DISSERTATION

ECOLOGICAL INTERACTIONS INVOLVING PLANT SELENIUM HYPERACCUMULATION

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2010

COLORADO STATE UNIVERISTY

June 29, 2010

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY COLIN FRANCIS QUINN ENTITLED ECOLOGICAL INTERACTIONS INVOLVING PLANT SELENIUM HYPERACCUMULATION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ECOLOGICAL INTERACTIONS INVOLVING PLANT SELENIUM HYPERACCUMULATION

Selenium hyperaccumulation is the phenomenon where plant species accumulate Se to concentrations multiple orders of magnitude higher than other species on the same site. Selenium hyperaccumulating species, found in the Asteraceae, Brassicaceae and Fabaceae families, can accumulate this potentially toxic element up to 1% of their dry weight. Selenium is not known to be an essential element for plants, and the functional significance of Se hyperaccumulation has long been unclear. In this dissertation four studies are presented that give insight into the functional significance of Se hyperaccumulation through investigating the interactions between Se hyperaccumulating plants and their ecological partners. The objectives of this dissertation are to: 1) determine if Se protects hyperaccumulating plants from cell disruptor herbivory, 2) determine if Se protects *S. pinnata* in its natural habitat from prairie dog herbivory, 3) investigate the effect of Se accumulation on pollination and reproductive fitness and 4) determine if Se effects leaf litter decomposition.

Results presented in this dissertation support the elemental defense hypothesis, which states that hyperaccumulation functions as an elemental defense against herbivores

and pathogens. The first study investigates the protective effect of elevated Se in two Se hyperaccumulating species, *Astragalus bisulcatus* (two-grooved milkvetch) and *Stanleya pinnata* (prince's plume), against two cell-disrupting herbivores, the two-spotted spider mite (*Tetranychus urticae)* and the western flower thrips (*Frankliniella occidentalis)*. Both herbivores preferred to feed on *A. bisulcatus* and *S. pinnata* containing less than 150 mg Se kg^{-1} instead of plants with Se concentrations of at least 650 mg Se kg^{-1}. Furthermore, within high-Se plants, these herbivores preferred to feed on older leaves, which contain lower concentrations of Se than younger leaves.

The second study is a 2-year manipulative field study to determine if Se protects *S. pinnata* from a mammalian herbivore, the black-tailed prairie dog (*Cynomys ludovicianus*). This long-term field study, the first of its kind, found that *S. pinnata* with elevated Se suffered less herbivory than *S. pinnata* with trace concentrations of Se, and that high-Se plants had higher survival rates than low-Se plants. Since prairie dogs, a keystone herbivore species, and several Se hyperaccumulating plant species are native to the same region this study gives insight into possible selection pressures leading to Se hyperaccumulation.

The third study compares Se distribution, concentration and speciation between the Se hyperaccumulator *S. pinnata* and the related non-Se hyperaccumulator *Brassica juncea*, which is an important crop species and is considered a secondary Se accumulator. Results of this study revealed that that *S. pinnata* preferentially allocates Se to flowers and that within flowers Se was concentrated in pollen and ovules. In contrast, *B. juncea* had higher Se concentrations in leaves than flowers and within flowers Se was diffusely distributed. These results suggest that *S. pinnata* is distributing Se to its most valuable

plant parts, possibly as a defense against herbivores or pathogens. We also investigated whether there are costs associated with Se accumulation, by determining pollen germination of high-Se and low-Se *S. pinnata* and *B. juncea* plants, and by determining whether increased Se levels in plants deter pollinators, specifically honey bees, from visiting *B. juncea*. Our results showed no difference in pollen germination rates between high-Se and low-Se *S. pinnata*; however, pollen from high-Se *B. juncea* plants germinated at a 2-fold lower rate than pollen from low-Se *B. juncea* plants. We found no difference in visits by honey bees or other pollinators, between *B. juncea* with flowers containing 271 mg Se kg^{-1} or 7 mg Se kg^{-1}. These results provide insight into possible costs, such as decreased pollen germination, associated with increased plant Se concentrations in non-hyperaccumulators and accumulators. In addition, these results demonstrate that it is unlikely that honey bees will avoid flowers with Se concentrations as high as 220 mg Se kg^{-1} which suggests Se-rich plants will be pollinated by the economically important honey bee.

In the final experimental chapter the effect of Se hyperaccumulation on leaf litter decomposition was investigated. Litter decomposition rates from two populations of *A. bisulcatus*, one with 350 mg Se kg^{-1} and the other with 550 mg Se kg^{-1}, were compared over a 12-month period to litter decomposition rates of the related *Astragalus drummondii* (Drummond's milkvetch) and *Medicago sativa* (alfalfa), each of which contained approximately 1-2 mg Se kg^{-1}. In addition, the decomposing community on each type of litter was compared. We found that the Se hyperaccumulator litter decomposed faster than litter from the non-Se hyperaccumulators in a seleniferous habitat and supported more micro-arthropods and microbes. A possible explanation for these

results is that decomposers/detrivores have evolved Se tolerance and prefer to feed on Se rich material, leading to faster decomposition of high-Se leaf litter.

The last chapter of this dissertation is a summarizing discussion of all studies to date on ecological aspects of plant Se accumulation, with a particular focus on their implications for the cultivation of Se-rich plants for phytoremediation and biofortification with a particular focus on plant-herbivore, plant-pollinator and plant-decomposer interactions as well as the role plants play in distribution of Se in the soil.

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Acknowledgements

I want to express my sincere thanks to Dr. Elizabeth Pilon-Smits for the opportunity to do this work and her assistance and guidance throughout. She has been an excellent mentor and advisor and this work could not have been completed without her assistance. I also want to thank Dr. Mark Paschke, Dr. Arathi Seshadri and Dr. David Steingraeber for serving on my committee and their assistance and insightful discussion and contributions throughout. All three of them have contributed to my growth as a scientist and provide excellent role models. I would also like to thank my many co-authors and collaborators who have assisted in this dissertation. In particular I want to thank Dr. John Freeman for collaborating with the first two chapters. Dr. Freeman is/will be a co-first author on any manuscripts that are published from those studies. I am grateful to the individuals who assisted in my experiments including Erin Quinn, Jeremy Shulman, Amanda Gross, Ray Reynolds, Stormy Lindblom, Karl Wyant and Miriam Galeas. I am also grateful to Dr. Marinus Pilon for his friendship, scientific discussion and the opportunity to work in his lab. I also want to thank all the members of both the Pilon and Pilon-Smits labs for their encouragement and providing an excellent working environment.

Lastly, I would like to thank my wife, Erin Quinn, who has been supportive, loving and helpful throughout this process.

TABLE OF CONTENTS

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Quinn CF, Freeman JL, Reynolds RJB, Cappa JJ, Fakra SC, Marcus MA, Lindblom SD, Quinn EK, Bennett LE and Pilon Smits EAH. 2010. Selenium hyperaccumulation offers protection from cell disruptor herbivores. Submitted to BMC Ecology.

Quinn CF, Wyant K, Wangeline AL, Shulman J, Galeas ML, Valdez JR, Self, JR, Paschke MW, and Pilon-Smits EAH. 2010. Enhanced decomposition of selenium hyperaccumulator litter in a seleniferous habitat – evidence for specialist decomposers? Plant and Soil, in press

Quinn CF, Lindblom SD, Pilon-Smits EAH. 2010. Ecological aspects of selenium phytoremediation. In: "Phytoremediation: Processes, Characteristics, and Applications." Nova Publishers. In press **Chapter 6 in this dissertation**

Pilon-Smits EAH, **Quinn CF**. 2010. Selenium Metabolism in Plants. In: "Cell Biology of Metal and Nutrients", Hell R, Mendel R, Springer, New York.

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Freeman JL*, **Quinn CF***, Lindblom SD, Klamper EM, Pilon-Smits EAH 2009. Selenium protects the hyperaccumulator Stanleya pinnata against black-tailed prairie dog herbivory in native seleniferous habitats. American Journal of Botany 96: 1075-1085 **Chapter 2 in this dissertation**
*shared first authors

Quinn CF, Freeman JL, Galeas ML, Klamper EM, Pilon-Smits EAH. 2008. Selenium protects plants from prairie dog herbivory – implications for the evolution of selenium hyperaccumulation. Oecologia 155:267-275 **Published in authors masters thesis**

Galeas ML, Klamper EK, Bennet L, **Quinn CF**, Freeman JL, Pilon-Smits EAH. 2008. Selenium hyperaccumulation reduces plant arthropod loads in the field. 177: 715-724.

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Freeman JL*, **Quinn CF***, Marcus M, Fakra S. Pilon-Smits EAH. 2006. Selenium tolerant diamondback moth disarms hyperaccumulator plant defense. Current Biology 16:2181-2192 **Published in authors masters thesis**
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Chapter 1

INTRODUCTION

Hyperaccumulation is the intriguing phenomenon where plants accumulate certain elements to levels several orders of magnitude higher than other plants growing on the same substrate. Elements that have been reported to be hyperaccumulated by plants include zinc (Zn), cadmium (Cd), copper (Cu), manganese (Mn), arsenic (As), aluminum (Al), lead (Pb) and selenium (Se) (Reeves and Baker 2000). Selenium hyperaccumulators have been defined as plants that accumulate more than 1,000 mg Se kg^{-1} in natural habitats (Reeves and Baker 2000); some hyperaccumulating plants have been reported to contain over 10,000 mg Se kg^{-1} (Galeas et al. 2007). Plant species that do not hyperaccumulate Se are classified into accumulators (containing levels of 100-1000 mg Se kg^{-1} in natural habitats) and non-accumulators (containing levels <100 mg Se kg^{-1}).

Selenium, a metalloid which is chemically similar to sulfur (S), is an essential trace element for many organisms, including most animals and bacteria, but is toxic at elevated concentrations. Selenium readily gets incorporated into the amino acid selenocysteine (SeCys). In mammals and other organisms that require Se, SeCys is present in the active site of selenoproteins such as the enzyme glutathione peroxidase. Glutathione peroxidase is an antioxidant enzyme responsible for preventing cell damage

by scavenging for free radicals (Steinbrenner and Sies 1990; Dickinson and Forman 2002). Recommended daily intake of Se for a healthy human adult is 55 μg Se per day (Institute of Medicine 2000). Selenium can be obtained either through Se rich food and water or through nutritional supplements. Some Se-rich foods include tuna and Brazil nuts, the latter of which may contain such high Se levels that overconsumption should be avoided to prevent Se poisoning (Cabañero et al. 2005; Parekh et al. 2008). A diet enriched in Se has been shown to have many beneficial health effects. Selenium prevents the onset of many types of cancer and heart disease and is known to have anti-aging capabilities (Goldhaber 2003; Shin et al. 2007; Anne-Marie and Tasnime 2007). Soils in many areas worldwide are low in Se, including parts of the USA, Russia and northern China, and in these areas Se deficiency poses problem in humans and livestock. Selenium deficiency in humans leads to a weak immune system and is known to cause Keshan disease, which results in an enlarged heart, and Kashin-Beck disease, which causes joint deformity (Chen et al. 1980, Hoffmann and Berry 2008, Li et al. 2009).

On the other hand, Se is toxic at elevated concentrations. Chronic consumption of food with elevated Se levels (> 1mg kg^{-1} DW) can lead to selenosis, which results in loss of hair, nails and teeth and can eventually lead to death (Haggerty and Curtis 2009). Many historians believe that General George Custer's pack horses were suffering from selenosis during 1876, possibly contributing to his defeat at the Battle of Little Bighorn (Hintz and Thompson 2000). Acute Se poisoning from a one-time ingestion of extremely elevated concentrations of Se can lead to death (Spiller and Pfiefer 2007). Ingestion of Se by livestock in the United States has been reported to result in an economic damage of over $330 million annually (Wilbur 1980).

Selenium is naturally occurring in Cretaceous shale, which is present in areas such as the Western United States, where oceans were present during the Cretaceous period around 65-145 million years ago (Kulp and Pratt 2004). Human activity, like some agricultural and mining practices as well as burning Se-rich fossil fuels, can promote the release of Se into waters and sediments, resulting in Se pollution (Kharaka et al. 1996; Lui et al. 2007). Disposal of Se-rich waste water (up to 200 μg Se L^{-1}) into Belews Lake in North Carolina, USA, was responsible for the elimination of 19 fish species from the lake in the early 1980s (Lemly 1998). Similarly, Se-rich drainage water was responsible for the well-known environmental tragedy in the early 1980s at Kesterson Reservoir in California, USA (Ohlendorf et al. 1990). The primary form of Se in soils is selenate (SeO_4^{2-}), but in anoxic environments Se is often found as selenite (SeO_3^{2-}); both forms are bioavailable to plants.

For plants, Se is not known to be an essential element, although some plants preferentially take up Se and Se has been reported to promote plant growth. For most plants, Se is toxic at elevated concentrations (Anderson 1993). Selenium toxicity in plants is associated with decreased growth and seed germination (Pezzarossa et al. 2009). The toxicity of Se is due to its similarity to S. Most plant species cannot distinguish the difference between Se and S and inadvertently take up Se and incorporate it into proteins, which results in a loss of protein function and overall toxicity (Stadtman, 1990; Smith et al. 1995; Terry et al 2000). Plants readily take up selenate and selenite via S transporters and incorporate it into organic forms of Se, primarily SeCys and Se-methionine. Plants can also volatilize Se in the form of dimethylselenide (DMeSe) (Zayed et al. 1998; de Souza et al. 2002).

Selenium hyperaccumulating plants can tolerate such high concentrations of Se because they methylate SeCys to form methylselenocysteine (MeSeCys), which is relatively non-toxic because it is not incorporated into proteins. Hyperaccumulating plants also volatilize Se, as dimethyldiselenide (DMeDSe) (Evans et al. 1968), which gives off a distinct odor making Se hyperaccumulating plants easy to identify. Hyperaccumulating species usually grow only on seleniferous soils, and are used as indicator plants to identify seleniferous habitats.

The functional significance of hyperaccumulation is an important focus of the research described in this dissertation. Five possible reasons for hyperaccumulation have been proposed by Boyd and Martens (1992): allelopathy, elemental tolerance, drought resistance, inadvertent uptake and as a defense against herbivore and pathogen attacks, termed the elemental defense hypothesis. Most studies to date have focused on, and lent support to the elemental defense hypothesis.

Hyperaccumulated Ni has been shown to protect plants from a variety of herbivores, including moth larvae, the root feeding cabbage maggot, the cell disruptor whitefly and grasshoppers (Jhee et al. 2005, 2006). Interestingly, elevated Ni concentrations do not appear to protect plants from vascular feeding herbivores (Jhee et al 2005). Arsenic hyperaccumulation has been shown to deter grasshopper herbivory in the arsenic hyperaccumulating Chinese brake fern (*Pteris vittata*) (Rathinasabapathi et al. 2007). Hyperaccumulated Zn and Cd also have been shown to protect plants from herbivores (Pollard and Baker 1997; Jiang et al. 2005). Elevated Se levels in *Brassica juncea*, not a Se hyperaccumulator, but a Se accumulator that can accumulate reasonably high concentrations of Se, was shown to be protected from two fungal pathogens, and the

economically important green peach aphid and cabbage white butterfly (Hanson et al. 2003, 2004). Furthermore, the Se hyperaccumulator *Stanleya pinnata* has been shown to be protected by Se from grasshoppers, moth larvae and prairie dogs (Freeman et al; 2006a, 2007; Quinn et al. 2008). *Astragalus bisulcatus*, another Se hyperaccumulating species, has been shown to be protected from prairie dogs (Quinn et al. 2008). Additionally, a field study by Galeas et al. (2008) revealed that Se hyperaccumulators harbor fewer arthropod individuals and arthropod species than similar non-Se hyperaccumulating species at the same site. Studies investigating Se hyperaccumulating plants revealed that they sequester Se in locations that are especially vulnerable to herbivore attacks, such as the periphery of the leaf, or in cells with known defensive functions, like leaf hairs. This is in contrast to non Se hyperaccumulating plants, which have a more diffuse distribution of Se (Freeman et al. 2006b). Furthermore, young leaves of Se hyperaccumulating plants have higher concentrations of Se than old leaves and Se is highest in aboveground tissues when the shoot begins to grow in early spring and is lowest in the fall, prior to senescence (Galeas et al. 2007).

As with any plant defense, this elemental defense has been disarmed by herbivores. A population of diamondback moth (*Plutella xylostella*) found thriving on the Se hyperaccumulator *S. pinnata*, was shown to have developed Se tolerance. In contrast, a population of diamondback moth collected from a Se-free area was found to be Se-sensitive. The Se tolerance mechanism was revealed through Se speciation studies. The Se tolerant moth population accumulated Se as MeSeCys, which is not easily incorporated into proteins and is the form found in the plant, and the Se sensitive

diamondback moth accumulated primarily SeCys, which is toxic because it is easily incorporated into proteins (Freeman et al. 2006a).

In this dissertation four experimental chapters are presented that address different ecological aspects of Se accumulation in plants. They are followed by a review chapter discussing the ecological implications of cultivating Se accumulating plants for Se phytoremediation (environmental cleanup using plants) or as fortified food. The first experimental chapter investigates how two cell disruptor herbivores, the two-spotted spider mite (*Tetranychus urticae*) and the western flower thrips (*Frankliniella occidentalis*), are affected by elevated Se in the Se hyperaccumulators *S. pinnata* and *A. bisulcatus*. The reason for doing these studies was that it is likely that Se hyperaccumulation is more effective as a defensive mechanism against some herbivores than others because of the localization of Se to certain tissues. This is the first study to investigate if Se hyperaccumulation is an effective defense against cell disrupting herbivores.

The second study is a two-year field study that investigates the role Se plays in protecting *S. pinnata* against black tailed prairie dogs (*Cynomys ludovicianus*). This study is ecologically relevant because many Se hyperaccumulating plants and prairie dogs are native to the same area and the results of this study provide insight into the selection pressures that led to Se hyperaccumulation. In addition, this is the first long-term field study to investigate the functional significance of hyperaccumulation and increases our understanding of how plants may benefit from increased Se in field conditions over a two-year period.

The third study investigates floral Se accumulation, speciation and localization in the Se hyperaccumulator *S. pinnata* and the non-hyperaccumulator *B. juncea*. In addition, we examine the effect of increased floral Se on pollen germination and pollination. The reason for doing this study was to explore possible reproductive costs of hyperaccumulation, and effects of floral Se on pollinators. Since so many herbivores are Se sensitive it is possible that pollinators have similar Se sensitivity and may be deterred from Se hyperaccumulating plants or suffer from Se toxicity when foraging on high-Se flowers.

The fourth experimental chapter in this dissertation describes a litterbag approach to investigate the effect of elevated Se on leaf decomposition in a seleniferous habitat. In addition, soil Se concentration underneath litter, and litter-associated microbes and micro-arthropods were compared between high-Se and low-Se leaf litter.

The experimental chapters are followed by a review chapter, summarizing the results and discussing their implications for cultivating Se accumulating plants for Se phytoremediation (environmental cleanup using plants) or as fortified food. Combined, the studies described in this dissertation provide a thorough, multi-faceted investigation of the ecology of Se hyperaccumulation in plants. They increase our understanding of the roles and effects of Se in interactions between Se hyperaccumulating plants and their ecological partners, and shed light on the selection pressures that may have driven the evolution of Se hyperaccumulation. The results from these studies may also have applications for the management of seleniferous habitats, pest management, and for cultivation of crops for Se phytoremediation or as fortified foods.

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Chapter 2

SELENIUM HYPERACCUMULATION OFFERS PROTECTION FROM CELL DISRUPTOR HERBIVORES

NOTES AND ACKNOWLEDGEMENTS

A version of this chapter has been submitted to *BMC Ecology*. Dr. John Freeman and Colin Quinn contributed equally to this work. Dr. John Freeman and Stormy Lindblom designed and carried out spider mite experiments using *A. bisuclatus*. Colin Quinn and Dr. John Freeman designed and conducted thrips experiments using A. bisulcatus. Colin Quinn, Ray Reynolds and Jennifer Cappa designed and carried out all experiments using S. pinnata. Dr. Elizabeth Pilon-Smits aided in experimental design. Colin Quinn, Dr. John Freeman and Dr. Elizabeth Pilon-Smits drafted the manuscript. This research was supported by grants #IOB-0444471 and #IOS-0817748 from the National Science Foundation to EAHPS.

ABSTRACT

Hyperaccumulation, the rare capacity of certain plant species to accumulate toxic trace elements to levels several orders of magnitude higher than other species growing on the same site, is thought to be an elemental defense mechanism against herbivores and pathogens. Previous research has shown that selenium (Se) hyperaccumulation protects plants from a variety of herbivores and pathogens. Selenium hyperaccumulating plants sequester Se in discrete locations in the leaf periphery, making them potentially more susceptible to some herbivore feeding modes than others. In this study we investigate the protective function of Se in the Se hyperaccumulators *Stanleya pinnata* and *Astragalus bisulcatus* against two cell disrupting herbivores, the western flower thrips (*Frankliniella occidentalis*) and the two-spotted spider mite (*Tetranychus urticae*).

Astragalus bisulcatus and *S. pinnata* with high Se concentrations (greater than 650 mg Se kg^{-1}) were more protected from thrips herbivory than plants with low-Se levels (less than 150 mg Se kg^{-1}). Furthermore, in plants containing elevated Se levels, leaves with higher concentrations of Se suffered less herbivore than leaves with less Se. Spider mites also preferred to feed on low-Se *A. bisulcatus* and *S. pinnata* plants rather than high-Se plants. Spider mite populations on *A. bisulcatus* decreased after plants were given a higher concentration of Se. Interestingly, spider mites could colonize *A. bisulcatus* plants containing up to 200 mg Se kg^{-1} dry weight, concentrations which are toxic to many other herbivores. Selenium speciation studies using x-ray absorption spectroscopy revealed that the spider mites accumulated primarily methylselenocysteine, the relatively non-toxic form of Se that is also the predominant form of Se in hyperaccumulators

This is the first study to investigate the protective effect of Se against cell-disrupting herbivores. The finding that Se protected the two hyperaccumulator species from both cell disruptors lends further support to the elemental defense hypothesis and increases the number of herbivores and feeding modes against which Se has shown a protective effect. Because western flower thrips and two-spotted spider mites are widespread and economically important herbivores, the results from this study also have potential applications in agriculture or horticulture, and implications for the management of Se-rich crops.

BACKGROUND

For many organisms, including mammals and many species of bacteria and algae, selenium (Se) is an essential trace element (Stadtman 1990). These organisms contain selenoproteins, some of which destroy free radicals that damage DNA (Steinbrenner and Sies 2009). In humans, Se supplementation has been shown to reduce the chance of getting cancer, including the devastating widespread lung and prostate cancers (Clark et al. 1996; Shin et al. 2007). In addition, Se plays an essential role in thyroid function (Kato et al. 2010). While Se is essential for many organisms, the level between deficiency and toxicity is narrow. Selenium toxicity can be both acute and chronic. Acute Se toxicity leads to "blind staggers" in livestock; the symptoms include staggered walking, impaired vision, paralysis and sometimes death. Chronic Se poisoning leads to hair and nail loss, fatigue, nausea and eventually death (Oliveira et al. 2007).

Selenium has no known essential function for higher plants, and elevated levels of Se are toxic to most plants (Anderson 1993). This toxicity is due to the chemical similarity of Se and sulfur (Stadtman 1990). Most plants inadvertently assimilate Se into proteins, leading to toxicity (Stadtman 1990). A few plant species have evolved to accumulate unusually large amounts of Se, as much as 1%, or 10,000 mg Se kg^{-1} dry weight (DW) (Beath et al. 1939; Freeman et al 2006a). These unique plants are called Se hyperaccumulators and avoid Se poisoning by methylating SeCys into methylselenocyteine (MeSeCys), which is relatively non-toxic because it does not get incorporated into proteins (Brown and Shrift 1981).

Hyperaccumulation is a phenomenon where plants accumulate particular elements to levels several orders of magnitude higher than other plant species growing on the same

substrate (Baker and Brooks 1989). Some other elements besides Se that can be hyperaccumulated by plants include aluminum (Al), arsenic (As), cadmium (Cd), manganese (Mn), nickel (Ni) and zinc (Zn) (Reeves and baker 2000). Feist and Parker (2001) defined Se hyperaccumulation as plants that contain more than 1,000 mg Se kg^{-1} DW. Most research investigating the functional significance of hyperaccumulation has focused on and lent support to the elemental defense hypothesis, which states that plants have evolved to hyperaccumulate these various toxic elements as protection against herbivore and pathogen attacks (Boyd and Martens 1992). Hyperaccumulated As, Cd, Ni, Zn and Se all have been shown to protect plants from herbivores and/or pathogens (Pollard and Baker 1997; Jhee et al. 1999; Boyd et al. 2002; Hanson et al. 2004; Rathinasabapathi et al. 2007).

To date, Se hyperaccumulation has been shown to protect plants from a mammalian herbivore, the black-tailed prairie dog (*Cynomys ludovicianus*), as well as from several arthropod herbivores and two fungal pathogens (Hanson et al. 2004; Freeman et al. 2006b; Quinn et al. 2008; Chapter 3). Additionally, Se hyperaccumulating plants harbored fewer arthropods and arthropod species than comparable non Se hyperaccumulators growing in the same, seleniferous habitat (Galeas et al. 2008). Moreover, Se hyperaccumulating plants sequester Se in organs and tissues that are most susceptible to herbivore attack. For example, the Se in hyperaccumulator *Astragalus bisulcatus* (two-grooved milk vetch) is predominantly present in the leaf hairs, and *Stanleya pinnata* (Prince's plume), another Se hyperaccumulator, sequesters Se in epidermal cells in the leaf margins (Freeman et al. 2006a). This uneven distribution of Se, which leaves some areas of the plant with lower concentrations of Se than others,

may allow some herbivores, depending on their feeding mode, to avoid this elemental defense. Indeed, some herbivore species were found living on Se hyperaccumulating plants in the field and apparently were feeding on the Se-rich plant material, in view of the fact that they contained higher Se concentrations than individuals collected from non-hyperaccumulators (Galeas et al. 2008). Thus, it is important to investigate the effect of feeding mode on the herbivores' ability to feed on Se hyperaccumulating plants. The effect of feeding mode on herbivore susceptibility to hyperaccumulated elements is illustrated by the study by Jhee et al. (2008) who found that the Ni hyperaccumulator *Streptanthus polygaloides* was protected from folivore herbivores but not vascular feeding herbivores.

This study investigates the protective effect of Se hyperaccumulation against cell disruptor herbivore species, specifically the two-spotted spider mite (*Tetranychus urticae*) and western flower thrips (*Frankliniella occidentalis*). Both herbivores have been observed feeding on *A. bisulcatus* and *S. pinnata* in the greenhouse and both feed by piercing the cell surface with their mouthparts and sucking out the cell contents (Tomczyk and Kropczynska 1985). This study, the first to examine cell disruptor herbivores' sensitivity to Se hyperaccumulation, is ecologically relevant because both of these herbivores share habitats with Se hyperaccumulating plants (Feist and Parker 2001; Strand 2006). Interestingly, western flower thrips and many Se hyperaccumulating plant species are native to the western United States and protection against thrips herbivory may have contributed to the evolution of Se hyperaccumulation. Both herbivores are also ecologically important pests. The two-spotted spider mites can have devastating effects on crop yields worldwide (Berlinger 1986). Outbreaks often occur after pesticide

application inadvertently kills their predators. Western flower thrips are native to the Western United States, but have been reported on all continents except Asia and Antarctica (Brunner and Frey 2010). Through a combination of their herbivory and their notorious ability to transfer disease and develop pesticide resistance, western flower thrips can significantly reduce crop yields (Immaraju et al. 1992; Williams 2006) In this study we report a significant effect of plant Se accumulation on the interaction of two Se hyperaccumulators, *A. bisulcatus* and *S. pinnata* with both cell disruptors, the western flower thrips and the two-spotted spider mite.

METHODS

Plant material

Seeds of *A. bisulcatus* were obtained from plants growing at Pine Ridge Natural Area in Fort Collins, CO, USA (40°32.70N, 105°07.87W). Pine Ridge Natural Area is a seleniferous habitat; the population of *A. bisulcatus* from which seeds were collected accumulates up to 10,000 mg Se kg^{-1} (Galeas et al. 2007). Seed germination and growth followed an arid western plant growth protocol previously used for Se hyperaccumulating plant growth and described by Sors et al. (2005). Plants were grown on pre-washed Turface MVP (Profile Products LLC, Buffalo Grove, IL) in 25 cm diameter pots in greenhouse conditions (24/20°C day/night, 16-h photoperiod, 300 μmol m^{-2} sec^{-1} photosynthetic photon flux). Three weeks after germination half of the plants received high-Se fertilizer treatments, 1 g of fertilizer (Miracle-Gro Excel, 15:5:15 Cal-Mag, The Scotts Co., Marysville, OH) per liter of water combined with 20 μM Na_2SeO_4, while the

other half received low-Se treatments, 1 g of fertilizer per liter of water with 2 μM Na_2SeO_4, three times a week. After 20 weeks of growth plants were used for thrips and spider mite experiments as described below.

