THESIS

EFFECTS OF ELEVATED PLANT SELENIUM LEVELS ON REPRODUCTION AND
ROOT-NEMATODE INTERACTIONS

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Spring 2011

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ABSTRACT

EFFECTS OF ELEVATED PLANT SELENIUM LEVELS ON REPRODUCTION AND
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Selenium is an important element in soils throughout the western United States,
and its presence has important consequences for the ecology of these seleniferous sites.
Some plants in seleniferous areas have evolved mechanisms to hyperaccumulate Se to 0.1
- 1% of their dry weight. Other plants accumulate moderate Se levels between 0.01 –
0.1% of their dry weight. In the studies described in this thesis, facets of the evolution of
Se accumulation and the associated ecology of Se hyperaccumulators are considered.
First, I examined the effect of increasing Se on reproductive parameters of Se
accumulators and hyperaccumulators. The reproductive parameters were measured
through cross-pollinations of greenhouse-grown accumulator plants receiving different Se
concentrations. In the accumulator *Brassica juncea*, increasing Se concentrations in plant
tissues caused decreases in biomass, pollen germination, seed weight, seed production,
and seed germination. In some cases, however, interactions of similar Se concentrations
in both parents actually proved beneficial to reproduction. The hyperaccumulator
Stanleya pinnata showed no effect of increased Se concentration on pollen germination.
These data provide interesting insight into the potential reproductive cost of Se
accumulation, and the apparent evolution of physiological tolerance mechanisms in
hyperaccumulators to avoid these reproductive problems.

To further investigate the effect of Se on reproduction, *S. pinnata* plants were taken to a field site with hives of the European honey bee (*Apis mellifera*) to examine the effect of Se in floral tissues on potential pollinators. The bees and other pollinators showed no preference for or against Se in the flower and foraged on both high- and low-Se plants equally. Because the bees showed no preference, the honey of bees in seleniferous sites was analyzed for the presence of Se, and there were small amounts (up to 2 mg kg^{-1} FW) of Se found in this honey. These findings are important for bee keepers in seleniferous areas, as they show no evidence of toxic Se levels in their honey and they may even market their honey as Se-enriched and beneficial for human health. The finding that bees do not discriminate between high and low-Se plants does warrant further studies on the potential health effects of the ingested Se on the pollinators and the movement of Se into the food chain.

Next, to further investigate the ecology of Se hyperaccumulators, I examined the interactions of hyperaccumulator roots with root-associated nematodes. Selenium hyperaccumulators *S. pinnata* and *Astragalus bisulcatus* growing in the field have root Se concentrations between 100 and 1,500 mg Se kg^{-1} DW, a toxic concentration to most above-ground herbivores. Therefore, it was expected that with increasing root Se concentrations, there would be reduced levels of nematodes associated with plants. There was no significant negative correlation with increasing Se concentration, and even roots containing >1,000 mg Se kg^{-1} Se harbored nematode herbivores. However, when nematodes extracted from field-harvested plants were used to inoculate greenhouse-grown *S. pinnata*, plants treated with Se did harbor significantly fewer nematodes several months later. These findings are of significance, both because they suggest the presence

of Se-tolerant and potentially Se-specialist nematodes in seleniferous sites, and for the possible use of Se as a pesticide for nematodes in non-seleniferous sites.

Furthermore, the roots of hyperaccumulators were examined for the spatial distribution and speciation of Se using X-ray Absorption Spectroscopy (XAS). The majority of the Se was found in the cortex and epidermis of the root, with lower levels in the wood. Organic Se of the C-Se-C type (Se bonded to two carbon atoms, similar to methyl-selenocysteine) was the predominant form of Se in the hyperaccumulator roots, together with a small fraction of inorganic selenite.

