and analyzed for Se as described below.

Non-choice feeding experiment – For each of the two species *A. ludoviciana* and *S. ericoides* two aquaria were prepared, each containing six plants: one aquarium contained (high-Se) plants collected next to *A. bisulcatus* and the other aquarium (low-Se) plants collected next to non-hyperaccumulator neighbors. Ten grasshoppers were added to each aquarium, and herbivory and grasshopper survivals were monitored over 6 days as described above.

Elemental analysis

Leaves, soil and animals collected as described above were acid-digested and analyzed for Se and S as described earlier (Galeas *et al.*, 2008). In short, the samples were dried at 50°C for 48 hours, weighed, and digested in nitric acid as described (Zarcinas *et al.*, 1987). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used as described by (Fassel 1978) to determine each digest's elemental composition.

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 (ANOVA) followed by a post-hoc Tukey Kramer test was used when comparing multiple means. For the grasshopper Se comparisons (Figures 17, 18) the live and dead animals did not show significantly different Se levels and therefore the data were pooled.

FIGURES

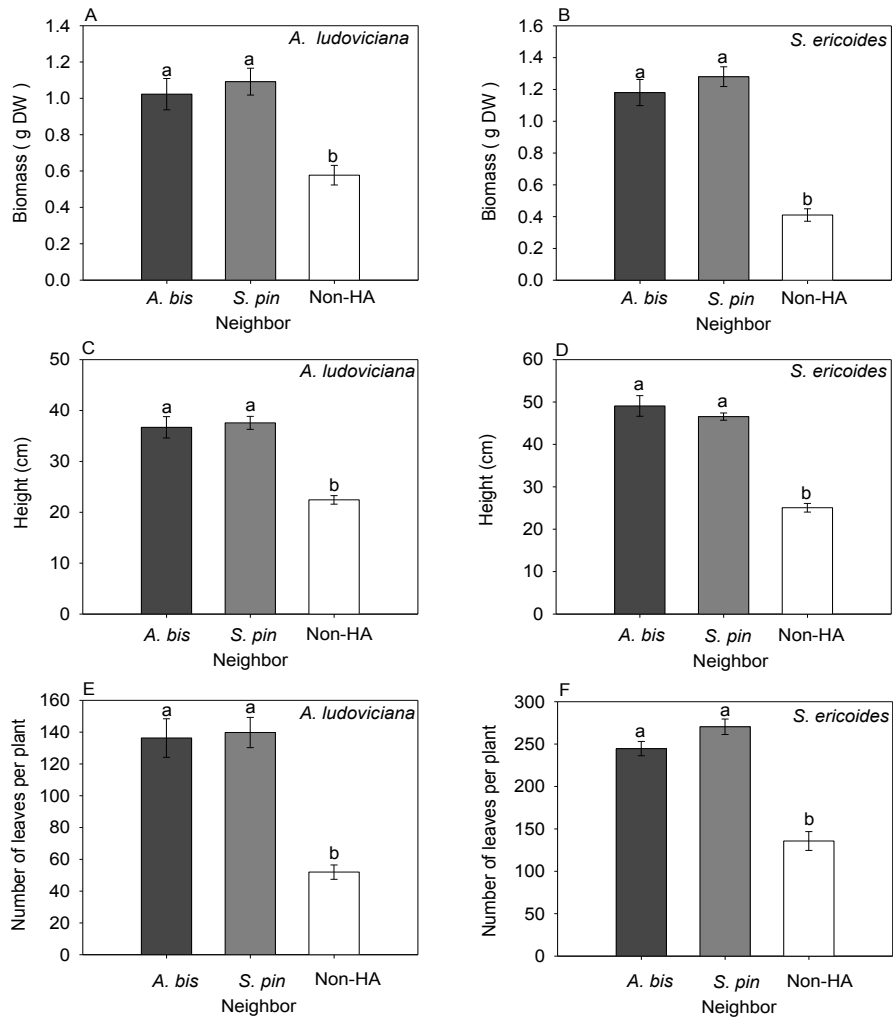


Figure 11: Size comparison of *A. ludoviciana* and *S. ericoides* growing around hyperaccumulator species (*A. bisulcatus* and *S. pinnata*) or far (>4m) from hyperaccumulator vegetation in seleniferous habitat (Fort Collins, Colorado, USA). (A, B): shoot biomass; (C, D): stem length; (E, F): number of leaves. Values shown represent means \pm SE (n= 16); different lower case letters above bars indicate significantly different means ($p < 0.05$).

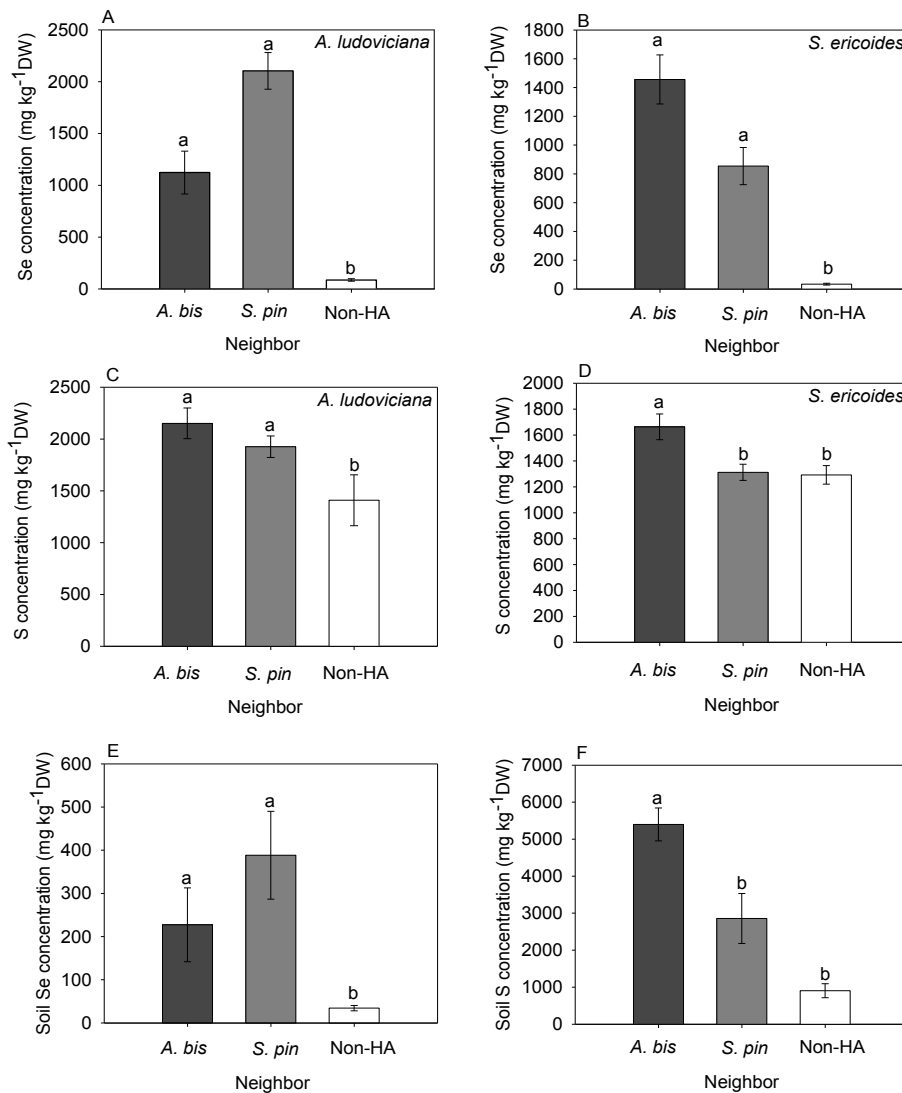


Figure 12: (A-D): Selenium and sulfur concentration in leaves of *A. ludoviciana* and *S. ericoides* collected from around hyperaccumulators (*A. bisulcatus* and *S. pinnata*) or from around non-hyperaccumulator vegetation in the same seleniferous habitat. (E, F): Soil Se and S concentration adjacent to the hyperaccumulators and non-hyperaccumulators. Values shown represent means \pm SE (n= 16); different lower case letters above bars indicate significantly different means ($p < 0.05$).

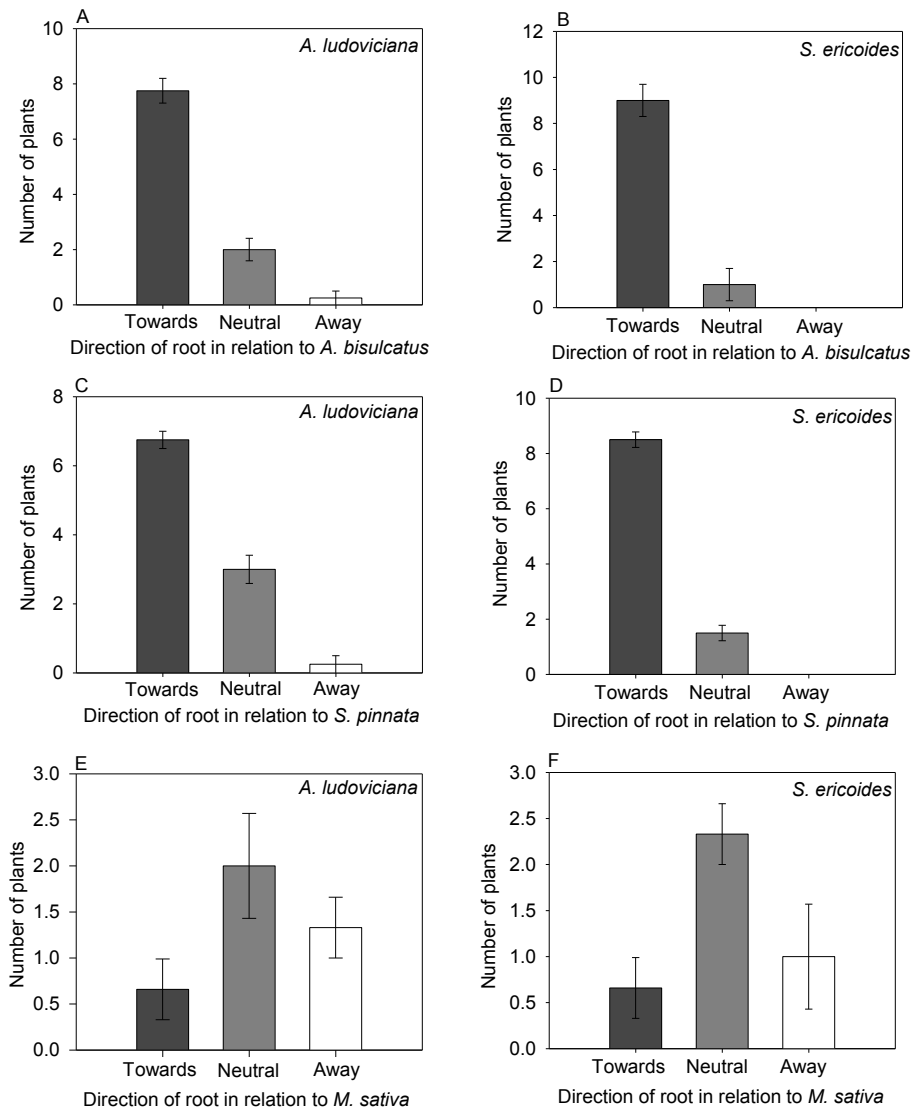


Figure 13: Root direction of (A) *A. ludoviciana* in relation to *A. bisulcatus*, (B) *S. ericoides* in relation to *A. bisulcatus*, (C) *A. ludoviciana* in relation to *S. pinnata*, (D) *S. ericoides* in relation to *S. pinnata*, (E) *A. ludoviciana* in relation to *M. sativa* and (F) *S. ericoides* in relation to *M. sativa*. Values shown represent means \pm SE ($n=10$ for A-D and $n=4$ for E, F); different lower case letters above bars indicate significantly different means ($p < 0.05$).

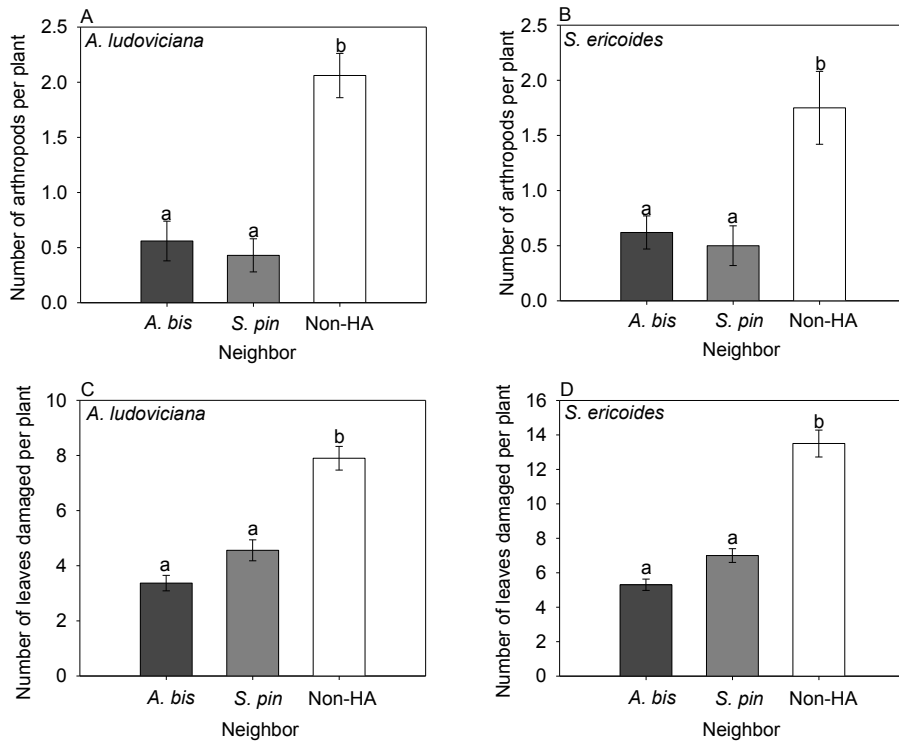


Figure 14: Number of arthropods per plant on (A) *A. ludoviciana* and (B) *S. ericoides*, and number of leaves damaged per plant of (C) *A. ludoviciana* and (D) *S. ericoides* when growing close to hyperaccumulator species (*A. bisulcatus* and *S. pinnata*) or away from hyperaccumulators (non-HA). Values shown represent means \pm SE (n= 16); different lower case letters above bars indicate significantly different means ($p < 0.05$).