Stanleya pinnata seeds were obtained from Western Native Seed (Coaldale, CO, USA) and were grown from seed in pre-washed Turface MVP. Thirty-six plants were grown in a growth room (24°C/20°C, 12 h/12 h light/dark, 120 μmol m^{-2} s^{-1} photosynthetic photon flux), 10 weeks after germination half of the plants were watered twice a week for 50 weeks with 1 g of fertilizer (Miracle-Gro Excel, 15:5:15 Cal-Mag, The Scotts Co., Marysville, OH) per liter of water and 20 μM Na_2SeO_4, the other half were watered with 1 g of fertilizer per liter of water as a control. Plants were used for thrips and spider mite experiments as described below.

Effects of Se on herbivory of *A. bisulcatus* by thrips

To investigate thrips toxicity to Se and their preference to feed on high- or low- Se plants, both non-choice and choice experiments were conducted. For non-choice experiments high- and low-Se *A. bisulcatus* were infected with western flower thrips by placing three excised *A. bisulcatus* leaves previously harboring large populations of thrips on each plant. Two high and low-Se plants were then placed in separate 20 g glass tanks that were covered with 0.2 mm^2 nylon mesh tops to prevent thrips transfer while still allowing gas exchange. For choice experiments plants were infected with thrips as described above and a high- and a low-Se plant were placed in the same glass tank. After three weeks of herbivory the percentage of young (mature leaves from the top five nodes), medium (leaves from middle nodes) and old (leaves from the bottom 3 nodes) leaves and

the percentage of leaflets per leave with thrips herbivory were calculated on each plant. Non-choice experiments were repeated 6 times and choice experiments were repeated 4 times for high-Se and 4 times for low-Se treatments. Selenium concentrations for young, medium and old leaves were measured as described below.

Effects of Se on herbivory of *A. bisulcatus* by spider mites

Spider mite non-choice and choice experiments were also conducted using *A. bisulcatus*. For non-choice experiments 10 high- or low-Se plants were placed in 20 L glass tanks covered with 0.2 mm^2 mesh and each plant was inoculated with 100 spider mites. The number of spider mites on plants were counted after 7, 14 and 21 days and the percent of the population change was calculated for each plant. For choice experiments spider mites were given a choice to feed on high- or low-Se plants. Seven high- and seven low-Se *A. bisulcatus* plants were placed in tanks and 100 spider mites were placed on each plant. The number of spider mites on each plant were counted after 7, 14 and 21 days and percent population change was calculated. Leaf Se concentrations of youngest mature leaves were compared between high- and low-Se plants as described below.

In addition to choice and non-choice experiments low-Se *A. bisulcatus* pre-infected with spider mites were provided with Se to determine if adding Se reduces established populations of spider mites. At the onset of the experiment low-Se *A. bisulcatus* plants that were being treated with 2 μm Se were infected with large spider mite populations. For three weeks eight of the plants were provide with 40 μm Se three times a week while eight others were provided with water as a control. The percent

population change in spider mite herbivory was recorded after 7, 14 and 21 days. Leaf Se concentration of the plants was measured before and after the experiment.

Spider mites from high-Se *A. bisulcatus* plants were collected and analyzed for Se speciation. Samples were washed and flash-frozen using liquid nitrogen. Samples were kept frozen to prevent Se metabolism, and Se speciation was determined using XANES as described by Marcus et al. (2004), using known selenocompounds as standards.

Se speciation, X-ray microprobe measurements

Spider mites from high-Se *A. bisulcatus* plants were collected and analyzed for Se speciation. Samples were washed and flash-frozen using liquid nitrogen. Samples were kept frozen to prevent Se metabolism, and Se speciation was determined using XANES as described earlier (Marcus et al. 2004; Freeman et al. 2006b), using well characterized selenocompounds as standards.

Effects of Se on herbivory of *S. pinnata* by thrips

Stanleya pinnata, another Se hyperaccumulating plant, was also used to determine if Se protects against cell disrupting herbivores. Thrips were given a choice to feed on either high- or low-Se *S. pinnata*. Eighteen high-Se and 18 low-Se plants were intermixed and placed in a growing room heavily infested with thrips. After 4 weeks of being exposed to thrips herbivory the percentage of leaves with herbivory was compared between plants with and without Se. In addition, for six of the *S. pinnata* plants treated with Se, two similar-aged leaves per plant, one with herbivory and one without herbivory, were collected and analyzed for elemental concentrations using ICP-AES, as described below.

To determine the variation in Se concentration with leaf age in plants not suffering herbivory, three leaves from consecutive nodes on the same high-Se *S. pinnata* plants were tested for Se concentration. This was repeated for 6 plants.

Effects of Se on herbivory of *S. pinnata* by spider mites

Ten high-Se and nine low-Se *S. pinnata* were interspersed in a 50 cm x 50 cm area on a greenhouse bench. Each plant was infected with spider mites by placing three leaves from other plants that harbored high concentrations of spider mites on each plant. Spider mites were allowed to forage for two weeks, and herbivory was then scored by counting the number of leaves on each plant with and without spider mite herbivory. The youngest mature leaves were collected from each plant and analyzed for Se concentration.

Elemental analysis

Elemental concentrations in leaves were determined by digesting approximately 100 mg DW of leaf material in 1 ml of nitric acid as described by Zarcinas et al. (1987). Using distilled water the samples were diluted to 10 ml and elemental concentrations were determined using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) as described by Fassel (1978).

Data analysis

The software package JMP-IN (3.2.6, SAS Institute, Cary, NC) was used for all data analysis. To compare differences in herbivory between high-Se and low-Se plants a

Student's t-test was used. A Student's t-test was also used to compare elemental concentrations of leaf samples.

RESULTS

Effects of Se on herbivory of *A. bisulcatus* by thrips

To investigate if the Se hyperaccumulator *A. bisulcatus* was protected from western flower thrips, both choice and non-choice studies were conducted with plants containing high and low concentrations of Se (the thrips are displayed in Figure 2.1 A, B; high- and low-Se leaflets exposed to thrips herbivory are shown in Figure 2.1 C, D).

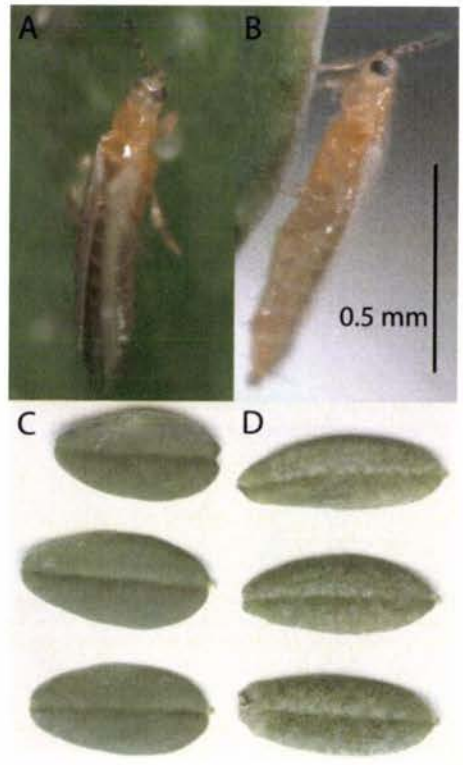

Figure 2.1: Western flower thrips feeding on *A. bisulcatus* (A, B). High-Se (C) and low-Se (D) *A. bisulcatus* leaflets exposed to thrips herbivory. On high and low-Se leaves thrips herbivory damage is apparent where there are white patches with black spots (C, D).

The non-choice experiments revealed that the fraction of leaves with herbivory was significantly lower for high-Se plants than low-Se plants ($p = 0.018$, $t = -2.832$, $n = 6$ for both high- and low-Se experiments), and that younger leaves suffered less herbivory than older leaves for both high- and low-Se plants (Figure 2.2A). In addition, fewer leaflets per leaf suffered thrips herbivory on high-Se than low-Se plants (Figure 2.2B; $p = 0.011$, $t = -3.095$, $n = 6$ for both high- and low-Se experiments). Young leaves from high-Se plants contained roughly 1.5-fold and 5-fold higher Se concentrations than medium-aged and old leaves of the same plants, ranging from 3,945 mg Se kg^{-1} for young leaves and 812 mg Se kg^{-1} for old leaves, while leaves from low-Se plants did not reach above 11 mg Se kg^{-1} (Figure 2.2C). When thrips were given a choice to feed on high- or low-Se plants they showed a significant preference to colonize low-Se plants. In these choice experiments low-Se leaves and leaflets suffered more herbivory than high-Se leaves and leaflets (Figure 2.2D, E; $p = 0.001$, $t = -5.926$ when comparing percent herbivory on leaves from high- and low-Se plants; $p < 0.001$, $t = -6.443$ when comparing percent herbivory on leafltes from high- and low-Se plants. $N = 4$ choice experiments). Within high-Se plants young leaves suffered less herbivory than older leaves (Figure 2.2D). Similar to what was found for plants used in the non-choice thrips experiments, young leaves of the high-Se plants contained more Se than old leaves, 3,000 mg Se kg^{-1} compared to 1,350 mg Se kg^{-1}, respectively (Figure 2.2F). While in the choice study high-Se plants had many fold higher Se concentrations than low-Se plants, leaves from low-Se plants also contained around 100 mg Se kg^{-1} DW in young leaves and approximately 50 mg Se kg^{-1} DW in medium-aged and old leaves (Figure 2.2F).

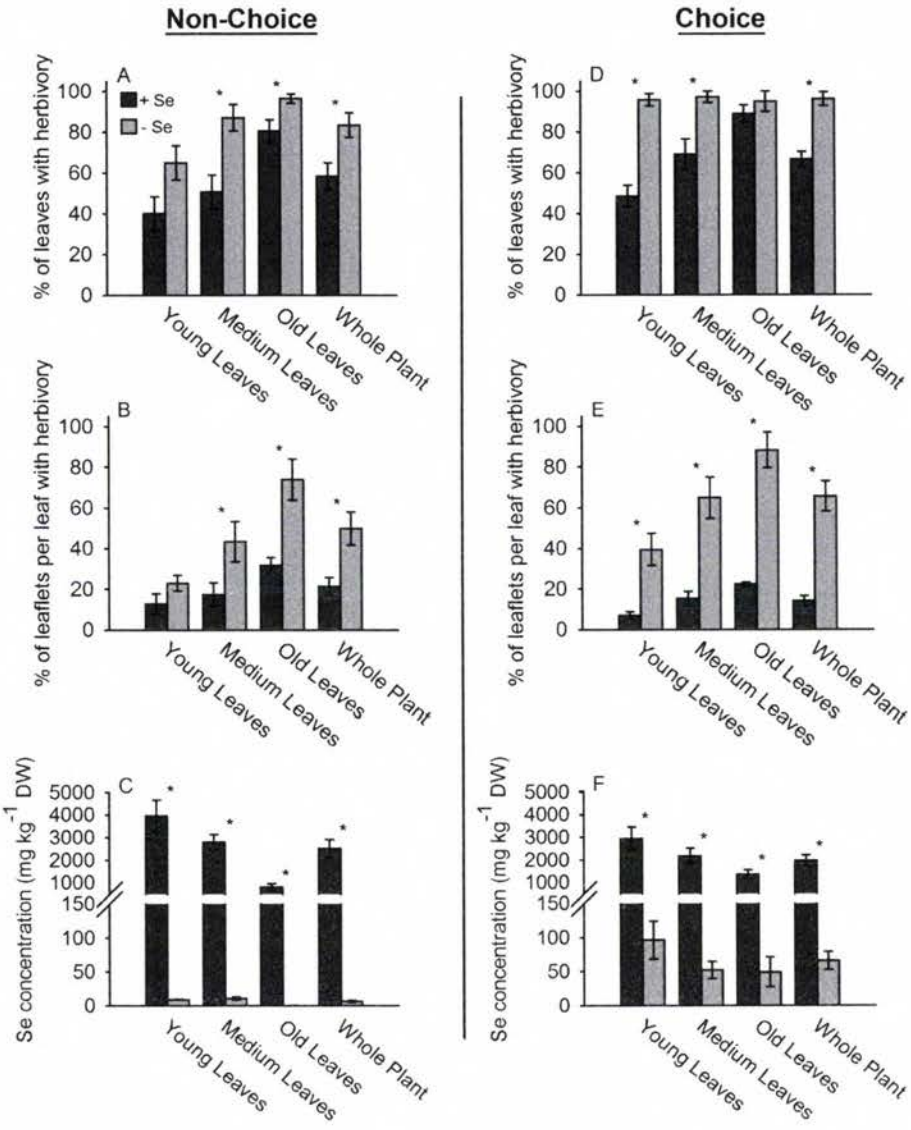

Figure 2.2: Thrips non-choice feeding (thrips were provided with either high-Se or low-Se plants) experiments showing the percent of *A. bisulcatus* young, medium and old leaves (A) and leaflets (B) from high-Se and low-Se plants (C) that suffered herbivory. Thrips choice feeding (thrips were provided with both high-Se and low-Se plants) experiments showing the percent of *A. bisulcatus* young, medium and old leaves (D) and leaflets (E) from high-Se and low-Se plants (F) that suffered herbivory. Values are means +/- SE. An asterisk above bars represents a significant difference in high-Se and low-Se treatments ($\alpha = 0.05$, n = 6 for both high-Se and low-Se non-choice experiments, n = 4 for choice experiments).

Effects of Se on herbivory of *A. bisulcatus* by spider mites

Non-choice and choice experiments were conducted to determine if Se effectively protected *A. bisulcatus* from another cell disruptor herbivore, the two-spotted spider mite. During a non-choice study spider mite populations only gradually increased in size on high-Se plants, whereas plants pre-treated with a low Se concentration showed an 800% spider mite population growth over three weeks (Figure 2.3A; $p < 0.001$, $t = 5.306$, $n =$ 10 high- and 10 low-Se plants). When spider mites were given a choice to feed on high- or low-Se plants they preferred low-Se plants. The protective effect of Se was already seen after one week, as populations of spider mites on high-Se plants decreased in size over time while populations on low-Se plants increased by over 200% after three weeks (Figure 2.3B; $p < 0.001$, $t = 6.004$, $n = 7$ high- and 7 low-Se plants). High-Se plants contained over 2,200 mg Se kg^{-1} DW and low-Se plants contained 110 mg Se kg^{-1} DW (Figure 2.3C; $p = 0.009$, $t = -4.792$, $n = 3$ high- and 3 low-Se plants).

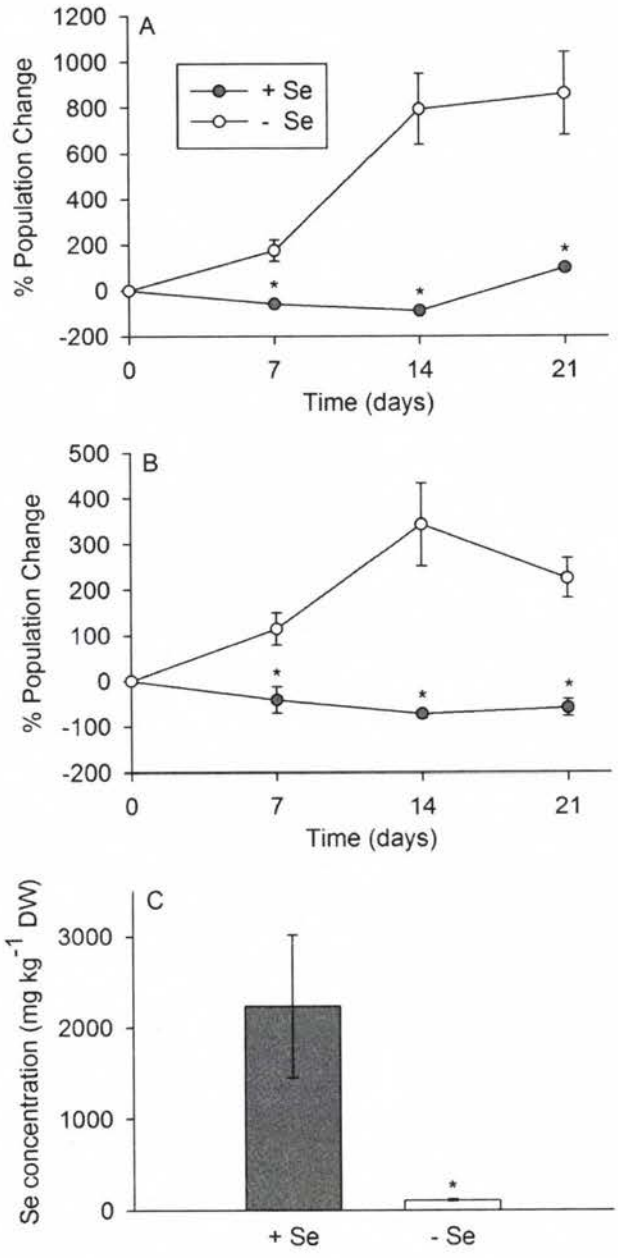

Figure 2.3: The percent population change of spider mite populations feeding on high-Se and low-Se *A. bisulcatus* during a non-choice feeding study (A) and a choice feeding study (B). Selenium concentrations in high-Se and low-Se *A. bisulcatus* used during the experiments. Values are means +/- SE. An asterisk between data points in the non-choice and choice feeding experiments or above bars comparing high-Se and low-Se in plants represents a significant difference ($\alpha = 0.05$).

Another experiment was conducted to investigate the effect of adding Se to *A. bisulcatus* pre-infested with spider mites. Half of the plants infested with spider mites were treated with Se and the other half was given water as a control. After seven days the Se treatment had resulted in a 50% reduction in the population of spider mites; in contrast, the population of spider mites on plants not treated with Se had increased by 50% during the same time period (Figure 2.4A, $p = 0.004$, $t = 3.416$, $n = 8$ high- and 8 low-Se plants). Three weeks after the start of the Se treatment the spider mite populations on the high-Se plants had decreased by almost 80% while the populations of spider mites on low-Se plants still showed an increase of 50% (Figure 2.4A, $p < 0.001$, $t = 12.807$, $n = 8$ high- and 8 low-Se plants). Prior to conducting the experiment, all *A. bisulcatus* plants contained between 100 – 200 mg Se kg^{-1} DW. After the three-week experiment the high-Se plants contained almost 800 mg Se kg^{-1} DW and the low-Se plants contained 100 mg Se kg^{-1} (Figure 2.4B, $p = 0.010$, $t = 2.870$, $n = 8$ high- and 8 low-Se plants).

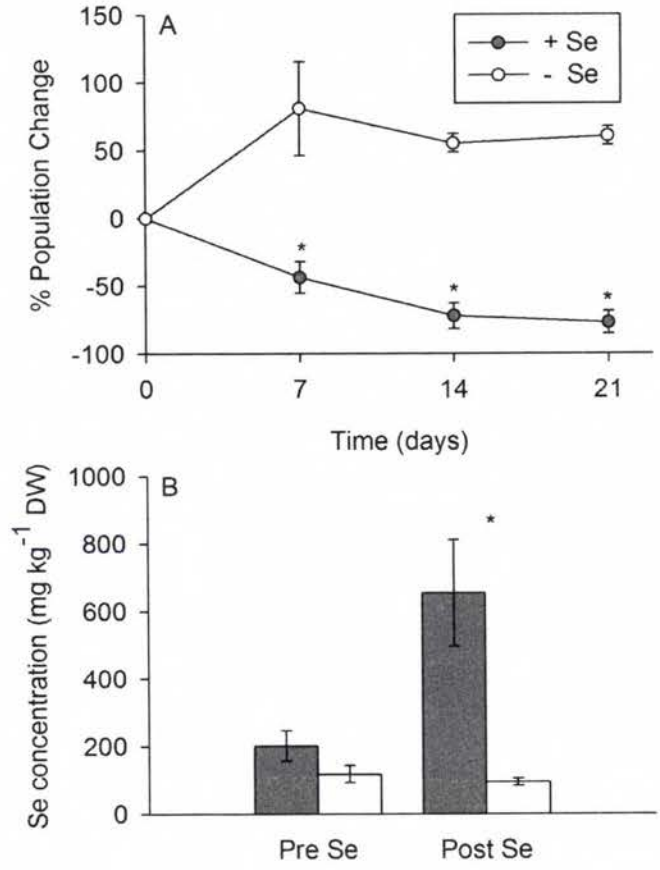

Figure 2.4: The percent population change in previously existing spider mite populations on *A. bisulcatus* after treating plants with high-Se or low-Se (A). Selenium concentration of plants at the beginning and end of the experiment (B). Values are means +/- SE. An asterisk between data points (B) or above bars (C) represents a significant difference ($\alpha = 0.05$, n = 10 for non-choice experiments, n = 7 for choice experiments).

Since the spider mites appeared to tolerate plant Se concentrations up to 150 mg Se kg^{-1} we collected spider mites off Se-treated plants to investigate the mechanism of their relatively high Se tolerance at the biochemical level. Selenium speciation studies using Se K-edge(X-ray absorption near-edge structure (XANES)) spectroscopy and least

square linear combination fitting (LCF) of the XANES spectra using standard compounds revealed that spider mites store Se primarily as an organic C-Se-C form similar to methylselenocysteine (MeSeCys) (Figure 2.5A-C).

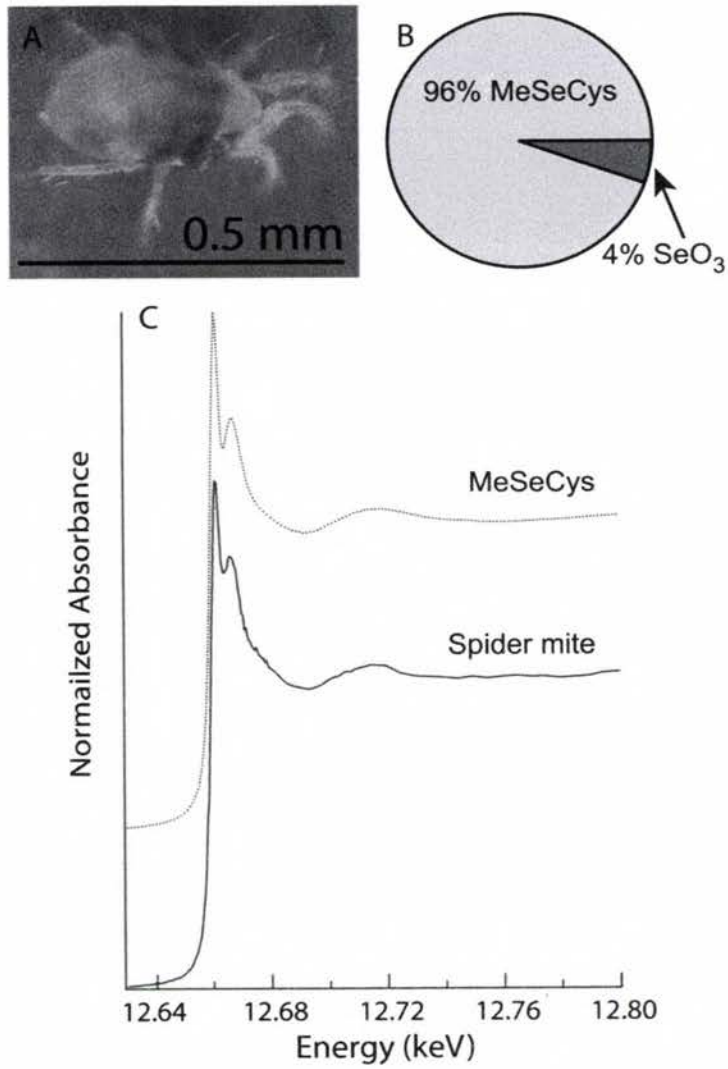

Figure 2.5: Selenium speciation results revealed that two-spotted spider mites (A) collected from Se-rich *A. bisulcatus* contained primarily methylselenocysteine (B). Spider mite selenium speciation spectra and methylselenocysteine standard spectra (C).

Effects of Se on herbivory of *S. pinnata* by thrips

To further investigate if Se hyperaccumulators are protected from cell disrupting herbivores we used another Se hyperaccumulating plants species, *S. pinnata,* and again used thrips in a choice herbivory experiment. The thrips preferred to feed on *S. pinnata* plants without Se when given a choice between high- and low-Se plants (Figure 2.6A, $p < 0.001$, $t = -10.333$, $n = 18$ high- and 18 low-Se plants). Within the Se-treated plants, leaves with elevated Se suffered less herbivory than similar-aged leaves on the same plants with lower Se levels (Figure 2.6B, $p = 0.012$, $t = -3.056$, $n = 6$ high- and 6 low-Se plants). Interestingly, the leaves that were compared had similar concentrations of other elements beside Se (Figure 2.6C).

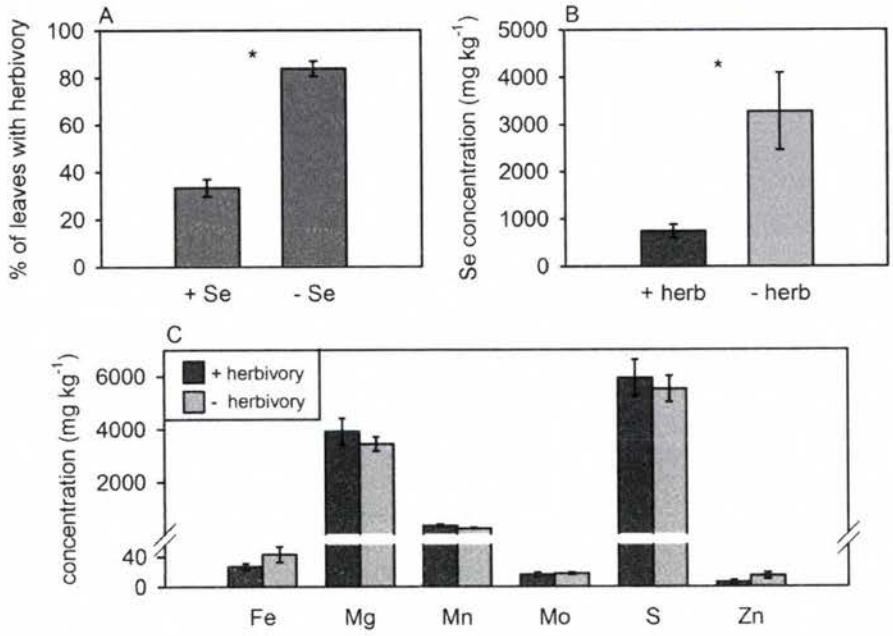

Figure 2.6: The percent of leaves per *S. pinnata* treated with or without Se that suffered thrips herbivory in a choice feeding experiment (A). Selenium concentrations in leaves from plants treated with Se that either experienced thrips herbivory or no herbivory (B). Elemental concentrations in leaves from plants treated with Se that suffered thrips herbivory (C). Values are means +/- SE. An asterisk over bars represents a significant difference ($\alpha = 0.05$, $n = 18$).

To determine if the difference in Se concentration found in each pair of leaves was a response to herbivory or rather a leaf age-related difference in Se concentration to which the herbivore responded Se concentration as a function of leaf age was investigated in more detail in plants without herbivory. Three leaves from consecutive nodes on each of six high-Se plants were analyzed for Se. In every plant sampled the youngest of the three leaves contained a higher Se concentration than the medium-aged leaf, which had again a higher concentration than the oldest of the three (Figure 2.7A-F).

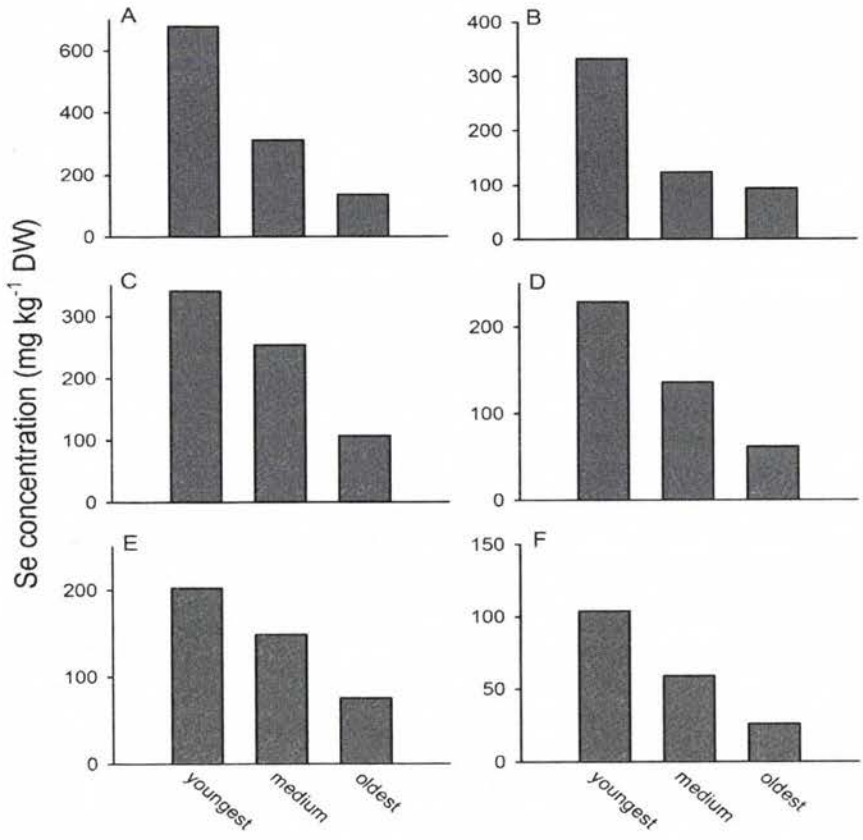

Figure 2.7: Selenium concentration in three young *S. pinnata* leaves from consecutive nodes from 6 plants (A-F).