The findings presented in this thesis may also have relevance for hyperaccumulators of other elements, such as arsenic, cadmium, nickel or zinc, as these metals may also protect roots from nematodes and other root herbivores, and may have similar effects on reproduction. Further investigations may reveal other herbivores that are deterred by root hyperaccumulation, as well as more evidence of specialist herbivores that have evolved tolerance in response to the hyperaccumulator's elemental defense. Beyond insight into the ecological and co-evolutionary relationships between roots and herbivores, the results presented here also have applications in agriculture. Since Se is both a nutrient and a toxin, depending on its concentration, Se could be used as an alternative to organic pesticides in controlling root nematode and herbivore levels in organic and subsistence farming. With careful monitoring, the resulting plants may be considered Se-fortified food with enhanced nutritional value. Finally, the findings presented here provide a framework for follow-up studies investigating the evolution of plant Se hyperaccumulation and the associated effects of (hyper)accumulated plant Se on ecological interactions in seleniferous habitats.

ACKNOWLEDGMENTS

I would very much like to thank the people that made the completion of this thesis

possible. Their support and knowledge were integral to the work I completed.

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CHAPTER 1

INTRODUCTION

Soils all over the world contain a variety of elements. These elements include nutrients like sulfur, calcium, and phosphorus that plants need to survive. These also include heavy metals and metalloids like arsenic and cadmium that can be toxic to plant growth even in relatively low concentrations. The sources of these elements vary, being naturally occurring or the result of industrial processes (De Jong *et al.*1977). Depending on the geologic source of the soil, different soils contain different levels of these toxic metals and metalloids. The element selenium (Se) is present at substantial levels in cretaceous shale and is prevalent in much of the western United States as well as parts of China, whereas it is much less abundant in the eastern United States and Europe (Fig 1.1) (Reeves and Baker 2000, Girling 1984).

Selenium is an essential element for humans and most animals. It is present in the Se-containing amino acids selenocysteine and selenomethionine which are present in selenoproteins (Brown and Arthur 2001). These selenoproteins have anti-oxidant and anti-carcinogenic functions (Ellis and Salt 2003). Selenium has also been shown to reduce susceptibility to the human immunodeficiency virus, HIV (Rayman 2000). The concentration range yielding these Se benefits, however, is a narrow one.   People experience Se toxicity (selenosis) above a daily Se intake of 3.2 mg, which is manifested by the loss of hair and nails, pain in extremities associated with abnormalities in the

nervous system, and skin lesions (Yang *et al.* 1983). On the opposite side of the spectrum, people living in non-seleniferous sites may not receive sufficient Se to produce adequate levels of selenoproteins, and experience Se-deficiency. Keshan disease and Kashin-Beck disease are caused by Se-deficiency, and are characterized by a weakened immune system and heart dystrophy. Insufficient levels of Se can also cause male infertility, since one selenoprotein is necessary for spermatogenesis. (Rayman 2000, Ellis and Salt 2003). Plants differ from animals with respect to Se because there is not yet a documented essential function of Se in plants (Ellis and Salt 2003). However, plants have been shown to benefit from the presence of low levels of Se (less than 100 mg Se kg^{-1} : they reach a higher biomass and show enhanced antioxidant activity (Hartikainen 2005, Pilon-Smits et al., 2009).

Some plants accumulate metals or metalloids to concentrations as high as macronutrients like sulfur and phosphorus; concentrations that would normally be toxic to most plants. There are over 500 reported taxa with 'hyperaccumulator' properties, which can accumulate arsenic, cadmium, nickel, selenium, or zinc; the defining concentrations for hyperaccumulation vary by the element in question (Krämer 2010). Selenium hyperaccumulators accumulate Se to levels above 1,000 mg Se kg^{-1} DW and up to 10,000 mg Se kg^{-1} DW (1% of the plant's DW) without showing signs of toxicity. Se hyperaccumulators occur in the plant families Fabaceae (*Astragalus* spp.), Brassicaceae (*Stanleya* spp.) and Asteraceae (*Oonopis, Xylorhiza* spp). Most plants accumulate less than 100 mg Se kg^{-1} dry weight (DW) Se before experiencing Se toxicity like chlorosis, stunted growth, and death (Terry *et al.* 2000). These plants are considered non-accumulators of Se, and include most crop species (Burau *et al.* 1988). Other plants

accumulate Se to concentrations between 100 and 1000 mg Se kg^{-1} DW before toxicity

symptoms are apparent. These plants are called Se accumulators, accumulators, or Se-

indicators (Terry *et al.* 2000, White *et al.* 2004). Accumulators commonly occur in

families that contain high S levels, such as Brassicaceae (cabbage, mustard) and Liliaceae

(onion, garlic). Table 1 summarizes the categories of Se accumulation in plants.