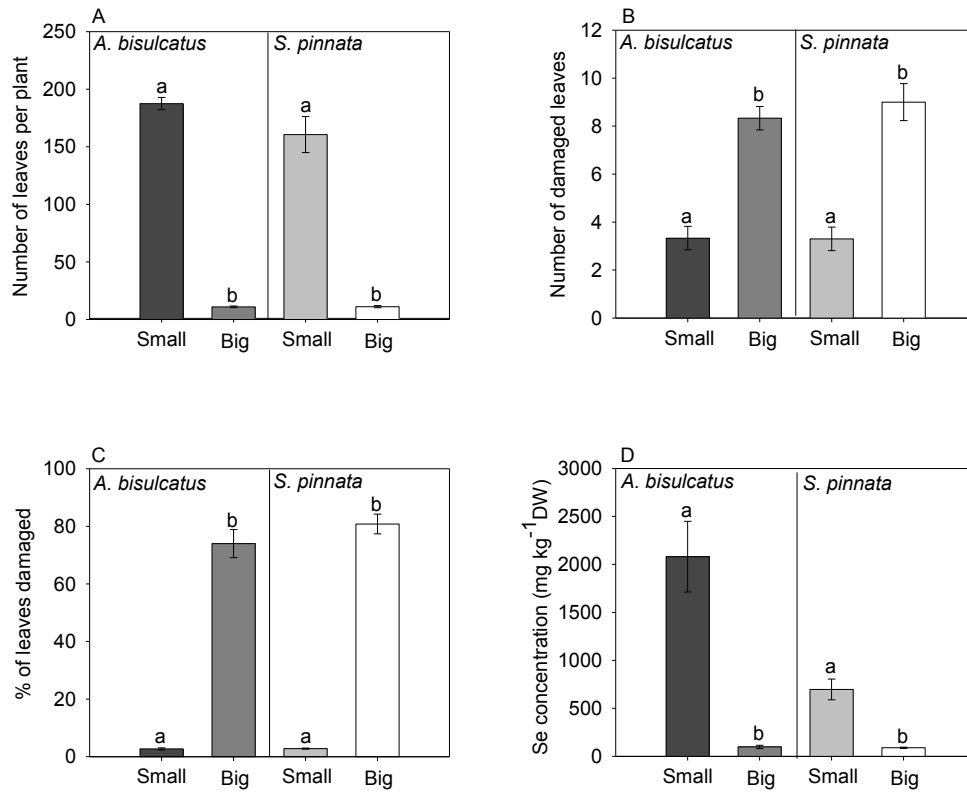


Figure 15: Leaf damage and Se concentration of the two leaf types of *S. ericoides* growing next to hyperaccumulators *A. bisulcatus* or *S. pinnata*. (A) Number of small and big leaves, (B) number of damaged small and big leaves, (C) percentage of small and big leaves damaged, (D) Se concentration in small and big leaves. Values shown represent means \pm SE (n=16); different lower case letters above bars indicate significantly different means ($p < 0.05$).

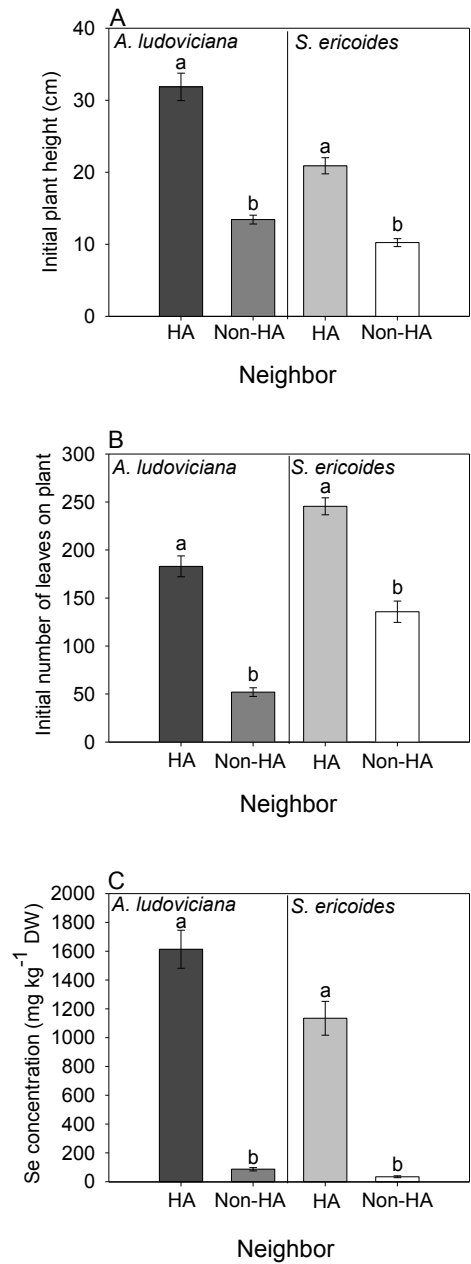


Figure 16: Characterization of *A. ludoviciana* and *S. ericoides* collected from the field for laboratory herbivory studies. (A) Plant height, (B) number of leaves, (C) leaf Se concentration. The plants were collected either next to the hyperaccumulator *A. bisulcatus* (HA) or next to non-hyperaccumulator species (non-HA). Values shown represent means \pm SE (n=15); different lower case letters above bars indicate significantly different means ($p < 0.05$).

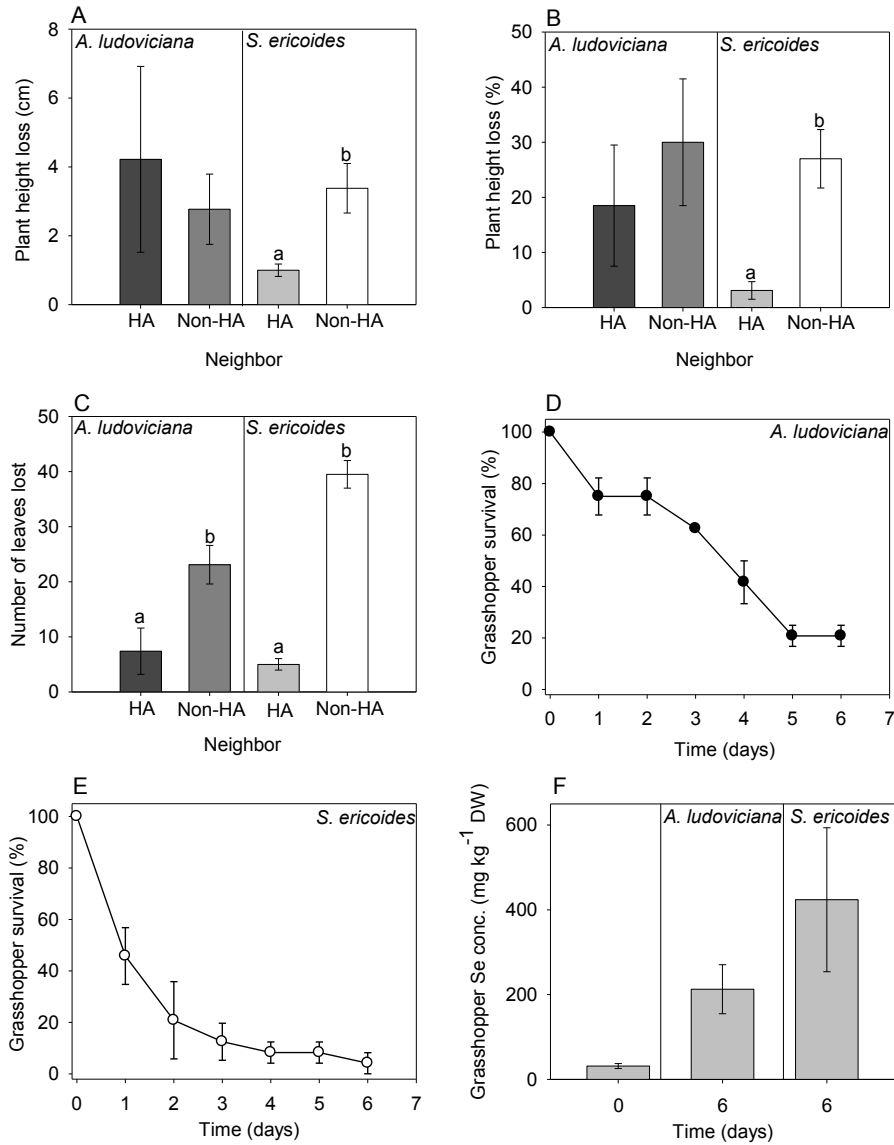


Figure 17: Choice experiment comparing herbivory, survival and Se accumulation of grasshoppers given the choice to feed on *A. ludoviciana* or *S. ericoides* plants collected either next to hyperaccumulator *A. bisulcatus* (HA) or next to non-hyperaccumulators (non-HA). (A) Absolute plant height loss, (B) relative plant height loss, (C) number of leaves lost, (D) grasshopper survival on *A. ludoviciana*, (E) grasshopper survival on *S. ericoides*, (F) grasshopper Se concentration in animals from the field at day 0 and in animals collected from *A. ludoviciana* or *S. ericoides* after 6 days of cocultivation. Values shown represent means \pm SE (n=9 for A-C; n=3 for D; E; n=6-8 for F). Different lower case letters above bars indicate significantly different means ($p < 0.05$).

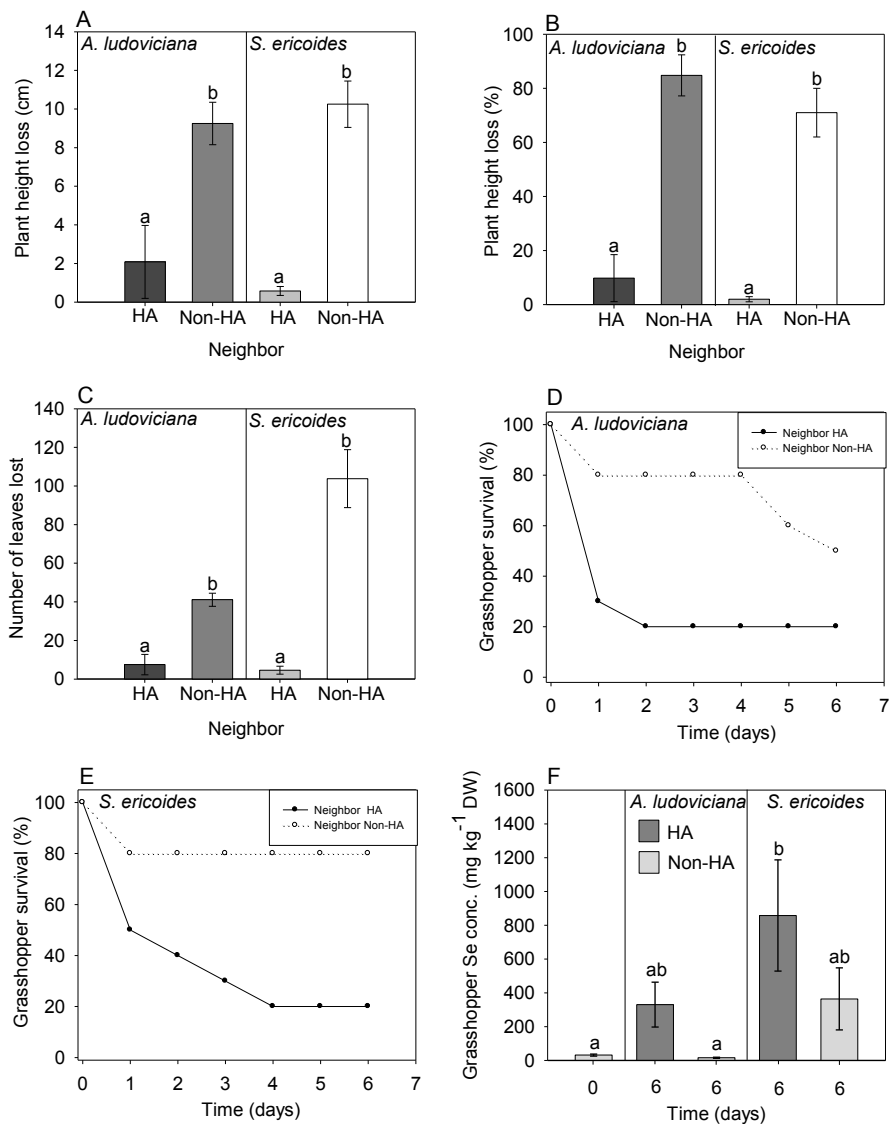


Figure 18: Non-choice experiment comparing herbivory, survival and Se accumulation of grasshoppers fed *A. ludoviciana* or *S. ericoides* plants collected next to hyperaccumulator *A. bisulcatus* (HA) or next to non-hyperaccumulators (non-HA). (A) Absolute plant height loss, (B) relative plant height loss, (C) number of leaves lost, (D) grasshopper survival on *A. ludoviciana*, (E) grasshopper survival on *S. ericoides*, (F) grasshopper Se concentration in animals from the field at day 0 and in animals collected after 6 days of cocultivation with *A. ludoviciana* or *S. ericoides*. Values shown represent means \pm SE (n= 6 for A-C; n=1 for D, E; n=6-8 for F). Different lower case letters above bars indicate significantly different means ($p < 0.05$).

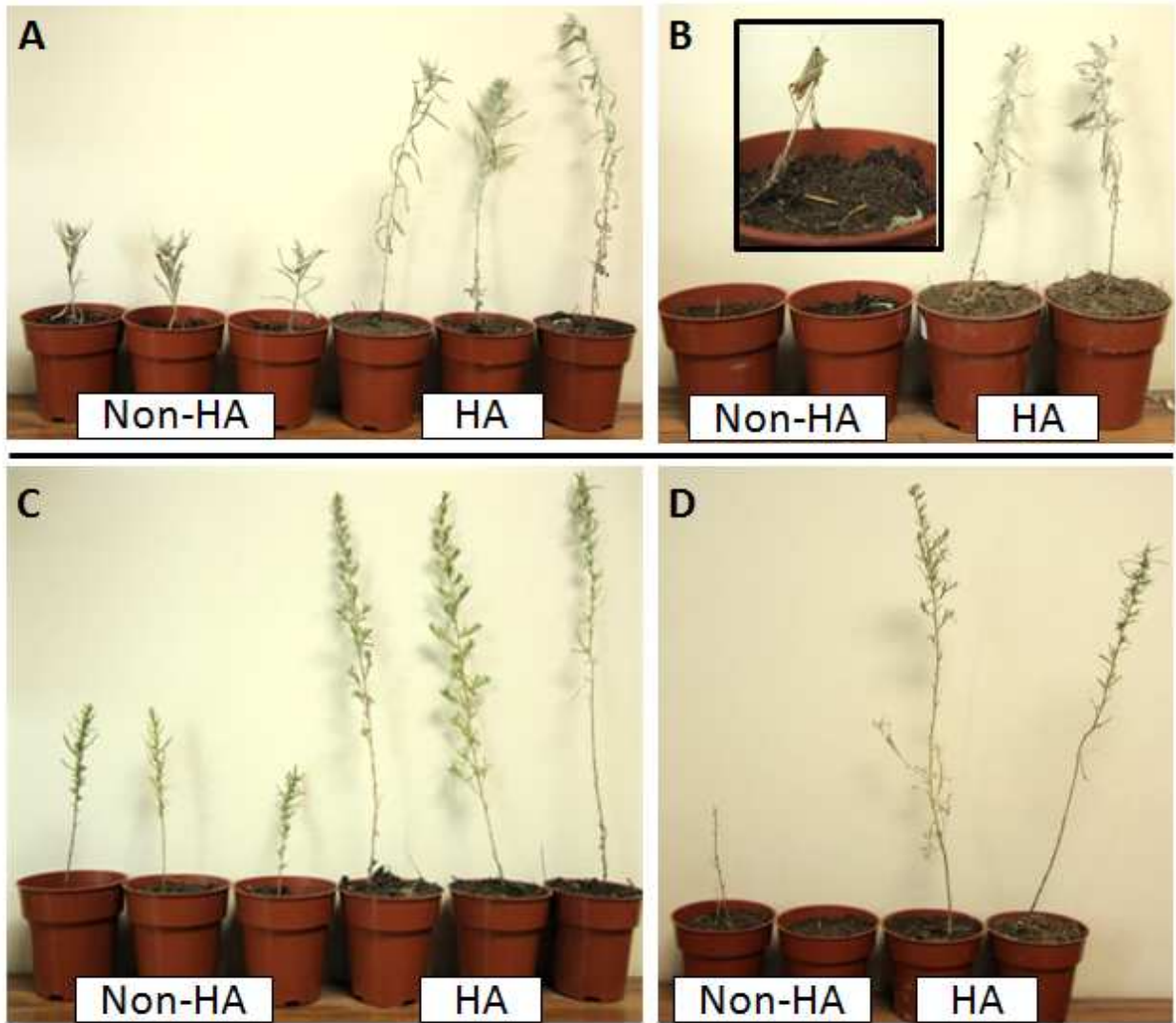


Figure 19: Representative *A. ludoviciana* (A, B) and *S. ericoides* (C, D) plants used in the laboratory grasshopper herbivory experiments. The plants were collected in the field next to hyperaccumulator *A. bisulcatus* (HA) or non-hyperaccumulator (non-HA) neighbors. A, C: Before exposure to grasshopper herbivory; B, D: after 6 days of exposure to grasshopper herbivory in the non-choice experiment (data shown in Fig. 18). The inset in panel B shows a representative grasshopper at the end of the experiment.