Effects of Se on herbivory of *S. pinnata* by spider mites

Spider mites were given a choice to feed on either high-Se or low-Se *S. pinnata* to determine if elevated Se concentrations protected *S. pinnata* from spider mite herbivory. On plants with elevated Se only 35% of leaves suffered spider mite herbivory while over 75% of leaves from low-Se plants suffered spider mite herbivory (Figure 8A, $p = 0.002$, $t = 3.617$, $n = 10$ high and 9 low-Se plants). High-Se plants contained 420 mg Se kg-1 compared to low Se plants, which only had 50 mg Se kg^{-1} (Figure 8B, $p = 0.007$, $t = -3.078$, $n = 10$ high and 9 low-Se plants).

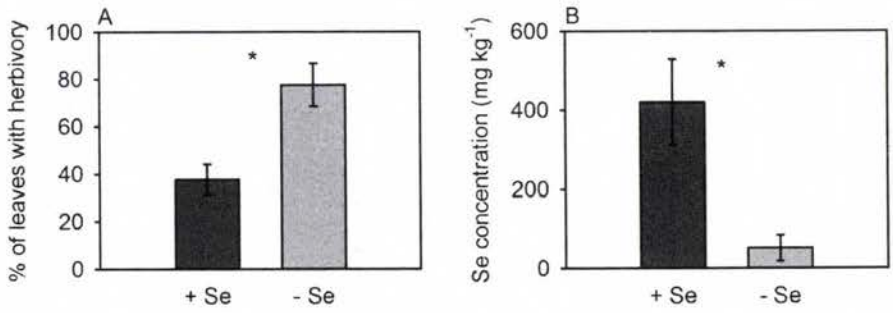

Figure 2.8: The percent of leaves from high-Se and low-Se *S. pinnata* that suffered spider mite herbivory (A). Selenium concentrations in leaves from plants with either high-Se or low-Se (Values are means +/- SE. An asterisk over bars represents a significant difference ($\alpha = 0.05$, $n = 10$ high- and 9 low-Se plants).

DISCUSSION

These results expand on previous studies investigating the functional significance of Se hyperaccumulation. The earlier studies have shown that elevated Se can protect plants from arthropod folivore herbivores (grasshoppers, caterpillars), grazing mammalian herbivores (prairie dogs), phloem-feeding arthropods (aphids) and leaf and stem/root fungal pathogens (Hanson et al. 2003; Hanson et al. 2004; Freeman et al. 2006b; Galeas

et al. 2008). This is the first study to show that Se protects hyperaccumulating plants from cell disrupting herbivores. This study provides evidence that two Se hyperaccumulating species, *S. pinnata* and *A. bisulcatus*, are protected against two ecologically relevant and economically important cell disruptor herbivores, the two-spotted spider mite and the western flower thrips, only when containing elevated Se concentrations. The non-choice studies showed that high-Se plants suffered less spider mite and thrips herbivory. The choice studies demonstrated that spider mites and thrips preferred low-Se *A. bisulcatus* and *S. pinnata* plants over high-Se plants. Furthermore, within a single plant, low-Se leaves suffered more thrips herbivory than high-Se leaves. Studies using *A. bisulcatus* showed that thrips preferred to feed on older leaves, which contained less Se. Studies with *S. pinnata* showed that leaves with high concentrations of Se suffered less thrips and spider mite herbivory than low-Se leaves and that younger leaves, even when only one node apart, had higher Se concentrations than older leaves. Those results suggest that these plants sequester Se in their younger leaves, which may be more valuable than older leaves because of higher photosynthesis rates (Kitajima et al. 2002), and in doing so are successful in protecting what may be considered their more valuable parts against these herbivores. Thus, these results lend further support to the hypothesis that Se hyperaccumulation serves as protection against herbivore attacks, and expands the list of herbivores against which Se is effective.

Herbivore feeding mode can be an important factor in plant-herbivore interactions. It is likely that some herbivores can circumvent plant defenses, including elemental defense, as a result of feeding modes (Gatehouse 2002; Karban and Agrawal 2002) and that different hyperaccumulating plants are protected from different groups of

herbivores. For example, Ni hyperaccumulation does not appear to protect plants from xylem and phloem feeding herbivores, while elevated Se, even at concentrations as low as 10 mg Se kg^{-1} DW, can protect plants from the phloem-feeding green peach aphid (Tomczyk and Kropczynska 2985; Hanson et al. 2004). Studies investigating Se distribution in Se hyperaccumulating plants suggests that they are better protected from some feeding modes than others. Leaves of *S. pinnata* sequester Se in the periphery of the leaves, in the epidermal cell layer, which is expected to be particularly effective against many folivores, like grasshoppers and caterpillars. *Astragalus bisulcatus* leaves sequester Se in trichomes, which may act as an initial defense mechanism against a variety of feeding types (Freeman et al. 2006a).

Interestingly, it appears that spider mites can tolerate plant Se concentrations in hyperaccumulators up to ~150 mg Se kg^{-1} DW, concentrations that are toxic to many other herbivores (Hanson et al. 2004; Quinn et al. 2008). Selenium speciation studies revealed that the spider mites accumulated an organic form of Se indistinguishable from MeSeCys. This form of Se is less toxic than many other forms of Se because it is not incorporated into proteins (Brown and Shrift 1981). The same form of Se was found in Se hyperaccumulator plants as well as in Se-tolerant herbivores found feeding on hyperaccumulators (Freeman et al. 2006b). If the spider mites accumulate MeSeCys as well, this may contribute to their tolerance of relatively high concentrations of Se. It should be noted, however, that XANES does not effectively distinguish between various C-Se-C compounds, including MeSeCys, selenomethionine, and Se-cystathionine (Brown and Shrift 1981) and therefore it cannot be excluded that the mites accumulated a more toxic form of Se, or a mixture of these organic selenocompounds. This would explain

why at higher Se levels, around 420 mg Se kg^{-1} DW for *S. pinnata* and 800 mg Se kg^{-1} DW for *A. bisulcatus*, Se effectively protected the plants, even against spider mites.

These results have important implications for managing Se-rich agricultural or natural areas and Se phytoremediation or biofortification crops. Crops in seleniferous habitats and plants used for Se phytoremediation often do not accumulate upwards of 150 mg Se kg^{-1} (Stapleton and Bañuelos 2009). While these plants may be protected by their low Se levels from folivore arthropods, they may still be susceptible to spider mite herbivory. On the other hand, Se hyperaccumulating plants, which typically contain more than 1,000 mg Se kg^{-1} DW (Galeas et al. 2008) likely are protected against both folivores and spider mites. This combined protective effect of Se accumulation against such a wide variety of herbivores may have been an important driving force for the evolution of Se hyperaccumulation.

CONCLUSIONS

Herbivores with different feeding modes may respond differently to hyperaccumulation in plants, as was suggested by Jhee et al. (2005). Because Se hyperaccumulating plants preferentially allocate Se to specific locations it may leave other locations vulnerable to herbivore attacks. This study shows that Se hyperaccumulating plants are protected from two economically important cell disrupting herbivores. The western flower thrips is considered a major pest because it is known to feed on plants in over 62 different families including many crop species (Tommasini and Maini 1995), they effectively transfer viruses to crop species (Cho et al. 1989) and they rapidly develop pesticide resistance (Herron and James 2005; Herron and James 2007). Two-spotted spider mites are also

known to target many crops, such as fruit trees and vegetables, and can also develop resistance to pesticides (Flexner et al. 1988) The results of this study provide support for the elemental defense hypothesis and have implications for management of seleniferous habitats and Se phytoremediation. Selenium may act as a natural pesticide in Se-rich crops and plants used for Se phytoremediation in areas such as the western United States, where two-spotted spider mites, western flower thrips and Se hyperaccumulators all occur and where Se-rich agriculture is present. The observed avoidance of Se-rich plants by herbivores may also reduce the probability of Se movement and bioconcentration in the food chain.

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Chapter 3

Selenium protects the hyperaccumulator *Stanleya pinnata* against black-tailed prairie dog herbivory in native seleniferous habitats

NOTES AND ACKNOWLEDMENTS

This study was published as the cover article of the *American Journal of Botany*, volume 96 pgs 1075-1085. Colin Quinn and Dr. John Freeman contributed equally to this work. Colin Quinn and Dr. John Freeman both contributed to experimental design, data collection, data analyses and preparation of the manuscript. Stormy Lindblom and Erin Klamper contributed to data collection. Dr. Elizabeth Pilon Smits contributed to experimental design and preparation of the manuscript. Funding for these studies was provided by NSF grant #IOB-0444471 to EPS.

ABSTRACT

Elemental hyperaccumulation in plants is hypothesized to represent a plant defense mechanism. The objective of this study was to determine whether selenium (Se) hyperaccumulation offers plants long-term protection from the black-tailed prairie dog (*Cynomys ludovicianus*). Prairie dogs are a keystone species. The hyperaccumulator *Stanleya pinnata* (prince's plume) co-occurs with prairie dogs in seleniferous areas in the Western U.S. *Stanleya pinnata* plants pretreated with high or low Se concentrations were planted on two prairie dog towns with different levels of herbivory pressure, and herbivory of these plants was monitored over a two-year period. Throughout this study, plants with elevated Se levels suffered less herbivory and survived better than plants with low leaf Se concentrations. This study indicates that the Se in hyperaccumulator *S. pinnata* protects the plant in its natural habitat from herbivory by the black-tailed prairie dog. The results from this study support the hypothesis that herbivory by prairie dogs, or similar small mammals, has been a contributing selection pressure for the evolution of plant Se hyperaccumulation in North America. This study is of significance since it is the first to test the ecological significance of hyperaccumulation over a long time period in a hyperaccumulator's natural habitat.

Key words: selenium, hyperaccumulation, *Stanleya pinnata*, elemental plant defense, black-tailed prairie dog, *Cynomys ludovicianus*.

INTRODUCTION

Certain specialized plant species growing on naturally enriched metalliferous soils often accumulate metals in their above-ground parts up to several orders of magnitude higher than other plants growing on the same soil (Baker and Brooks 1989). These so-called hyperaccumulators, can accumulate various elements, including arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), manganese (Mn), nickel (Ni), lead (Pb), selenium (Se) and zinc (Zn), and are indicators of naturally enriched metalliferous soils (Brooks 1987; Reeves and Baker 2000; Guerinot and Salt 2001). The functional significance of metal hyperaccumulation in plants is still obscure, but the current hypotheses are that hyperaccumulation confers drought tolerance, competitive advantages over other plant species through allelopathy, or prevents herbivore or pathogen attacks, also called the elemental defense hypothesis (Boyd and Martens 1992). There is mixed evidence for metal hyperaccumulation providing drought resistance and allelopathy (Whiting Neumann and Baker 2003; Bhatia et al. 2005; Zhang et al. 2007). The elemental defense hypothesis, however, has received significant support. Cadmium, Ni, Se and Zn can all effectively protect plants from various invertebrate herbivores and fungal pathogens (Pollard and Baker 1997; Jhee et al. 1999; Vickerman and Trumble 1999; Bañuelos et al. 2002; Boyd et al. 2002; Martens and Boyd 2002; Hanson et al. 2003, 2004; Freeman et al. 2006a). Thus, herbivory may have been an evolutionary driver of metal hyperaccumulation. Studies so far have mainly been conducted in the laboratory and with invertebrate herbivores. There is a need to investigate the ecological role that hyperaccumulation plays in natural ecosystems, and also to study the elemental defense hypothesis in relation to mammalian herbivores (Boyd 2007). The results from such

studies may shed light on the evolution of hyperaccumulation, its ecological significance, and its impacts on the local ecosystem.

In the Western U.S.A. the genera *Astragalus* and *Stanleya* are known to hyperaccumulate Se in their shoot tissues, up to 1% for *Astragalus bisulcatus* and 0.35% for *Stanleya pinnata* (Beath et al. 1934; Byers 1935; Galeas et al. 2007). Elevated Se in leaves can protect plants from herbivory by lepidoptera larvae, green peach aphids (*Myzus persicae*) and grasshoppers, as well as from fungal attacks by *Alternaria brassicicola* and species of the genus *Fusarium* (Hurd-Karrer and Poos 1938; Hanson et al. 2003, 2004; Freeman et al. 2006b, 2007). Further suggesting a role in defense, Se was found localized in hyperaccumulator plant organs, tissues and cells that are associated with plant defenses and are crucial for reproduction (Freeman et al. 2006a; Galeas et al. 2007).

While Se is an essential trace element for many organisms (Combs and Gray 1998; Ellis and Salt 2003; Goldhaber 2003) it is toxic at elevated levels due to the chemical similarity between Se and sulfur (S) and the associated non-specific replacement of S by Se in proteins (Stadtman 1990; Birringer et al. 2002; Ellis and Salt 2003). Ingestion of Se hyperaccumulator plants by animals can cause chronic or acute Se poisoning, commonly called alkali disease, selenosis and blind staggers (Draize and Beath 1935; Cosgrove 2001). While consumption of Se-enriched forage clearly results in toxicity in animals, one aspect of the elemental defense hypothesis that has received relatively little attention is whether plant Se accumulation actually deters mammalian herbivory in the wild. The long term manipulative field study presented here investigates

the role Se plays in protecting the hyperaccumulator *Stanleya pinnata* (prince's plume, Brassicaceae) from black-tailed prairie dog (*Cynomys ludovicianus*) herbivory.

It is possible that prairie dogs influenced the evolution of plant Se hyperaccumulation. Prairie dogs are considered ecosystem engineers that have historically had large impacts on surrounding plant communities (Whicker and Detling 1988; Weltzin et al. 1997). Prairie dogs have affected vast areas for at least 2 million years in the Western plains, including Se hyperaccumulator habitats, where *S. pinnata* and *C. ludovicianus* are found to often naturally coexist (Quinn 2006). The two-year study described here was aimed to provide insight into the role hyperaccumulation plays in native Se hyperaccumulator habitat, and into the role of prairie dog herbivory as an environmental pressure that may have influenced the evolution of Se hyperaccumulation.

MATERIALS AND METHODS

Field sites —To test if elevated Se concentrations in hyperaccumulator plants protect them from prairie dog herbivory, two field sites were selected in Fort Collins, Colorado, U.S.A. Both had similar vegetation and native species of Se hyperaccumulators, including *Stanleya pinnata*, but with clear differences in prairie dog herbivory pressure, based on observed prairie dog activity at the sites, as well as average number of active prairie dog burrows in a 10 m radius from each plot at each site. The site with high prairie dog herbivory pressure (11 ± 3 burrows, SE) had five test plots and was located in south Fort Collins, at Prairie Dog Meadows Natural Area (40°30.37N, 105°03.69W) (Fig. 3.1a). The other site, North College Lake in North West Fort Collins (40°67.42N,

105°14.86W) had medium prairie dog herbivory pressure (6 ± 2 burrows) and ten test plots (Fig. 3.1b). Soil was collected from the top 2 cm at 5-8 plots at each site to analyze for Se concentration. Both sites had 4-8 μg Se g^{-1} DW in the soil, and the presence of the native hyperaccumulators *Astragalus bisulcatus* and *S. pinnata* indicated the presence of Se. The dominant vegetation at both sites consisted of native grasses and forbs. Judging from long term observations, droppings, and from the presence of prairie dog tooth marks on the leaves of *S. pinnata,* prairie dogs were the primary, if not only, mammalian herbivore foraging on *S. pinnata* on both sites.

Figure 3.1: Black-tailed prairie dogs (*Cynomys ludovicianus*) and their burrows directly adjoining two prairie dog herbivory test plots (a, b). Herbivore test plots with *S. pinnata* plants containing high or low selenium directly after planting (c, d).

Plant material—*Stanleya pinnata* seeds from two different accessions were obtained from Western Native Seed (WNS, Coaldale, Colorado, USA) and Plants of the Southwest (POSW, Sante Fe, New Mexico, USA). The *S. pinnata* WNS variety, published as the CO4 ecotype, was collected south west of Denver Colorado and has been shown to accumulate Se to ~2 fold greater levels than the *S. pinnata* ecotype obtained from POSW which was collected from San Juan county New Mexico (Feist and Parker 2001). This difference in Se hyperaccumulation ability makes these two accessions interesting for comparison in the field. Seeds were germinated with distilled water on filter paper and planted in Scotts Metro Mix 350. Plants were grown in a growth room at 24°C/20°C, 10 h/14 h light/dark, 120 μmol m^{-2} s^{-1} photosynthetic photon flux, and watered 2 times weekly with a 1/4 X Hoagland's nutrient solution (Hoagland and Arnon, 1938). After one month half of the plants were treated with 40 μM Na_2SeO_4 in the nutrient solution while the other half was treated with 2 μM Na_2SeO_4. After 12 weeks of treatment, *S. pinnata* plants were sampled for leaf Se concentration, taken out to the field on the thirteenth of May in 2005 and transplanted along with the majority of soil that was firmly held by their roots. Because we carefully transplanted these plants along with a large amount of previously fertilized soil and because the field sites received two weeks of rain in the spring of 2005, no transplant shock was noticed in any plant. Initial plant sizes (height x diameter in cm^2) which is the best representation of the total plant surface area were as follows: WNS 40μM Se = 386 ± 14, WNS 2μM Se = 372 ± 15, POSW 40μM Se = 496 ± 28, POSW 2μM Se = 475 ± 25.

Long term manipulative field studies—To determine if elevated concentrations of Se affected prairie dog herbivory, both accessions of *S. pinnata* pre-treated with high (40 μM SeO_4) or low (2 μM SeO_4) Se were planted at each of the two test sites. In total, fifteen test plots were selected on the high prairie dog herbivory (Prairie Dog Meadows) and medium prairie dog herbivory sites (North College Lake). Eight *S. pinnata* were planted in each of the 1 m^2 plots (Fig. 1c, d). Four plants with high Se (40 μM SeO_4, two WNS & two POSW) were grouped together on one half and four plants with low Se (2 μM SeO_4, two WNS & two POSW) were grouped together on the other half (Fig. 1c, d). These different treatments and accessions represented four different experimental groups (n = 50 plants for each experimental group, 200 plants in total) throughout this study. All plots were at least 15 m apart from one another and oriented so that the nearest prairie dog burrows were equal distance from the high-Se and low-Se pretreated plants. Most plots were completely surrounded on all four sides with prairie dog trails often intersecting in the center of test plots. Because in previous experiments prairie dogs sometimes clipped vegetation for consumption and sometimes just to maintain an unobstructed view (Quinn 2006), we measured clipping and eating separately.

The same plants were left in the field for two growing seasons, 2005 and 2006, and various parameters were measured to determine prairie dog herbivory and plant damage. Clipping was calculated by counting the number of leaves that were removed from each plant by prairie dogs, and herbivory was calculated by determining the percentage of the clippings that were removed from the area around each plant. Plant damage was also calculated by counting the total leaf numbers per plant, and by estimating plant sizes (height x diameter in cm^2). For the first growing season, measurements were taken every

two weeks for the first 10 weeks, then again at 20 weeks. After the first year the percentage of plant survival per experimental group was determined. For the second growing season, plant damage was measured for the experimental groups at the beginning (week 0) and after 20 weeks. In addition, during the second season plant survival percentage per experimental group was determined along with flowering success. Two of the youngest mature leaves, when available, were harvested from all plants present in each experimental group at the beginning and end of each growing season to determine plant tissue Se concentrations. The final two year leaf number, plant size, survival percentage and flowering success were also recorded. In addition, the medium prairie dog herbivory site had a mammalian exclusion cage with 1.5 cm steel wire mesh covering one plot of 8 plants, 4 with elevated and 4 low leaf Se.

Leaf Se concentrations—Leaf tissue samples were rinsed with distilled water and dried at 50°C for 48 hours. One hundred mg of each sample was digested with 1 mL of concentrated nitric acid according to Zarcinas et al. (1987). The samples were then diluted with distilled water and analyzed for Se by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) as described by Fassel (1978).

Data analyses—Statistical analyses were performed using the software package JMP-IN version 3.2.6 from SAS institute (Cary, North Carolina, USA) and SigmaPlot for Windows version 10.0 from Systat Software, Inc. (San Jose, California, USA). A Tukey Kramer test was used when more than two means were compared and the statistical differences were denoted by letters. A chi-squared test was used to compare the survival

percentage of plants after one and two years. A Student t-test was used when only two means were compared, and the statistical differences were denoted in the figures by asterisks.

RESULTS

Season 1— At the beginning and end of the first growing season Se leaf concentrations were higher for plants treated with 40 μM Se than in plants of the same accession treated with 2 μM Se (Fig. 3.2a-d). In the course of this first growing season, leaf Se concentrations increased, especially in plants pre-treated with high levels of Se (Fig. 3.2a-d). This may be a result of the high Se concentration in the soil that was transplanted with these plants.

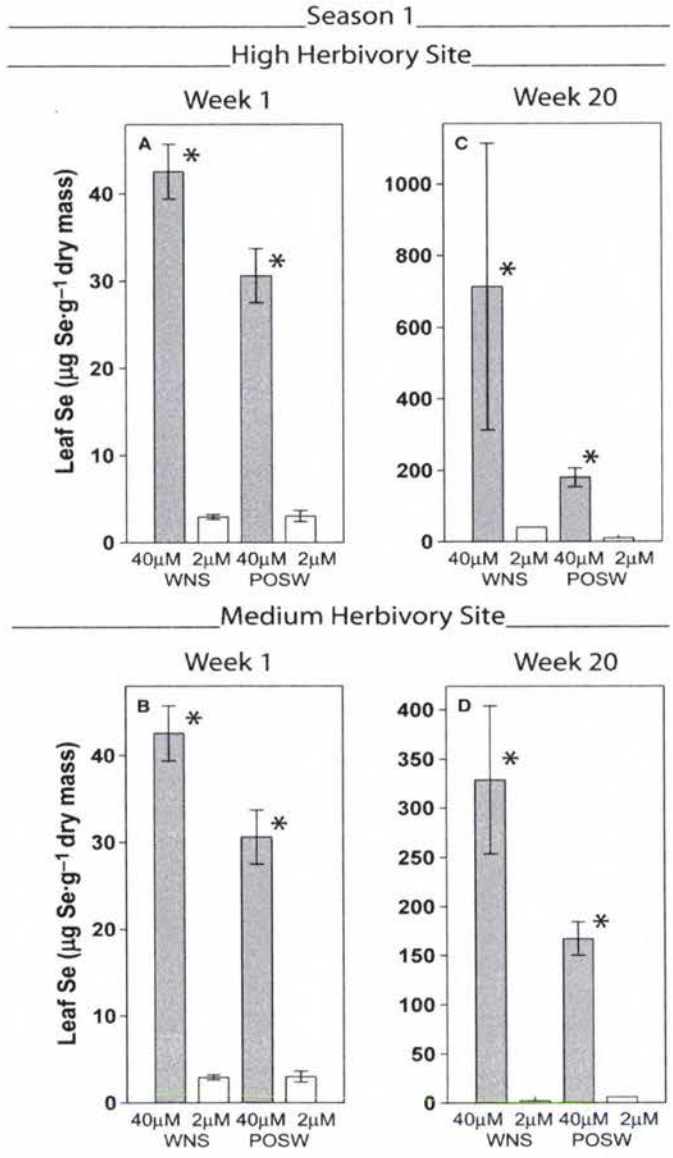

Figure 3.2: *Stanleya pinnata* leaf Se concentration in young mature leaves at the beginning (a, b) and the end (c, d) of the first growing season on a high prairie dog herbivory pressure site (a, c) and a medium prairie dog herbivory pressure site (b, d). Values are means ± SE. An asterisk denotes a significant difference between the two Se treatments (40 µM Se and 2 µM Se) of the same accession (WNS or POSW, $p < 0.05$).

The high prairie dog herbivory site, at Prairie Dog Meadows, showed a clear difference between the *S. pinnata* groups with respect to the percentage of plants clipped during the first 7 weeks (Fig. 3.3a). The *S. pinnata* groups with the highest (WNS 40µM) and intermediate (POSW 40µM) leaf Se concentrations were clipped less compared to the

corresponding genotypes containing low Se levels (WNS 2μM & POSW 2μM). After 10 weeks this site had undergone extreme prairie dog herbivory and most low Se plants had been clipped at least once. This high clipping frequency subsided after week 10.

The medium prairie dog herbivory site, at North College Lake, showed a similar Se-related difference in clipping frequency during weeks 0-10 (Fig. 3.3b). Here, the *S. pinnata* plants with the highest levels of Se (WNS 40μM) were clipped less when compared to those with intermediate Se concentrations (POSW 40μM) and plants with low Se concentrations (WNS 2μM & POSW 2μM).

The percentage of leaves eaten on the high prairie dog herbivory site was lower for the *S. pinnata* groups with high Se (< 20%, WNS 40μM) and intermediate Se (< 30%, POSW 40μM), compared to the corresponding low-Se groups (~50%, WNS 2μM & ~ 80%, POSW 2μM), indicative of Se deterrence (Fig. 3.3c). The clipping and consumption patterns were different in weeks 8 and 10 because plants were clipped but not eaten (Fig. 3.3a, c). This difference was most pronounced for the high-Se treatments, which were clipped, but not eaten as often as the low-Se experimental groups.

On the medium prairie dog herbivory site the findings were similar, indicating Se deterrence and avoidance. The percentage of leaves eaten was lower for the experimental treatment with the highest Se concentration (WNS 40μM) compared to the treatment with intermediate Se (POSW 40μM) and the two low-Se groups (WNS 2μM & POSW 2μM) (Fig. 3.3d). Due to both clipping and eating, the number of leaves decreased over the growing season. On the high prairie dog herbivory site the *S. pinnata* treatment with the highest Se levels (WNS 40μM) maintained the highest number of leaves until week 8, when many clipping events occurred (Fig. 3.3e). The POSW 2μM group showed the

largest decrease in leaf number, while the other experimental groups had intermediate leaf numbers.

Leaves per plant on the medium prairie dog herbivory site were greatest in number for the experimental group with the highest Se level (WNS 40μM), and lowest for POSW 2μM (Fig. 3.3f). The experimental group with intermediate leaf Se levels (POSW 40μM), had an intermediate leaf number over weeks 8-20. At most time points, the two high-Se groups (WNS 40μM & POSW 40μM) had significantly more leaves than the corresponding low-Se groups (WNS 2μM & POSW 2μM). As a control, the medium prairie dog herbivory site had a mammalian exclusion cage covering one plot of 8 plants, 4 with elevated and 4 with low leaf Se. The plants in this mammalian exclusion cage showed 100% survival after growing season 1.

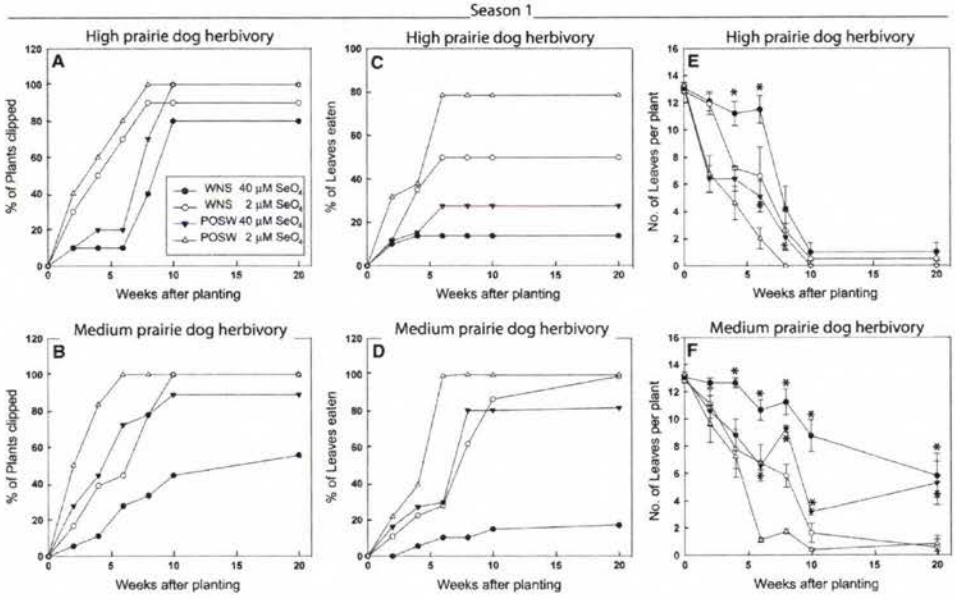

Figure 3.3: *Stanleya pinnata* prairie dog damage after the 1st growing season. Percentage of plants clipped and eaten by prairie dogs over time for high (a, c) and medium (b, d) prairie dog herbivory sites. *S. pinnata* leaf # over time for high (e) and medium (f) prairie dog herbivory test sites. Values are means ± SE. An asterisk denotes a significant difference between the two Se treatments of the same accession ($p < 0.05$). Values are means ± SE. An asterisk denotes a significant difference between the two Se treatments of the same accession ($p < 0.05$).

At the end of season 1 the plant sizes were measured at the different sites. At the high prairie dog herbivory site the final plant sizes were small and in several cases zero (Fig. 3.4a). On the medium prairie dog herbivory site the two high-Se treatments (WNS 40μM & POSW 40μM) gave larger final plant sizes than the corresponding low-Se treatments WNS 2μM & PoSW 2μM (Fig. 3.4b).