Selenium is chemically related to sulfur (S), and Se accumulation is thought to

have originated because of this similarity (Daniels 1996). Plants growing in seleniferous

areas mistake Se for S and take up Se into the root. As Se enters the root tissues, it is

translocated to the shoot, and is metabolized via the sulfur assimilation pathway (Fig. 1.2)

(Terry *et al.* 2000). Selenium is toxic to most plants at low quantities because it gets

metabolized into the amino acids methionine and cysteine that normally contain S,

transforming them to Se-methionine (SeMet) and Se-cysteine (SeCys). These Se-

containing amino acids then get incorporated into proteins on accident and cause mis-

folding and dysfunction of proteins (Brown and Shrift 1988). Hyperaccumulators, on the

other hand, have an additional step in their metabolism and add a methyl group to SeCys,

forming methyl-SeCys (MeSeCys). This methyl group precludes the amino acid from

being used in proteins, preventing toxicity (Neuhierl and Böck 2004). The MeSeCys in

hyperaccumulator leaves is preferentially allocated to leaf margins, leaf hairs and

specialized storage structures along the leaf edges (Fig 1.3a) (Freeman *et al.* 2006a).

Furthermore, some of the accumulated MeSeCys can be volatilized in the form of

dimethyldiselenide (DMDSe), which is a gaseous form of Se and escapes from leaves

through the stomata (Terry *et al.* 2000). Non-hyperaccumulators store Se more in

inorganic form, and do not have specialized storage structures (Fig 1.3b). They can also

volatilize Se, but as dimethylselenide (DMSe) (Terry et al., 2000; Freeman et al. 2006a).

Why do some plants accumulate Se to levels up to 1% of their DW? There are

five hypotheses describing an evolutionary reason for hyperaccumulation. The first is

that plants accumulate Se, mistaking it for S because of their chemical similarity and ac.

The second hypothesis states that these plants hyperaccumulate toxic elements for use in

elemental allelopathy, inhibiting the growth of competitor plants. The third hypothesis is

that by hyperaccumulating extra solutes including Se, the plant can better take up water

via osmosis, giving the plant better drought resistance. The fourth hypothesis states that

hyperaccumulation is a way to tolerate the metal in the soil. The plant can accumulate

the element then dispose of the concentrated element by shedding plant parts (Boyd

2004). The fifth hypothesis is that plants hyperaccumulate metals as a form of defense

against herbivores or pathogens. This hypothesis is called the elemental defense

hypothesis (Boyd and Martens 1998; Boyd 2007). This is the hypothesis that has

received the most experimental support from research. Selenium accumulation has been

found to be effective against aphids, caterpillars, spidermites, thrips, grasshoppers,

crickets, two pathogenic fungi, and prairie dogs (Hanson *et al.* 2003, 2004; Freeman *et al.*

2007, 2009; Quinn *et al.* 2008, 2010). Selenium hyperaccumulators also harbored lower

levels of arthropods in field sites, compared to non-hyperaccumulators (Galeas *et al.*

2008). Similar results have been found for other accumulated elements. In one study

there was a complete deterrence of *Pieris brassicae* by plants containing high levels of

zinc (Pollard and Baker 1997). In another, the root herbivore cabbage maggot (*Delia*

radicum) was deterred by high nickel (Ni) levels of the Ni-hyperaccumulator

Strepthanthus polygaloides. All of the maggot larvae placed on low-Ni plants survived after 7 days and none of the maggot larvae placed in high-Ni plants survived (Jhee *et al.* 2005). Hyperaccumulation, therefore, appears to confer an evolutionary advantage in reducing herbivory *via* both deterrence and toxicity.