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CHAPTER 4:

INTERACTIONS OF SELENIUM HYPERACCUMULATORS AND NON-ACCUMULATORS DURING CO-CULTIVATION ON SELENIFEROUS OR NON-SELENIFEROUS SOIL-THE IMPORTANCE OF HAVING GOOD NEIGHBORS⁴:

This study investigated how selenium (Se) affects relationships between Se hyperaccumulator and non-accumulator species, particularly how plants influence their neighbor's Se accumulation and growth.

Hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* and non-accumulators *A. drummondii* and *S. elata* were co-cultivated on seleniferous or non-seleniferous soil, or on gravel supplied with different selenate levels. The plants were analyzed for growth, Se and S accumulation, and Se speciation. Also, root exudates were analyzed for Se content and soil Se extraction capacity.

The hyperaccumulators performed 2.5-fold better on seleniferous than non-seleniferous soil, and grew up to 4-fold better with increasing Se supply, while the non-accumulators showed opposite results. Both hyperaccumulators and non-accumulators could affect growth (up to 3-fold) and Se accumulation (up to 6-fold) of neighboring plants. Non-accumulators *S. elata* and *A. drummondii* accumulated predominantly (88-95%) organic C-Se-C; the remainder was selenate. *S. elata* accumulated more C-Se-C and less selenate next to *S. pinnata*. Hyperaccumulator roots released more Se than non-accumulators, mainly as C-Se-C. Root exudates from non-accumulators, however, extracted more Se from seleniferous soil.

These results show that soils contains high concentrations of selenium or even low concentration with hyperaccumulator plants for instance *S. pinnata* or *A. bisulcatus* may affect competition and facilitation between plants, and give insight into why hyperaccumulators are found predominantly on seleniferous soils.

⁴El Mehdawi AF, Cappa JJ, Fakra SC, Self J, Pilon-Smits EAH (2012) *New Phytologist* 194: 264–277. Co-authors Jennifer Cappa and Sirine Fakra helped collect and analyze x-ray absorption spectroscopy data. James Self helped with soil characterization.

INTRODUCTION

Selenium (Se) is an essential element for humans and animals, but toxic at higher levels (Terry *et al.*, 2000). There is a narrow margin between Se deficiency and toxicity in animals (Stadtman 1990). As an essential element Se is required for the production of selenoproteins, some of which function in scavenging free radicals (Zhang and Gladyshev, 2009). Selenium deficiency may promote cancer and cause other diseases such as white muscle disease, which may be fatal (Cosgrove, 2001). Selenium toxicity is thought to be due to the similarity of Se to sulfur (S); substitution of S by Se in proteins disrupts protein structure and function (Stadtman, 1990). For plants, Se is also toxic at high levels (Anderson 1993). The essentiality of Se for higher plants is still unproven, but Se is considered a beneficial nutrient for many plant species (Pilon-Smits *et al.*, 2009), perhaps due to better oxidative stress resistance (Cartes *et al.*, 2005; Hartikainen, 2005). Plants readily take up and assimilate Se, a capacity that may be used to alleviate both Se deficiency and toxicity in animals and humans. Plants can be used to clean up excess Se from polluted areas (phytoremediation), and Se-enriched plant material may be considered fortified food (biofortification) (Terry *et al.*, 2000).

Plants mainly take up Se from soil in the form of selenate (SeO_4^{2-}), which is taken up inadvertently via sulfate transporters, and metabolized via the S assimilation pathway (for a review see Sors *et al.*, 2005). In this pathway, selenate is reduced to selenite (SeO_3^{2-}), which can undergo further reduction to selenide (Se^{2-}). This may be incorporated into the organic forms selenocysteine (SeCys), selenocystathionine (SeCysth) and selenomethionine (SeMet). Plant species differ in their capacity to accumulate Se. While most plant species accumulate Se to levels below $100 \text{ mg Se kg}^{-1}$ dry weight (DW), even when growing on Se-rich (seleniferous) soils, some plant species native to seleniferous soils can accumulate Se to levels as high as $10,000 \text{ mg Se kg}^{-1}$ DW (Beath *et al.*, 1934, 1939; Galeas *et al.*, 2007). These are called Se hyperaccumulators; examples are *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae). The Se levels in hyperaccumulators are typically 1000-fold higher than those in seleniferous soil, and 100-fold higher than those in other vegetation on the same soil (Galeas *et al.*, 2007). The Se levels found in

hyperaccumulators would be toxic to other plant species. A clue to the tolerance mechanism of hyperaccumulators was found using micro-focused X-ray fluorescence (μ XRF) mapping and micro X-ray absorption near edge structure (μ XANES) spectroscopy, which revealed a stark contrast in spatial distribution and chemical speciation of Se between hyperaccumulators and non-accumulators. While non-hyperaccumulator plants were found to accumulate Se primarily in the leaf vasculature as selenate (de Souza *et al.*, 1998; Freeman *et al.*, 2006a), Se hyperaccumulators accumulated Se predominantly in their leaf epidermis as MeSeCys (Freeman *et al.*, 2006a). Thus, hyperaccumulators avoid Se toxicity by storing Se in peripheral tissues and converting it to methyl-selenocysteine (MeSeCys), a non-protein amino acid. The enzyme mediating this conversion is SeCys methyltransferase, SMT (Neuhierl & Böck, 1996).

Since hyperaccumulators are found predominantly on seleniferous soils, they appear to have a physiological or ecological need for Se (Beath *et al.*, 1934). There is ample evidence that Se serves ecological functions for hyperaccumulators. Selenium has been shown to protect plants from a wide variety of invertebrate and vertebrate herbivores, due to a combination of deterrence and toxicity (Hurd-Karrer & Poos, 1936; Vickerman *et al.* 2002; Hanson *et al.*, 2003, 2004; Freeman *et al.*, 2006b, 2007, 2009; Galeas *et al.*, 2008; Quinn *et al.*, 2008, 2010). In addition to herbivores, Se can protect plants from Se-sensitive fungal pathogens (Hanson *et al.*, 2003). Thus, hyperaccumulators may have an ecological dependency on Se for protection from various biotic stresses. In addition to elemental defense, Se may be used by hyperaccumulators for elemental allelopathy. The soil adjacent to hyperaccumulators was found to be 7-13 fold enriched in Se compared to soil on the same site collected >4 m away from hyperaccumulators (El Mehdawi *et al.*, 2011a,b). Accordingly, neighboring vegetation of hyperaccumulators contained 2-20 fold elevated Se levels compared to plants from the same species growing on the same site but >4 m away from hyperaccumulators (El Mehdawi *et al.*, 2011a,b). The higher Se levels in neighbors of hyperaccumulators may have an allelopathic effect if they are Se-sensitive. Indeed, the percentage vegetative ground cover was on average 10% lower around hyperaccumulators than around comparable non-accumulator species (El Mehdawi *et al.*, 2011a). Moreover, soil collected next to hyperaccumulators yielded significantly lower

germination and growth of the Se-sensitive model plant *A. thaliana*, and higher Se accumulation, compared to soil collected around non-hyperaccumulator species (El Mehdawi *et al.*, 2011a). Based on controlled experiments using agar medium supplied with different levels of Se, the Se levels in the soil were high enough to explain the observed inhibitive effect on *A. thaliana* germination (El Mehdawi *et al.*, 2011a).

Interestingly, in some cases hyperaccumulators can also have a positive effect on their plant neighbors (facilitation), if these neighbors are Se tolerant. In field studies, *Symphytotrichum ericoides* and *Artemisia ludoviciana* were 2-fold bigger when growing next to hyperaccumulators compared to when they were growing next to non-accumulators (El Mehdawi *et al.*, 2011b). This benefit appeared to be at least in part due to enhanced protection from herbivory: *S. ericoides* and *A. ludoviciana* harbored fewer herbivores in the field and exhibited less herbivory. Moreover, when taken to the lab and used in controlled herbivory studies with grasshoppers collected from the same field site, the high-Se *S. ericoides* and *A. ludoviciana* plants collected next to hyperaccumulators were eaten less than their low-Se counterparts collected next to non-accumulators (El Mehdawi *et al.*, 2011b).

Several questions remain regarding the effects of hyperaccumulated Se on plant-plant interactions. First, is the higher Se concentration in soil around hyperaccumulators due to litter deposition, root exudation, or both? In an earlier study it was found that high-Se litter decomposed readily in a seleniferous habitat, harbored more microbial and micro-arthropod decomposers than low-Se litter, and led to enrichment of the underlying soil with Se (Quinn *et al.*, 2011a). Release of Se by hyperaccumulator roots via exudation and turnover has never been tested, but may also be substantial, since hyperaccumulator roots can contain Se levels around 0.3% of DW (Galeas *et al.*, 2007). Second, are the higher Se levels in neighboring plants solely due to higher total soil Se levels, or also to different chemical Se speciation in soil around hyperaccumulators (which may affect Se bioavailability), and/or to the presence of Se chelators? Do hyperaccumulators affect the speciation of Se in their local environment, including neighboring plants? Third, how is the competition between hyperaccumulators and non-hyperaccumulators affected by the Se level of the soil? In the study described here we aim to address these questions.

The approach taken in this study was to co-cultivate plants pair-wise in pots on seleniferous or non-seleniferous soil, and on gravel medium supplied with different Se levels. The pairs of plants were either of the same or different species, and either hyperaccumulators or non-hyperaccumulators. The co-cultivated plants were analyzed for growth, Se and S accumulation, and Se speciation, as well as for root exudation of selenocompounds or Se chelators. The results from this research give better insight into the effects of Se on plant-plant interactions of hyperaccumulators and non-accumulators, and the mechanisms responsible for phytoenrichment.

MATERIALS AND METHODS

Soil collection and characterization

Soil was collected in June 2010 from two sites on the West side of Fort Collins, CO, USA: Pine Ridge Natural Area (40°32.70N, 105°07.87W), and Cloudy Pass (40°37.33N, 105°12.38W). Pine Ridge Natural Area is a seleniferous area with soil composed of Se-rich Cretaceous shale. This semi-arid shrubland harbors at least two species of Se hyperaccumulating plants: *A. bisulcatus* and *S. pinnata* (Galeas *et al.*, 2007). Cloudy Pass is a non-seleniferous area 10 miles North West of Pine Ridge Natural Area and similar in altitude, climate and vegetation except that no Se hyperaccumulators are present. Cloudy Pass does contain the non-hyperaccumulator *Astragalus* species *A. drummondii*. Soil samples from 0-5 cm depth were collected to determine soil properties and elemental concentrations.

Soil pH and electroconductivity (EC) were determined as described using a saturated soil paste (Soil Survey Laboratory Methods Manual, 2004). Soil texture was determined as described by Gee and Bauder (1986) using a hydrometer method for sand, silt and clay. Soil organic matter (SOM) was determined using a modification of the Walkley Black method, by means of a Spectronic 20 (Milton Roy Co.) at 610 nm (Soltanpour and Workman 1981). Soil calcium carbonate (CaCO₃) was quantified using gravimetric determination from CO₂ evolution (Soil Survey Laboratory Methods Manual, 2004). Soil elemental analysis was performed as described below.

Plant material

Seeds from *A. bisulcatus* and *A. drummondii* were obtained from Western Native Seed, Coaldale, CO, USA. *Stanleya pinnata* seeds were collected from seleniferous soil at Pine Ridge Natural Area in Fort Collins, CO (elevation 1,510 m). *Stanleya elata* seeds (accession #113) were collected from non-seleniferous soil in Nevada at N 37°26.699 W 117°21.896, at an elevation of 1,515 m.

Cocultivation experiment on seleniferous and non-seleniferous soils

The soil collected from Pine Ridge Natural Area (seleniferous) and from Cloudy Pass (non-seleniferous) was sieved to remove large stones and organic material, and mixed 3:1 with Turface® (Buffalo Grove, Illinois, USA) to make the aeration adequate and to enhance drainage. A thin layer of coarse gravel and sand was placed in the bottom of 10 x 10 cm pots, and the soil-Turface® mixture placed on top. Each pot was placed on an individual tray to catch leachate and keep it available for the plants.

Stanleya pinnata and *S. elata* seeds were surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10-min rinses in sterile water. The *A. bisulcatus* and *A. drummondii* seeds were first scarified with sand paper and then surface-sterilized. The seeds were germinated on sterilized, wet filter paper under continuous light at 23°C in a plant growth cabinet. The emerging seedlings were carefully transferred to the pots. Two plants were placed in each pot. For each soil type the following seven species combinations were created, using six replicates per treatment: (1-4) two plants of the same species, either *A. bisulcatus*, *S. pinnata*, *A. drummondii* or *S. elata*, (5-7) two plants of different species, either one hyperaccumulator and one non-hyperaccumulator (5, *A. bisulcatus* and *A. drummondii*; 6, *S. pinnata* and *S. elata*), or two hyperaccumulators (7, *A. bisulcatus* and *S. pinnata*). The plants were watered twice a week with water and once a week with 0.5-strength Hoagland solution (Hoagland and Arnon, 1938). After two months, when the plants became bigger, they were transferred to 14 cm diameter round pots. Again, a thin layer of gravel and sand was placed on the bottom, and the area around the transplanted soil was filled up with a similar mixture of soil (from the same source as originally) and Turface®. The plants were cultivated for an additional four months and then harvested. At harvest, the plants were rinsed, divided into shoot and

root, dried, and then measured for shoot and root biomass. At that point shoot and root samples were collected for elemental analysis as described below.