At the end of the first year under high prairie dog herbivory pressure there were no statistically significant differences in survival percentage between the four experimental treatments (Fig 3.4c). In contrast, on the medium prairie dog herbivory pressure site plants with high tissue Se concentration survived better than plants of the same accessions (WNS or POSW) with low Se levels (Fig. 3.4d). On the medium prairie dog herbivory site both high-Se treatments (WNS 40μM & POSW 40μM) had a 45% survival rate after year one. This is a 9- and 3-fold higher survival rate than the corresponding low-Se treatments, which had 5% and 15% survival, respectively (Fig. 3.4d).

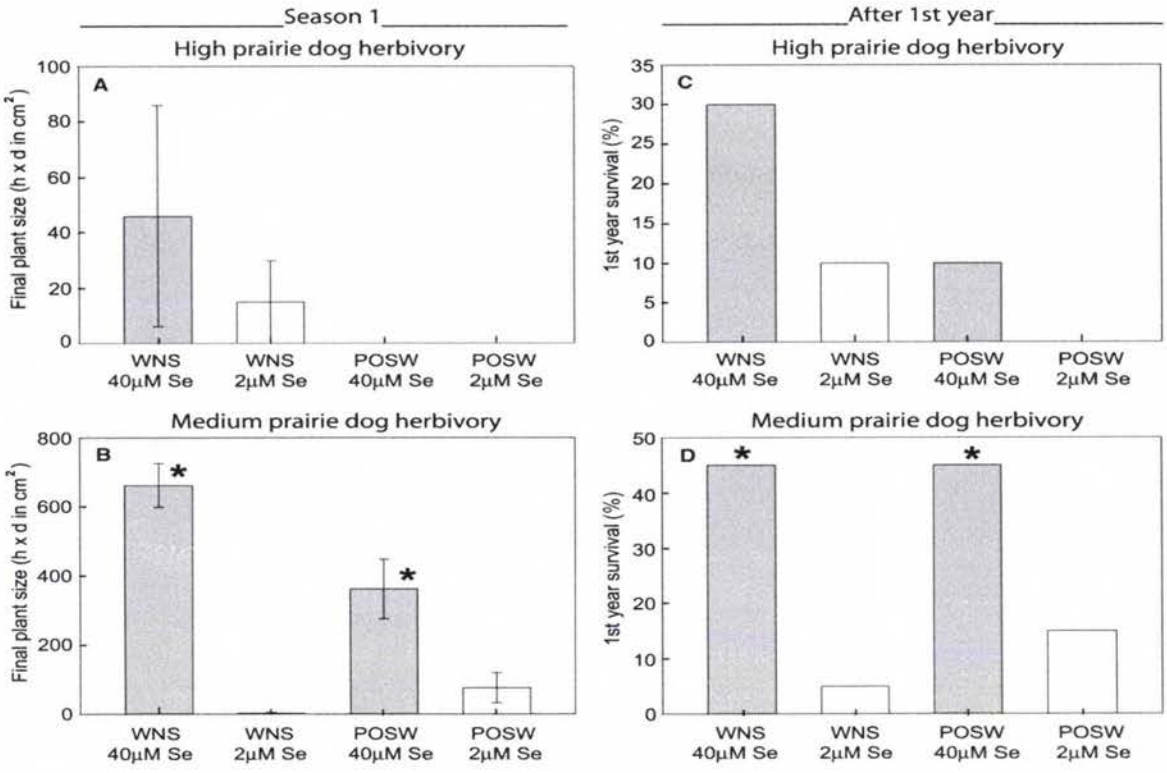

Figure 3.4: *Stanleya pinnata* final plant size and survival % after the 1st growing season for high (a, c) and medium (b, d) prairie dog herbivory test sites. Values are means ± SE. An asterisk denotes a significant difference between the two Se treatments of the same accession ($p < 0.05$).

Season 2—The long-term protective effect of plant Se against herbivory was again measured during the second growing season. Incidentally, the data from the plot with the mammalian exclusion cage could not be used during growing season 2 because the cage was destroyed.

At the beginning of the second season, on the high prairie dog herbivory site the only experimental group that had more than one plant with enough material to sample for leaf Se concentration was *S. pinnata* WNS pre-treated with 40μM Se (Fig. 3.5a). At the end of the second growing season this was the only experimental group with enough material to measure Se leaf concentration at all (Fig. 3.5c). On the medium prairie dog herbivory site, both at the beginning and end of season two, plants pre-treated with high

Se had a higher leaf Se concentration than plants of the same accession pre-treated with low Se (Fig. 3.5b, d).

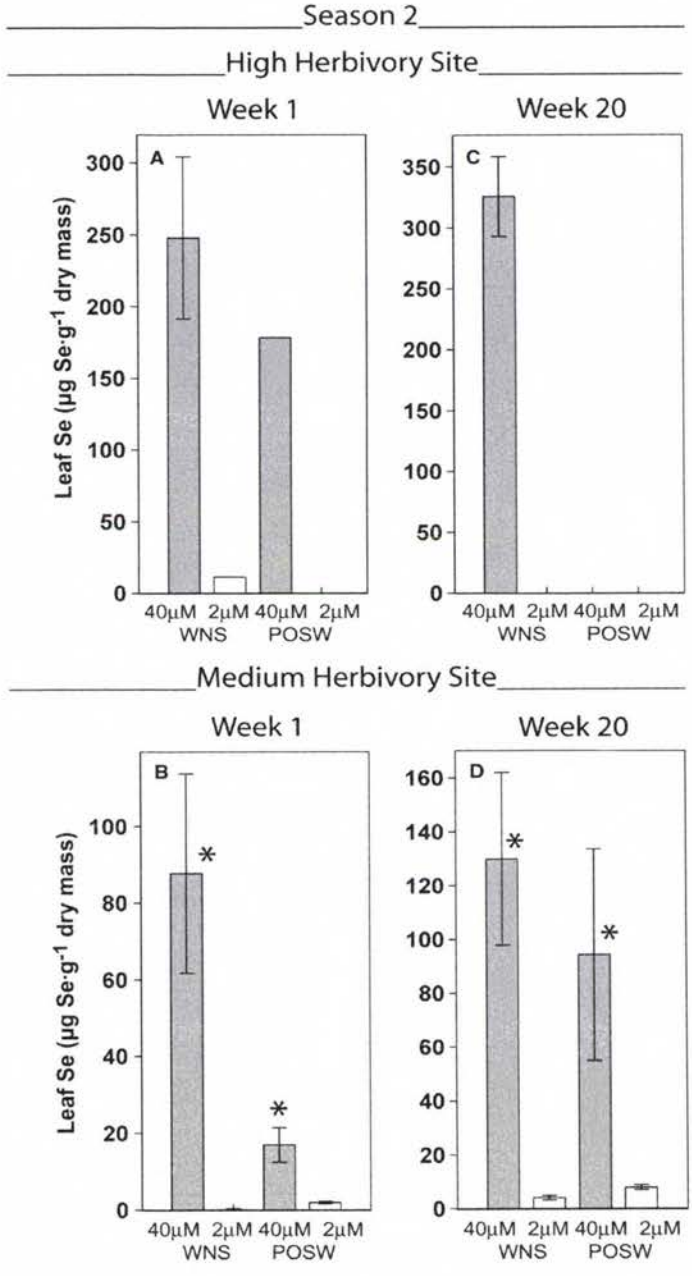

Figure 3.5: *Stanleya pinnata* leaf Se concentration at the beginning (a, b) and the end (c, d) of the second growing season on a high prairie dog herbivory pressure site (a, c) and a medium prairie dog herbivory pressure site (b, d). Values are means ± SE. An asterisk denotes a significant difference between the two Se treatments of the same accession ($p < 0.05$).

The leaf number, plant size and survival percentage of all four *S. pinnata* treatments were recorded at the beginning and end of the second growing season. On the high prairie dog herbivory site, there were no differences between the experimental groups with respect to leaf number and survival percentage at the beginning or end of the second growing season. There was no significant difference in the size of high-Se and low-Se POSW on the high prairie dog herbivory site, however, at the same site, high-Se *S. pinnata* WNS were bigger than low-Se *S. pinnata* WNS when measured at the first week of the second growing season (Fig 3.6a-f). This lack of significance is in part caused by the fact that many plants from each treatment did not survive to the second growing season on the high prairie dog herbivory pressure site. At the end of the second growing season, the high-Se WNS group was the only one that survived, showing a 30% survival rate (Fig. 3.6f).

On the medium prairie dog herbivory site, the high-Se groups of both accessions were again protected when compared with the low-Se groups. High-Se plants had the same number of leaves as low-Se plants of the same ecotype at the beginning of the second growing season (Fig. 3.6g); by the ednd of the seond growing season low-Se plants had significantly (6-fold) fewer leaves than their high-Se counterparts (Fig. 3.6h). High-Se and low-Se *S. pinnata* WNS were the same size at the beginning of the second growing season, however, high-Se *S. pinnata* POSW were bigger than low-Se *S. pinnata* POSW at the same time point (Fig. 3.6i). At the end of the growing season high-Se *S. pinnta* WNS were bigger than low-Se S. pinnata WNS and high-Se and low-Se S. pinnata POSW plant size was not significantly different (Fig. 3.6j).

On the medium prairie dog herbivory site, both high-Se treatments (WNS 40μM & POSW 40μM) survived ~3 times better when compared with their respective low-Se treatment (Fig. 3.6k, l). After the second growing season less than 10% of the original plants from the low-Se groups were alive (Fig. 3.6l).

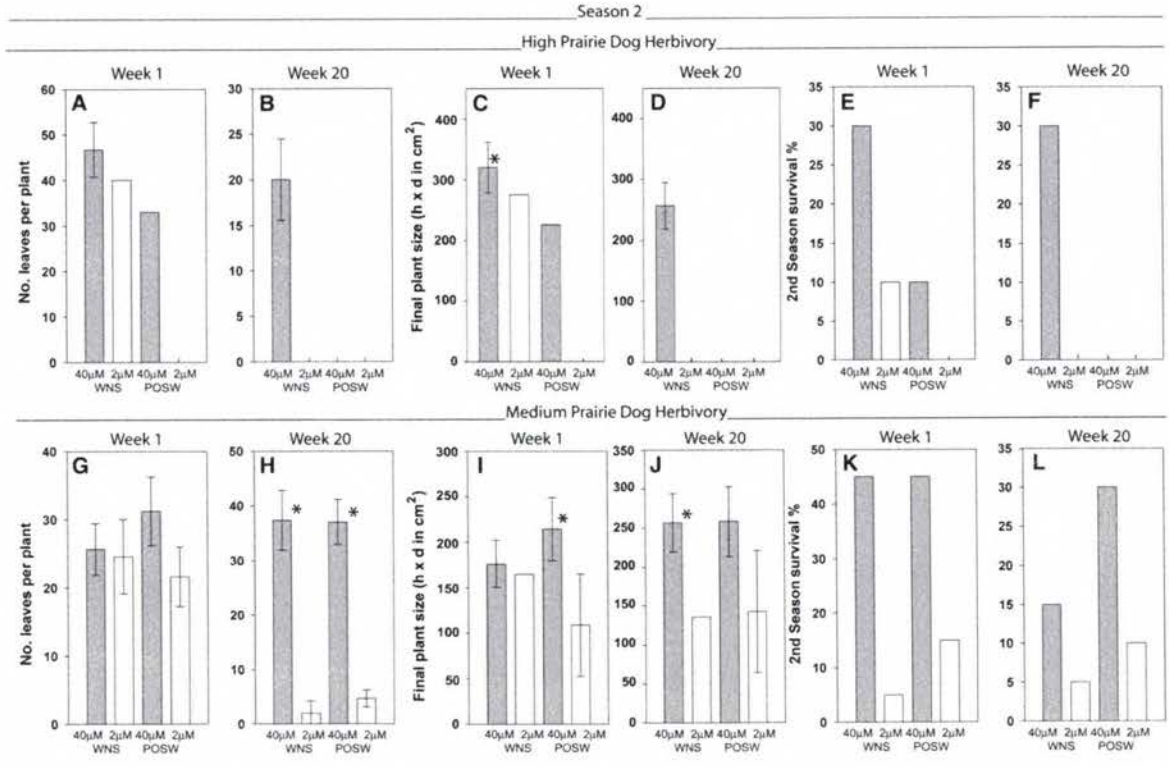

Figure 3.6: *Stanleya pinnata* prairie dog damage after the second growing season. *Stanleya pinnata* leaf number per plant, plant size, and survival percentage over time, for high (a, b, c, d, e, f) and medium (g, h, I, j, k, l)) prairie dog herbivory test sites. Values are means ± SE. An asterisk denotes a significant difference between the two Se treatments of the same accession ($p < 0.05$).

Two years after planting—Herbivory was again analyzed at the beginning of season three which was at the end of this two-year study. On the high prairie dog herbivory pressure site the WNS 40μM Se plants had more than 60 leaves per plant and plants were bigger than 350 cm^2 (Fig. 3.7a, c). This was the only group that survived on the high

prairie dog herbivory site after two years, and their survival percentage was 30% (Fig. 3.7e), the same as at the beginning of season 2.

At the medium prairie dog herbivory pressure site the POSW 40μM Se plants fared better than the others after two years. The POSW 40μM Se plants had more than 40 leaves per plant and were bigger than 300 cm^2 (Fig. 3.7b, d). The difference in leaf Se concentration between the high and low Se pretreated plants remained, and may have contributed to survival (Fig. 3.7f). The difference in Se concentration between the low-Se and high-Se treatments did not decrease over the course of this 2-year experiment; this was probably due to the different Se levels in the transplanted soil.

After two years the flowering rate on the high prairie dog herbivory site was 30% for WNS 40 μM meaning that all the remaining high-Se plants were flowering. On the medium prairie dog herbivory site the high-Se *S. pinnata* treatment (POSW 40μM Se) also had a flowering rate of 30% while none of the low-Se *S. pinnata* plants were flowering.

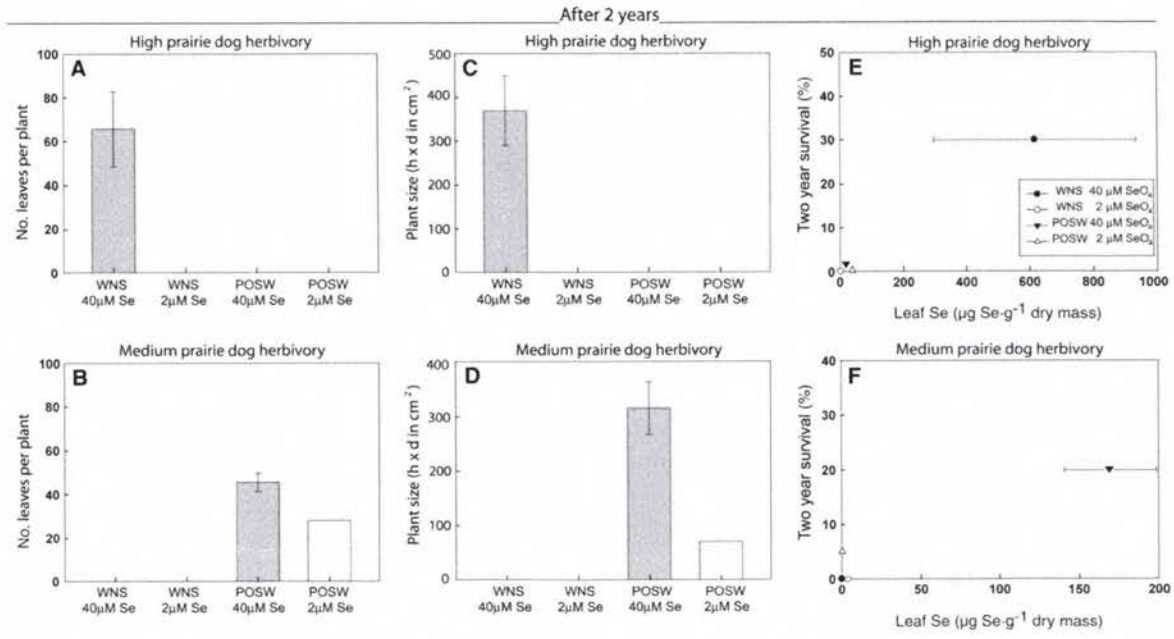

Figure 3.7: *S. pinnata* prairie dog damage, and survival percentage in relation to leaf Se concentration, two years after planting. Shown are *S. pinnata* leaf # per plant (a, b), final plant size (c, d), and two year survival percentage vs. Se concentration (μg g^{-1} dwt) in leaf tissue (e, f) for both high and medium prairie dog herbivory test sites. Values are means ± SE. An asterisk denotes a significant difference between the two Se treatments of the same ecotype ($p < 0.05$).

DISCUSSION

A previous manipulative field study has shown that prairie dogs avoid Se-rich plants (Quinn et al. 2008), however, this study is the first to demonstrate that elevated Se levels protect hyperaccumulator plants against mammalian herbivory in their natural habitat over a long time period. Over a 2-year manipulative field study on two sites with different black-tailed prairie dog (*C. ludovicianus*) herbivory pressure, two *Stanleya pinnata* (prince's plume) experimental groups with elevated (50-750 μg g^{-1} d wt) shoot Se levels suffered less herbivory than experimental groups with low (<10 μg g^{-1} d wt) leaf Se concentration. Both plant groups containing elevated Se levels were clipped and

eaten less, and were bigger than low-Se groups. Experimental groups with elevated Se also had higher survival rates than plants with low Se. This study shows that elevated Se in plants deters prairie dog herbivory. The animals may be deterred by volatile Se emitted by the plants; in addition, any ingested Se-rich plants may have caused toxicity, leading to subsequent avoidance. The Se in *S. pinnata* was found previously to occur in the organic forms methylselenocysteine (MeSeCys) and selenocystathionine (SeCysth) (Shrift and Virupaksha 1965), both of which were toxic to insects when present in *S. pinnata* leaves at elevated levels (Freeman et al. 2006b; 2007). Furthermore, *S. pinnata* volatilizes dimethyldiselenide (DMDSe) which is hypothesized to be a chemical deterrent due to its pungent repellent odor (Terr et al. 2000; Birringer et al. 2002; Ellis and Salt 2003; Sors et al. 2005).

The findings of this study are of significance for several reasons. Although previous experiments have explored plant metal hyperaccumulation as an elemental defense, this study is the first to test the ecological significance of Se hyperaccumulation over a long time period in a hyperaccumulator's natural habitat. While laboratory studies and short term field studies help identify possible ecological advantages of Se hyperaccumulators, only long term field studies provide sufficient evidence that Se plays a significant role in protecting hyperaccumulating plants from herbivory in their natural environment. Moreover, no long-term manipulative field studies have tested the protective effect of hyperaccumulation against mammalian herbivory. Field studies by Martens and Boyd (2002) did suggest a protective effect for Ni in the hyperaccumulator *Streptanthus polygaloides* against a variety of insect herbivores, but in a field study, these plants were unfortunately eaten entirely by larger unknown mammalian herbivores and

the end results were difficult to interpret. Another recent field study was also inconclusive and suggested that Zn accumulation was not correlated with protecting the Zn hyperaccumulator *Thlaspi caerulescens* in the wild from native gastropods (slugs and snails) (Noret et al. 2007).

The plant-herbivore interactions observed in this study have ecological relevance because prairie dogs are native to Se hyperaccumulator habitat, and are known plant ecosystem engineers that have historically had a large impact on plant communities (Whicker and Detling 1988; Weltzin et al. 1997). Thus, Se hyperaccumulators may enjoy reduced competition and survive better on prairie dog colonies, as a result of the intense prairie dog grazing on other plant species. The results presented here may suggest that that herbivory by mammals such as prairie dogs and their ancestors have been one of the contributing selection pressures for the evolution of hyperaccumulation. In this context it is interesting to note that the plant Se levels in this field study were an order of magnitude lower than those which have been found in hyperaccumulators in the field (Galeas et al. 2007). If such low tissue Se levels are already protective (the Se levels right after planting were only ~50 $\mu g\ g^{-1}$ d wt), this may explain how hyperaccumulation could have gradually evolved, driven by herbivore protection (the "defensive enhancement hypothesis", Boyd 2007). Moreover, the protective effect of Se may be even more pronounced in well-established hyperaccumulator populations that contain 10-fold higher levels than those observed in this study.

The findings from this study do not only provide insight into the ecological significance of hyperaccumulation, but are also of interest to rangeland managers and ranchers, and for the cultivation of plants with elevated Se. The aversion to Se-

containing plant material displayed by this native mammalian species, the black-tailed prairie dog, contrasts with the feeding preference of several non-native livestock species which are known to often suffer selenosis from ingestion of Se-rich plants (Magg and Glen 1967), even if there is other vegetation to eat. It would be interesting to investigate whether other native mammals from seleniferous areas (e.g. bison) have an aversion to seleniferous vegetation, similar to the prairie dog. If so, selection of suitable grazers may be a strategy to avoid Se poisoning. Se-accumulating plants may be cultivated to clean up Se-contaminated areas (phytoremediation). Another reason to cultivate Se-accumulating plants is that Se-enriched plant material has added nutritional value, since Se is an essential nutrient for mammals. A concern related to the cultivation of Se-accumulating plants is that the plant Se may enter the food chain and cause toxicity. Our finding that Se levels as low as 50 $\mu g\ g^{-1}$ d wt are already sufficient to deter herbivory from this native mammal suggests that herbivores may avoid Se-rich plants. Indeed, we found a similar deterrence by Se for a variety of invertebrate herbivores (for a review see Quinn et al. 2007). Therefore, the study described here has important implications for the risks of cultivation of plants with elevated Se for either phytoremediation or as Se-fortified food. Based on our results, the probability of this risk appears to be relatively low.

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Chapter 4

Ecological aspects of selenium accumulation in flowers – effects on pollination

NOTES AND ACKNOWLEDGMENTS

Dr. Elizabeth Pilon-Smits and Dr. Arathi Sheshadri contributed to experimental design. We would like to thank Dr. Dhruba Naug for access to honey bee hives. We would also like to thank Dr. Patricia A. Bedinger and Paul Covey for assistance with pollen germination experiments. Ray Reynolds aided in the pollen germination experiments. Amanda Gross aided in the pollinator experiments. We would also like to thank the Colorado Native Plant Society for their aid in funding this research. Funding for these studies was also provided by the National Science Foundation grant # IOS-0817748.

ABSTRACT

Selenium (Se) hyperaccumulator plants grow exclusively on seleniferous soils and typically accumulate Se to concentrations two orders of magnitude higher than other species growing on the same site. The functional significance of hyperaccumulation may be as an elemental defense, since Se-rich plants are protected from a variety of herbivores and pathogens. Negative consequences associated with Se hyperaccumulation, such as decreases in pollinator visits or pollen viability, remain to be explored. In addition, the localization and speciation of Se in flowers is not known. In this study we investigated Se concentration, distribution and speciation in flowers of the Se hyperaccumulator *Stanleya pinnata* and the related non-hyperaccumulator *Brassica juncea*, an important crop and phytoremediation species. In addition, we explored the reproductive costs associated with Se accumulation by measuring pollen germination rates and pollinator visitation frequency of high- and low-Se plants.

S. pinnata stored more Se in flowers than in leaves (3,621 and 1,458 mg Se kg^{-1} dry weight (DW), respectively). The predominant form of Se in flowers was methylselenocysteine, a relatively non-toxic form of Se. Within the flower the Se concentration was higher in stamens and pistils than in sepals and petals, with the highest concentration in ovules and pollen. In contrast, *B. juncea* accumulated more Se in leaves than flowers (371 and 229 mg Se kg^{-1} DW, respectively) and within flowers Se was evenly distributed. The main form of Se found in *B. juncea* flowers was the toxic selenite. Elevated Se concentrations seemed to have a positive, if any, effect on *S. pinnata* pollen germination. In contrast, *B. juncea* plants containing 2,210 mg Se kg^{-1}

DW showed a significant decrease in pollen germination rate compared to plants with trace Se concentrations.

Pollination studies comparing high-Se (220 mg Se kg^{-1} DW) and low-Se concentration *B. juncea* plants placed in close proximity to a honey bee hive in a non-seleniferous area found no difference in visitation frequency or duration from honey bee and total pollinators. These results have implications for the management of Se-rich agricultural and natural areas and for phytoremediation of Se-polluted sites.

Keywords: *Stanleya pinnata*, *Brassica juncea*, selenium, hyperaccumulator, pollen, pollinator

INTRODUCTION

Selenium (Se), named after the Greek word for moon, selene, was first discovered in 1817 by the Swedish chemist Jöns Jacob Berzelius. Since its discovery, Se has been identified both as an essential element as well as a devastating toxin. The gap between Se deficiency and toxicity is narrow and both are problems worldwide. Sufficient Se in humans is known to reduce the chances of cancer and heart disease and is a valuable element for regulating thyroid function (Goldhaber 2003, Shin et al. 2007, Kato et al. 2010).

Selenium toxicity is also a well-documented problem across the globe. Selenium is toxic to organisms at elevated concentrations because of its chemical similarity to sulfur (S). Selenium is inadvertently incorporated into essential S proteins resulting in a loss of protein function (Stadtman 1990). Chronic Se toxicity, called selenosis, results in loss of hair and nails and can eventually lead to death (Oliveira 2007; Steinbrenner and Sies 2009). If selenosis is correctly diagnosed most symptoms can usually be reversed with a decreased daily intake of Se. Acute Se poisoning due to a one-time ingestion of a high dosage of Se may result in death within 48 hours (Salyi et al. 1993).

While in plants Se serves no known essential function, Se has been reported to be a beneficial element to many plants. Remarkably for a non-essential element, some so-called Se hyperaccumulating plants regularly accumulate Se to levels of more than 1,000 mg Se kg^{-1}; they may even contain levels as high as 10,000 mg Se kg^{-1}. Selenium hyperaccumulators are found in the Brassicaceae, Asteraceae and Fabaceae families (Terry 2000, Galeas et al. 2007). Plants that accumulate Se between 100 and 1,000 mg Se kg^{-1} are called Se accumulators while other plant species, called non-Se accumulators,

only accumulate trace concentrations of Se when grown on seleniferous soils (Terry 2000). Non-Se hyperaccumulating plant species suffer Se toxicity when grown on elevated concentrations of Se; these plants inadvertently take up Se via the S assimilation pathway and incorporate Se into essential S proteins, causing toxicity (Anderson 1993). Research investigating Se speciation in the leaves of Se hyperaccumulating and non-hyperaccumulating plants has revealed that Se hyperaccumulators accumulate primarily methylselenocyteine (MeSeCys), which is not incorporated into proteins and therefore not toxic , in contrast to the toxic selenate and selenocysteine that are more commonly found in non-hyperaccumulators (Brown and Shrift 1981, Neuhierl et al. 1999, Freeman et al. 2006). Plants can also volatilize Se, creating a strong distinctive odor which often makes Se hyperaccumulating plants easy to identify. Non-Se hyperaccumulating plants volatilize Se as dimethylselenide while Se hyperaccumulators volatilize Se as dimethyldiselenide (Lewis et al. 1966, Kubachka et al. 2007).

Multiple hypotheses for the functional significance of hyperaccumulation have been proposed, including increased drought resistance, allelopathy and as an elemental defense against herbivores and pathogens (Boyd and Martens 1992). Most research has focused on and provided support for the elemental defense hypothesis. Elevated Se concentrations were shown to protect *Brassica juncea* (Indian mustard), an important Se accumulating crop species, from aphids, Lepidoptera larvae and fungal pathogens (Hanson et al. 2003, 2004). Studies have shown that Se hyperaccumulating species, such as *Astragalus bisulcatus* and *Stanleya pinnata*, are protected from a variety of arthropod herbivores and prairie dogs, a mammalian herbivore native to the same region of many Se hyperaccumulator species (Freeman et al 2006b, 2007, Quinn et al. 2008). Moreover,

a field survey revealed that Se hyperaccumulators harbored fewer arthropods and fewer arthropod species than similar non-Se hyperaccumulator species (Galeas et al. 2008). Like any arms race between herbivores and plant defense mechanisms, this elemental defense has been disarmed by herbivores. A population of diamondback moths observed feeding on *S. pinnata* was shown to have evolved Se tolerance, apparently by losing its capacity to demethylate MeSeCys (Freeman et al. 2006b).

Besides the benefits of hyperaccumulation, there may also be costs associated with Se hyperaccumulation, as suggested by the fact that hyperaccumulators are rarely found on non-seleniferous soils. For instance, it is possible that elevated plant Se concentrations affect reproductive success through decreased pollen viability and/or pollination. To date, no studies have been published regarding the effects of plant Se on pollinator visits or health, nor on any effects of Se on pollen viability and growth. The role of elevated Se in flowers has recently received some media attention due to its possible toxicity to the economically important honey bee (Reilly 2009). Since Se is toxic to many herbivores it can be speculated that pollinators may also be Se sensitive, and suffer toxicity when foraging on high-Se flowers. In seleniferous areas, on the other hand, Se tolerant pollinators may exist that are attracted to flowers with high amounts of Se because they provide decreased foraging competition or possibly because the elevated Se provides some health benefit to the pollinator. Volatile Se may serve as a cue to pollinators to identify plants high in Se, either as an attractant for specialist pollinators or as a deterrent for generalists.

In this study we investigate Se distribution, localization and speciation in the flowers of the Se hyperaccumulator *S. pinnata* and the related Se accumulator *B. juncea*.

We also investigate how elevated Se levels affect pollen viability and how floral Se in *B. juncea* affects pollination by honey bees and other pollinator species.