Like any plant defense against herbivores, the Se-based elemental defense has been overcome, since some herbivores have evolved Se tolerance. One such herbivore, the diamondback moth, has disarmed the defense of Se hyperaccumulator *Stanleya pinnata* (Freeman *et al.* 2006b). The tolerance of the moth appears to be due to a loss of the ability to demethylate the ingested MeSeCys, since the tolerant moth accumulated MeSeCys while a Se-sensitive relative accumulated SeCys (Freeman *et al.*, 2006b). In a Ni hyperaccumulator, non-choice feeding trials were conducted with aphids (*Acyrthosiphon pisum*) that were provided with either high- or low-Ni plants. There were no significant differences in deterrence or mortality between high- and low-Ni treatments (Boyd and Martens 1999). While most studies so far have focused on hyperaccumulator shoots, roots can also contain high metal levels, and the soil around hyperaccumulators may be enriched in the metal as well (El Mehdawi et al, 2011). High levels of heavy metals in soils may increase the tolerance of rhizosphere organisms. For example, nematodes sampled from copper (Cu) polluted soils had a higher tolerance to Cu than nematodes from a non-polluted site (Millward and Grant 2000). Thus, both above- and below-ground parts of hyperaccumulators are likely using the accumulated elements to evade herbivory, but also are caught in an evolutionary arms race with select organisms that have evolved tolerance to toxic concentrations of metals.

Several parts of the world, including the Western United States (Fig 1.1), have highly seleniferous soils, and in these areas there may be ecological implications of Se accumulation and hyperaccumulation. Many seleniferous sites in the Western United States support irrigated agriculture. The irrigation leaches Se from the seleniferous soils, into ditches and rivers (Clark 1987). These end up in stagnant bodies of water like lakes and ponds. The Se concentration in these water bodies increases over time by agricultural runoff and by the evaporation of water from the surface. As a result, many of these ponds have toxic levels of Se, and the fish and waterfowl in the ecosystem suffer Se toxicity and death (Ohlendorf *et al.* 1986). This is a significant problem, and one solution to it is to plant accumulator plants in highly seleniferous areas to remove Se from the soil and keep it in plant tissues. This practice of removing toxic contaminants from the field using plants is called phytoremediation. Plants used in this way usually reach concentrations around 50 mg Se kg^{-1} DW (Bañuelos *et al* 2005). Herbivores and pollinators interact with these phytoremediation fields, and the plant-accumulated Se may have a negative effect on these associated organisms. The exposure to high-Se plants may also drive evolutionary processes in the ecological partners, leading to Se tolerance (Pilon-Smits 2005). In addition to the use of plants for phytoremediation of Se, farmers in seleniferous areas are experimenting with growing crops to contain low Se concentrations (from 1.5-10 mg Se kg^{-1}DW, depending on the plant and plant part), which may be considered fortified food (Bañuelos and Lin 2010). The Se-fortified crops may be shipped to low-Se areas, where livestock or people suffer Se deficiency.

At this point in time, studies primarily have focused on the ecological interactions of the stem and leaf portion of hyperaccumulators (Fig 1.3). According to Galeas and

colleagues (2007), Se is reallocated in the late summer from leaf tissue to developing reproductive tissues. Some of the highest Se concentrations plants achieve are in the reproductive tissues, where Se is allocated primarily to the pollen, anthers, and ovules, and secondarily to petals and sepals (Fig 1.3a) (Quinn, *unpublished data*). One study described in this thesis (chapter 2) focuses on the effects of Se on floral reproductive functions, overall reproductive output, and the influence of plant Se on pollinator visitation (Fig 1.4). In this study the pollen germination rate in both an accumulator and hyperaccumulator were compared to assess the effect of Se on pollen function. The total number and weight of seeds produced, the average individual seed weight, and seed germination were quantified in plants treated with a range of Se levels, to assess reproductive output. Finally, the effects of Se on plant-pollinator interactions were evaluated by comparing pollinator visitation to hyperaccumulators receiving Se treatments or not receiving Se treatment, and by analyzing the Se content in local honey supplies. The findings of this study shed more light on the potential evolutionary constraints acting on Se accumulation/hyperaccumulation, and the potential effects of high-Se plants on pollinators. Moreover, by evaluating the effects of plant Se on pollinator visitation, the potential ecological implications for pollinators around phytoremediation fields can be evaluated.