Co-cultivation experiment on Turface® supplied with different Se concentrations

Essentially the same experimental outline was followed as described above for the soil co-cultivation experiments, with the difference that the plants were cultivated in 100% Turface® growth medium, and treated once a week with different concentrations of Se (0, 10, 20, 40 or 80 $\mu\text{M Na}_2\text{SeO}_4$), and twice a week with 0.5-strength Hoagland solution. Also, five (rather than six) replicates were planted for each of the seven plant species combinations and Se concentration. At harvest, the youngest mature leaf was collected from *A. drummondii* and *S. elata* and immediately flash-frozen using liquid nitrogen for X-ray microprobe analyses as described below.

For root exudate collection, plants of all four species were grown on Turface® with two plants of the same species per pot (n=3) and treated with 20 $\mu\text{M Na}_2\text{SeO}_4$ as described above. The plants were harvested after 6 months, gently washed, and transferred to 50 mL of distilled water. After two days the plants were transferred to another container with 50 mL water. After three more days, this second volume of water and root-released compounds (which will below be referred to as exudate) was collected and analyzed for Se and S concentration as described below. Furthermore, some of the exudate fractions were frozen for Se speciation as described below. In addition, the exudate fractions were used to extract some Pine Ridge Natural Area soil. To 2 g of soil, 6 mL of exudate was added, and after mixing by rotation for 1h at room temperature, allowed to settle overnight at 4°C. The liquid fraction was then removed and used for elemental analysis and X-ray microprobe analyses as described below.

Selenium distribution and speciation

Selenium speciation was compared in leaf material of *S. elata* grown next to *S. elata* and *S. elata* grown next to *S. pinnata*, as well as in leaves of *A. drummondii* growing next to *A. drummondii* and *A. drummondii* grown next to *A. bisulcatus*. Root exudates and extract from seleniferous (Pine Ridge) soil

collected using these exudates were also analyzed for Se speciation. Selenium distribution and local speciation were determined using μ XRF mapping and μ XANES spectroscopy, respectively, both as described by Quinn *et al.* (2011b).

Elemental analysis

Entire youngest mature leaves were collected from *A. bisulcatus*, *S. pinnata*, *A. drummondii* and *S. elata* for Se and S concentration analysis. Samples were rinsed with distilled water to remove any external Se and S and then dried at 45° C for 48 hours. The samples were then digested in nitric acid as described by Zarcinas *et al.* (1987). Soil samples were dried, sieved, and extracted with ammonium bicarbonate-diethylenetriaminepentaacetic acid (AB-DTPA) as described by Soltanpour and Schwab (1977). Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used as described by Fassel (1978) to determine Se and S concentration.

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 (ANOVA) followed by a post-hoc Tukey Kramer test was used when comparing multiple means. Correlation analysis and linear regression were used to correlate plant biomass rate with substrate Se concentration.

RESULTS

When pairs of plants from the same species were grown together in one pot, the total biomass attained by Se hyperaccumulators *A. bisulcatus* and *S. pinnata* was 2-3 fold larger on seleniferous (PR) soil than on non-seleniferous (CP) soil (Fig.20A). In contrast, non-hyperaccumulator species *A. drummondii* and *S. elata* attained a somewhat (5-20%) larger biomass on non-seleniferous soil than on seleniferous soil (Fig.20A, NS). When growth on each soil was compared between the four plant species, there was a pronounced difference between hyperaccumulators and non-accumulators with respect to their performance on seleniferous soil: the average shoot and root dry weight (DW) of *A. bisulcatus* and *S. pinnata* was 2- to

4-fold larger compared to *A. drummondii* and *S. elata* (Fig. 20B). On non-seleniferous soil there were no significant differences in growth between hyperaccumulators and non-hyperaccumulators (Fig.20C).

When two plants from different species, one hyperaccumulator and one non-hyperaccumulator from the same genus, were grown together in one pot on seleniferous soil, the hyperaccumulators were bigger than the non-hyperaccumulators in both cases (Fig. 21A, B). *A. bisulcatus* was 2- to 3-fold larger than *A. drummondii* (Fig. 21A); the average DW of hyperaccumulator *S. pinnata* root was 2-fold larger than that of *S. elata*; the *S. pinnata* shoot was on average 20% bigger than *S. elata* but this was not significant (Fig. 21B). Figure 21 C-F show the biomass of each of the four species on seleniferous soil as influenced by which neighbor was in the same pot. The shoot and root biomass of hyperaccumulator *A. bisulcatus* was the same when grown with another *A. bisulcatus* plant as compared to when it was grown with non-hyperaccumulator *A. drummondii*; however, the *A. bisulcatus* biomass was 2-fold smaller when grown with hyperaccumulator *S. pinnata* (Fig. 21C). The shoot and root biomass of hyperaccumulator *S. pinnata* were significantly larger when grown with another *S. pinnata* plant than when grown with non-hyperaccumulator *S. elata* or with hyperaccumulator *A. bisulcatus* (Fig. 21D). The shoot and root biomass of non-hyperaccumulator *A. drummondii* was 2-fold lower when grown next to another *A. drummondii* than when grown next to hyperaccumulator *A. bisulcatus* (Fig. 21E). On average the shoot biomass of non-hyperaccumulator *S. elata* was 30% lower when grown next to another *S. elata* than when grown next to *S. pinnata* (Fig. 21F, NS).

When a hyperaccumulator and a non-hyperaccumulator from the same genus were co-cultivated on non-seleniferous soil, the non-hyperaccumulators were bigger than the hyperaccumulators (Fig. 22A, B). *A. bisulcatus* produced around 2-fold less biomass than *A. drummondii* (Fig. 22A). While the average shoot and root DW of hyperaccumulator *S. pinnata* were 15-20% lower than those of *S. elata*, this was not significantly different (Fig. 22B). Interestingly, the average shoot and root DW of hyperaccumulator *A. bisulcatus* were 2-fold higher when grown with hyperaccumulator *S. pinnata* than when grown with another *A. bisulcatus* or with non-hyperaccumulator *A. drummondii* (Fig. 22C). Similarly, there was a pronounced increase in the average shoot and root DW of hyperaccumulator *S. pinnata* when it was grown with

hyperaccumulator *A. bisulcatus* as compared to when grown with another *S. pinnata* plant or with non-hyperaccumulator *S. elata* (Fig. 22D). Non-hyperaccumulator *A. drummondii* was bigger when grown next to *A. bisulcatus* than when grown next to another *A. drummondii* (Fig. 22E). The same trend appears to be present for *S. elata*, which produced on average 25% more shoot biomass next to *S. pinnata* than next to *S. elata* (Fig 22F, NS).

Figure 23 shows the Se concentration in shoot and root of each of the four species on seleniferous soil, as influenced by which neighbor was in the same pot. The only significant neighbor effect was found for hyperaccumulator *S. pinnata*, whose shoot and root Se concentration was 8-10 fold higher when grown in a pot with non-hyperaccumulator *S. elata* than when grown with another *S. pinnata* or with hyperaccumulator *A. bisulcatus* (Fig. 23B). It is also worth noting that the Se concentration in *A. bisulcatus* was ~50% higher when growing next to another *A. bisulcatus* than when growing next to *A. drummondii* or *S. pinnata* (Fig. 23A, NS), and similarly the Se concentration in *S. pinnata* was ~50% higher when growing next to another *S. pinnata* than when growing next to *A. bisulcatus* (Fig. 23B, NS). If these Se concentration effects are combined with the biomass effects (Fig. 21C,D), and total Se accumulation is calculated per plant, the total Se per plant was about 3-fold higher when the hyperaccumulators were grown next to a hyperaccumulator from the same species as compared to a hyperaccumulator of the other species.

Since S is chemically similar to Se and taken up and metabolized via the same pathway, the neighbor effects on the S levels were also compared for the plants grown on seleniferous soil (Fig.24). Shoot S levels in *A. bisulcatus* were significantly lower when grown next to another *A. bisulcatus* than when grown next to *S. pinnata* (Fig.24A), which was opposite to the trend observed for Se (Fig. 23A). In *S. pinnata* the S levels were significantly lower in roots of plants grown next to another *S. pinnata* than in plants grown next to *S. elata* or *A. bisulcatus* (Fig. 24B); in shoots the S levels were also ~20% lower next to *S. pinnata*, but this was not significant. Thus, the neighbor effect of *A. bisulcatus* on *S. pinnata* was opposite for Se and S (reducing Se levels while increasing S levels), while the neighbor effect of *S. elata* on *S. pinnata* was similar (in both cases enhancing elemental concentrations). For both non-hyperaccumulators *A. drummondii* and *S. elata*, the shoot S concentration was about three-fold higher when

grown next to a plant of the same species than when growing next to a hyperaccumulator (Fig. 24C,D); the S levels were also 2-fold higher in the roots for *A. drummondii*, but this was not significant.

The Se concentration in shoot and root of the four species when grown on non-seleniferous soil is shown in Figure 25. As expected the Se levels were substantially lower than in the plants grown on seleniferous soil, but still measurable. The shoot Se concentration in hyperaccumulator *A. bisulcatus* was two- to three-fold higher when growing in the same pot with non-hyperaccumulator *A. drummondii* or with hyperaccumulator *S. pinnata* as compared to when growing with another *A. bisulcatus* plant; there were no differences in root Se concentration (Fig 25A). The root and shoot Se concentration in hyperaccumulator *S. pinnata* was 5-10 fold higher when grown in same pot with non-hyperaccumulator *S. elata* than when grown with another *S. pinnata* or with hyperaccumulator *A. bisulcatus* (Fig. 25B), which is similar to the results found on seleniferous soil (Fig. 23B). Root Se concentration of non-hyperaccumulator *A. drummondii* was two-fold lower when growing in a pot with another *A. drummondii* than when growing with hyperaccumulator *A. bisulcatus*; the shoot concentration was also 40% lower next to *A. drummondii* but this was not significant (Fig 25C). The same trend was seen for non-hyperaccumulator *S. elata*: the root Se levels were about five-fold lower when growing next to *S. elata* than next to *S. pinnata* (Fig. 25D, $p < 0.05$), and the shoot Se levels were about 40% lower (NS).

The shoot S concentration in hyperaccumulator *A. bisulcatus* was significantly (5-fold) higher when growing in the same pot with hyperaccumulator *S. pinnata* as compared to when growing with another *A. bisulcatus* (Fig. 26A). *A. bisulcatus* S levels were on average also 2-3 fold higher when growing next to non-hyperaccumulator *A. drummondii*, but this was not significant (Fig. 26A). There were no significant differences in *A. bisulcatus* root S concentration, although the average was 1.5-fold higher in the plants growing next to *S. pinnata* (Fig.26A). The root and shoot S concentration in hyperaccumulator *S. pinnata* was 20-40% lower when grown next to non-hyperaccumulator *S. elata* than when grown with another *S. pinnata* (Fig 26B); shoot S levels in *S. pinnata* were elevated when growing next to hyperaccumulator *A. bisulcatus*, but root S levels were reduced as compared to when *S. pinnata* was the neighbor (Fig 26B). Shoot and root S levels in non-hyperaccumulator *A. drummondii* were 25% lower when growing in a pot

with another *A. drummondii* than when growing with hyperaccumulator *A. bisulcatus* (Fig 26C, NS). Similarly, in non-hyperaccumulator *S. elata* the root S levels were about 35% lower when growing next to *S. elata* than next to *S. pinnata* (Fig 26D, $p < 0.05$) and the shoot S levels were about 20% lower (NS).

To be able to tease apart the effect of Se on plant-plant interactions from other factors (other soil properties, microbial composition), a second co-cultivation experiment was carried out using Turface® growth medium supplied with different concentrations of Na_2SeO_4 . When pairs of plants from the same species were grown together in one pot, there was an opposite growth response in Se hyperaccumulators and non-accumulators. Total plant biomass showed a positive correlation ($p < 0.05$) with increasing external Se concentration for hyperaccumulators *A. bisulcatus* and *S. pinnata*, which were 4.5-fold and 2-fold bigger, respectively, when treated with $80 \mu\text{m Na}_2\text{SeO}_4$ than in the absence of Se (Fig 27A,B). In contrast, the biomass of non-hyperaccumulators *A. drummondii* and *S. elata* decreased 6- and 15-fold, respectively, with increasing Se concentration ($p < 0.05$, Fig 27C, D). Thus, the hyperaccumulators were not only Se tolerant to but even benefited from increasing Se supply, while the non-accumulators were Se-sensitive, showing 50% growth inhibition at external Se levels between 5 and $15 \mu\text{m}$.

When two plants from different species, one hyperaccumulator and one non-hyperaccumulator from the same genus, were grown together in one pot, similar growth responses to Se were observed. Hyperaccumulator *A. bisulcatus* increased 3-fold in size with increasing Se treatment, while the co-cultivated non-hyperaccumulator *A. drummondii* decreased more than 10-fold in size (Fig. 27E). *Stanleya pinnata* increased 2-fold in size with increasing Se supply while non-hyperaccumulator *S. elata* decreased over 60-fold in size (Fig. 27F). As a result of these differential growth responses to Se, the non-accumulators were bigger than the hyperaccumulators in the absence of Se, while the hyperaccumulators outgrew the non-accumulators above external Se levels of 3 and $8 \mu\text{m Na}_2\text{SeO}_4$, respectively, for the *Astragalus* and *Stanleya* pairs (Fig. 27E, F). When the two hyperaccumulator species were grown together in one pot, their growth responses were also similar to those observed when grown individually: the biomass of *A. bisulcatus* and *S. pinnata* increased 2.5- and 4-fold respectively, with increasing Se supply (Fig 27G).

Figure 28A-D shows the shoot Se concentration for each of the four species grown on Turface®, as influenced by which neighbor was in the same pot. The Se concentration in hyperaccumulator *A. bisulcatus* was 2-fold higher when growing with non-hyperaccumulator *A. drummondii* than when growing with another *A. bisulcatus* (Fig. 28A). The Se concentration in hyperaccumulator *S. pinnata* was up to 20-fold higher when growing with non-hyperaccumulator *S. elata* than when growing with another *S. pinnata* (Fig. 28B). Non-hyperaccumulators *A. drummondii* and *S. elata* showed increasing tissue Se concentration with increasing Se supply, which was not significantly different between plants grown with a hyperaccumulator or a non-accumulator neighbor (Fig. 28C, D).

To obtain better insight into the mechanism responsible for the observed effects of neighboring plants on plant Se accumulation, root exudate was collected from each of the four species after being grown on Turface® and treated with 20 μm Na_2SeO_4 . The shoot and root Se levels in hyperaccumulators *A. bisulcatus* and *S. pinnata* were 2-3 fold higher than those in non-hyperaccumulators *A. drummondii* and *S. elata* (Fig 29A,B, NS). The Se levels in root exudates were about 6-fold higher for the two hyperaccumulators, than the two non-accumulators (Fig. 29C); here it is worth noting that the hyperaccumulator plants were 2-3 fold larger than the non-accumulators (Fig. 27A-D). Surprisingly, when these root exudates were used to extract seleniferous (Pine Ridge) soil, the extract obtained using hyperaccumulator-derived exudates contained about 2-fold lower Se levels than extract obtained using non-hyperaccumulator exudates (Fig. 29D). After interacting with the seleniferous soil, the hyperaccumulator exudates had decreased in Se concentration while the non-accumulator exudates had increased in Se. For comparison, the S levels were also determined in the plant tissues, exudates and soil extracts (Fig. 30). The shoot and root S levels were somewhat higher (20-40%) in the hyperaccumulators than in non-accumulators (Fig. 30A,B, NS) and the S levels in the hyperaccumulator exudates were 4-5 fold higher (Fig. 30C, NS), all similar to the Se trends. However, the extract obtained using hyperaccumulator-derived exudates contained about 3-4 fold higher S levels than extract obtained using non-hyperaccumulator exudates (Fig. 30D, NS); in all cases the exudates had lost S after interacting with the soil.