METHODS

Plant material

Stanleya pinnata flowers and leaves, and bumble bees (*Bombus* sp) foraging on *S. pinnata* flowers, were collected from Pine Ridge Natural Area (40°32.70N, 105°07.87W) in South West Fort Collins, CO, USA during June 2008. Pine Ridge Natural Area is a seleniferous habitat with soil composed of Se-rich Cretaceous shale and harbors at least two species of Se hyperaccumulating plants: *S. pinnata* and *A. bisulcatus* (Galeas et al. 2008). The population of *S. pinnata* sampled is known to accumulate high concentrations of Se (Freeman et al. 2006b; Galeas et al. 2007). Samples collected were analyzed for Se and S concentration, distribution and speciation as described below.

Surface-sterilized *B. juncea* and *S. pinnata* seeds (Pilon-Smits et al. 1999) were germinated in Pro Mix BX in a greenhouse with natural light and a 12 hour photoperiod (24°C/20°C). Two weeks after germination half of the plants were treated with high-Se, either 80 μm Na_2SeO_4 (selenate is the dominant form of bioavailable Se in most soils) for pollen germination experiments or 20 μm Na_2SeO_4 for pollinator studies, while the other half were given water as a control. *Brassica juncea* plants flowered 5-6 weeks after germination and were used for pollen germination and pollinator studies as described below. After 18 months of growth, *S. pinnata* plants were placed in a cold room for 4 weeks to induce flowering. After flowering, *S. pinnata* plants were used for pollen

germination experiments. Leaf and flower samples were collected and analyzed for Se and S concentration, distribution and speciation as described below.

Se concentration, speciation and distribution

Whole mature flowers, flower parts (sepals, petals, stamens, pistils and immature seeds) and the youngest mature leaves were collected from *S. pinnata* and *B. juncea* for Se and S concentration analysis as described below. Bumble bees foraging on Se rich *S. pinnata* were also analyzed for Se concentration. Samples were rinsed with distilled water to remove any external Se and S and then dried at 45° C for 48 hours. 100 mg DW of each sample was then 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 Se concentration.

Selenium speciation and distribution were analyzed using the Advanced Light Source beamline 10.3.2 at the Lawrence Berkeley Laboratory as described by Marcus et al. (2004). Selenium speciation and distribution was determined for flowers and flower parts of *S. pinnata* and *B. juncea* as well as for bumble bees. In short, samples used for Se speciation were immediately frozen using liquid nitrogen after collection and remained frozen until analysis was complete to prevent changes in Se distribution and speciation. Samples were scanned using an X-ray at 13,000 eV to map Se distribution. The speciation of Se at particular points in the sample was investigated using Se-K XANES. Each flower part, including pollen and ovules, of both *B. juncea* and *S. pinnata* was analyzed for Se speciation. In the bumble bee the thorax and abdomen, as well as

pollen sacs on rear legs, were analyzed for Se speciation. Known forms of Se were used as standards.

Pollen germination studies

Brassica juncea and *S. pinnata* plants grown as described above were used in pollen germination studies to determine if elevated floral Se affected pollen viability. Anthers of high-Se and low-Se plants were collected and pollen was placed on semi-solid agar media containing 18% sucrose, 0.01% boric acid, 1mM $CaCl_2$, 1mM $Ca(NO_3)_2$ and 1mM $MgSO_4$. After 24 hours of growth the total number of pollen and the number of pollen grains that had germinated were counted, and the % pollen germination calculated.

Pollinator studies

The pollinator experiment was conducted with a colony of the European honey bees (*Apis cerana*) at a non-seleniferous field site. We provided the honey bees with a choice between high-Se and low-Se *B. juncea* grown as described above. Groups of 18 high- or low-Se flowering plants were placed 10 m from the hive and 10 m from each other. To investigate if pollinators preferred high-Se or low-Se plants the percent of plants whose flowers were visited by arthropods, visited by honey bees and the number of individual honey bees that visited each group of plants was recorded simultaneously for high and low-Se plants for 45 minute time intervals. In addition, quality bee visits were determined by counting the number of visits that were longer than 5 seconds at each flower. All of the pollinator experiments were conducted between 9:30 am and 12:30 pm on sunny days when bees were actively foraging, between June 25 and July 30, 2008. For

each time period one person collected data from high-Se plants and another individual from low-Se plants. To neutralize any effects of environmental conditions, such as wind direction or location of the sun, the groups of plants were removed from the site after the 45 minute observation period. After 15 minutes the plants were returned to the field site with the high- and low-Se locations switched, and the experiment was repeated. The total observation time was 1 hour 30 minute (two 45 minute time intervals) each day the experiments were conducted.

Data 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 Se and S concentrations of leaves and flowers of *S. pinnata* and *B. juncea* and high- and low-Se *B. juncea* used for pollinator studies. A linear regression was used to correlate plants Se concentration and *S. pinnata* pollen germination rates. A student's t-test was also used to compare % of pollen germination between high- and low-Se *B. juncea* and the % of plants visited by pollinators and honey bees, number of honey bees visiting each group and quality bee visits to high and low-Se plants during pollinator studies. When comparing differences between Se concentrations in floral parts a Tukey-Kramer test was used.

RESULTS

Se concentration, speciation and distribution

To investigate the concentration, distribution, speciation and location of Se in Se hyperaccumulating plants and non-Se hyperaccumulating plants leaf and flower samples were analyzed for the Se hyperaccumulator *S. pinnata* and the non-hyperaccumulator *B. juncea* (Fig. 4.1).

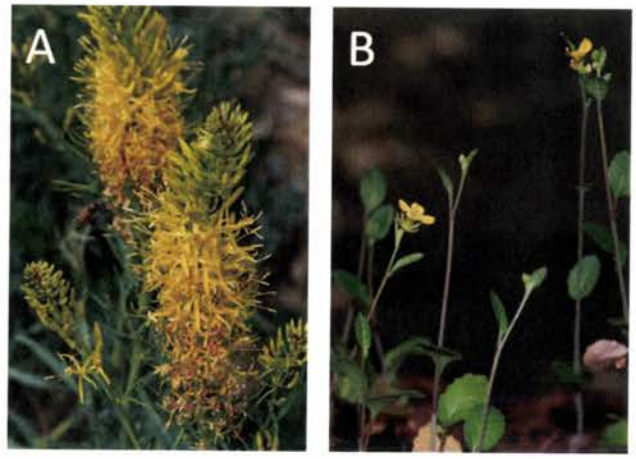

Figure 4.1: The Selenium hyperaccumulating plant *S. pinnata* (A) and the related non Se hyperaccumulator *B. juncea* (B).

Selenium distribution maps (μXRF) of *S. pinnata* flowers (Fig. 4.2A) revealed that Se was primarily localized in the ovules in the pistil (Fig. 2B) and in pollen grains on the tips of the anthers (Fig. 4.2C). Selenium in petals and sepals of *S. pinnata* was distributed in a more diffuse pattern (Fig. 4.2D). XANES analysis showed that flowers of *S. pinnata* primarily accumulated Se in the form of an organic C-Se-C compound, consistent with the MeSeCys standard (Fig. 4.2E, similar Se speciation results were found for all flower parts). This the same form was found previously in *S. pinnata* leaves as well as in other Se hyperaccumulating species, and was found by liquid chromatography-mass spectrometry to be MeSeCys (Burnell 1981, Freeman et al. 2006a). *Brassica juncea* flowers (Fig. 4.2F) showed a diffuse distribution of Se in all of their flower parts including the pistil (Fig. 4.2G), stamen (Fig. 4.2H), petal (Fig. 4.2I) and

sepal (Fig. 4.2J). Selenium speciation studies revealed that *B. juncea* flowers contained a variety of forms of Se including 26% selenite, which is toxic to plants at elevated concentrations (Hopper and Parker 1999).

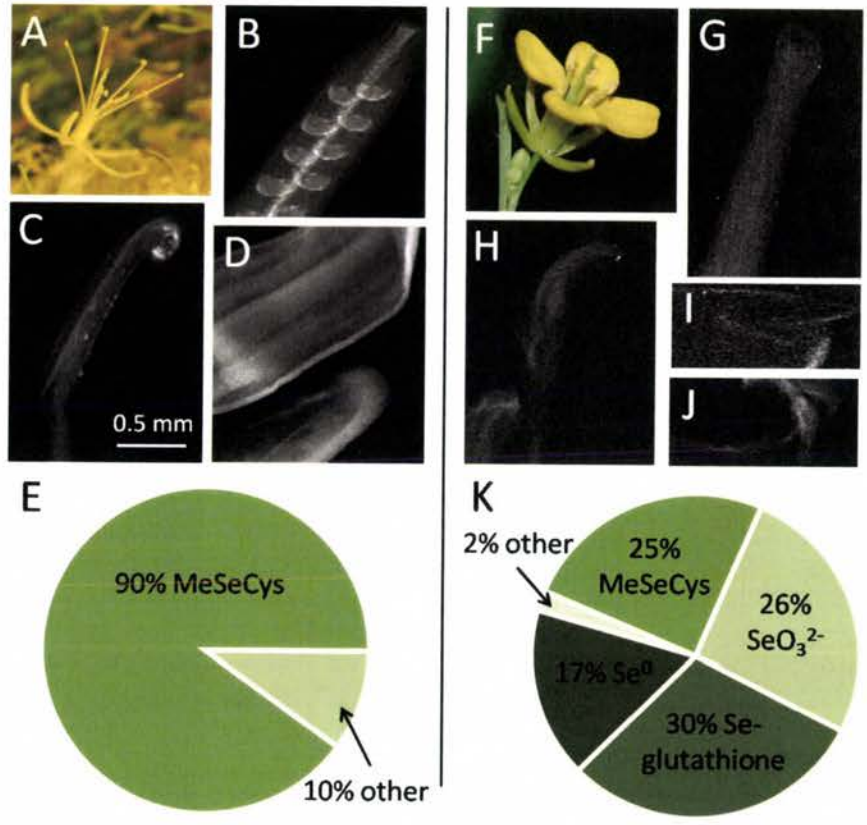

Figure 4.2: In *S. pinnata* flowers (A) Se, shown in white using X-ray absorption spectroscopy, was found to be concentrated in the ovules of the pistil (B), in pollen grains on the anther of the stamen (C) and diffusely distributed in petals and sepals (D). The primary form of Se found in *S. pinnata* flowers was MeSeCys (E). Selenium in a *B. juncea* flower (F) was diffusely distributed in the pistil (G), stamen (H), petal (I) and sepals (J).Selenium in the *B. juncea* flower was composited of a variety of selenocompounds (K).

Bumble bees observed foraging on Se-rich *S. pinnata* (Fig. 4.3A) were collected to determine Se distribution, speciation and concentration. Distribution studies revealed that Se was distributed throughout the body of the bumble bee with a relatively high

concentration on the rear legs, most probably in pollen sacs (Fig. 4.3B). Two individual bumble bees foraging on *S. pinnata* flowers with 3,621 mg Se kg^{-1} contained 228 and 274 mg Se kg^{-1} dry weight (DW), respectively. The bumble bees contained the same form of Se found in *S. pinnata*, the non-toxic MeSeCys (Fig. 4.3C), both on their legs and in their body.

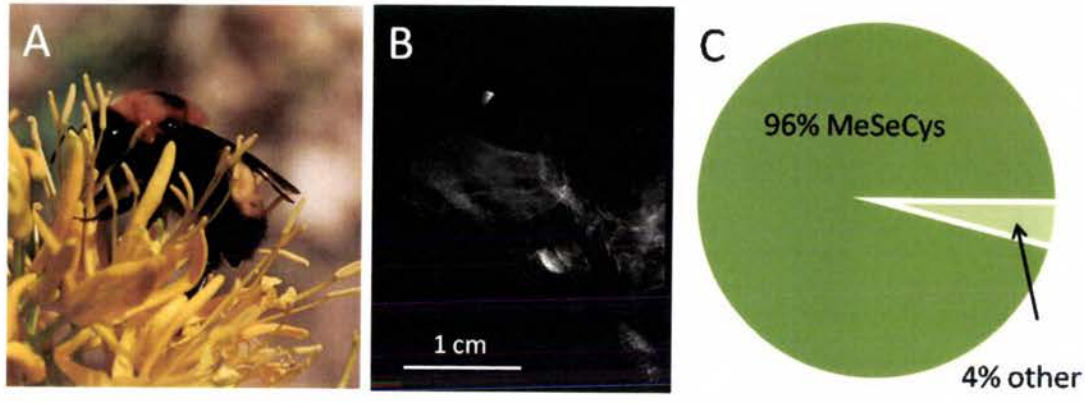

Figure 4.3: Bumble bees foraging on Se-rich *S. pinnata* (A) were found to have Se distributed throughout their body and in pollen collected (B), primarily in the form of MeSeCys (C), the same form found in *S. pinnata* flowers (as shown in Fig. 2E).

Further investigation of Se concentration and distribution revealed differences between Se allocation of *S. pinnata* and *B. juncea*. *Stanleya pinnata* contained more Se in flowers than in leaves, averaging 3,621 ± 126 and 1,458 ± 246 mg Se kg^{-1} DW, respectively (Fig. 4.4A, student's t-test, $p < 0.001$, $t = 6.73$, $n = 10$ repetitions of either leaves or flowers each from a different plant). Within the *S. pinnata* flower Se was preferentially allocated to the sex parts and to the immature seeds: the stamen, pistil and immature seeds had higher concentrations of Se, between 3,400 and 4,400 mg Se kg^{-1} DW, than the petals and sepals, which contained between 2,200 and 2,400 mg Se kg^{-1} DW (Fig. 4.4B, Tukey-Kramer test, $n = 9$ different repetitions for each flower part, each

repetition is from a different flower and different plant). Interestingly, the opposite trend was observed in *B. juncea*, where leaves contained more Se than flowers, at 371 and 229 mg Se kg^{-1} DW, respectively (Fig. 4.4C, student's t-test, $p = 0.02$, $t = -2.40$, $n = 20$ repetitions of either leaves or flowers each from a different plant). The sepals, the most vegetative-like part of the flower, had more Se (1,400 mg Se kg^{-1} DW) than the petals, stamens, pistils and immature seeds, which all had less than 700 mg Se kg^{-1} DW (Fig. 4.4D, Tukey-Kramer test, $n = 5$ repetitions for each flower part, each repetition is from a different flower and different plant).

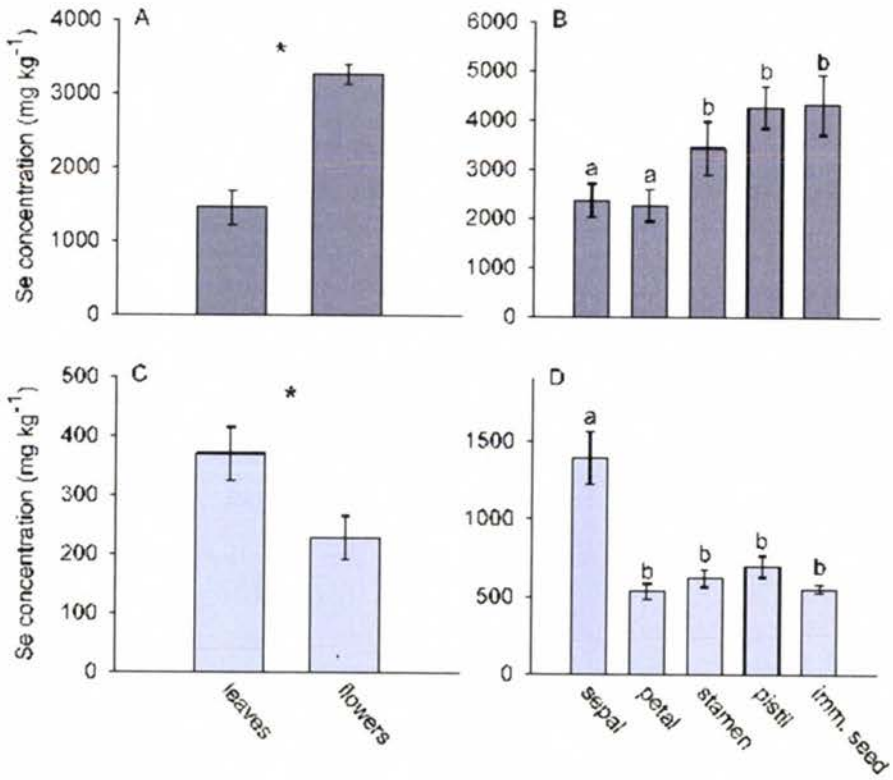

Figure 4.4: Selenium concentration in *S. pinnata* leaves and flowers (A, $n = 10$) and flower parts (B, $n = 9$). *Brassica juncea* Se concentration in leaf and flower (C, $n = 20$) and flower parts (D, $n = 20$). Values are means +/- SE; an asterisk or a different letter above bars represents a significant difference ($\alpha = 0.05$).

Interestingly, S concentrations in leaves and flowers of *S. pinnata* were similar (Fig. 4.5A, student's t-test, p = 0.77, t = -0.29, n = 10 repetitions of either leaves or flowers each from a different plant) and within the flowers *S. pinnata* was more evenly distributed than Se (Fig. 4.5B, Tukey-Kramer test, n = 9 repetitions for each flower part, each repetition is from a different flower and different plant). *Brassica juncea* flowers contained less S than leaves, a similar trend observed for Se (Fig. 4.5C, student's t-test, p < 0.001, t= -5.16, n = 20 repetitions of either leaves or flowers each from a different plant). Within flowers, sepals had higher concentrations of S than petals, and all other flower parts had similar concentrations of S (Fig. 4.5D, Tukey-Kramer test, n = 20 repetitions for each flower part, each repetition is from a different flower and different plant).

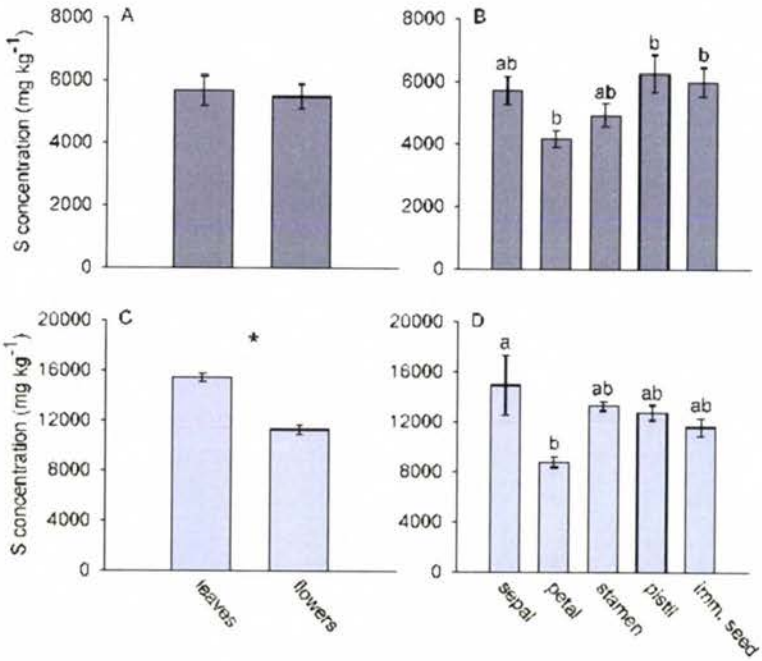

Figure 4.5: Sulfur concentration in *S. pinnata* leaves and flowers (A, n = 10) and flower parts (B, n = 9). *Brassica juncea* S concentration in leaf and flower (C, n = 20) and flower parts (D, n = 20). Values are means +/- SE; asterisks or different letters above bars represent significant differences ($\alpha = 0.05$).

Pollen germination studies

Pollen germination was weakly correlated with Se concentration in *S. pinnata* and compared between high-Se and low-Se *B. juncea* to investigate whether elevated Se affects plant fitness. There was an extremely weak positive correlation between increased leaf Se concentration and pollen germination rates in *S. pinnata* (Fig. 4.6A, r^2 = 0.11, n = 7 flowers from different plant's pollen that was analyzed for pollen germination). Pollen collected from high-Se *B. juncea* plants (2,100 mg Se kg^{-1} DW in flower) had approximately 2-fold lower germination rates than pollen from flowers containing 2 mg Se kg^{-1} DW (Fig 4.6B p = 0.026, t =2.60, n = 6 high-Se and 6 low-Se flowers from different plant's pollen that was analyzed for pollen germination; Fig 6C, p < 0.001 , t = 11.30, n = 3 for low-Se and 6 for high-Se flowers from different plants that were analyzed for Se concentration).

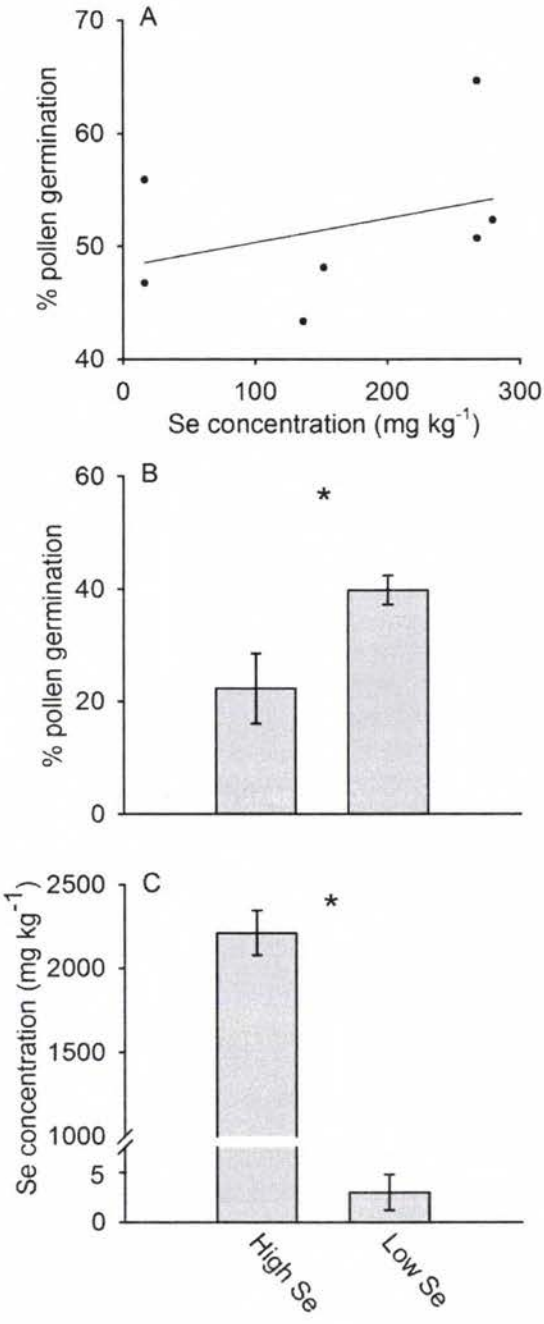

Figure 4.6: Pollen germination rate correlated with *S. pinnata* leaf Se concentration (A, n = 7), and pollen germination rate (B) and flower Se concentration (C) of high- and low-Se *B. juncea* (n = 6). Values are means +/- SE; an asterisk between bars represents a significant difference ($\alpha = 0.05$).

Pollinator studies

To investigate the effect of elevated floral Se concentrations on pollination by honey bees, pollinator visits were compared between *B. juncea* plants containing significantly different floral Se concentrations of 229 and 7 mg Se kg^{-1} DW, respectively (Fig. 4.7A, student's t-test, $p < 0.001$, $t = -5.94$, $n = 20$). The same percentage of high- and low-Se plants were visited by arthropods (primarily belonging to the order Hymenoptera) as well as by honey bees (Fig. 4.7B, student's t-test, $p = 0.86$, $t = 0.178$, $n = 13$; Fig. 4.7C, student's t-test, $p = 0.54$, $t = 0.662$, $n = 13$). The same number of honey bees visited high- and low-Se plants (Fig. 4.7D, student's t-test, $p = 0.95$ $t = 0.057$, $n = 13$). In addition, the number of quality foraging visits were the same between high- and low-Se plants, averaging 21 and 22 per 45 minutes, respectively (student's t-test, $p = 0.815$, $t = -0.24$, $n = 13$).

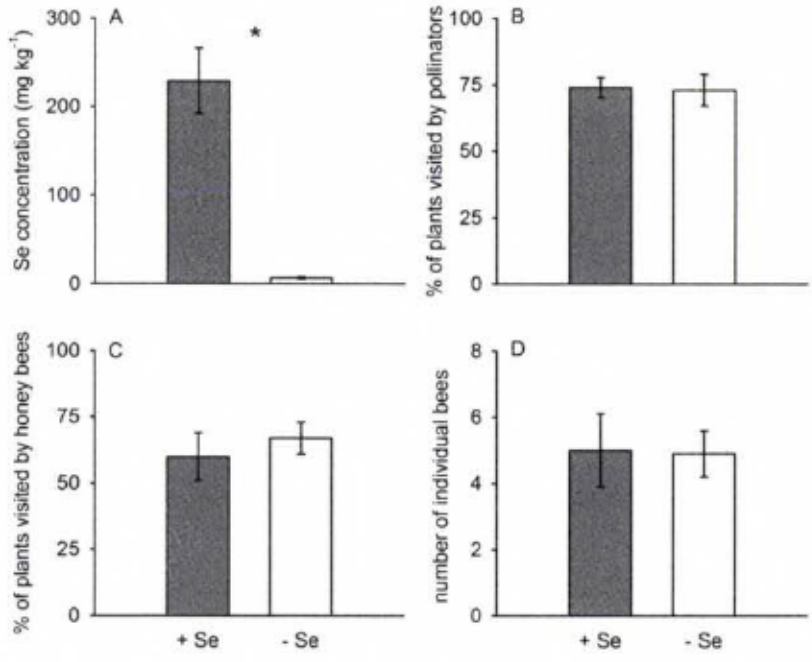

Figure 4.7: Selenium concentration in flowers of high- and low-Se *B. juncea* used for pollination studies (A, n = 20). The percent of plants visited by any potential pollinator (B), by honey bees (C) and the number of individual honey bee visits per group of plants (D) during 45 minute time intervals. Values are means +/- SE; an asterisk between bars represents a significant difference ($\alpha = 0.05$).

DISCUSSION

This study is the first to investigate Se localization and speciation in flowers of Se hyperaccumulators and non-hyperaccumulators. In addition, this is the first study to investigate the potential costs associated with hyperaccumulation by examining the effects of elevated Se on pollen germination rates and plant-pollinator interactions. *Stanleya pinnata*, a Se hyperaccumulator native to the Western USA, was compared to *B. juncea*, an important crop species often used for phytoremediation of Se. *Brassica juncea* is in the same family as *S. pinnata* and is considered a Se accumulator, but not a hyperaccumulator.

We show that Se accumulation in flowers is substantial, particularly in the hyperaccumulator S. pinnata, which appears to concentrate Se preferentially in the reproductive organs, stamens and pistils, and in ovules within pistils and pollen on anthers. Elevated floral Se did not affect pollen germination rates of *S. pinnata*, but negatively affected pollen germination in *B. juncea*. Thus, Se accumulation in non-hyperaccumulators may carry a cost in terms of reproductive fitness. Floral Se concentrations averaging 229 mg Se kg^{-1} DW did not deter pollinator visits to *B. juncea*. These data are important for management of seleniferous habitats because they give insight into pollinator behavior when foraging on Se-rich plants. In addition, these data aid in risk assessment related to the cultivation of Se-rich plants for phytoremediation or as fortified foods because they provide insight into how Se effects plant fitness about plant fitness when.

An interesting difference between the Se hyperaccumulator *S. pinnata* and the non-hyperaccumulator *B. juncea* was that the hyperaccumulator contained higher Se concentrations in flowers than leaves while the non-hyperaccumulator showed the opposite pattern. Moreover, within the flower the hyperaccumulator concentrated its Se in the reproductive parts while *B. juncea* did not show such localized sequestration. A possible explanation for this uneven distribution of Se is that *S. pinnata* preferentially allocates Se to valuable tissues, in order to protect them against pathogens and/or herbivores. Interestingly, floral and leaf S concentrations in *S. pinnata* were the same, and thus S and Se showed different distribution patterns in the hyperaccumulator. Floral S concentrations in leaves and flowers of *B. juncea* followed the same pattern as its Se concentrations. Non Se hyperaccumulators like *B. juncea*, in contrast to Se

hyperaccumulating species, cannot discriminate between S and Se due to the chemical similarity of the two elements. Thus, the Se distribution in *B. juncea* is likely following S accumulation patterns while the Se in *S. pinnata* is specifically transferred to specific locations. Previous studies have shown that young leaves of Se hyperaccumulating plants have more Se than old leaves and that Se is located in areas that are either vulnerable to herbivore attacks, like the edges of leaves, or have a known defensive function, such as leaf hairs (Freeman et al. 2006a, Galeas et al. 2007).

The tissues where Se was most concentrated in *S. pinnata*, ovules and pollen, are directly responsible for passing down genes to future generations. Elevated Se in seeds, a product of these two reproductive tissues, might give offspring a better chance of survival because they are protected from herbivores and pathogens during the vulnerable stages of germination and primary growth. Indeed, developing *S. pinnata* seeds had similar Se concentrations as stamens and pistils. A possible selection pressure leading to increasingly elevated concentrations of Se in pollen and ovules could be that seeds with higher concentrations of Se give rise to plants with higher survival and reproduction rates than seeds with low Se. *Stanleya pinnata* flowers accumulated Se in the form of MeSeCys. Methyl-SeCys is relatively non-toxic compared to the inorganic forms of Se commonly found in non-hyperaccumulators, because it does not get incorporated into proteins. Methylation of SeCys is thought to be one of the mechanisms that allow Se hyperaccumulators to accumulate such high concentrations of Se without suffering toxicity (Neuhierl et al., 1999).