Another facet of hyperaccumulator ecology that has to this point received little attention is root interactions (Fig 1.4). Hyperaccumulator Se levels fluctuate seasonally, moving from the shoots and flowers to the roots in the late summer through the winter (Galeas *et al.* 2007). Therefore, roots contain high levels of Se during the winter and fall. The location and form of the stored Se is unknown. A second study described in this

thesis (chapter 3) investigated the Se concentration, distribution, and speciation in

hyperaccumulator roots. Like the above-ground tissues, hyperacumulator roots encounter

herbivores. While Se hyperaccumulation has been shown to deter above-ground

herbivores and reduce general arthropod levels (Galeas *et al.* 2008), the below-ground

herbivore-deterrent potential has not been investigated. This study examines

hyperaccumulator – root nematode interactions, both in a natural seleniferous site and via

manipulative greenhouse experiments. This study is the first to yield knowledge of the

effects of Se on root herbivory and may lead to new options in agricultural treatments for

nematode eradication.

FIGURE LEGENDS

Fig. 1.1 Distribution of selenium in the United States. Areas in white represent high-Se areas, areas in grey are variable, and areas in black have low Se. Black dots represent areas where Se hyperaccumulators occur.

Fig 1.2 Sulfur and selenium metabolic pathways. The S pathway is shown in blue, and the Se pathway is shown in black.

Fig 1.3 Distribution of Se in floral tissues of a hyperaccumualtor and accumulator.*

A) X-ray absorption spectroscopy (XAS) analysis of Se distribution in the leaf, petals, sepal, pistil, and anther of *S. pinnata.* i: XAS scan of leaf margin of *S. pinnata*; ii: Scanning Electron Micrograph of *S. pinnata* leaf; iii: Energy-dispersive X-ray analysis (EDX) of *S. pinnata* leaf shown in, showing Se distribution; iv: *S. pinnata* inflorescence v: individual *S. pinnata* flower vi: XAS scan of ovules vii: XAS scan of immature anter viii: XAS scan of *S. pinnata* sepals and petals

B) XAS analysis of Se distribution in the leaf, petals, sepal, pistil, and anther of *B. juncea*. i: stem and flower of *B. juncea* ii: flower of *B. juncea* iii: XAS scan of ovules iv: XAS scan of leaf margin v: XAS scan of *B. juncea* vi: XAS scan of *B. juncea* petal vi: XAS scan of *B. juncea* sepal.

*Note: Fig 1.3 A i, ii and iii and B iv are found in Freeman *et. al* 2006a; all other images are by Quinn *et. al*, unpublished data.

Fig 1.4 Ecological considerations of Se hyperaccumulators. Possible ecological

interactions are listed on the right. Interactions considered in this thesis are listed in red,

and previously considered interactions are in black.

FIGURES

Fig 1.1

Fig 1.2

Fig 1.3

A)

B)

Fig 1.4

Se volatilization

Se accumulation

Se translocation

Se uptake

Se

Se

Se

Se

Se

Se

Volatile Se may attract or deter ecological partners like pollinators

Does Se in flowers influence pollinators and reproduction?

Se in tissues may prevent herbivory and pathogen attacks

Herbivores/pathogens may evolve Se resistance

Changes in soil Se distribution may influence soil microbial communities, root herbivores, and neighboring plants

Does Se in root tissue prevent herbivory by nematodes?