In addition to affecting the total Se concentration in neighboring plants, it is also feasible that plants can affect their neighbor's Se speciation (i.e. the chemical composition of the selenocompounds). To investigate the Se speciation in non-hyperaccumulator species *A. drummondii* and *S. elata* as affected by their neighbor in the same pot, Se K-edge XANES spectra were collected in leaves of plants grown on Turface® and treated with 20 µM Na₂SeO₄ (Table 7). The Se in both non-accumulators consisted primarily (89-95%) of an organic C-Se-C compound, indistinguishable from the standards selenomethionine and methyl-selenocysteine; the remainder was selenate (SeO₄²⁻) (Table 7). The relative abundance of C-Se-C and selenate were similar in *A. drummondii* leaves collected from plants growing next to *A. drummondii* or growing next to *A. bisulcatus* (Table 7). However, speciation in *S. elata* leaves appeared to be affected by its neighboring plant: *S. elata* that was grown next to *S. pinnata* showed a 3.5-fold lower selenate fraction and a concomitant increase in C-Se-C abundance compared to *S. elata* grown next to another *S. elata* (Figure 31, Table 7). The Se speciation in the root exudates and soil extracts obtained using root exudates was also analyzed by XANES. Only the *A. bisulcatus* exudate provided useful Se spectra; the main selenocompound (83%) in the exudate was organic Se of a C-Se-C type, and the remainder was selenite (Table 7).

DISCUSSION

The objective of this study was to investigate how soil Se affects the relationships between hyperaccumulator and non-accumulator plants, and to obtain some insight into the mechanisms by which the plants influence each other's growth and Se accumulation. The two hyperaccumulator species grew about two-fold larger on their native seleniferous soil than on non-seleniferous soil. The two non-accumulator species did not show a significant difference in growth between the two soils, although they attained 5-30% more biomass on the non-seleniferous soil. The finding that Se hyperaccumulators perform better on seleniferous soil may indicate that they benefit from the Se. The growth promotion may also in part be due to soil microbes. The Turface® experiment showed that the two hyperaccumulators indeed grew several fold better with increasing Se concentration, which supports the hypothesis that the Se in the

seleniferous soil was responsible for the better growth of the hyperaccumulators. The beneficial effect of Se on hyperaccumulator growth was mentioned earlier by Shrift (1969). A possible mechanism may be enhanced antioxidant activity, as was found for non-hyperaccumulator species (Cartes *et al.*, 2005; Hartikainen 2005).

In the same Turface® experiment the non-accumulators grew worse when Se supply increased, reaching 50% inhibition around 10-20 μM sodium selenate (0.8-1.6 ppm Se), corresponding with a tissue Se concentration around 200-250 mg kg^{-1} DW. This is similar to what was found earlier for *Arabidopsis thaliana* (El Mehdawi *et al.*, 2011a). However, the form of Se in *A. drummondii* and *S. elata* was mainly organic C-Se-C (89-95%), while other non-accumulator species including *A. thaliana* accumulate mainly selenate with a minor fraction of C-Se-C (de Souza *et al.*, 1998; Van Hoewyk *et al.*, 2005; Freeman *et al.*, 2006a). Based on XANES data alone the C-Se-C compound in *A. drummondii* and *S. elata* could be MeSeCys, SeMet or SeCysth; these cannot be distinguished. MeSeCys was found earlier to be the predominant form of Se in hyperaccumulators *S. pinnata* and *A. bisulcatus*, which explains their Se tolerance, since MeSeCys does not enter proteins. The intermediate Se accumulator *Stanleya albenscens*, on the other hand, accumulated mainly SeCysth and was fairly Se sensitive (Freeman *et al.*, 2010). The Se sensitivity in *A. drummondii* and *S. elata* could be due to accumulation of the more toxic forms SeMet or SeCysth, or to the fact that the remainder of their Se was selenate (4-11%). This form of Se is toxic when accumulated, due to pro-oxidant activity (Grant *et al.*, 2011).

The opposite growth responses to Se may affect competition between hyperaccumulators and non-accumulators: the two likely have different competitive strength depending on soil Se levels. When co-cultivated in Turface® at different Se levels, the threshold above which the hyperaccumulators started to out-compete the non-accumulators was around 5 μM sodium selenate (\sim 0.4 ppm Se). In seleniferous soil the Se levels are often above this threshold (e.g. in the Pine Ridge soil used here the level was 1.5 ppm bioavailable Se), allowing hyperaccumulators to grow well and thus be relatively competitive. The fact that hyperaccumulator growth is impaired in the absence of Se may explain why we find hyperaccumulators

primarily on seleniferous soil. In addition to the physiological benefits observed here, hyperaccumulators have already been found earlier to derive ecological benefits from Se accumulation in the form of herbivory and pathogen protection, and allelopathic effects on Se-sensitive plant neighbors.

Thus, the hyperaccumulators may also have an ecological dependency on Se for their negative biotic interactions. During the evolution of Se hyperaccumulation any or all of these physiological and ecological benefits may have played a role as selective pressures.

In addition to the growth responses of individual plant species to Se, it was observed here that plants may affect their neighboring plant's growth and Se accumulation. Both hyperaccumulator and non-accumulator species could affect their neighbor in terms of growth (up to 3-fold) and/or Se accumulation (up to 6-fold). The biggest effect was observed for *S. elata*, which appeared to enhance the shoot and root Se levels in neighboring *S. pinnata* plants 3-6 fold. This was found on seleniferous soil, on non-seleniferous soil, as well as in Turface®. The mechanism for this positive effect is not readily apparent. *Stanleya elata* roots were shown to release some Se, but these levels were much lower than the Se release from hyperaccumulators. It was interesting, however, that the *S. elata* exudate extracted two-fold more Se from seleniferous soil compared to *S. pinnata* exudate. Thus, *S. elata* exudate may somehow enhance Se bioavailability for *S. pinnata*. The mechanism is not clear but could for instance involve a Se chelator. It appears to be a Se-specific effect because *S. elata* exudate extracted less S from seleniferous soil compared to *S. pinnata* exudate, and *S. elata* did not enhance S levels in *S. pinnata* when co-cultivated on either of the two soils. The enhanced Se levels in *S. pinnata* associated with growing next to *S. elata* would be expected to have a positive growth effect. Indeed, a modest positive growth effect was observed on non-seleniferous soil. On seleniferous soil, however, the effect of *S. elata* on growth of *S. pinnata* appeared to be negative.

The effect of *S. pinnata* on growth and Se accumulation of *S. elata* was slightly positive on both soils (NS); the Se-enrichment effect might be due to root Se release as observed in *S. pinnata* exudates. On Turface® there was no effect of *S. pinnata* on *S. elata* growth or Se accumulation. Interestingly, *S. pinnata*

did seem to affect Se speciation in *S. elata*: *S. elata* contained relatively more organic Se when its neighbor was *S. pinnata* compared to when it was another *S. elata*. This may be due to root release of organic Se by *S. pinnata*. In support of this hypothesis, *S. pinnata* roots were shown here to exude significant levels of Se, and the form of Se in roots of *S. pinnata* was shown recently to be C-Se-C (Pilon-Smits, unpublished results). While the exudate of *S. pinnata* did not have a strong enough Se signal to obtain reliable speciation information from XANES, *A. bisulcatus* exudate was shown by XANES to contain predominantly C-Se-C. Thus, hyperaccumulators may exude organic Se and since the main form of bioavailable Se in soil is thought to be inorganic selenate the root release of organic Se may affect local Se speciation, and with that Se bioavailability and Se uptake and speciation by neighboring plants. Enhanced bioavailability of Se around hyperaccumulators was also suggested by the earlier finding that while the soil around hyperaccumulators was 7-13 fold enriched with Se, the neighboring plants were enriched up to 20-fold. The finding that hyperaccumulators release Se from their roots supports the hypothesis that hyperaccumulators can phytoenrich their surrounding soil with Se, and that root release of Se is one of the mechanisms for phytoenrichment. Litter deposition and decomposition likely is another mechanism, as indicated by an earlier study (Quinn *et al.*, 2011a).

Hyperaccumulator *A. bisulcatus* had a positive effect on growth of non-accumulator *A. drummondii* on both soils. Conversely, *A. drummondii* appeared to have a modest positive effect on *A. bisulcatus* growth on both soils, but this was not significant. The neighbor effects between the *Astragalus* species with respect to Se accumulation varied. On non-seleniferous soil where Se levels were very low, *A. bisulcatus* plants growing next to *A. drummondii* contained 5-fold higher Se levels than *A. bisulcatus* plants growing next to *A. bisulcatus*; *A. drummondii* also contained elevated Se levels when growing next to *A. bisulcatus* compared to another *A. drummondii*. No such effects were seen on seleniferous soil, but on Turface® *A. drummondii* also appeared to stimulate Se accumulation in *A. bisulcatus* at all Se concentrations. The positive effect of *A. drummondii* on Se uptake by *A. bisulcatus* when external Se levels were low may be due to the release of Se chelators by *A. drummondii* roots, whose presence is suggested from the observation that *A. drummondii* exudate did not contain much Se but released more Se from soil than *A. bisulcatus*.

exudate. The presence of chelators would not explain the stimulatory effect of *A. drummondii* on Turface®, however, since the Se supply is not likely to be limiting there.

The two hyperaccumulators *A. bisulcatus* and *S. pinnata* appear to affect each other's growth and Se accumulation negatively on seleniferous soil, but positively on non-seleniferous soil. For comparison, the hyperaccumulators affected each other's S accumulation positively on seleniferous soil as well as on non-seleniferous soil. The reason for these differences is not clear, but may be due to differences in soil properties. The soils differed with respect to Se but also some other properties: the pH of the Cloudy Pass soil was 5.8 while that of Pine Ridge soil was 7.6. This may affect nutrient bioavailability. Soil organic matter was 11% in Pine Ridge soil but 6% in Cloudy Pass soil. This will affect nutrient concentration and bioavailability, as well as microbial communities. Iron levels were about 4-fold lower in Pine Ridge soil. Calcium was mainly present as calcium carbonate in Pine Ridge soil, but in another form in Cloudy Pass soil. In addition to affecting plants directly, soil properties may also affect plant-plant interactions, for instance by affecting the bioavailability of exuded compounds. This may explain some of the observed differences in plant-plant interactions between the two soils.

These studies have provided better insight into the role of Se in plant-plant interactions, particularly between hyperaccumulators and non-accumulators. Hyperaccumulator species perform better on seleniferous than non-seleniferous soil, and in general grow better with increasing Se supply. Non-accumulator species grow worse with increasing Se levels. It appears that both hyperaccumulators and non-accumulators can affect the growth and Se accumulation of neighboring plants. Roots of hyperaccumulators can exude significant levels of Se, mainly in organic form, which may lead to higher fractions of organic Se in non-accumulator neighbors. Non-accumulators on the other hand may be able to enhance soil Se bioavailability, and with that Se levels in their neighbors. These results are of significance since they give insight into how Se affects competition and facilitation between plants, and why hyperaccumulators are found almost exclusively on seleniferous soils.

Table 6: Soil properties (0–5 cm depth) at the study sites, Pine Ridge Natural Area and Cloudy Pass, Fort Collins, CO.

Sample ID	Pine Ridge	Cloudy Pass
Texture	Sandy Loam	Sandy Loam
pH	7.6	5.8
EC (mmhos cm ⁻¹)	0.3	0.2
SOM (%)	10.8	6.3
CaCO ₃ (%)	21.1	0.14
NO ₃ -N (mg kg ⁻¹)	5.1	5.2
Al (mg kg ⁻¹)	0.07	0.76
Ba (mg kg ⁻¹)	0.72	2.4
Ca (mg kg ⁻¹)	291	299
Cd (mg kg ⁻¹)	0.56	0.14
Cr (mg kg ⁻¹)	0.02	0.05
Cu (mg kg ⁻¹)	5.5	1.9
Fe (mg kg ⁻¹)	12	53.4
K (mg kg ⁻¹)	425	360
Mg (mg kg ⁻¹)	129	159
Mn (mg kg ⁻¹)	5.6	5.9
Mo (mg kg ⁻¹)	0.04	0.01
Na (mg kg ⁻¹)	22.7	22.7
Ni (mg kg ⁻¹)	1.2	0.50
P (mg kg ⁻¹)	12.4	11.8
Pb (mg kg ⁻¹)	2.6	1.6
S (mg kg ⁻¹)	18.6	13.6
Se (mg kg ⁻¹)	1.46	0.17
V (mg kg ⁻¹)	1.3	0.1
Zn (mg kg ⁻¹)	3	3

Table 7: Selenium speciation in plant leaf material and root exudates determined from XANES LSQ fitting. Plants were grown in pairs on Turface® (gravel) and supplied with selenate. SS: Sum of Squares; ND: not detectable. C-Se-C: MeSeCys/SeMet/SeCystathionine (indistinguishable). Forms of Se that were not detected in any of the samples and therefore not tabulated: red and gray Se⁰, Se-cysteine, Se-cystine, Se(GSH)₂.