Our XANES studies show that *B. juncea* flowers contained a wide array of Se species, including the toxic selenite (Hopper and Parker 1999). Since *B. juncea* is such

an important crop and because they are often grown in seleniferous habitats, either for phytoremediation or for agriculture (Bañuelos et al. 2000, 2007), pollinator tests were carried out to determine the effect of Se on pollination. We found that *B. juncea* with leaves containing 371 mg Se kg-1 DW and flowers containing 229 mg Se kg^{-1}, concentrations that deter many generalist herbivores (Vickerman and Trumble 1999, Quinn et al. 2008), did not decrease overall pollinator visits or honey bee visits in particular. Similarly, a variety of pollinators including honey bees and native bumble bees were observed visiting the hyperaccumulator *S. pinnata* in its native seleniferous habitat. Thus, our observations and data provide no evidence that Se deters pollinators.

This study may suggest that these bumble bees, native to seleniferous habitat, can tolerate elevated Se concentrations because they contained up to 271 mg Se kg^{-1} DW without sign of toxicity. The bumble bees contained Se in the form of MeSeCys Previous studies have shown that an arthropod herbivore (*Plutella xylostella*) found feeding on these same plant species on the same site were also Se tolerant and also accumulated MeSeCys, while a population of the same species from a non-seleniferous area was Se-sensitive and accumulated primarily SeCys (Freeman et al. 2006b). It is reasonable to assume that ecological partners of hyperaccumulator plants have evolved Se tolerance to take advantage of the niche provided by these plants that are toxic to other species.

Most studies to date have investigated and demonstrated the beneficial effects of Se (hyper)accumulation in plants. This study indicates a potential cost of Se accumulation, in the form of reduced pollen germination. These potential reproductive costs and reduced fitness associated with Se accumulation was only found in the non-

hyperaccumulator; hyperaccumulator pollen appeared to be Se-tolerant. The key to this difference in Se tolerance between *B. juncea* and *S. pinnata* pollen appeared to be the form of Se accumulated: while *B. juncea* flower parts contained a variety of Se species, *S. pinnata* flower parts, including pollen, contained almost exclusively non-toxic MeSeCys.

The apparent cost, as reduced pollen germination, of Se accumulation in flowers of accumulators, but not hyperaccumulators is interesting when considering the possible selection pressures during gradual evolution of Se hyperaccumulation. As was shown in earlier studies, moderate Se accumulation (as low as 10 mg Se kg^{-1} DW, Hanson et al., 2004) can already give non-hyperaccumulator plants the benefit of reduced herbivory, but at the same time there may be a cost in terms of reproductive fitness. Depending on the degree of herbivory pressure, the benefits may outweigh the cost. At some point in evolution hyperaccumulators may have overcome this cost via changes in Se metabolism, or alternatively, hypertolerance may have evolved before hyperaccumulation.

The results from this study are especially relevant for agriculture in seleniferous environments and for Se phytoremediation of polluted areas or cultivation of Se-fortified crops. Most crops and phytoremediation species used in these settings have Se concentrations lower than those in the plants used in these pollinator studies (371 mg Se kg^{-1} DW), which did not lead to a decrease in pollinator visits (Dhillon and Dhillon 2009). In the United States, honey bee benefits to agriculture are estimated to be $14 million annually and worldwide estimates are over $200 billion annually (Gallai et al. 2009). If Se-rich crops were unable to be pollinated by honey bees, this would have a drastic negative influence on agricultural production in Se-rich habitats, like California's Central valley. Based on our studies, however, Se accumulation in flowers up to 371 mg

Se kg^{-1} DW does not appear to deter pollinator visits. This raises the question, however, whether the Se accumulated by honey bees from Se-rich crops affects the health of the bees. Such an effect may be either positive or negative. Selenium is thought to be an essential element for insects (Zhang and Gladyshev, 2009) and thus Se ingestion may have a beneficial effect on insects at low levels, as is the case for mammals, while being toxic at higher levels. If indeed honey bees do not discriminate between high- and low-Se flowers, this warrants a further investigation of the effect of foraged Se on bee health. In such studies both hyperaccumulator and non-hyperaccumulator plants should be included, since they accumulate different forms of Se in their flowers, as shown here. In addition, investigating Se concentrations in honey from bees foraging on Se rich plants would be beneficial.

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Chapter 5

Enhanced decomposition of selenium hyperaccumulator litter in a seleniferous habitat – evidence for specialist decomposers?

NOTES AND ACKNOWLEDGMENTS

A version of this manuscript is in press at the journal *Plant and Soil* as of June 2010. Dr. Elizabeth Pilon-Smits and Dr. Mark Paschke contributed to experimental design and drafting the manuscript. Jeremy Shulman contributed to data collection. We would like to thank Colin Pinney for assistance with C:N analysis and Laurie Kerzicnik for aid with Berlese funnels. We would also like to thank Dr. Robert Boyd and 2 anonymous reviewers for reviewing the manuscript. Funding for these studies was provided by the National Science Foundation grant # IOS-0817748 to Elizabeth A. H. Pilon-Smits.

ABSTRACT

Selenium (Se) hyperaccumulation, when plant species accumulate upwards of 1,000 mg Se kg^{-1} dry weight (DW), protects plants from a variety of herbivores and pathogens. The objective of this study was to determine the effect of plant Se concentration on the rate of litter decomposition by invertebrates and microbes in a seleniferous habitat.

Decomposition, Se loss, the decomposer community and soil Se concentration beneath leaf litter were compared between litter from two populations of the Se hyperaccumulator *Astragalus bisulcatus* (one population with 350 and the other with 550 mg Se kg^{-1} DW) and from the related non-accumulator species *Astragalus drummondii* and *Medicago sativa* containing 1- 2 mg Se kg^{-1} DW using a litterbag method.

High-Se litter decomposed faster than low-Se litter and supported more microbes and arthropods than low-Se leaf litter after 8 and 12 months, respectively. Soil collected from under high-Se litter had higher Se concentration than soil from beneath low-Se litter after 8 months.

The higher decomposition rate and abundance of decomposers in high-Se litter indicates the presence of Se-tolerant decomposers in this seleniferous habitat that may have contributed to increased decomposition rates of high-Se litter.

Keywords: *Astragalus bisulcatus,* hyperaccumulating plant, litterbag, detrivore, decomposer

INTRODUCTION

Leaf litter decomposition is an important ecosystem process that plays an integral role in chemical cycling (Aerts 2006). The decomposition rate of leaf litter is influenced by a number of factors, including: 1) climate, such as mean annual temperature, mean annual precipitation and annual actual evapotranspiration (Aerts 1997; O'Neill et al. 2003), 2), geographic variables such as latitude and altitude (Silver and Miya 2001), 3) litter quality, which depends on the chemical composition of litter (Swift et al. 1979) and 4) the local decomposer community (Smith and Bradford 2003). Evidence suggests that a combination of these factors determines litter decomposition rate, usually with litter quality playing the most important role (Swift et al. 1979). In past studies litter quality has primarily been measured as percent N or the C:N ratio. The effects of metals and metalloids on litter decomposition remain largely unexplored (Yavitt and Fahey 1986; Berg and Ekbohm 1991).

Hyperaccumulator species can accumulate metals or metalloids in their tissues up to 1% or more of their dry weight. For instance, some plant species native to seleniferous areas in the Western United States, such as *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae) can hyperaccumulate selenium (Se) to levels as high as 10,000 mg Se kg^{-1} dry weight (Beath et al. 1939; Galeas et al. 2007). Selenium is an essential trace element for many organisms, but becomes toxic at elevated concentrations (Wilbur 1980; Stadtman 1990). For higher plants Se has no known essential function, however, most plant species suffer toxicity when grown in the presence of high levels of Se (Anderson 1993).

The functional significance of Se hyperaccumulation may be to protect plants from herbivory and microbial infection. Hyperaccumulated Se has been shown to act as a strong elemental defense against a variety of herbivores and pathogens (reviewed by Quinn et al. 2007). The crop species *Brassica juncea* was shown to be protected from aphid herbivory at leaf Se levels as low as 10 mg Se kg^{-1} DW due to both deterrence and toxicity (Hanson et al. 2004). Plants containing around 800 mg Se kg^{-1} DW were protected from a variety of invertebrate herbivores as well as two fungal pathogens (Hanson et al. 2003, 2004; Freeman et al. 2006, 2007). Selenium also protected plants from black-tailed prairie dog (*Cynomys ludovicianus*) herbivory, a native herbivore in seleniferous habitats. Prairie dogs avoided eating Se hyperaccumulator *Stanleya pinnata* leaves containing as little as 37 mg Se kg^{-1} DW but did eat this same species when it was not supplied with Se (Quinn et al. 2008). Thus, Se in plant tissues appears to protect plants from generalist herbivores. However, there is evidence that some herbivores in seleniferous habitats are able to feed on hyperaccumulator plants without ill effects: a diamondback moth population was found thriving on *S. pinnata* plants containing 2,000 mg Se kg^{-1} DW (Freeman et al. 2006). Selenium tolerant herbivores have overcome this elemental plant defense and are able to occupy the specialized niche that hyperaccumulators provide.

There have been very limited studies to date on the effect of hyperaccumulation of any element on litter decomposition rate. Boyd et al. (2008) found that leaf litter from the Ni hyperaccumulator *Senecio coronatus* containing 15,000 mg Ni kg^{-1} decomposed slower than *S. coronatus* litter with 9,200 mg Ni kg^{-1} or less. However, litter with 9,200 mg Ni kg^{-1} decomposed at the same rate as leaf litter with Ni levels as low as 16 mg Ni

kg^{-1}. After only 1 month of decomposition 72-91% of Ni was already lost from the litter (Boyd et al. 2008). In a laboratory study Boucher et al. (2005) found that the decomposition of Zn-enriched *Arabidopsis halleri* litter led to increased Zn concentrations and decreased microbial biomass in soil, suggesting that increased litter Zn concentrations are toxic to microbial communities.

In the case of Se, no studies have investigated the effects of Se hyperaccumulation on litter decomposition. Because of the general toxicity of Se at elevated concentrations it may be hypothesized that litter with elevated Se is toxic to, or may deter, generalist decomposers. If this is the case, litter from Se hyperacccumulating species will decompose slower than litter from related species that do not accumulate Se. On the other hand, it is also possible that Se-tolerant and perhaps even Se-specialist decomposers have evolved that thrive in seleniferous habitats, facilitating the rate of decomposition of Se hyperaccumulator litter. Past studies have indicated that decomposers in an ecosystem are adapted to litter common to that ecosystem (Hunt et al. 1988). Recently a number of fungi, - an important class of decomposer- collected from the root zone of Se hyperaccumulators growing in seleniferous soils were shown to be highly Se tolerant (Wangeline et al. 2007). In addition, fungi isolated from the roots of plants growing in seleniferous habitats showed significantly higher Se tolerance than fungi from non-seleniferous habitats (Wangeline and Pilon-Smits, unpublished). The decomposition of high-Se plant litter may be mediated by a specialized, Se-tolerant microbial community and be an important factor in Se cycling.

The objectives of this study were to 1) elucidate the effect of elevated Se concentrations on the rate of leaf litter decomposition, 2) determine if decomposition of

high-Se litter increases associated soil Se concentrations and 3) determine how litter Se concentration may affect decomposer community structure.

MATERIALS AND METHODS

Study site

This study was conducted at Pine Ridge Natural Area (40°32.70N, 105°07.87W) in South-West Fort Collins, CO, USA. The study area is a semi-arid shrubland located just east of the Rocky Mountains at 1525 m altitude with a dry climate (average precipitation 382 mm/year) and annual maximal temperatures varying between 29.5 °C (July) and 5.2 °C (January) (Western Regional Climate Center, 2009). Pine Ridge Natural Area is a seleniferous habitat with soil containing up to 10 mg Se kg^{-1} (Galeas et al. 2007) and harbors the native Se hyperaccumulating species *Astragalus bisulcatus* (two-grooved milkvetch, Fabaceae) and *Stanleya pinnata* (prince's plume, Brassicaceae), both indicators of seleniferous soils. Other vegetation at this site consists of native and introduced grass and forb species.

Litter collection

Green mature leaves were collected from three species of the Fabaceae family growing at Pine Ridge Natural Area in June 2006 to compare in decomposition experiments: the Se hyperaccumulator *A. bisulcatus* (referred in our study to as *A. bisulcatus* CO) and the non-Se hyperaccumulators *Astragalus drummondii* (Drummond's milkvetch) and *Medicago sativa* (alfalfa). For an additional comparison, leaf material was collected in

June 2006 from another population of *A. bisulcatus* (referred to in our study as *A. bisulcatus* WY) from a seleniferous site near Laramie, WY, USA (42°51.17N, 106°31.07W). While litter was collected before typical leaf senescence, Se concentrations in *A. bisulcatus* were consistent with Se concentrations found during leaf senescence (Galeas et al. 2007). All four populations of plants had similar sized leaflets. Leaves were removed from plants and air dried at 33°C in dry conditions under lamps for 1 week before being placed in litterbags. Prior to being placed in the field, litter was analyzed for Se concentration and C:N ratio as described below.

Litterbag preparation and mass loss experiment

For each plant population we filled forty 10 X 10 cm aluminum mesh bags (with 1.5 mm^2 mesh holes), with 5–6 g of dried leaf material each. The mesh was large enough to allow micro-arthropods and microorganisms access to the leaf material, while preventing excessive leaf loss (Bradford et al. 2002). A 2 m^2 level area was selected at Pine Ridge Natural Area where bags were placed 2 cm apart directly on the soil in July 2006. Bags were secured in place to prevent animal tampering by nailing stainless steel screens with 7 mm^2 holes over each bag. Half of the litterbags for each treatment were collected after 8 months, in March 2007, and the other half after 12 months, in July 2007.

Soil particles were carefully removed from the bags before removing litter. After collecting litterbags at both time points, 5 bags of each treatment were set aside for bacterial and fungal analysis. Of the remaining litterbags, half of the samples were air dried under lamps at 33° C for 96 hours and weight was recorded. The other half was placed under lamps in Berlese funnels, which were used for micro-arthropod extraction,

and weight was recorded. Micro-arthropod weight lost from litter was negligible and did not significantly change the weight of the litter. In addition, the top 2 cm of soil was collected from under 7 bags of each treatment after 8 months to compare soil Se concentration.

Micro-arthropod, bacterial and fungal analysis

Micro-arthropods were collected by placing leaf litter in a Berlese – Tullgren funnel for 5 days. A bulb was used as a heat and light source causing micro-arthropods to move toward the base of the funnels where they were collected in 85% ethanol. Micro-arthropods were then counted and identified to order.

In addition, five samples from each treatment were used for bacterial and fungal analysis after 8 and 12 months. One gram was extracted from each of the five samples and slurry was created by adding 1 ml of distilled water. The slurry was diluted by factors of 10^4 and 10^6 and spread on both potato dextrose agar (PDA) and Luria-Bertani broth (LB) agar plates. Plates were incubated at 30° C for 3 days and then colony forming units were counted on each plate. For the 8-month time point fungi growing on plates were identified to genus using morphological characteristics.

Leaf litter analysis

Leaf litter quality was determined by pooling dried leaf litter for each treatment and analyzing for total carbon and nitrogen using a LECO CHN1000 analyzer. Selenium concentration was determined by digesting 100 mg of litter in 1 ml of nitric acid as follows. The sample was heated to 60° C for 2 hours and then 130° C for 6 hours.

Samples were subsequently diluted to 10 ml with distilled water and analyzed for Se using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (Fassel 1978). Five samples from each treatment were ground and ashed at 550° C in a muffle furnace to determine ash content and organic matter mass and to correct for contamination.

Data Analyses

All statistical analyses were carried out using the software JMP-IN (3.2.6, SAS Institute, Cary, NC). When comparing all four populations of plants a Tukey-Kramer test was used. When pooling treatments with Se and comparing them to pooled treatments without Se an unpaired Student t-test was used.

RESULTS

Weight loss experiments

We tested the effect of litter Se concentration on its decomposition by conducting a leaf litterbag experiment in a seleniferous habitat, Pine Ridge Natural Area. We compared decomposition of leaf litter collected from four plant populations with various Se concentrations. The litter from two populations of *A. bisulcatus* had greater concentrations of Se (350 and 550 mg Se kg^{-1} DW for the CO and WY population, respectively) than the litter from populations of *A. drummondii* and *M. sativa* (1 – 2 mg Se kg^{-1} DW) (Fig. 5.1a; Tukey-Kramer test, $\alpha = 0.05$). After 8 months of decomposition, we found that the *A. bisulcatus* litter with the highest Se concentration, *A. bisulcatus* WY, had lost more weight and thus presumably had decomposed faster than both types of leaf

litter with low-Se, and the *A. bisulcatus* litter from CO decomposed faster than that of *M. sativa* (Fig. 5.1b; Tukey-Kramer test, $\alpha = 0.05$). At the 12-month time point *M. sativa* litter had lost less weight than the other three litter types (Fig. 5.1c; Tukey-Kramer test, $\alpha = 0.05$). The positive effect of Se on decomposition was more significant when both treatments of high-Se litter were pooled and statistically compared to both treatments of low-Se litter. After both 8 and 12 months the high-Se litter had lost more mass than low-Se litter (Fig. 5.1b, c; t-test; 8 months $t = -4.6$, $p < 0.001$; 12 months $t = -3$, $p = 0.0053$).

To test whether decomposition of high-Se leaf litter affects soil Se concentration we measured soil Se concentration under the litter from each treatment after 8 months. Soil Se concentration below high-Se litter was twice as high (3 mg Se kg^{-1}) as that below low-Se litter (1.5 mg Se kg^{-1}) (Fig. 5.1d; t-test, $t = -2.3$, $p = 0.0338$).

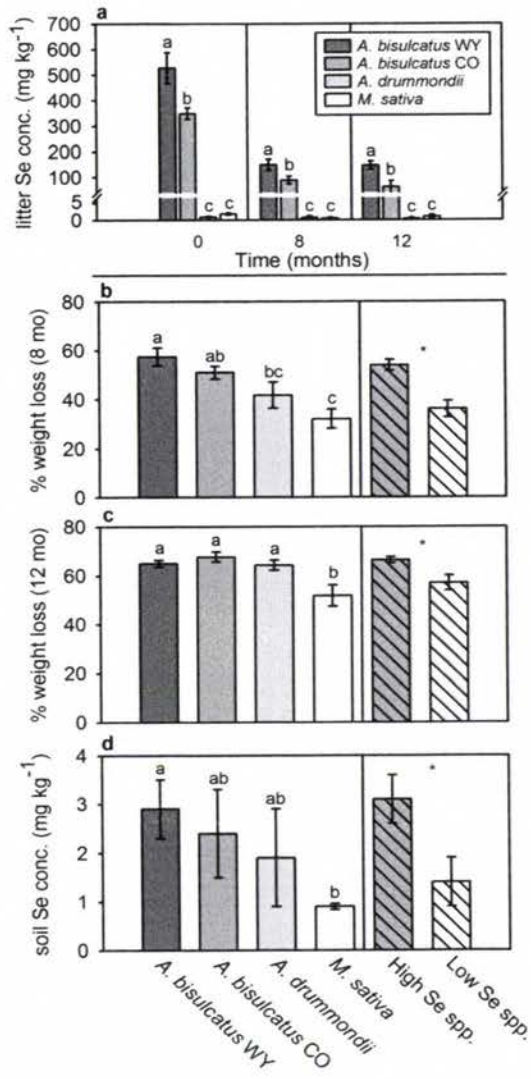

Figure 5.1: Leaf litter Se concentration, litter mass loss and soil Se concentration under high-Se litter, *A. bisulcatus* WY and *A. bisulcatus* CO, and low-Se litter *A. drummondii* and *M. sativa*. Selenium concentration of leaf litter after initial collection of litter and after 8 and 12 months of litter decomposition at Pine Ridge Natural Area in Fort Collins, CO, USA (a). The percent of weight that was lost from all four treatments of leaf litter and when both treatments of high-Se litter were pooled (grey stripped bars) and when both treatments of low-Se litter were pooled (white stripped bars) after 8 months (b) and 12 months (c). Soil Se concentrations under each treatment of leaf litter after 8 months (d). Values shown are means ± Se, n = 20 for *A. bisulcatus* WY and *A. bisulcatus* CO for initial Se concentration, n = 10 for Se concentration for *A. bisulcatus* WY and *A. bisulcatus* CO after 8 and 12 months of decomposition, n = 5 for Se concentration of *A. drummondii* and *M. sativa* at all time points, n = 10 for weight change for all litter treatments except for *A. drummondii*, for which n = 7. N = 7 for soil Se concentration under litter bags. A different letter or an asterisk above bars represents a significant difference ($\alpha < 0.05$).

Leaf litter quality was determined by measuring the C:N ratio of each litter treatment prior to placing litter in the field and after 8 and 12 months of decomposition. Prior to being placed in the field *A. bisulcatus* from CO had the lowest C:N ratio followed by *A. bisulcatus* from WY. The two low-Se litter treatments had higher C:N ratios than the high-Se treatments, with *M. sativa* having a higher C:N ratio than *A. drummondii* (Fig. 5.2a; Tukey-Kramer test, $\alpha = 0.05$). After 8 months of decomposition both treatments of *A. bisulcatus* litter had the same C:N ratio, which was less than the C:N ratios of *A. drummondii* and *M. sativa*. After 12 months of decomposition this difference in C:N ratio between high- and low-Se litter was largely gone (Fig 5.2a; Tukey-Kramer test, $\alpha = 0.05$). However, when high-Se litter was grouped and compared to low Se litter, high-Se litter still had a lower C:N ratio (Fig. 5.2b; t-test; 0 months $t = 5.8$, $p < 0.0001$; 8 month $t = 13.9$, $p < 0.001$; 12 month $t = 2.6$, $p = 0.0166$).

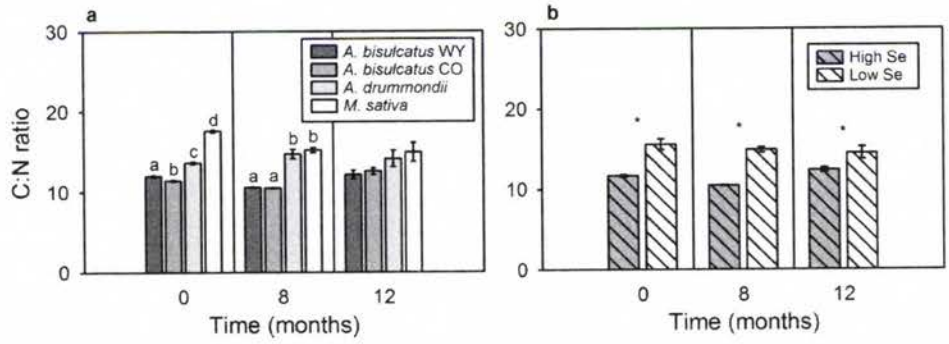

Figure 5.2: Carbon to nitrogen ratio for *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* when first collected and after 8 months and 12 months of decomposition (a). Carbon to nitrogen ratio of high-Se litter, of *A. bisulcatus* WY and *A. bisulcatus* CO pooled (grey stripped bars), compared to low-Se litter, *A. drummondii* and *M. sativa* pooled (white stripped bars) (b). Values shown are means ± SE, n = 5. A different letter or an asterisk above bars represents a significant difference ($\alpha < 0.05$).

Decomposer community analysis

To further understand what caused the observed differences in decomposition rate between high- and low-Se litter we compared the abundance and composition of micro-arthropods and microbes in high and low-Se treatments. After 8 months we did not find a difference in the number of micro-arthropods or the number of orders of micro-arthropods between high- and low-Se litter (Fig. 5.3a, b). However, after 12 months we found more arthropods and more arthropod orders in high-Se litter than in low-Se litter (Fig. 5.3c, d; t-test; micro-arthropod number $t = 2.2$, $p = 0.0393$; micro-arthropod orders $t = 2.2$, $p = 0.0449$). At both the 8 and 12 month time points most of the arthropods found were mites, particularly Oribatid mites, which are often abundant in soils and usually have chewing mouthparts.

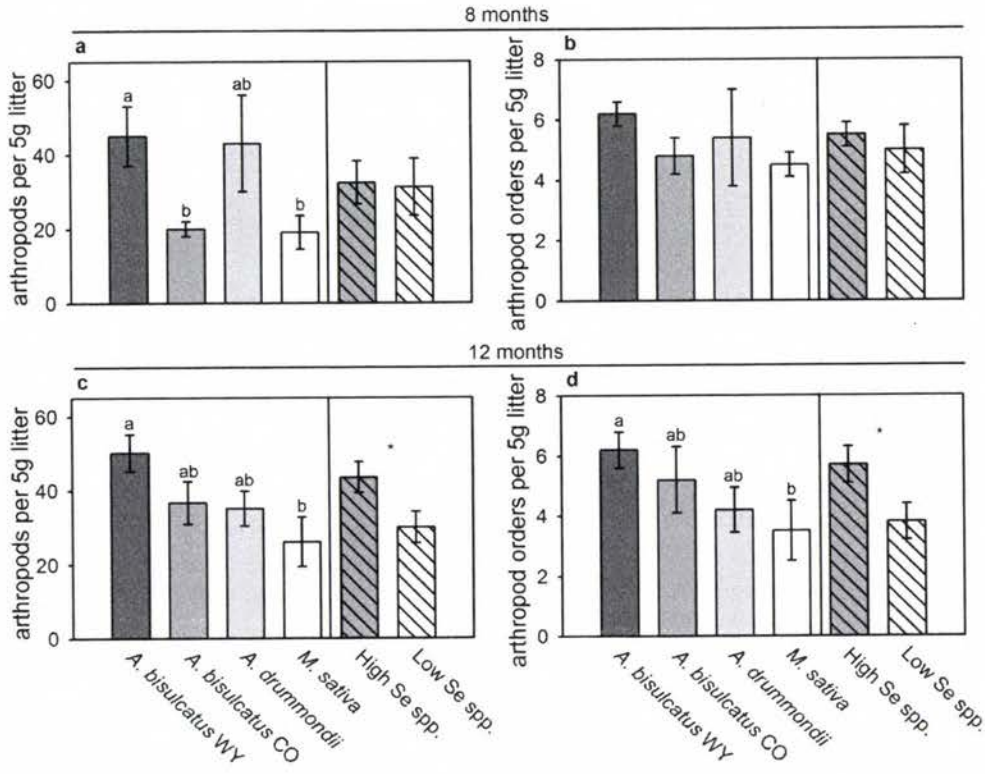

Figure 5.3: Total number of micro-arthropod individuals (a) and micro-arthropod orders (b) collected from 5 g of *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* litter after 8 months of decomposition. Total number of micro-arthropod individuals (c) and micro-arthropod orders (d) collected from 5 g of *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* litter after 12 months of decomposition. Grey stripped bars represent high Se litter treatments, *A. bisulcatus* WY and *A. bisulcatus* CO pooled, and white stripped bars represent low-Se litter, *A. drummondii* and *M. sativa* pooled. Values shown are means ± SE, n = 5. A different letter or an asterisk above bars represents a significant difference ($\alpha < 0.05$).

In addition, mites belonging to the order Prostigmata, which are known plant pests with sucking mouthparts, and mites in the Mesostigmata order, often predators, were abundant (Fig. 5.4a, b). While most micro-arthropods were mites, the differences observed in micro-arthropods after 12 months were actually the result of the presence of more non-mite arthropods in high-Se litter than in low-Se litter (Fig. 5.4b), particularly Collembola and Lepidoptera (Table 1).

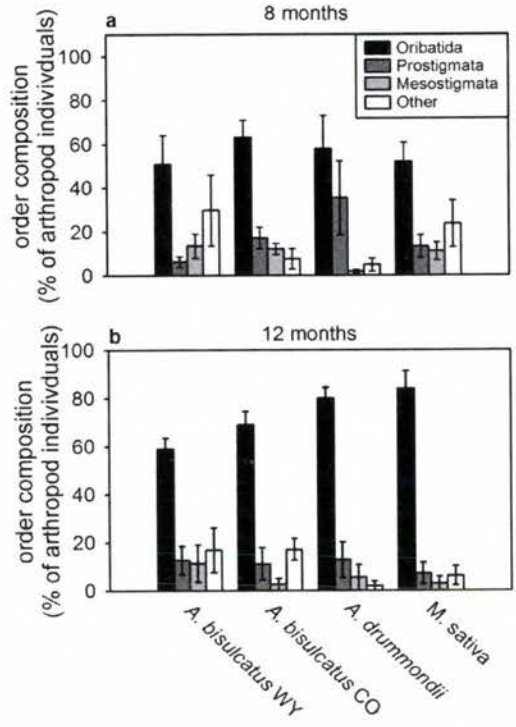

Figure 5.4: Micro-arthropod order composition collected from *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* litter after 8 months (a) and 12 months (b) of decomposition. All orders that are not classified as "other" are mite orders. Values shown are means ± SE, n = 5.