Table 1: Categories of Se accumulation in plants			
Categories	Levels Accumulated	% DW	Species Examples
Non-accumulators	0 - 100 mg Se kg^{-1} DW	0 - 0.01%	*Arabidopsis thaliana*
Accumulators or Secondary accumulators	100 - 1000 mg Se kg^{-1} DW	0.01 - 0.1%	*Medicago sativa* *Brassica juncea* *Brassica napus*
Hyperaccumulators	1,000 – 10,000 mg Se kg^{-1} DW	0.1 - 1%	*Astragalus bisulcatus* *Stanleya pinnata*

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CHAPTER 2

REPRODUCTIVE COSTS OF SELENIUM ACCUMULATION IN PLANTS

INTRODUCTION

Selenium (Se) is a trace element naturally occurring in seleniferous soils, the most common source being shale originating from the Cretaceous and early Tertiary periods (White *et al*. 2004). Places like the Western United States have elevated levels of Se in soils, sometimes in excess of 10 mg Se kg^{-1} whereas places like the Northeastern United States have little or no Se in soils (Reeves and Baker 2000). Like most trace elements, Se is shown to be necessary for animal and human health, but can be toxic in even moderate quantities (Rayman 2000, Brown and Arthur 2001, Goldhaber 2003). Because of the dangers of Se to animals, phytoremediation efforts have been implemented, using plants to remove or retain the Se on seleniferous site (Banuelos *et al.* 2005, 2007, 2010). In places experiencing excess soil Se, agricultural strategies for Se enrichment are also being implemented to produce crops that accumulate some levels of Se to ship to places experiencing Se deficiencies (Gissel-Nielsen 1998, Gomez-Galera *et al.*2010). In addition, the tissues of the plants grown in phytoremediation fields can also be chopped up and used as a Se fertilizer in Se-deficient areas.

Plants differ from animals with regards to Se because there is no demonstrated need for Se. However, Se can be beneficial to plant growth at low levels and detrimental at high levels (Pilon-Smits *et al.* 2009). Not all plants respond to Se in the same way.

Because of its chemical similarity to sulfur (S), plants take up Se in addition to S through the S assimilation pathway (Arvy 1993). Most plant species are considered Se non-accumulators, and show signs of toxicity (i.e. decreased biomass, chlorosis, death) between 10-100 mg Se kg^{-1} dry weight (DW). Accumulators, also called indicators or secondary accumulators, are plants that can tolerate moderate levels of Se, up to 1,000 mg Se kg^{-1} DW, before showing signs of toxicity. A small group of plants are considered Se hyperaccumulators and show upwards of 10,000 mg Se kg^{-1} DW in plant tissues without visible consequence, even in field settings (Terry *et al.* 2000, White *et al.*2004, Seppänen *et al.* 2010). When plants accumulate Se, they readily translocate it from the roots to the shoots, and from the shoots to the reproductive tissues. Therefore when the plant is flowering, Se levels are concentrated in petals, anthers, and pistils. Any organism coming in contact with floral tissues of hyperaccumulators may be faced with a toxic concentration of Se (Terry *et al.* 2000, Quinn *et al, unpublished data*).

If Se is not essential for plant growth, why would a plant accumulate moderate to high levels of Se in its tissues? Several studies have shown that Se accumulation can serve as a form of defense against herbivores such as caterpillars (*Pieris rapae*), grasshoppers (*Orthoptera*), crickets (*Orthoptera*), aphids (*Myzus persicae*) and two kinds of fungi (*Fusarium, Alternaria*) (Hanson *et al.* 2003, 2004; Freeman *et al.* 2007). This elemental defense can offer plants an evolutionary benefit even at low Se concentration, and thus herbivory may have been a positive selection pressure toward higher plant Se accumulation. This leads to an interesting question: Are there any evolutionary selection pressures that would act against Se accumulation in addition to toxicity? A possible negative effect of Se accumulation is on potential pollinators coming into contact with

high-Se reproductive tissues. Also, physiological processes involved in plant growth and reproduction may be affected at high Se levels. In a recent study by Quinn *et al.* (*unpublished data*), the European honeybee (*Apis mellifera*) showed no preference for or against the presence of Se in the accumulator *Brassica juncea* (Brassicaceae). Furthermore, the honeybee and a native bumblebee (*Bombus sp.*) contained elevated levels of Se when collected from hyperaccumulator flowers, but mostly in a non-toxic form.