	SS (x 10 ⁻⁴)	C-Se-C %	SeO ₄ ²⁻ %	SeO ₃ ²⁻ %
<i>A. drummondii</i> grown next to <i>A. drummondii</i>				
1	7.8	95.8	5.8	ND
2	10.9	96.0	5.9	ND
3	8.9	90.0	8.9	ND
Average ± SE		93.9 ± 1.9	6.8 ± 1.0	ND
<i>A. drummondii</i> grown next to <i>A. bisulcatus</i>				
1	5.1	94.9	6.2	ND
2	5.4	93.9	6.9	0.4
3	4.5	90.8	9.5	ND
Average ± SE		93.2 ± 1.0	7.5 ± 1.0	ND
<i>S. elata</i> grown next to <i>S. elata</i>				
1	5.1	88.7	9.8	ND
2	4.0	89.7	11.0	ND
3	4.7	88.3	11.9	ND
Average ± SE		88.9 ± 0.3	10.9 ± 0.6	ND
<i>S. elata</i> grown next to <i>S. pinnata</i>				
1	3.5	94.0	3.7	ND
2	4.0	95.4	3.5	ND
3	3.4	96.4	2.4	ND
Average ± SE		95.2 ± 0.6	3.2 ± 0.6	ND
<i>A. bisulcatus</i> root exudate				
1	25.6	70.7	ND	33.5
2	6.4	90.6	ND	11.9
3	11.6	96.7	ND	4.5
Average ± SE		86 ± 8.0	ND	16.6 ± 8.8

FIGURES

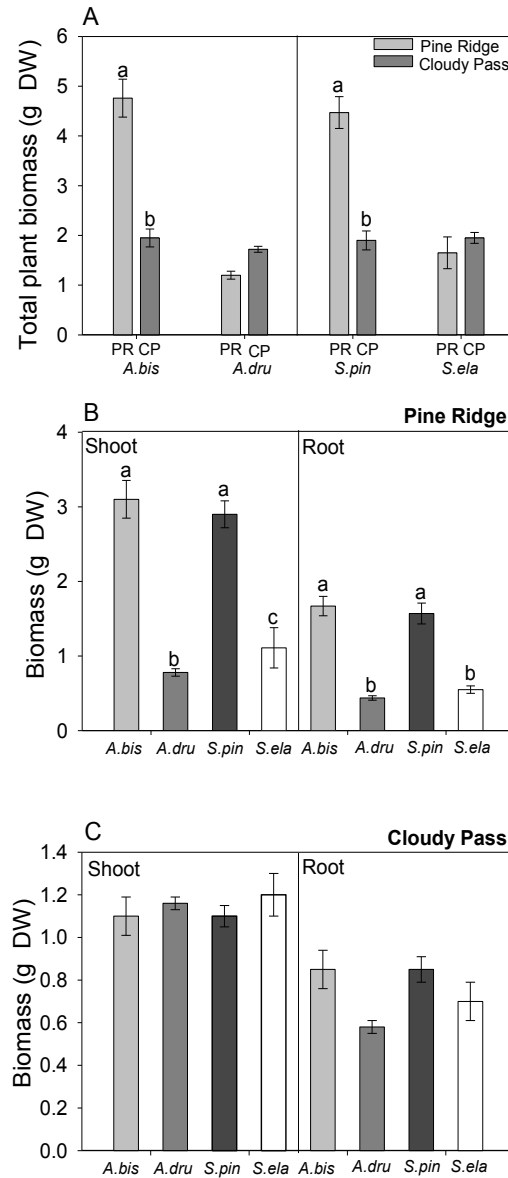


Figure 20: Comparison of (A) total plant biomass or (B,C) shoot and root biomass (g DW) between hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* and non-accumulators *A. drummondii* and *S. elata* grown in pots on seleniferous soil from Pine Ridge Natural Area (PR) or non-seleniferous soil from Cloudy Pass (CP). Two plants from the same species were grown per pot. Shown are the average and standard error of the mean (SEM) from six replicates. Different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

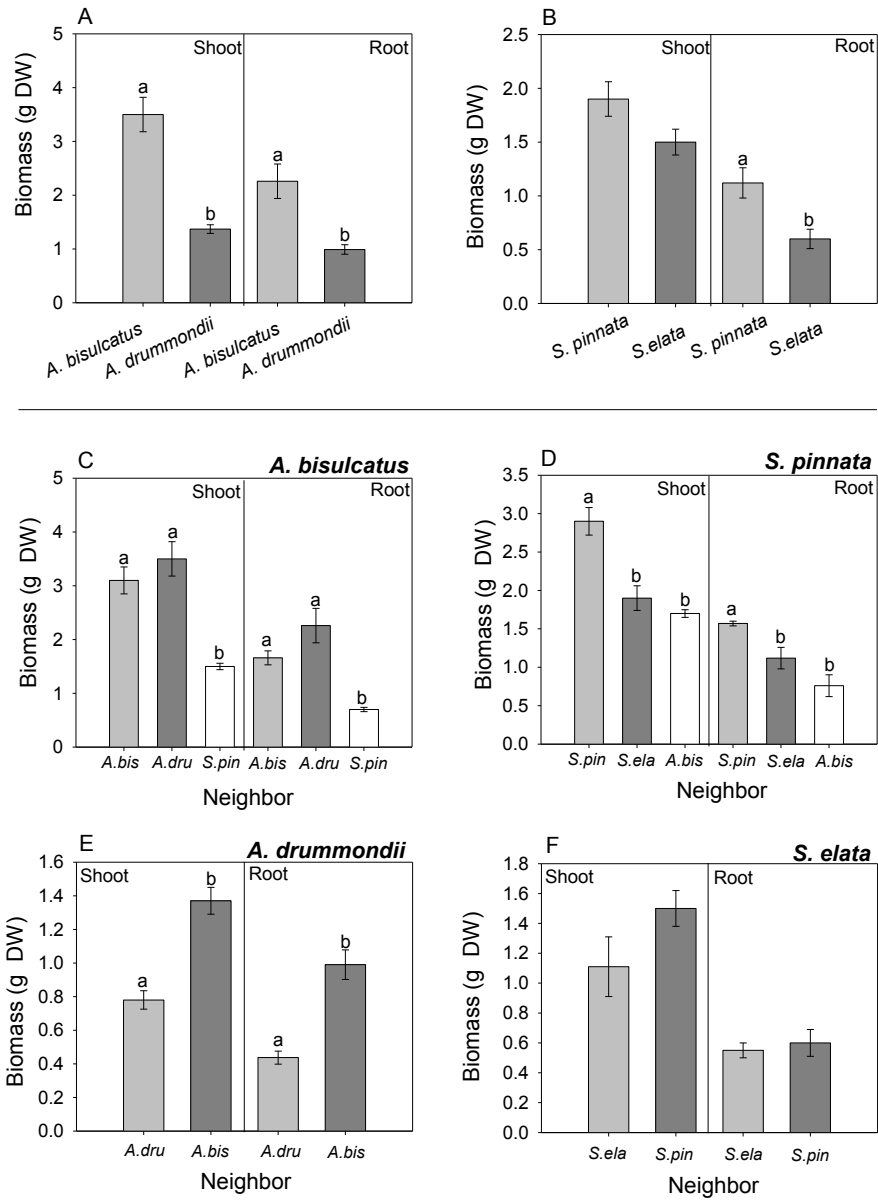


Figure 21: Shoot and root biomass (g DW) of hyperaccumulator plants *Astragalus bisulcatus* and *Stanleya pinnata* and non-accumulators *A. drummondii* and *S. elata* grown in pots on seleniferous soil from Pine Ridge Natural Area. Two plants from the same or different species were grown per pot. (A, B) Biomass of each of two neighbors that was co-cultivated in one pot. (C- F) Growth of each of the four species as influenced by which neighbor was in the same pot. Values shown represent means \pm SE (n= 6); different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

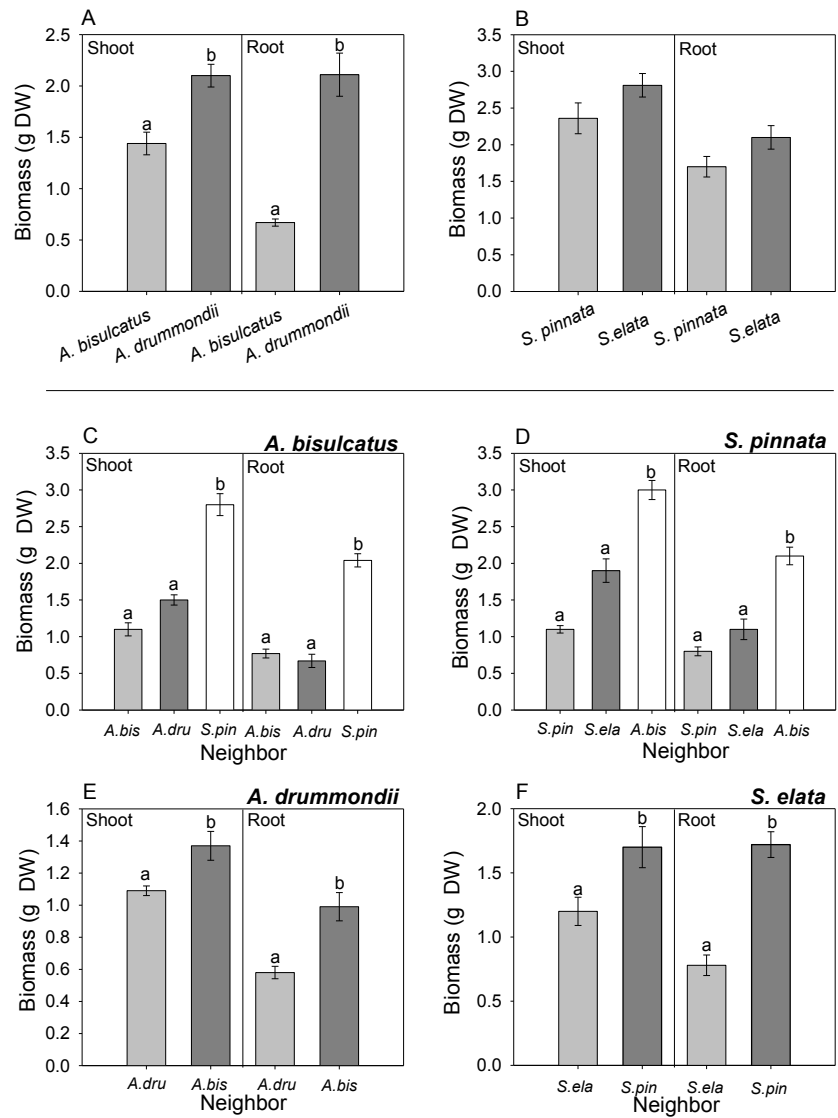


Figure 22: Shoot and root biomass (g DW) of hyperaccumulator plants *Astragalus bisulcatus* and *Stanleya pinnata* and non-accumulators *A. drummondii* and *S. elata* grown in pots on non-seleniferous soil from Cloudy Pass. Two plants from the same or different species were grown per pot. (A, B) Biomass of each of two neighbors that were co-cultivated in one pot. (C-F) Growth of each of the four species as influenced by which neighbor was in the same pot. Values shown represent means \pm SE ($n=6$); different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

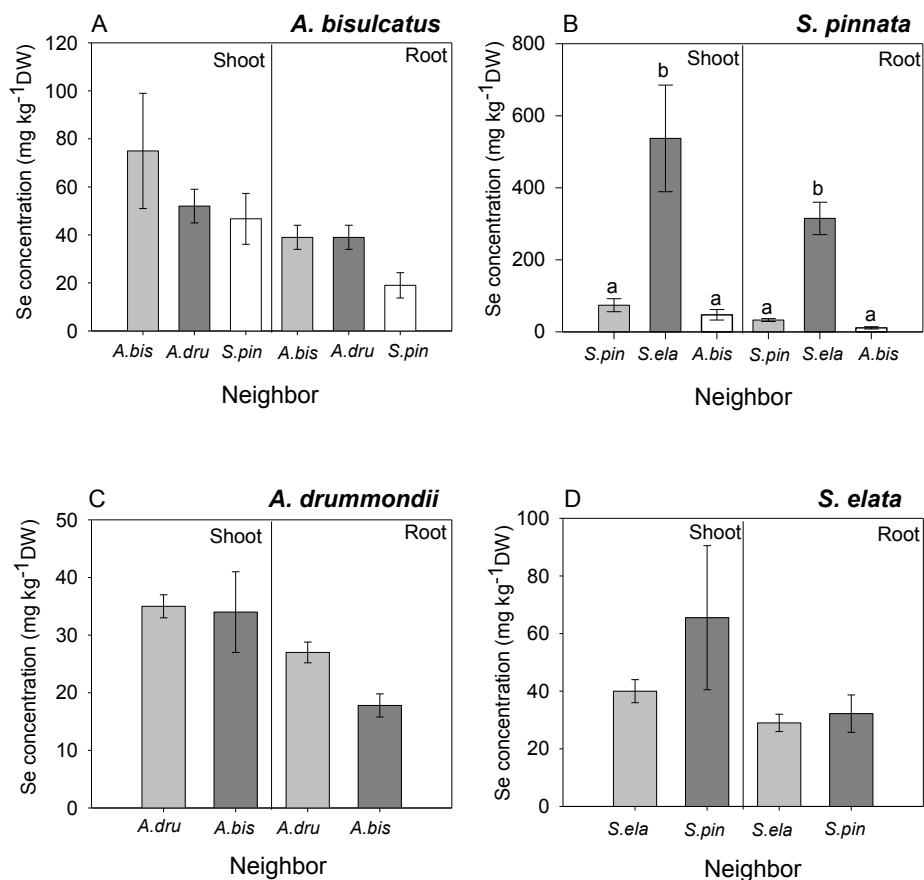


Figure 23: Selenium concentration ($\text{mg kg}^{-1} \text{DW}$) in shoot and root of hyperaccumulators *Astragalus bisulcatus* (A) and *Stanleya pinnata* (B) and non-accumulators *A. drummondii* (C) and *S. elata* (D) after being grown in pots on seleniferous soil from Pine Ridge Natural Area with either another plant from the same species or one from a different species as neighbor. Values shown represent means \pm SE ($n=6$), different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

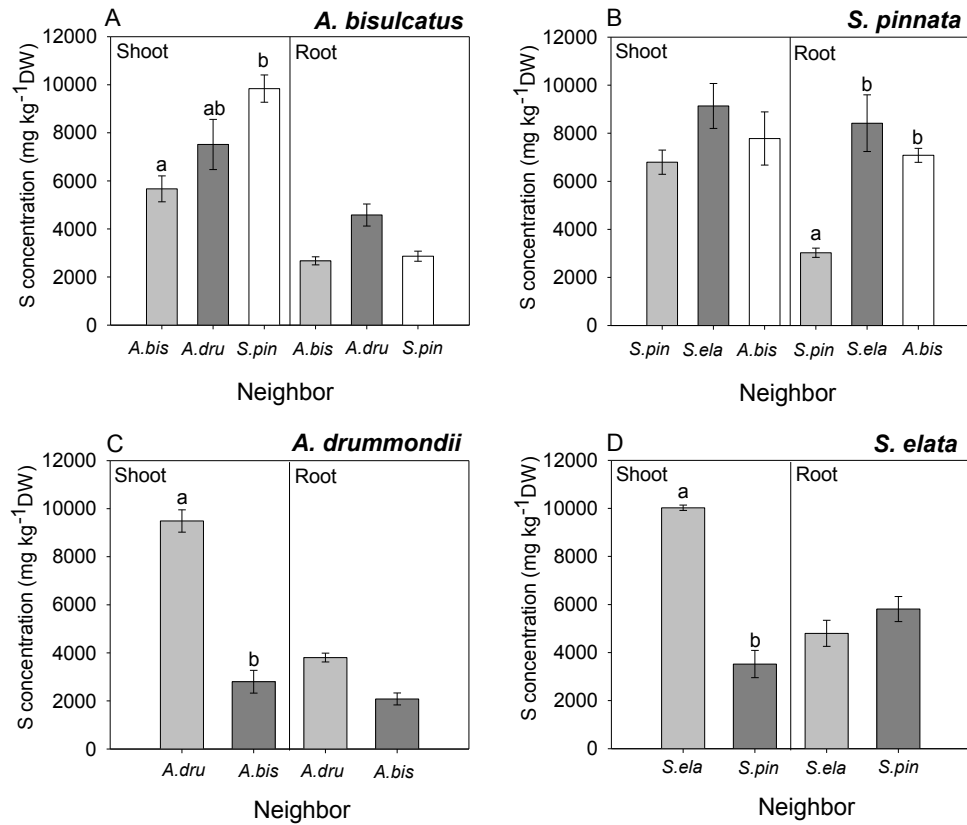


Figure 24: Sulfur concentration ($\text{mg kg}^{-1} \text{DW}$) in shoot and root of hyperaccumulators *Astragalus bisulcatus* (A) and *Stanleya pinnata* (B) and non-accumulators *A. drummondii* (C) and *S. elata* (D) after being grown in pots on seleniferous soil from Pine Ridge Natural Area with either another plant from the same species or one from a different species as neighbor. Values shown represent means \pm SE ($n=6$), different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