Table 1: Micro-arthropod composition collected from high-Se litter, *A. bisulcatus* WY and *A. bisulcatus* CO, and low-Se litter, *A. drummondii* and *M. sativa*, after 8 and 12 months of decomposition on a seleniferous site. Values represent mean number of arthropod order per 5 g of litter and mean percentage of each order of arthropod per litter treatment ± SE, n = 5.

	8 months							
	A. bisulcatus WY		*A. bisulcatus* CO		*A. drummondii*		*M. sativa*	
	# per 5 g	%	# per 5 g	%	# per 5 g	%	# per 5 g	%
Coleoptera	0.8 ± 0.8	3	0.3 ± 0.3	1	0	0	0	0
Collembola	0.5 ± 0.5	2	0.3 ± 0.3	1	0	0	0.2 ± 0.2	1
Hemiptera	0	6	0.3 ± 0.3	1	0	0	0.6 ± 0.4	3
Lepidoptera	14 ± 11	25	0.8 ± 0.8	5	2 ± 2	4	3 ± 2	20
Mesostigmata	7 ± 3	14	2 ± 1	12	1 ± 1	2	1 ± 1	11
Oribatida	20 ± 5	51	13 ± 2	63	32 ± 12	58	12 ± 5	52
Prostigmata	3 ± 1	6	3 ± 1	17	8 ± 2	35	2 ± 1	13
Other	0	0	0	0	0	0	0	0
	12 months							
	# per 5 g	%	# per 5 g	%	# per 5 g	%	# per 5 g	%
Coleoptera	0	0	0	0	0	0	0	0
Collembola	4 ± 3	8	1 ± 1	5	0	0	0	0
Hemiptera	0	0	3 ± 2	7	0	0	2 ± 1	6
Lepidoptera	8 ± 5	8	4 ± 4	4	0	0	0	0
Mesostigmata	6 ± 4	11	1 ± 1	3	2 ± 2	5	1 ± 1	3
Oribatida	30 ± 4	59	25 ± 4	69	28 ± 4	80	21 ± 4	84
Prostigmata	5 ± 2	13	4 ± 3	11	5 ± 3	13	2 ± 1	7
Other	0.3 ± 0.3	1	0.5 ± 0.5	1	0.6 ± 0.6	2	0	0

In addition to micro-arthropods, we compared the abundance of bacteria and fungi on high- and low-Se litter. We obtained more bacterial and fungal colony forming units from high-Se litter compared to low-Se litter after 8 months, but found no difference after 12 months (Fig. 5.5a – d).

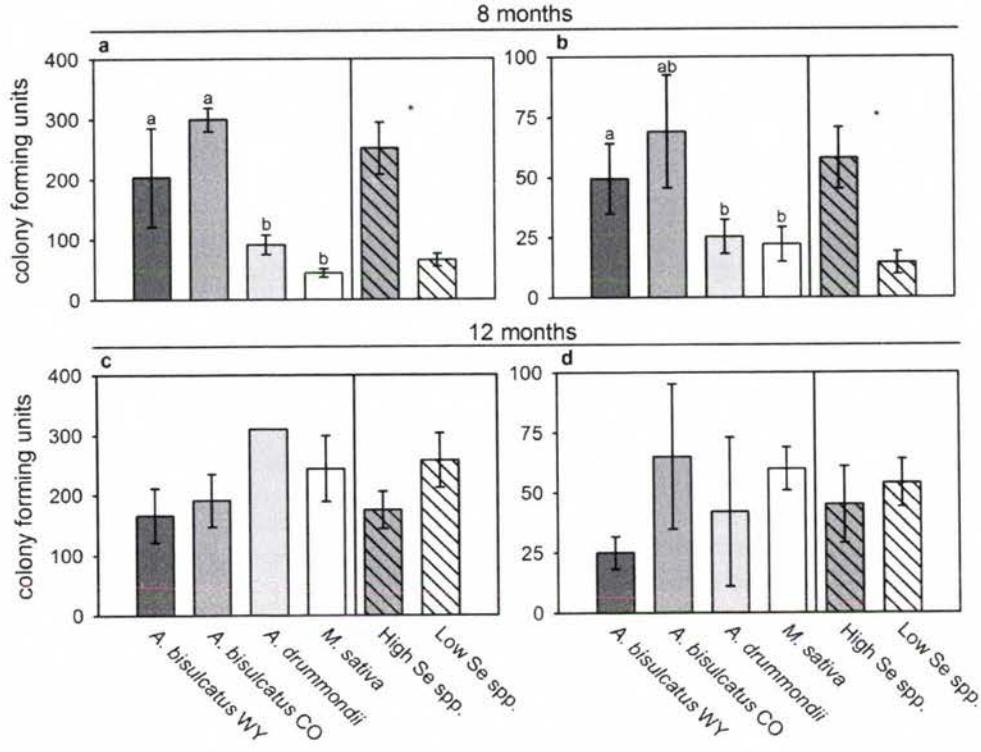

Figure 5.5: The number of colony forming units cultured on LB (a) and PDA (b) agar from *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* litter after 8 months of decomposition. The number of colony forming units cultured on LB (c) and PDA (d) agar from *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* litter after 8 months of decomposition. Values shown are means ± SE, n = 5. A different letter or an asterisk above bars represents a significant difference ($\alpha < 0.05$).

To further investigate the role of fungi in decomposition we identified fungi growing on all litter populations after 8 months to genus. We found there to be no difference in the number of genera per g of litter between high and low-Se litter (Fig. 5.6a). The three most abundant genera of fungi found in decomposing litter were *Alternaria*, *Cladosporium* and *Fusarium* (Fig. 5.6b).

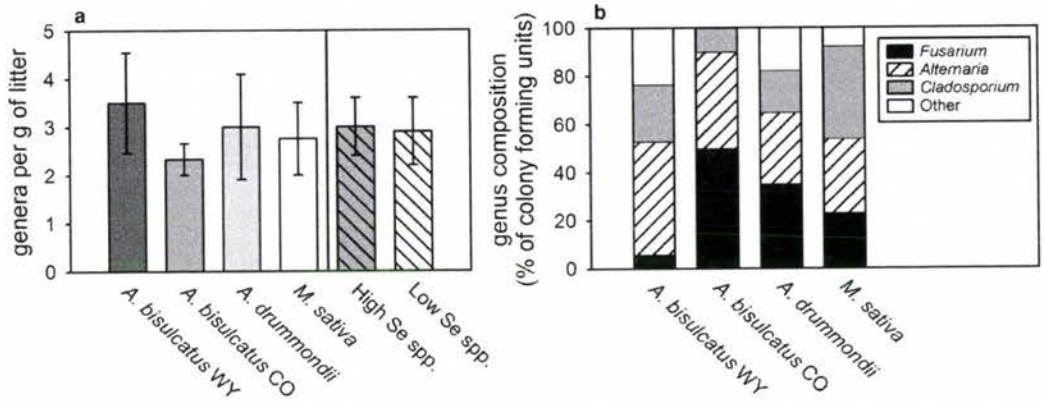

Figure 5.6: Total number of fungi genera cultured on PDA agar per g of *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* litter (a) and the fungi genus composition found on *A. bisulcatus* WY, *A. bisulcatus* CO, *A. drummondii* and *M. sativa* litter after 8 months (b). Values shown are means ± SE, n =5.

DISCUSSION

This is the first study to investigate how elevated leaf Se concentrations affect litter decomposition. Results from this study show that high-Se litter from hyperaccumulator *A. bisulcatus* (350-550 mg Se kg^{-1} DW) lost more weight than low-Se litter (1-2 mg Se kg^{-1} DW) from *A. drummondii* and *M. sativa* after 8 and 12 months of decomposition in the seleniferous habitat Pine Ridge Natural Area. Since the high-Se litter Se levels are toxic to most invertebrates and many microbes (for a review see Trumble and Sorensen

2008), it appears that Se-tolerant decomposers have evolved in this seleniferous habitat and these may specialize in decomposing high-Se leaf litter. Such Se-tolerant decomposers may even benefit from the ingested Se via protection from predators or pathogens, or metabolically (Se can protect many organisms against toxic free radicals (Iman et al. 1999). In the only other field study investigating the role of hyperaccumulation on litter decomposition, Boyd et al. (2008) found that litter from the Ni hyperaccumulator *Senecio coronatus* decomposed slower than low-Ni *S. coronatus* on low-Ni sites. However, on high-Ni sites *S. coronatus* with high- and low-Ni decomposed at the same rate. Thus, some decomposers and detrivores at high-Ni sites may have developed Ni tolerance similar to the possible Se tolerance observed in this study.

Our analyses of litter quality and the presence and role of detrivores and decomposers on each litter treatment shed some light on the mechanisms responsible for the observed increased decomposition rate of the high-Se litter. Both high-Se litter treatments had lower C:N ratios than the low-Se treatments, indicating that they had more N and presumably higher litter quality (Pérez-Harguindeguy et al. 2000). Studies that have added external N to litter, which should increase decomposition rates if N is a limiting factor of decomposition, have shown varied results. In some studies added N had no effect on decomposition, while in other studies an increase or a decrease in decomposition was found (Pastor et al. 1987; Hunt et al. 1988; O' Connell 1994). If increased N does increase litter quality of high-Se material, as commonly thought, the high-N Se hyperaccumulator litter is an especially attractive food source for detrivores and decomposers. However, for these organisms to enjoy the benefits of feeding on this high-quality litter they must be able to tolerate Se at concentrations that are toxic to most

herbivores and pathogens. The benefit of being able to utilize this nutritious, high-Se litter may have helped drive the evolution of Se tolerance in specialist decomposers/detrivores, in addition to the benefits of having access to an exclusive food source that is toxic to competing detrivores, and the possible defensive or metabolic advantages derived from the ingested Se.

There was a larger number of culturable microbes (bacteria and fungi) on high-Se litter relative to low-Se litter after 8 months of decomposition, but after 12 months the numbers of colony forming units obtained were the same for all litter treatments. The higher microbial density on high-Se litter after 8 months of decomposition may have contributed to the observed higher rate of decomposition over the first 8 months. There were no apparent differences in numbers of fungal genera in each litter treatment, and thus there was no correlation between fungal diversity and Se concentration. There was a similar genus composition in the three litter treatments that were collected from Pine Ridge Natural Area, i.e. *A. bisulcatus* CO, *A. drummondii* and *M. sativa*. The three dominant fungal genera found on these litter treatments were *Fusarium*, *Alternaria* and *Cladosporium*. All of these fungi are commonly found living in plant litter and are typical decomposers (Tiunov and Scheu 2000; Thormann et al. 2004). Interestingly, *A. bisulcatus* WY, which was not collected from Pine Ridge Natural Area, contained a small percentage of *Fusarium* spp. compared to other litter treatments. A possible explanation is that *Fusarium* living at Pine Ridge Natural Area has not adapted to live on *A. bisulcatus* from other locations. Research has shown that some microbes do not interact with plants introduced from another location (Dighton et al. 1997). Another possible

explanation is that each leaf litter treatment maintained fungi previously living on the plants prior to being placed in litterbags.

It is interesting that there were more decomposer colony forming units on high-Se litter treatments than on low-Se litter at the 8-month time point but not at the 12-month time point. An important driver of decomposer biomass and composition is environmental conditions, particularly temperature and water availability (Sulkava et al. 1996). Temperature and water availability varied greatly between the 8-month time point and the 12-month time point. At the 8-month time point, in March, the average maximum temperature was 12° C and litter was moist when collected from the field as a result of recent snow melt; after the 12-month time point, in July, with an average temperature of 30° C, the litterbags were dry when collected. Many microbes thrive in moist environments and the differences in water availability and temperature may have contributed to the difference in colony forming units between the 8- and 12-month time points by enhancing microbial biomass and magnifying differences between high- and low-Se litter. In addition to environmental conditions, an important biotic driver of decomposer composition is the presence of detrivores (Duarte et al. 2009).

Detrivores break down organic material, increasing surface area for decomposers to feed on, which can increase decomposer biomass and diversity (Begon et al. 2006). We found no difference in micro-arthropod order diversity and abundance after 8 months, but more micro-arthropods individuals and orders were present in high-Se litter than low Se litter after 12 months of decomposition. The increased number of individuals and orders of micro-arthropods in high-Se litter may have contributed to the larger weight loss of the high-Se litter compared to low-Se litter after 12 months. The higher number

of micro-arthropod individuals and orders was likely due to higher numbers of Collembolla (springtails) and Lepidoptera (butterflies and moths) in high-Se litter compared to low-Se litter. In a previous study a Lepidoptera species, the diamondback moth (*Plutella xylostella*), was shown to be able to disarm Se as a plant defense and to tolerate feeding on plants with high concentrations of Se (Freeman et al. 2006). It is possible that some of the micro-arthropods found living on high-Se litter have also evolved tolerance to high-Se diets and may even prefer to feed on food rich in Se for protection against predators. This might help explain why high-Se litter has higher decomposition rates than low-Se litter in this seleniferous habitat.

In this study we also investigated the rate at which Se is lost from decomposing litter and how the decomposition of high-Se litter changes soil Se concentration. We found that 70% of Se was lost from litter after 8 months of decomposition. Interestingly, only trace amounts of Se were lost from litter between the 8 and 12 month time points. Similar results were seen in studies with other elements: both Ni and Zn were lost from litter rapidly during decomposition (Boyd et al. 2008; Boucher et al. 2005). The rapid release of Se may be due to both leaching and litter consumption by detrivores and decomposers. The Se fraction left after the initial rapid decrease in Se concentration may be less bioavailable, e.g. incorporated into macromolecules rather than present as selenoaminoacid or inorganic Se. Soil collected from directly below high-Se litter had a higher Se concentration than soil from below low-Se litter, indicating that Se from plant decomposition affects soil Se concentration under the canopy of hyperaccumulators. The soil Se concentrations below high-Se litter were not very high: only 3 mg Se kg^{-1}. It is possible that Se released from high-Se litter leaches beyond the first 2 centimeters of soil,

and that high-Se litter is consumed and Se is moved to another location through the consumer's movements; some litter Se may also be volatilized by microbial decomposers.

The results from this study help increase our understanding of Se cycling and the effect Se has on litter decomposition. In this seleniferous habitat, high levels of Se in plant litter did not impede decomposition, as might be hypothesized, but rather was associated with enhanced weight loss, presumably due to decomposition. Both microbial and micro-arthropod levels were higher in the high-Se litter at some time point, and thus both groups likely contributed to the decomposition.

The results from these studies provide direction for future research. To further increase our understanding of the effect of Se on litter decomposition, a similar study could be conducted at a non-seleniferous habitat. In addition, studies could be conducted to investigate detrivore/decomposer Se tolerance in seleniferous and non-seleniferous areas, by providing detrivores/decomposers with Se-rich leaf litter and measure growth over time. In addition, to increase our understanding of Se cycling, studies could be carried out that investigate the fate of Se from Se-rich litter.

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Chapter 6

Ecological aspects of selenium phytoremediation

NOTES AND ACKNOWLEDGMENTS

Colin Quinn, Stormy Lindblom and Dr. Elizabeth Pilon-Smits drafted this manuscript which has been accepted to be published as a chapter in "Phytoremdiation: Processes, Characteristics, and Applications." by Nova Publishers.

ABSTRACT

Selenium is essential for many organisms but is toxic at elevated concentrations. The window between nutritious and toxic levels of Se is narrow, and both Se deficiency and toxicity are problems worldwide. For plants Se serves no known essential function, and uptake of Se by plants can lead to toxicity due to the similarity of Se to sulfur (S) and the incorporation of Se into S proteins. However, many plants readily take up Se and can benefit from increased Se due to increased growth and/or as an elemental defense.

In relation to Se, plants can be classified into three categories: 1) non-Se accumulators 2) Se accumulators, and 3) Se hyperaccumulators. Non-Se accumulators do not accumulate Se, or only accumulate trace concentrations of Se, even when growing on seleniferous soils, Se accumulators can accumulate up to 1,000 mg Se kg^{-1} and Se hyperaccumulators accumulate upwards of 1,000 mg Se kg^{-1} and as much as 15,000 mg Se kg^{-1}.

Elevated tissue Se levels can protect plants from a variety of herbivores and pathogens, including fungi, arthropods and mammals. This elemental plant defense may act as a convenient pesticide when using plants for Se phytoremediation, and may also help prevent toxic Se concentrations from entering the ecosystem. Selenium as a defense has been disarmed in at least one instance, by a population of diamondback moth (*Plutella xylostella*), and probably has been disarmed on other occasions. Understanding the mechanisms that have led to the disarmament of Se as a defense is important to better understand how plant Se may enter higher trophic levels. In addition, many decomposers in seleniferous environments appear to have evolved Se tolerance, resulting in increased decomposition rates of Se-rich plant material and possibly faster release of Se into soil.

Selenium may also influence pollination. There is evidence that Se accumulation changes flower phenotype characteristics and that important reproductive tissues, such as pistils, stamens, nectar and pollen, accumulate Se. Another interesting ecological aspect of plant Se accumulation is the role of rhizosphere and endophytic-microbes in Se (hyper)accumulation; there is evidence that rhizosphere microbes can increase plant Se accumulation and volatilization.

Investigating the ecological implications of Se accumulation in plants is crucial to managing phytoremediation of Se-polluted sites. Moreover, studies on the effects of Se on plant ecology may serve as a model for ecological implications of plant accumulation of other elements during phytoremediation or production of fortified foods.

INTRODUCTION

Selenium is an essential micronutrient for many organisms including humans, but is toxic at elevated concentrations. The gap between Se deficiency and toxicity is narrow and both are problems worldwide. Selenium is essential in the active site of redox-active selenoproteins such as the enzyme glutathione peroxidase, which protects cells from free radicals (Steinbrenner and Sies 2009). Sufficient Se helps prevent a variety of cancers such as lung and prostate cancer, assists with the detoxification of heavy metals such as lead and mercury, and is essential for thyroid function (Clark et al. 1996; Birringer et al. 2000; Shin et al. 2007; Kato et al. 2010). The recommended dietary intake of Se for humans is 55 -100 μg Se a day (Lyon et al. 1989; Sriram and Lonchyna 2009). Selenium deficiency can lead to Keshan disease, a lethal heart disease named after the county in northeast China of the same name where the disease was first observed (Chen et al. 1980). Long before the essential function of Se was discovered, Se was famous for its toxicity.

Selenium is toxic due to its similarity to sulfur (S). Selenium readily replaces S in essential S proteins, interfering with their function (Stadtman 1990). In humans, chronic Se intake of more than 400 μg Se a day can lead to toxic symptoms, which include loss of hair and nails, gastrointestinal complications and eventually death (Oliveira 2007; Steinbrenner and Sies 2009). In the Western United States, where soils have elevated Se concentrations, chronic ingestion of high-Se plants by livestock has been reported to result in $330 million in losses annually (Rosenfield and Beath 1964; Wilbur 1980). A one-time ingestion of upwards of 1000 μg Se for a healthy human adult can lead to acute Se poisoning, and even death (Rosenfield and Beath 1964). Famously, in 2009, 21

Argentinean polo horses mysteriously died shortly before their match in the U.S. Open Polo Championship. The death of the horses, whose value was estimated at $2 million collectively, was a result of acute Se poisoning due to accidental elevated concentrations of Se in their vitamins.

Selenium serves no known essential function in plants, although Se is beneficial to many plants because it increases growth and antioxidant activity and protects against a wide variety of herbivores and pathogens (Hanson et al. 2003; Freeman et al. 2006a). Plants are classified as either non Se accumulators, plants that do not take up Se when grown on seleniferous sites, Se accumulators, plants that take up to 1000 mg Se kg^{-1} when grown on seleniferous sites, and Se hyperaccumulators, plants that accumulate upwards of 1000 mg Se kg^{-1} at seleniferous sites and have been show to accumulate up to 15,000 mg Se kg^{-1} (Terry et al. 2000). Selenium accumulators have traditionally been used for phytoremediation more often than Se hyperaccumulators because they yield more biomass, grow faster and some are crop species (e.g. *Brassica* spp.). However, Se hyperaccumulators, found in the families Asteraceae, Brassicaceae and Fabaceae, are gaining popularity in phytoremediation as a result of increased understanding of their physiology and taxonomy (see Figure 6.1 for an example). Transgenic crops expressing genes from hyperaccumulators that are responsible for Se tolerance, uptake and volatilization are also promising for Se phytoremediation (for a review see Pilon-Smits and Leduc, 2009).

Figure 6.1: A field of the Se hyperaccumulator *Stanleya pinnata* that was seeded in seleniferous soil in the Fort Collins, CO, USA as part of a restoration project after the construction of an irrigation pipe (Pine Ridge Natural Area).

Most soils contain low Se concentrations: less than 1 mg Se kg^{-1}. However, natural Se deposits and human activity both contribute to Se pollution and can cause widespread health problems and economic devastation. During the warm Cretaceous period (approximately 100 million years ago) oceans covered many of the lower elevations of the earth's continents. When these oceans retreated they left shale high in Se concentration. Use of these seleniferous soils for agriculture leads to accelerated release of this naturally occurring Se into the environment. Mining, burning of seleniferous coal, and refining and burning of seleniferous oil also contribute to Se pollution of water, soil and air (Diaz et al. 1996; Blagojevic et al. 1998; Senesi et al. 1999; Lemly et al 2004; Xu et al. 2005). In the early 1980's agricultural drainage water with high Se concentrations was responsible for the death of fish and migrant bird species

in the Kesterson reservoir in California's central agriculture valley (Ohlendorf et al.1986; Saiki and Lowe 1987).

Plants are an effective tool to clean up Se-polluted soil or water. In a constructed wetland system established in the San Joaquin Valley of California, selenate-contaminated agricultural drainage water was treated effectively by stands of cattail (*Typha latifolia)* or rabbitsfoot grass (*Polypogon monspeliensis*), reducing Se levels by about 90% (Lin et al., 2000). A similar Se reduction level was found for a constructed wetland used to treat selenite-containing industrial wastewater from an oil refinery in the San Francisco Bay Area (Hansen et al., 1998). In both cases the Se removal was due to accumulation in plant tissues, immobilization in sediment, and volatilization. In another study Se-polluted water passing through a constructed wetland of common reed (*Phragmites australis)* lost 100% of Se in 25 days, and constructed wetlands with broadleaf cattail removed over 50% of Se in the same time period (Shardendu et al. 2002). Growing Se-accumulating terrestrial plants such as members of the *Brassica* genus (Indian mustard, canola) has been shown to effectively clean up Se-polluted agricultural fields in the San Joaquin Valley in California (Banuelos et al., 2002a).

Since Se is not only a toxin at high levels but also an essential nutrient at low levels, phytoremediation of high-Se areas provides a unique opportunity to use plants to remove a toxic element from one area and use the concentrated Se as a mineral in Se deficient areas. Prior to using this technology it is important to consider biotic and abiotic ecological consequences of the large-scale growth of Se accumulating plants. This is the main focus of this chapter.

MOVEMENT OF SELENIUM

Into and within plants

In soils, Se is most commonly found as selenate (SeO_4^{2-}), which plants readily take up and assimilate utilizing sulfate transporters and the S assimilation pathway (for a review see Pilon-Smits and Quinn, 2009). In short, selenate is taken up and reduced to selenite and selenide, respectively, and then combined with O-acetylserine into selenocysteine (SeCys). SeCys can be further converted to selenomethionine (SeMet). Both SeCys and SeMet can be non-specifically incorporated into proteins, which is toxic. Hyperaccumulator plants can methylate SeCys, and accumulate most of their Se as methyl-SeCys (Neuhierl et al., 1999). Most non-hyperaccumulator plants accumulate primarily selenate when supplied with selenate; the reduction of selenate to selenite appears to be a rate-limiting step, as plants can quickly reduce selenite to organic SeCys (de Souza et al. 1998). Both selenate and SeCys are toxic, the latter more so due to its inadvertent incorporation into S amino acids, which leads to a loss of function (Stadtman 1990). Plants also can convert SeMet to volatile Se as dimethyselenide (DMSe), a large component of atmospheric Se; they can also absorb atmospheric Se (Lewis et al. 1966; Haygarth et al 1995). Selenium hyperaccumulating plants differ from non-hyperaccumulators in that they preferentially take up Se over S, have increased biomass when grown with elevated Se, show positive chemotropism of their roots to Se in soil and can accumulate up to 15,000 mg Se kg^{-1} from soils with Se concentrations as low as 2-5 mg Se kg^{-1} (Freeman et al. 2006a; Lyons et al. 2009; Pilon-Smits et al. 2009). The ability to accumulate such high levels of Se and avoid toxicity is due to the unique Se

metabolism of Se hyperaccumulating plants: as mentioned above, they store Se as non-toxic methylselenocysteine (MeSeCys), which is not incorporated into proteins (Neuhierl et al. 1999; Pilon-Smits and Quinn 2010) (see Figure 6.2 for a comparison of Se accumulators and hyperaccumulators). Hyperaccumulating plants can also convert MeSeCys to volatile Se as dimethyldiselenide (DMDSe), which is not often found in the atmosphere because it is unstable and returns to the soil as organic Se soon after volatilization (Martens and Suarez 1999; Kubachka et al. 2007). Adding microbes to terrestrial and aquatic ecosystems play an important role in Se accumulation and volatilization, which is discussed in more detail below.

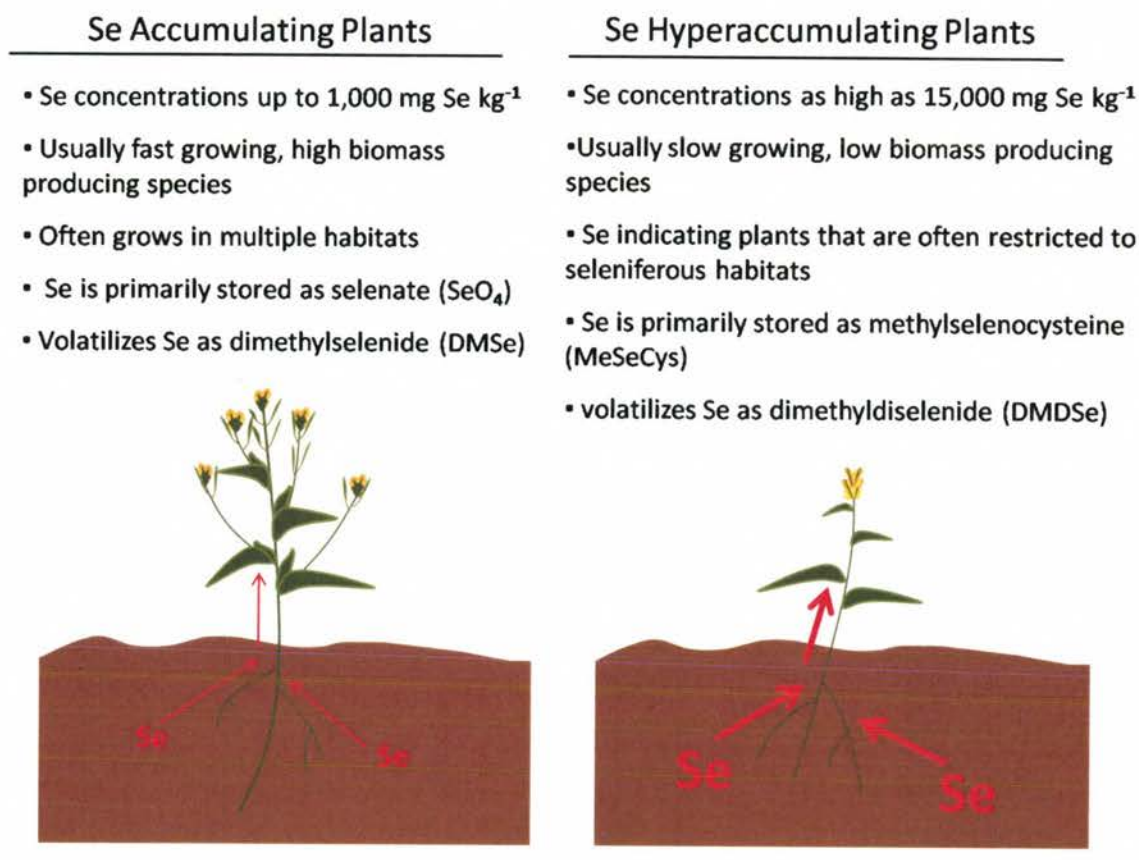

Figure 6.2: Comparison of traits associated with Se accumulating plants (left column) and Se hyperaccumulating plants. Although Se hyperaccumulating plants take up more Se, Se accumulating plants have traditionally been used for phytoremediation due to their fast growth and high biomass production.