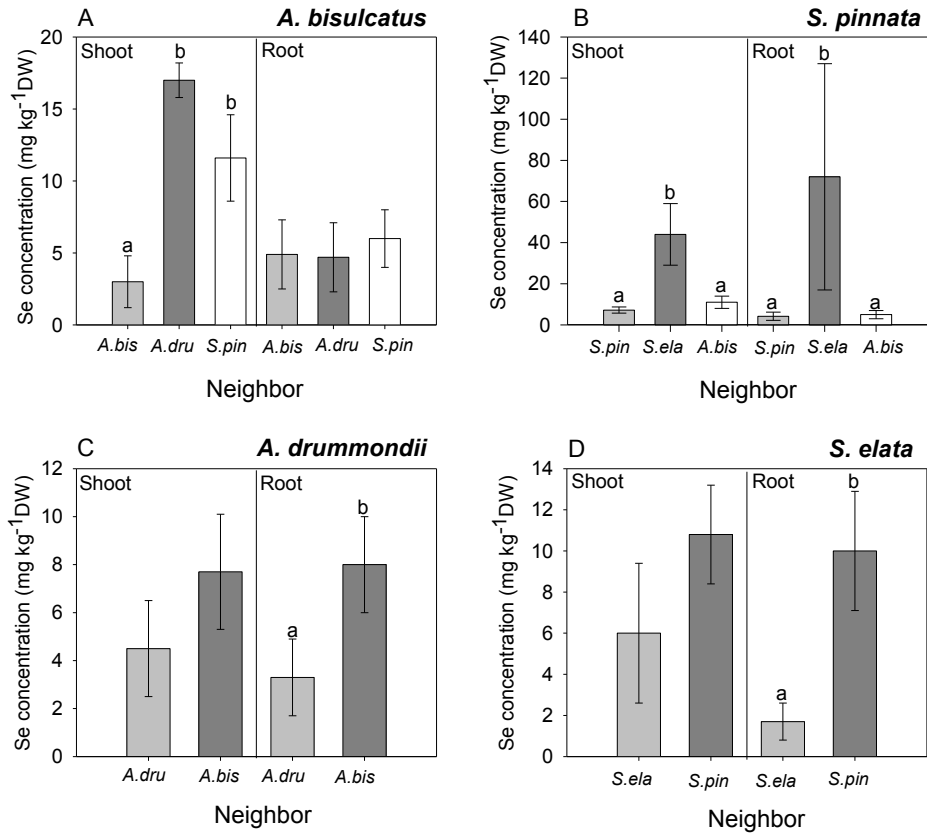


Figure 25: Selenium concentration (mg kg^{-1} DW) in shoot and root of hyperaccumulators *Astragalus bisulcatus* (A) and *Stanleya pinnata* (B) and non-accumulators *A. drummondii* (C) and *S. elata* (D) after being grown in pots on non-seleniferous soil from Cloudy Pass with either another plant from the same species or one from a different species as neighbor. Values shown represent means \pm SE ($n=6$), different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

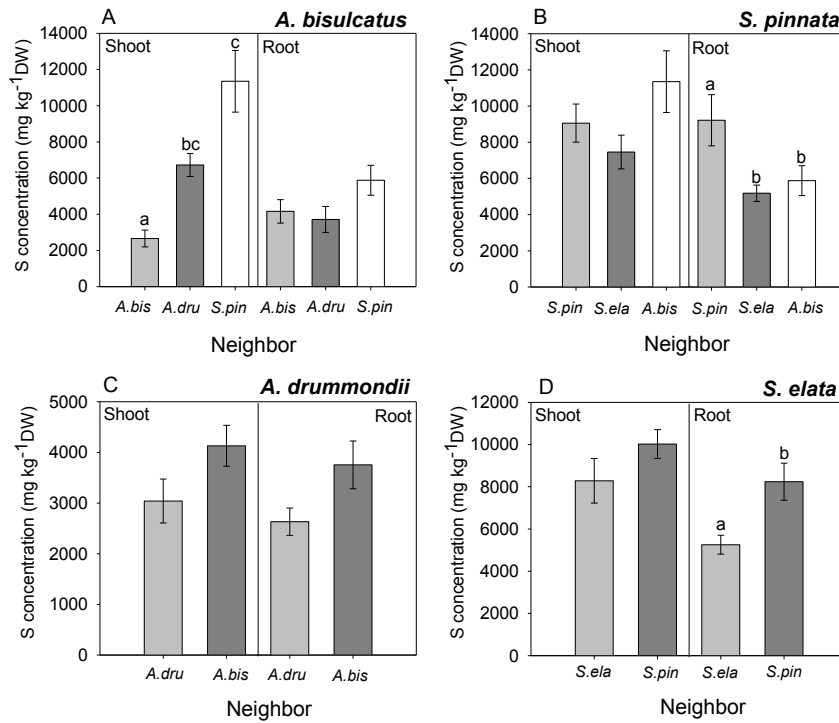


Figure 26: Sulfur concentration ($\text{mg kg}^{-1} \text{DW}$) in shoot and root of hyperaccumulators *Astragalus bisulcatus* (A) and *Stanleya pinnata* (B) and non-accumulators *A. drummondii* (C) and *S. elata* (D) after being grown in pots on non-seleniferous soil from Cloudy Pass with either another plant from the same species or one from a different species as neighbor. Values shown represent means \pm SE ($n=6$), different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

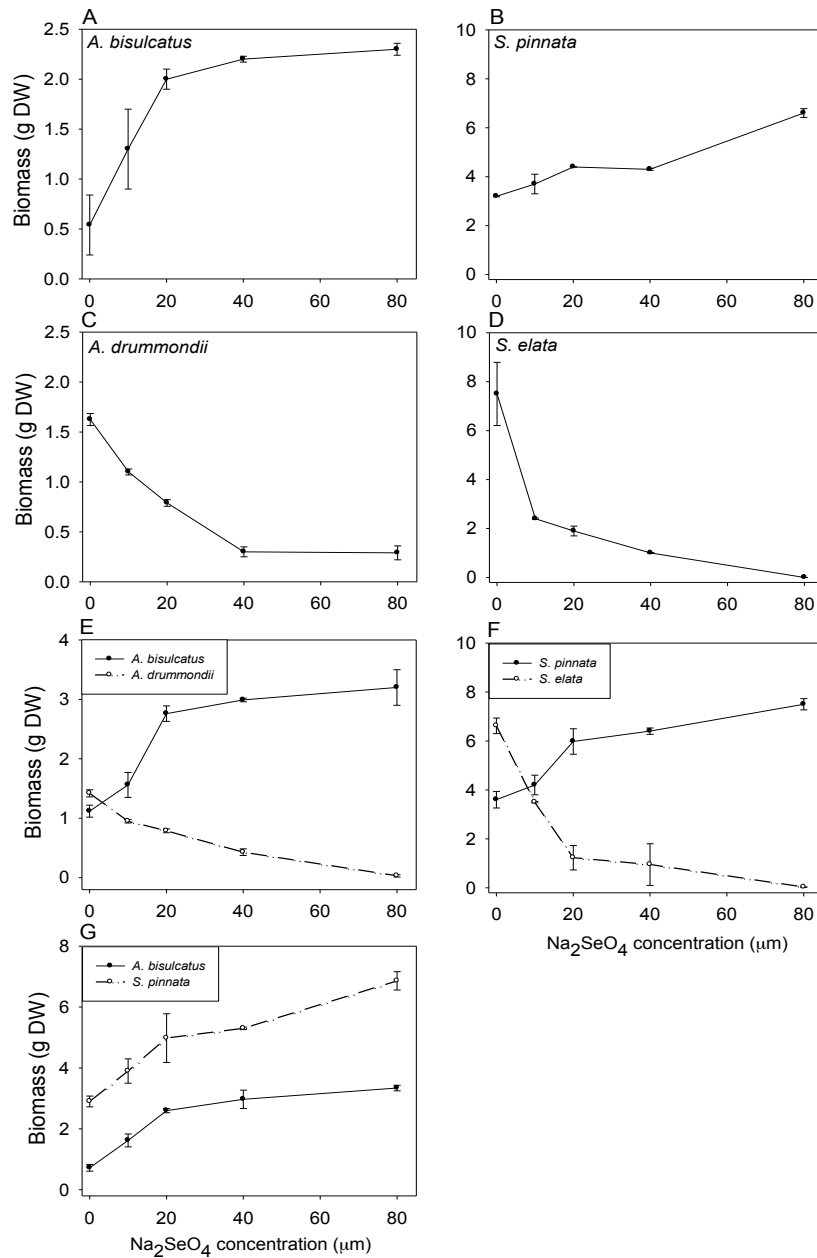


Figure 27: Total plant biomass (g DW) of hyperaccumulator plants *Astragalus bisulcatus* and *Stanleya pinnata* and non-accumulators *A. drummondii* and *S. elata* grown on Turface® growth medium and treated with different concentrations of Na₂SeO₄. (A-D) Two plants from the same species grown in one pot. (E-G) Two plants from different species grown in one pot. Values shown represent means ± SE (n= 5), different lower case letters above bars indicate significantly different means (ANOVA, α = 0.05).

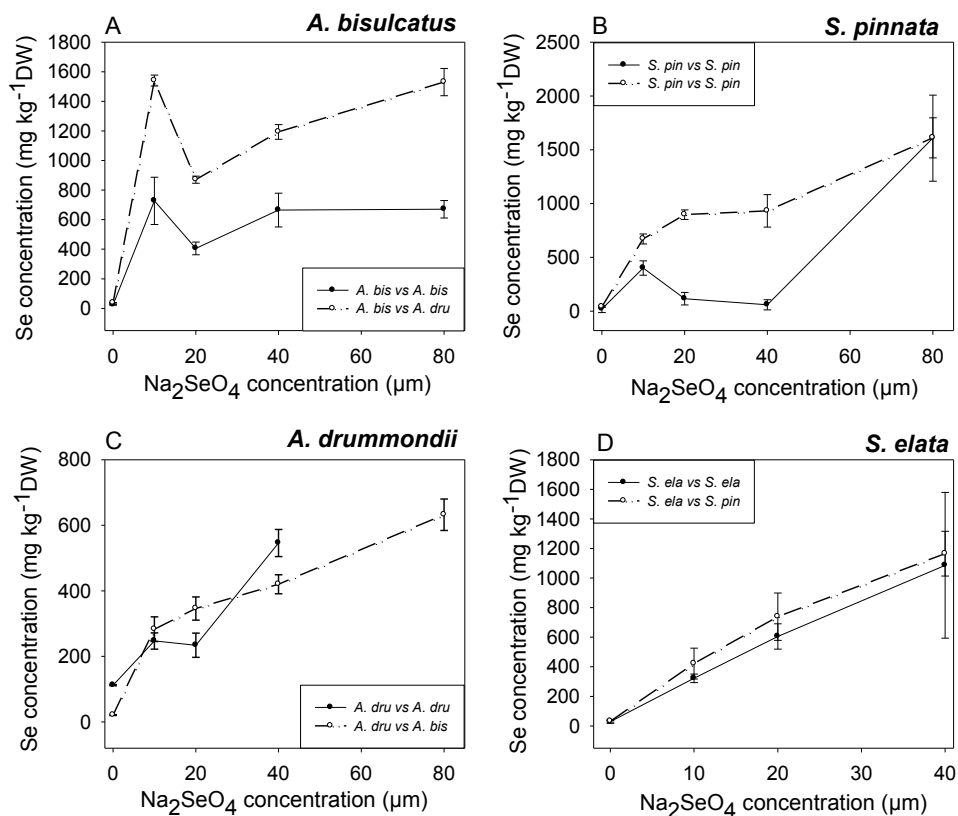


Figure 28: Shoot Se concentration (mg kg^{-1} DW) in hyperaccumulators *Astragalus bisulcatus* (A) and *Stanleya pinnata* (B) and non-accumulators *A. drummondii* (C) and *S. elata* (D) grown on Turface® growth medium supplied with different concentrations of Na_2SeO_4 . Two plants were grown per pot, either from the same or different species. Values shown represent means \pm SE ($n=5$), different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$). Note: In some cases no data are shown for the 80 μm treatment for the non-accumulators because there was not enough plant material.

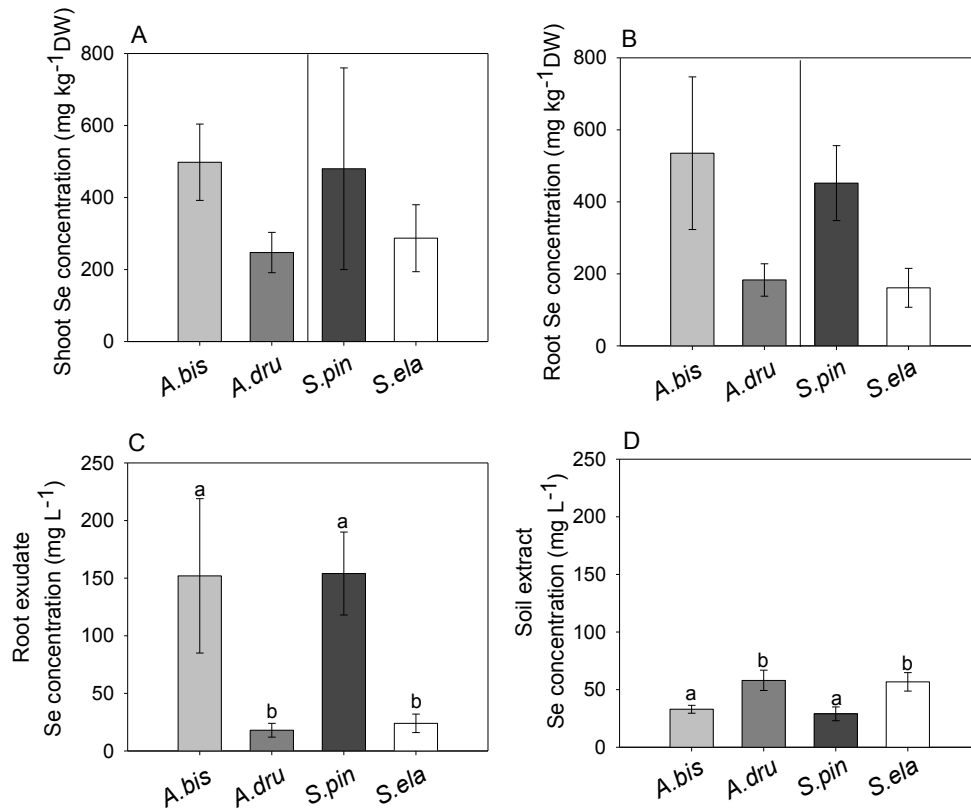


Figure 29: (A, B) Selenium concentration (mg kg⁻¹ DW) in shoot and root of hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* and non-accumulators *A. drummondii* and *S. elata* grown in pots on seleniferous soil from Pine Ridge Natural Area, used for collection of root exudate. (C, D) Se concentration in root exudate and in Pine Ridge soil extract obtained using this exudate. Values shown represent means \pm SE (n= 6), different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

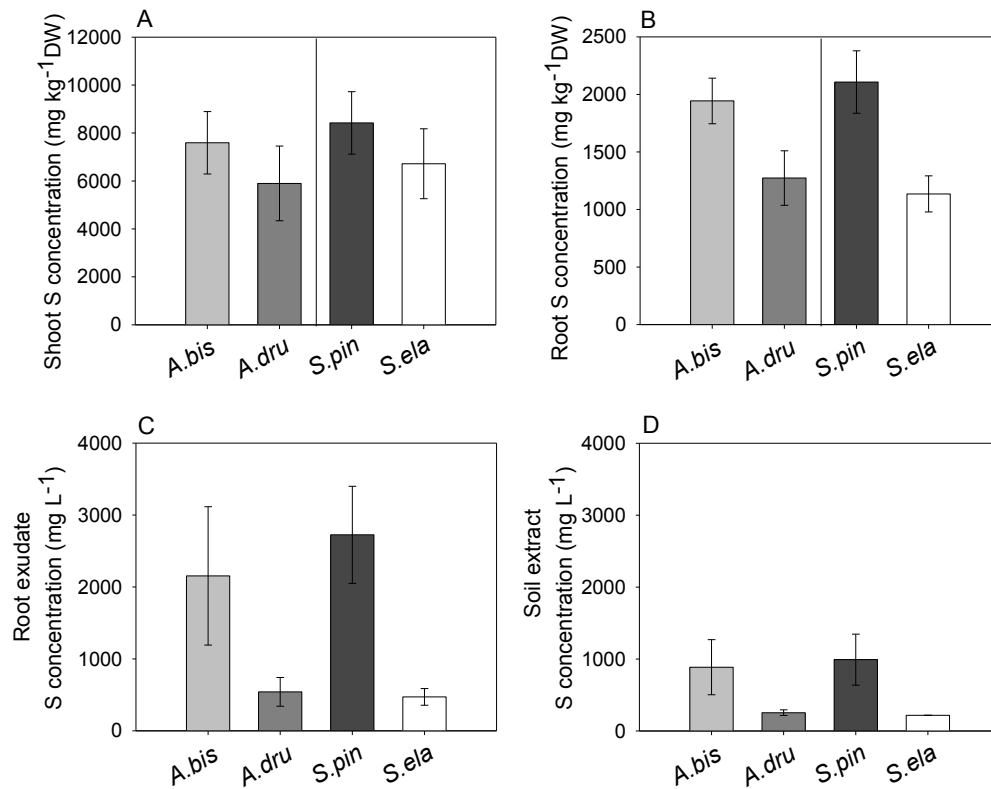


Figure 30: (A, B) Sulfur concentration (mg kg⁻¹ DW) in shoot and root of hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* and non-accumulators *A. drummondii* and *S. elata* grown in pots on seleniferous soil from Pine Ridge Natural Area, used for collection of root exudate. (C, D) Sulfur concentration in root exudate and in Pine Ridge soil extract obtained using this exudate. Values shown represent means \pm SE (n= 6), different lower case letters above bars indicate significantly different means (ANOVA, $\alpha = 0.05$).

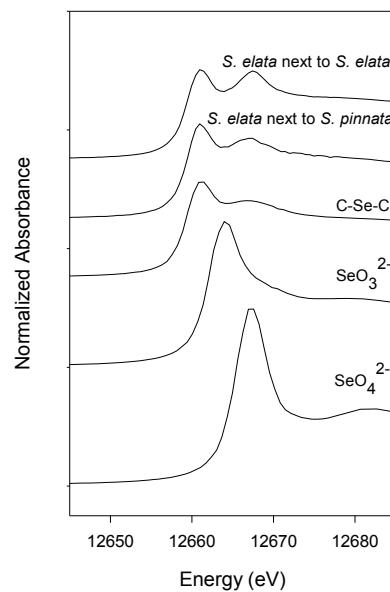


Figure 31: Selenium Se K-edge μ XANES spectra obtained from leaves of *S. elata* grown in Turface® (gravel) supplied with selenate. Top two spectra: *S. elata* grown next to another *S. elata*, and *S. elata* grown next to *S. pinnata*, respectively. Bottom three spectra: selenocompounds selenomethionine, selenite and selenate, respectively. Note: the SeMet spectrum was virtually indistinguishable from that of another C-Se-C compound, MeSeCys (not shown).

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CHAPTER 5:

SUMMARIZING DISCUSSION

Phytoenrichment is the process by which plants concentrate certain elements in their surrounding soil, and may be the result of deposition of litter, root exudation and turnover, or both (Morris *et al.* 2009). Since Se hyperaccumulators typically concentrate soil trace elements around 1,000 fold, and are perennials that yearly shed their leaves, they may be hypothesized to phytoenrich their surrounding soil. In support of this hypothesis, the Se concentration in soil surrounding hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* was 7-13 fold higher (up to 266 mg Se kg⁻¹) than Se in soil surrounding non-hyperaccumulators *M. sativa* and *H. pumilus* growing on the same site (El Mehdawi *et al.* 2011a,b). Moreover, the levels of Se were up to 20-fold higher in neighboring species *Artemisia ludoviciana* and *Symphyotrichum ericoides* when growing around hyperaccumulators than when growing >4 m away from Se hyperaccumulators (El Mehdawi *et al.* 2011a,b). These enhanced Se levels may be the result of phytoenrichment by the hyperaccumulator, although it cannot be excluded that soil Se distribution is simply heterogeneous and that Se hyperaccumulators are more abundant in Se “hot spots”.

Proximity to hyperaccumulators affects Se-sensitive and Se-tolerant neighbors differently

The high Se levels in Se hyperaccumulator plants and their associated soil may be hypothesized to have a negative effect on germination, growth and Se accumulation of Se-sensitive neighboring species. Indeed, soil collected around hyperaccumulators significantly reduced the germination and growth of Se-sensitive species *Arabidopsis thaliana*, and enhanced its Se levels compared to soil collected from non-hyperaccumulators (El Mehdawi *et al.* 2011a,b). This may point to elemental allelopathy, processes by which plants concentrate toxic elements as a means to outcompete their neighbors. Elemental allelopathy is one of the hypothesized functions of hyperaccumulation (Boyd and Martens 1992). If hyperaccumulation serves an allelopathic function, we would expect to find a difference between the plant community growing around Se hyperaccumulators compared with non-hyperaccumulators on the same site. The vegetative

ground cover was ~10% less and species diversity slightly lower around Se hyperaccumulator species *A. bisulcatus* and *S. pinnata* than around nonaccumulators (El Mehdawi *et al.* 2011a,b). Thus, it is feasible that Se hyperaccumulators benefit from their accumulated Se through decreased competition from surrounding vegetation; they may also affect the species composition in this vegetation.

Interestingly, some neighbors such as the abovementioned *A. ludoviciana* and *S. ericoides*, appear to thrive next to hyperaccumulators, despite or perhaps even owing to their elevated Se levels (El Mehdawi *et al.* 2011b). Both *A. ludoviciana* and *S. ericoides* were 2-fold larger, harbored fewer arthropods and showed less herbivory damage when growing in proximity (<1 m) to hyperaccumulators, as compared to plants of the same species growing away (>4 m) from hyperaccumulators. The Se-enriched neighbors of hyperaccumulators, which contained over 1,000 mg Se kg⁻¹ DW, were used in controlled herbivory studies in comparison with their low-Se counterparts collected next to non-accumulators. In choice experiments, grasshoppers collected from the same site preferred to feed on the low-Se *A. ludoviciana* and *S. ericoides* plants, and when given no choice the grasshoppers showed high Se accumulation and mortality after feeding on the high-Se plants. Therefore, the Se phytoenrichment associated with growing next to Se hyperaccumulators appears to facilitate Se-tolerant neighbors, via reduced herbivory (El Mehdawi *et al.* 2011a,b). In addition to this ecological benefit, the *A. ludoviciana* and *S. ericoides* plants may also experience a physiological benefit from their hyperaccumulator-mediated Se enrichment. Selenium has been shown to enhance growth for a variety of higher plant species (Pilon-Smits *et al.* 2009). Indeed, growth of *S. ericoides* also responded favorably to selenate treatment in herbivore-free greenhouse experiments, similar to hyperaccumulators *A. bisulcatus* and *S. pinnata* (El Mehdawi *et al.*, 2012 and unpublished results). Therefore, it appears that Se hyperaccumulators facilitate their Se-tolerant neighbors *A. ludoviciana* and *S. ericoides* by promoting their growth and decreasing their herbivory, both due to enhanced Se supply. It is interesting that the same process of Se phytoenrichment by hyperaccumulators can have either a negative or positive effect on the neighboring plants, depending on whether the neighbor is sensitive or tolerant to Se.

The patterns observed for the effects of plant Se on plant-plant interactions are similar to those observed for other types of ecological interactions. Selenium hyperaccumulators show a general trend to have a negative effect on Se-sensitive ecological partners, while offering a niche for, and potentially even facilitating Se-tolerant partners. This has now been found for herbivores, plants, microbes, and perhaps also pollinators. Selenium in or around hyperaccumulators deters or is toxic to Se-sensitive neighboring organisms, but may benefit Se-tolerant organisms via enhanced growth or stress resistance. Through these negative and positive effects, Se hyperaccumulators may affect the plant, microbial and animal species composition and species richness in the area that is under their influence. Selenium hyperaccumulators may even be keystone species, if they indeed profoundly affect their local ecosystem, favoring Se tolerant species at different trophic levels. It appears that specialized Se-tolerant herbivores, detritivores, microbial symbionts, and perhaps also pollinators have evolved to live in symbiosis with Se hyperaccumulators. Via their tendency to locally concentrate Se, change its speciation and then disperse it to its ecological partners the Se hyperaccumulators likely play an important role in the Se cycling through seleniferous ecosystems.

Implications for the evolution of hyperaccumulators and their ecological partners

Selenium hyperaccumulation has evolved independently in different plant lineages. The selection pressures that have driven this evolution may be one or more, and may be different or the same in different genera. Selenium hyperaccumulators are found predominantly or even exclusively on seleniferous soil, suggesting they cannot survive or effectively compete without sufficient Se. This Se dependency may have a physiological and/or ecological basis. So far, Se hyperaccumulation has been shown to benefit plants via enhanced growth (physiological benefit), allelopathy of Se-sensitive neighboring plant species (ecological benefit), and protection from Se-sensitive herbivores and pathogens (ecological benefit). Any or all of these benefits may have acted as selection pressures at any given time in the different plant lineages. Some of these benefits may act already at low Se levels, such as growth promotion and herbivory protection, while other benefits may not become significant until higher levels are achieved. Among non-Se accumulator species (such as *A. thaliana*), plants that naturally contain somewhat elevated Se levels, e.g. because they

have high levels of sulfate/selenate transporters, may have been the evolutionary starting point for the evolution of Se accumulators (such as *B. juncea*), which may have subsequently evolved into true hyperaccumulators (such as *S. pinnata*). For this later transition, Se hypertolerance must have evolved with Se hyperaccumulation, since hyperaccumulator Se levels (>0.1% of DW) impair growth and reproductive functions in non-hyperaccumulators (Prins *et al.* 2011). So far there is no evidence of an evolutionary cost of Se hyperaccumulation in Se hyperaccumulators: overall plant growth is not impaired but rather promoted by Se, reproductive functions are not impaired, and high-Se plants are not avoided by pollinators (Quinn *et al.* 2011a). Specialized Se-tolerant symbionts appear to have evolved that fulfill beneficial functions for hyperaccumulators: there is evidence of Se-tolerant nitrogen-fixing Rhizobia, Se-tolerant endophytic and rhizospheric microbes and litter decomposers, and Se-tolerant pollinators (Quinn *et al.* 2010, 2011; Lindblom *et al.* 2012; Valdez-Barilla *et al.* 2012).

Selenium hyperaccumulators appear to promote the evolution of Se-tolerant symbionts, as evidenced at the microbial, animal and plant level. The toxic Se levels associated with hyperaccumulators select against Se-sensitive individuals and favor Se-tolerant ones. Thus, ecological partners of Se hyperaccumulators may co-evolve with their host/neighbor. Evolution of Se tolerance in these partners not only enable them to live in the Se-rich environment created by the hyperaccumulator, but may even benefit in the form of better growth or stress resistance, as was observed for Se-tolerant neighboring plants. Some neighbors of Se hyperaccumulators even reach hyperaccumulator Se levels themselves (>0.1% of DW), but only when growing next to hyperaccumulators. They benefit from their higher Se levels due to reduced herbivory and perhaps also a physiological growth response to the Se. It is an interesting thought that hyperaccumulators may in this way facilitate the evolution of hypertolerance and hyperaccumulation in neighboring plant species, as hyperaccumulators promote the reproductive success of those individuals among their neighbors that accumulate and tolerate Se best.

Implications for managing seleniferous habitats and applications in agriculture and phytoremediation

Selenium is both an essential trace element and a toxin, dependent on the concentration. Selenium deficiency and toxicity are both problems worldwide. Plants may be used both to remove Se from natural or polluted Se-rich areas and as a food source to alleviate Se deficiency in humans or animals. The first process is called phytoremediation, the second biofortification. The two may even be combined: plants that have accumulated Se from polluted soil may be used as fortified food. To optimize these technologies and avoid environmental problems, it is important to have better knowledge of the ecological implications of growing Se-rich plants. For example, if Se in flowers negatively affects honey bee health this may have serious consequences for honey bee populations and agricultural productivity. It is also possible that Se positively affects bee health, e.g. protecting the bees from pathogens. Since Se is toxic to generalist herbivores and pathogens, Se-rich crops may have reduced need for pesticides and fungicides, and higher overall productivity. If Se hyperaccumulators enhance Se levels in neighboring plants, this may be utilized in the form of co-cropping or intercropping. The Se-tolerant microbes that are found in association with Se hyperaccumulators may perhaps be utilized for bio- or phytoremediation, by themselves or in concert with plants. If Se-rich plants form a portal for Se into the ecosystem, and perhaps also change the form of Se in the environment, this may have complex ecological implications as well. When using Se accumulating plants in agriculture or environmental restoration we have to consider these various positive and negative ecological implications and incorporate them into the management plan.

Prospects

The same ecological and evolutionary implications observed for Se hyperaccumulation may serve as a model for other hyperaccumulated elements such as As, Ni, Cd and Zn. These elements have already been shown to protect hyperaccumulators from herbivores, and there may be other parallels with the effects of Se. As for Se hyperaccumulator ecology, it will be interesting to look in more detail at ecosystem-wide effects such as the effects of the hyperaccumulators on species composition at different trophic levels, and

Se cycling through the food chain and ecosystem. In future studies it will also be interesting to investigate rhizosphere processes in more detail, such as the mechanisms of phytoenrichment, root-root interactions, and interactions with rhizosphere and endophytic microbes. From an applied perspective, future research may focus on the potential effects of plant Se on herbivory, pollination and pollinator health in the context of biofortification and phytoremediation. In addition, different co-cropping practices may be explored between hyperaccumulators and crops. If hyperaccumulators should not only increase their neighbor's Se concentration but also change the form of Se they accumulate to more organic Se, this could be beneficial for biofortification since organic Se has higher nutritional value. Finally, Se-tolerant microbes isolated from Se hyperaccumulators may be investigated for their capacity to affect plant growth, Se accumulation and speciation.

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