Within plants, Se levels are usually similar in shoot and root, particularly if the plant uses selenate as a source of Se (the predominant bioavailable form of Se in terrestrial habitats) and younger leaves have higher Se levels than older leaves (de Souza et al. 1998). In most plants the Se in leaves is highest in vascular tissues (Freeman et al. 2006b). Interestingly, the distribution of Se in organs and tissues of Se hyperaccumulating plants is different from non-hyperaccumulators, and may point to the functional significance of Se hyperaccumulation as a defensive mechanism. Hyperaccumulator *Stanleya pinnata* stores Se in globular structures along the margin of

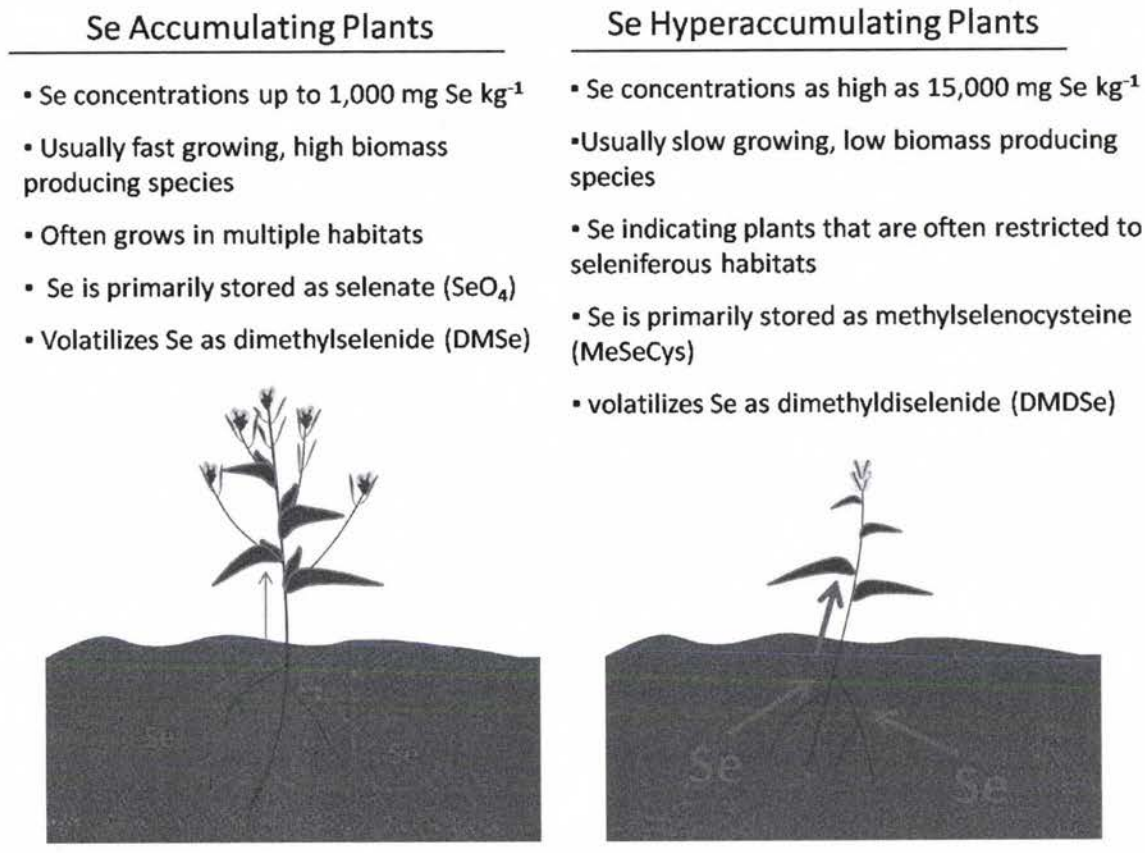

Figure 6.2: Comparison of traits associated with Se accumulating plants (left column) and Se hyperaccumulating plants. Although Se hyperaccumulating plants take up more Se, Se accumulating plants have traditionally been used for phytoremediation due to their fast growth and high biomass production.

Within plants, Se levels are usually similar in shoot and root, particularly if the plant uses selenate as a source of Se (the predominant bioavailable form of Se in terrestrial habitats) and younger leaves have higher Se levels than older leaves (de Souza et al. 1998). In most plants the Se in leaves is highest in vascular tissues (Freeman et al. 2006b). Interestingly, the distribution of Se in organs and tissues of Se hyperaccumulating plants is different from non-hyperaccumulators, and may point to the functional significance of Se hyperaccumulation as a defensive mechanism. Hyperaccumulator *Stanleya pinnata* stores Se in globular structures along the margin of

leaves and primarily in epidermal cells, and hyperaccumulator *A. bisulcatus* stores Se primarily in trichomes (Freeman et al. 2006b). Thus, in leaves of hyperaccumulators Se is concentrated in areas and cells that are often the first part of the plant to be consumed by generalist herbivores, and are generally associated with defensive functions. In a seasonal field study, younger leaves of these same two hyperaccumulator species had higher Se concentrations than older leaves and Se concentration in leaves peaked during early spring, when plants invest most in growth and development. Selenium levels steadily decreased in leaves until senescence in the fall months, when the Se appeared to be redistributed to the reproductive tissues and back to the roots (Galeas et al. 2007). Related non-hyperaccumulators growing on the same site showed a peak in leaf Se levels in mid-summer, and S levels also peaked in summer for both non-hyperaccumulators and hyperaccumulators (Galeas et al., 2007). Flowers of Se hyperaccumulating plants, particularly male and female sex organs, the pistil and stamen, respectively, have the highest concentrations of Se, together with the seeds (see chapter 4 of this dissertation). Flowers of the hyperaccumulator *Stanleya pinnata* have more than twice as much Se as leaves, and flowers of *A. bisulcatus* have 1.5 times as much Se in flowers compared to leaves; S levels were comparable in both organs. Flowers of non-Se hyperaccumulator species, like *B. juncea*, have less Se than leaves. Thus, it appears that hyperaccumulating plants are able to distinguish between Se and S, and specifically partition Se to plant parts that are most valuable, particularly plant parts essential to plant fitness, and to tissues that are most effective locations for storage of defense compounds.

From plants to the abiotic environment

When utilizing phytoremediation technologies to clean up Se-polluted areas it is important to consider the role the plants being used play in the ultimate fate of Se. As a result of their Se accumulation and volatilization, plants play a vital role in Se ecosystem cycling (Wen and Carignan 2007). High-Se plants, particularly Se hyperaccumulators, create small pockets of elevated Se within seleniferous habitats due to their uptake and redistribution of Se in the soil, decomposition of high-Se plant material and Se volatilization. Soil collected from under the canopy of hyperaccumulating species *A. bisulcatus* and *S. pinnata* has higher Se concentrations than bulk soil or soil collected under the canopy of comparable non hyperaccumulator species from the same site . Moreover, soil under decomposing *A. bisulcatus* leaf material had higher Se concentrations than soil under decomposing leaf material from non hyperaccumulator species (see chapter 5 of this dissertation). In addition, the Se concentration in rhizospheric soil from *A. bisulcatus* was higher than in surface soil under the canopy of *A. bisulcatus* and higher than in rhizospheric soil of comparable non-hyperaccumulator species at the same site. This type of redistribution of Se in soil creates high-Se micro-habitats that may be toxic to many organisms, but also may create a niche for Se tolerant organisms. The interactions between organisms and high-Se micro habitats are discussed in more detail later in this chapter.

The ability of plants to volatilize Se contributes to atmospheric Se, which is becoming an increasingly important pollution problem due to the continued burning of seleniferous coal. Atmospheric Se leads to Se deposition in aquatic and terrestrial ecosystems (Wen and Carignan 2007). Plants may help remove Se from the atmosphere

through absorption and metabolism of atmospheric Se, creating a valuable Se sink (Zieve and Peterson 1986). During phytoremediation, it is important to consider the amount of volatile Se being released by the plant compared to how much Se the plant removes from the atmosphere. While plants may produce both DMSe and DMDSe, most organic atmospheric Se from plants is probably DMSe since DMDSe is unstable and its ultimate fate is likely to either be metabolized by nearby plants or re-deposited in nearby soil (Martens and Suarez 1999). To minimize negative effects of translocation and redistribution of Se caused by plants used for phytoremediation it is best to remove high-Se biomass from the site, to optimize Se removal and prevent further Se pollution.

ECOLOGICAL PARTNERS

Microbes

While Se is toxic to most microbes, some bacteria and fungi live in the rhizosphere of Se hyperaccumulating plants where Se can be upwards of 100 mg Se kg^{-1} (Jose Rodolfo Valdez, personal communication). These microbes appear to have evolved mechanisms to overcome the toxic effects of Se. Some of these rhizosphere microbes may also play a role in plant Se accumulation. It has been shown that the presence of rhizosphere bacteria enhances Se accumulation and volatilization in Indian mustard, a Se accumulating plant (de Souza et al. 1999a) as well as certain wetland species (de Souza et al. 1999b). The activity of microbes in the rhizosphere may make Se more bioavailable to plants, stimulate plant Se uptake and assimilation and stimulate plant root growth leading to a larger Se uptake capacity. Some plant-associated microbes are microbial decomposers

that break down dead plant material that contains Se and release it back into the soil for reuptake by the plant.

In addition to free living microbes in the soil surrounding Se accumulators, there are microbes that live inside of the plant tissue: these are called endophytes. Most plants tested so far contain multiple bacterial and fungal endophyte species, which can colonize all plant tissues and be transmitted horizontally (to neighboring plants) or vertically (via the seeds) (for reviews see Saikkonen 1998; Sturz 2000). **E**ndophytes have been found in Se hyperaccumulators (Lindblom and Pilon-Smits, unpublished results). It is feasible that endophytes with high Se tolerance, accumulation or volatilization facilitate plant Se accumulation, volatilization or tolerance. Increased understanding of the role microbes play in plant Se accumulation and hyperaccumulation will prove to be a valuable tool when designing phytoremediation projects and when working towards biofortification of crops with Se.

Some microorganisms living on or inside plants are plant pathogens, or can become pathogenic under conditions of plant stress. Similar to plant-herbivore interactions, plants and microbial pathogens participate in an arms race. Plants often produce chemical defenses that microbes evolve to disarm. Selenium may function as a plant defense compound against microbial pathogens. Plants that were treated with Se had reduced disease when infected with the fugal pathogens *Alternaria alternata* and *Fusarium sp.* (Hanson et al. 2003). This may have important implications for Se phytoremediation: when growing Se-accumulating plants there may be less need for microbial pesticides and less biomass loss due to microbial pathogens. The total losses in the US of barley and wheat crops due to *Fusarium* head blight and seedling rot between

1991 and 1996 have been estimated at $3 billion (Priest and Campbell 2003). In addition, it is estimated that at least 20% of agricultural loss can be attributed to *Alternaria* plant pathogens. *Pseudomonas syringae*, a prevalent bacterial pathogen on plants has also been shown to be Se-sensitive (Lindblom and Pilon-Smits, unpublished results). It is encouraging for Se phytoremediation and biofortification projects that plants with elevated Se are protected from a wide range of devastating fungal and bacterial pathogens. However, in view of the chemical arms race between plants and microbial pathogens there are likely also many microbial pathogens that have evolved to overcome the toxic effects of Se. Such microbes may have co-evolved with Se hyperaccumulating plants. There is indeed a report of a *Fusarium sp.* isolated from a hyperaccumulator plant that is extremely Se tolerant and may even grow better in the presence of Se (Wangeline 2007). The further investigation of the effects of Se on positive and negative plant-microbial interactions and, conversely, the effects of microbes on plant Se accumulation and volatilization will be an interesting area of further study.

Herbivores and higher trophic levels

Since Se is toxic to many herbivores at concentrations found in hyperaccumulator plants, it has been hypothesized that the functional significance of Se hyperaccumulation is elemental defense – termed the elemental defense hypothesis by Boyd and Martens (1992) (see Figure 6.3 for an overview of Se uptake and interaction with ecological partners). There is mounting evidence that Se serves as an elemental defense against many herbivores. *Brassica juncea*, a Se accumulator and an important crop species often used for phytoremediation, was protected by elevated Se from important arthropod pests

as well as prairie dog herbivory (Bañuelos et al. 2002b; Hanson et al. 2003, 2004; Quinn et al. 2008). The Se hyperaccumulators *A. bisulcatus* and *S. pinnata* have also been shown to be protected from arthropod and mammalian herbivory (Freeman et al. 2006a, 2009). Selenium added to an artificial diet proved to be toxic to the herbivore *Spodoptera exigua* (beet armyworm) and the fly detrivore *Megaselia scalaris* (Vickerman and Trumble 1999; Jensen et al. 2005). Moreover, a field study comparing hyperaccumulating plants with non-hyperaccumulating plants found more arthropod individuals and arthropod species living on non-hyperaccumulating plants than hyperaccumulating plants (Galeas et al. 2008). Herbivores may use the distinct smell of volatile Se as an indicator of plants with elevated Se, and avoid them; they may also dislike the taste of Se-rich plants (Hanson et al. 2004; Freeman et al. 2007).

Like most arms races between plants and their herbivores/pathogens this elemental defense has been disarmed: Se-tolerant organisms have evolved that are able to occupy the niches provided by high-Se plants. Micro-arthropods and microbes responsible for decomposition were found living in Se hyperaccumulator leaf litter with toxic Se concentrations (see chapter 5 of this dissertation). In addition, a possible specialist diamondback moth (*Plutella xylostella*) has been discovered that feeds on *S. pinnata* in the seleniferous Western United States (Freeman et al. 2006a). Selenium tolerance studies comparing this population of diamondback moth with a population of diamondback moth collected from a non-seleniferous habitat in the Eastern United States confirmed that the population of diamondback moth found thriving on *S. pinnata* was Se tolerant and the diamondback moth from the non-seleniferous habitat was Se sensitive. The mechanism of Se tolerance was revealed through Se speciation studies. The Se-

tolerant diamondback was shown to accumulate Se in the form of MeSeCys, the same form found in the plants, which is not toxic. The Se-sensitive diamondback moth accumulates SeCys, which is toxic. This Se-tolerant diamondback moth has the ability to feed on high-Se plants that are toxic to many generalist herbivores, which may decrease browsing competition and provide a browsing niche. The elevated concentration of Se in the Se-tolerant diamondback moth may even protect it from Se-sensitive predators – this has not been studied yet. Interestingly, a parasitic wasp (*Diadegma insulare*) was found living on the Se-tolerant diamondback moth. This parasitic wasp also accumulates Se concentrations that are toxic to many organisms, and appears to have developed the same Se tolerance mechanism as the Se tolerant diamondback moth because it also accumulates MeSeCys (Freeman et al. 2006a).

Understanding the interactions between high-Se plants and their herbivores, in addition to how Se affects higher trophic levels, aids in management of Se phytoremediation sites. Selenium has the ability to act as an elemental pesticide in Se-polluted areas. However, if a Se-tolerant herbivore/pathogen evolves this may reduce the productivity of Se phytoremediation plants. Furthermore, if Se specialists prefer high-Se plant material then it is possible that monocultures of Se phytoremediation plants risk large biomass reduction.

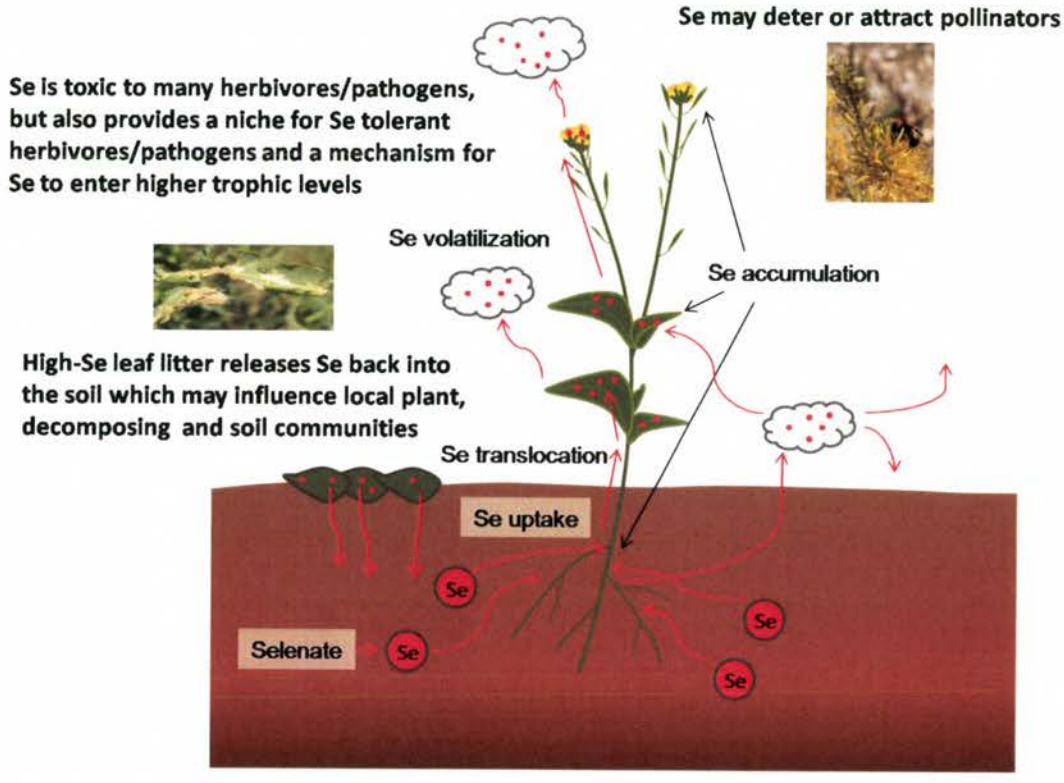

Figure 6.3: Overview of Se uptake, translocation and release of Se by plants and the effect high-Se plants have on ecological partners.

Pollinators

Since Se is toxic to many herbivores and pathogens, it may also affect pollination of plants with elevated Se. For plants, pollination is a key process in passing down genes and evolution through natural selection (Parra-Tabla and Vargas 2004). Elevated floral Se concentrations may act as a deterrent to Se sensitive pollinators, like it does to many herbivores. Alternatively, if pollinators benefit from Se (e.g. as a nutrient or antioxidant, or as a defense against pathogens or predators) or another characteristic unique to high-Se plants, Se may act as a cue to certain pollinators.

The role Se plays in pollination has large economic and ecological implications for Se phytoremediation. With the recent increase of sudden hive death syndrome in

honey bee hives the possible effect Se has on pollinators has gained increased interest, especially in the seleniferous central valley of California that economically relies on honeybee pollination (Reilly 2009). Pollinators of high-Se plants are likely exposed to Se because both hyperaccumulating and non-hyperaccumulating plants accumulate Se in flowers and flower parts that are particularly important to pollinators, specifically in pollen grains and nectar. Initial speciation studies have shown that *S. pinnata* flowers accumulate primarily the less toxic MeSeCys (see chapter 4 of this dissertation). There are currently no data on Se speciation in flowers of non-Se hyperaccumulators, but it is likely that they accumulate primarily selenate, as found in their other tissues (Freeman et al. 2006b). Pollinator studies investigating the role Se plays in pollination found that honeybee and other pollinator visits were the same on *B. juncea* flowers with less than 10 mg Se kg^{-1} and *B. juncea* flowers with 230 mg Se kg^{-1} (see chapter 4 of this dissertation). Future studies exploring how Se affects flower characteristics and pollination are important for phytoremediation.

Plant-plant interactions

To date, little is known about the role Se plays in plant-plant interactions. Selenium pollution may prevent many plant species from living on a once habitable ecosystem, particularly anthropogenic Se pollution because plants are unlikely to have previously evolved Se tolerance on these sites. Introducing plants that remediate Se pollution may also play an important role in redistributing Se in the soil and/or utilizing Se in allelopathic chemicals. Since it is known that plants with elevated Se increase local soil Se concentrations around the plant and around the rhizosphere of the roots, it is

reasonable to assume that this increased Se will affect Se-sensitive plant species growing in the same area. Indeed, *Arabidopsis thaliana*, a Se-sensitive plant, had lower germination rates and grew less biomass on high-Se soil that was collected from under the canopy of Se hyperaccumulating plants than on low-Se soil collected under the canopy of non-Se hyperaccumulators from the same site (El-Mehdawi, Quinn and Pilon-Smits, unpublished results). Managing Se phytoremediation sites requires consideration of how high-Se plants will interact with other plant species and alter soil characteristics that may affect the local vegetation.

FUTURE DIRECTIONS

There are many potential ecological impacts of Se phytoremediation that deserve further study. The interactions between Se accumulating or hyperaccumulating plants and neighboring plants are only beginning to be investigated. Hyperaccumulators contain extremely high Se levels in seeds, and Se released from germinating seeds may inhibit the germination of other, Se-sensitive species nearby, offering an additional selective advantage to Se-tolerant species. Another ecological question related to Se accumulation is the role volatile Se plays in plant-plant interactions. If Se accumulation is an induced defense then plants may volatilize Se as a signaling compound alerting nearby plants of eminent attack by pathogens or herbivores. This is an area that has not yet been studied, but will be a very interesting and ecologically significant research topic.

It is becoming more evident that many plant species can tolerate elevated Se in their tissues without suffering negative effects. Future studies may focus on the

movement of this plant Se through the ecosystem, particularly to higher trophic levels. It is essential to know how these Se (hyper)accumulating plants may alter the ecosystems that they inhabit prior to utilizing them for phytoremediation.

Interactions between Se accumulating plants and microbes also deserves further attention. We have just begun to grasp an understanding of the relationships between microbes and Se accumulating plants. Very little is known about the mechanism of Se detoxification in microbes that have evolved Se tolerance. Only very few microbes have been identified that associate intimately with Se accumulating or hyperaccumulating plants. Studies in this area are hampered by the fact that very few microbes can be cultured in artificial media: perhaps less than 1% of soil microbes are culturable (Torsvik and Ovreas 2002). Molecular tools such as DNA and RNA amplification and sequencing will be valuable in identifying many unique microbes never before identified or characterized. The possible importance of such microbes for Se tolerance, accumulation and volatilization in the plants they associate with has been documented by de Souza et al (1999a, 1999b) where rhizosphere bacteria enhanced plant Se accumulation and volatilization. More studies are needed to identify microbes that can enhance Se accumulation and volatilization, and the mechanisms responsible. Gaining this understanding has the potential to greatly increase the success of phytoremediation projects by simple inoculation with beneficial microbes. A potential additional benefit of Se phytoremediation besides cleaning up excess Se from the environment is the creation of Se-enriched food products. Since Se is an essential nutrient for humans and other mammals, Se-enriched plants can be used to combat Se deficiency worldwide. The crops themselves have the potential for use as food for humans, or for the production of

supplements, or for being processed into feed for animals. There are already many crops that are being fortified with Se, such as wheat, garlic and broccoli (Lintschinger et al. 2000; McSheehy et al. 2000; Roberge et al. 2003). Future research investigating Se concentration and speciation in crops used for phytoremediation will aid in understanding how best to use Se-enriched food products.

CONCLUSION

Elevated Se concentrations in soil, watersheds and the atmosphere occur naturally but are increasingly due to human activities such as agricultural practices and burning of seleniferous fossil fuels. Selenium toxicity can have devastating effects on ecosystems as was seen at the Kesterson Reservoir in central California, USA in the early 1980's when many fish and migratory bird species died due to Se poisoning. Phytoremediation can be an effective and inexpensive tool to clean up Se- polluted terrestrial and aquatic habitats. In terrestrial habitats plants can remove Se from soil through accumulation in plant shoots and volatilization. In aquatic systems constructed wetlands can remove Se from drainage or surface waters. Phytoremediation of Se is especially attractive because Se-enriched plants can help combat Se deficiency in low-Se areas. Like any remediation strategy, it is important to consider ecological interactions and ecosystem consequences when utilizing Se phytoremediation.

Traditionally, Se accumulating plants have been preferred over Se hyperaccumulating plants for phytoremediation because they are typically fast growing and are sometimes crop species. However, Se hyperaccumulating plants also have potential uses for phytoremediation because of their ability to accumulate extremely high

concentrations of Se, in a form that is highly anti-carcinogenic (MeSeCys). The genes of hyperaccumulator plants are also useful to help develop transgenic crop plants. Both Se accumulating and hyperaccumulating plants may change the distribution of Se in seleniferous habitats, and the Se accumulated in these plants has been shown to influence their interactions with ecological partners.

Elevated Se concentrations in plants may increase biomass yield and Se removal from a site because plant-accumulated Se acts as a pesticide through both deterrence of and toxicity to a variety of generalist herbivores and pathogens; moreover Se can be a beneficial element for plants, promoting plant growth and stress resistance. However, high-Se plants provide a niche for Se tolerant herbivores/pathogens, which may even prefer to feed on Se-rich plant material and have the potential to cause large biomass losses to Se phytoremediation plants. Se tolerant herbivores also provide a mechanism for Se to enter higher trophic levels in the ecosystem. In addition to herbivores and pathogens, high-Se plants may influence soil microbial communities and local plant communities. By creating micro-habitats of Se rich areas plants force microbial communities to adapt Se tolerance or live in another location lower in Se. Similarly, plants that are Se sensitive may not be able to survive around Se rich plants that have concentrated Se in a small area.

Selenium phytoremediation has a promising future and we are beginning to understand the interactions between Se-accumulating plants and their ecological partners. Selenium may provide a useful model element to aid in understanding how phytoremediation of other inorganics affect local ecosystems.

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Chapter 7

Conclusion

The ecology of Se hyperaccumulating plants is largely unexplored and in this dissertation my overall goal is to shed light on the ecological aspects of plant Se hyperaccumulation. It is thought that plants have evolved to hyperaccumulate Se as a protection against herbivores and pathogens, which has been termed the elemental defense hypothesis. Recent research investigating ecological interactions between Se hyperaccumulating plants and their partners has provided support for the elemental defense hypothesis. However, since only 20 plant species are known to hyperaccumulate Se it is reasonable to assume that there is a cost associated with Se hyperaccumulation. In order to investigate ecological aspects of Se hyperaccumulation my specific objectives are to: 1) determine if Se protects hyperaccumulating plants from cell disruptor herbivory, 2) determine if Se protects *S. pinnata* in its natural habitat from prairie dog herbivory, 3) investigate the effect of Se accumulation on pollination and reproductive fitness and 4) determine if Se effects leaf litter decomposition.

The first two research chapters of this dissertation (chapters two and three) provide additional support for the elemental defense hypothesis and expand the suite of herbivores that Se hyperaccumulating plants are against. In chapter two I show that Se

hyperaccumulating plants are protected from two cell disruptor herbivores: thrips and spider mites. This is the first study to show that Se hyperaccumulating plants are protected from herbivores with this feeding mode. Earlier invertebrate herbivores against which Se accumulation offers protection include phloem suckers (aphids) and both arthropod and mammalian folivores (grasshoppers, crickets, butterfly/moth larvae, prairie dogs). Chapter three provides evidence that Se protects the hyperaccumulator *Stanleya pinnata* from prairie dog herbivory over a two year period in its natural environment. This is the first study to show that Se hyperaccumulating plants are protected from herbivory over a long time period in the field. In addition, this is one of only a few studies that test the role of Se in protecting plants from mammalian herbivory.

Since Se hyperaccumulation protects plants from a variety of herbivores and pathogens, yet only a few species of plants hyperaccumulate Se, it is reasonable to assume that there is a cost associated with Se hyperaccumulation. I investigated a possible cost associated with Se hyperaccumulation by examining the effect of Se hyperaccumulation on pollinator visitation and pollen viability. I found that the hyperaccumulator *S. pinnata* and the non-hyperaccumulator *Brassica juncea* sequester Se differently in flowers. *Stanleya pinnata* stores Se primarily in stamens and pistils and in the form of methylselenocysteine while Se in *B. juncea* flowers is evenly distributed and found in a variety of organic and inorganic forms. Elevated Se in *B. juncea* did not affect pollination by honey bees and other potential pollinators. However, elevated Se in *B. juncea* did reduce pollen germination, which provides evidence for a cost of Se accumulation in non-hyperaccumulator plants, which may prevent more plant species from evolving Se hyperaccumulation.

In the final research chapter of this dissertation (chapter five) I investigated the effect elevated Se has on litter decomposition. Interestingly, I found that elevated Se increased leaf litter decomposition in a seleniferous habitat. This finding is important because it suggests that the decomposing community in this environment is Se tolerant and can feed on Se-rich litter.

Combined, these results provide a better understanding of ecological interactions involving Se hyperaccumulating plants. The Se in these plants appears to profoundly affect its ecological interactions. Most invertebrate herbivores of all feeding modes tend to be deterred by Se-containing plants and suffer toxicity when they feed on them, even at levels well below those of hyperaccumulators. Prairie dogs also avoid Se-rich plants. These results provide insight into the benefits and possible evolutionary pressures driving Se hyperaccumulation: protection from herbivory may have been an important selection pressure in favor of hyperaccumulation. I also show a potential reproductive cost associated with Se accumulation, which may shed light on why more plant species have not evolved Se hyperaccumulation: pollen viability is reduced in high-Se plants from non-hyperaccumulator species. Pollinators showed no aversion to collect pollen and nectar from high-Se plants. Some pollinators from seleniferous habitat were found to accumulate substantial Se levels in their tissues without apparent ill effects.

These results also increase our understanding of how Se from hyperaccumulating plants enters the food chain and affects decomposing communities and soil Se distribution. Herbivores that feed on Se rich food material provide a vector for Se to enter higher trophic levels and decomposing communities. Predators and decomposers that feed on herbivores that have consumed Se rich material potentially accumulate

elevated Se concentrations. Based the results in this dissertation it is reasonable to assume that decomposers in seleniferous habitats have evolved Se tolerance. In addition the predators of these Se tolerant decomposers have a competitive advantage if they are also Se tolerant. I also show that the decomposition of high Se leaf litter increases the Se concentration in the soil directly below the leaf litter, which may influence the soil community. Through the increased knowledge of ecological interactions involving Se hyperaccumulating plants we have better understanding of how to manage natural or polluted seleniferous habitats. It is important to consider the ecological significance of growing Se fortified crops or using plants for Se phytoremediation and these results shed light on possible consequences of growing Se rich plants, which are discussed in detail in chapter 6. Lastly, these results provide a framework for future research investigating the ecology of hyperaccumulating plants.