    Here I describe a study conducted with the native hyperaccumulator *Stanleya pinnata* (Brassicaceae) to investigate effects of hyperaccumulator levels of Se in floral tissues on potential pollinators. Bees are sensitive to smell, and because Se can be volatilized by plants in the form of the highly odoriferous dimethylselenide or dimethyldiselenide (Terry *et al.* 2000), the Se odor produced has potential to deter bees (Wetherwax 1986). This study investigates that potential deterrence of pollinators by volatile Se from flowers. Furthermore, I conducted a survey of honey produced in high-Se areas to investigate if the Se is further carried by the bees into the honey produced. Several metals have been previously detected in honey produced by bees foraging on flowers, depending on the geological and geochemical aspects of the foraging area (Petrovic *et al.*1994, Pisani *et al.* 2008).   If indeed Se from hyperaccumulators is passed on through the pollinators to the honey, the resulting naturally Se-fortified honey could have enhanced nutritional value.

    As another part of this study I investigate the effects of Se accumulation on plant productivity and reproduction, comparing hyperaccumulator *S. pinnata* with the related accumulator *B. juncea.* The goal is to obtain insight into potential physiologically based

evolutionary selection pressures against Se accumulation. So far, no parameters of

reproduction have yet been studied for Se accumulators/hyperaccumulators as a function

of Se concentration; in fact, hardly anything is known in this respect for any elemental

hyperaccumulators. In lead (Pb) hyperaccumulators, there was an overall decrease in

seed germination rate with increasing Pb concentration (Xiong 1998). In another study

by Searcy and MacNair (1990), copper-tolerant plants had decreased seed production

when grown on increasing Cu concentrations, but only when cross-pollinated with a Cu-

sensitive plant. The parameters examined in my investigation include pollen germination,

seed weight, seed germination, total seed production, and vegetative and reproductive

biomass. The findings from this study give further insight into the evolution of Se

accumulation and have broader ecological implications for cultivation of plants for

phytoremediation fields and Se fortified foods and products.

MATERIALS AND METHODS

Plant Material

Stanleya pinnata plants were grown in the greenhouse (24/20°C day/night,16-h

photoperiod, 300 μmol m^{-2} sec^{-1} photosynthetic photon flux). The plants used were

maintained in greenhouse conditions for 3 years on a 50:50 mix of Pro mix BX (Premier

Horticulture, Quakertown, PA) and Turface MVP (Profile Products LLC, Buffalo Grove,

IL). The plants were split into a high-Se treatment and a control (low-Se) treatment. The

high-Se treatment received once a week high-Se fertilizer containing 1 g of fertilizer

(Miracle-Gro Excel, 15:5:15 Cal-Mag, The Scotts Co., Marysville, OH) with 80 μM

Na_2SeO_4 and once a week 80 μM Na_2SeO_4. The control treatment received fertilizer

once a week and water once a week. To induce flowering in *S. pinnata*, plants from both

treatments were placed in a cold room (4°C day/night, 16-h photoperiod, 75 µmol m^{-2}s^{-1} photosynthetic photon flux) for 4 weeks. Se treatments were continued throughout the cold treatment, and the plants began flowering within 4 weeks of returning to regular greenhouse conditions. Leaves and flowers were sampled for Se analysis, and pollen was collected for pollen germination studies, all as described below.

Brassica juncea seeds were germinated in 10 x 10 cm pots (T.O. Plastics, Clearwater, MN) filled with Pro mix BX (Premier Horticulture, Quakertown, PA), thinned to one per pot, and placed in the greenhouse (conditions as listed above). Water was supplied until the first true leaves appeared, then Se treatments began. All plants received fertilizer once a week (1 g L^{-1} of Miracle-Gro Excel, 15:5:15 Cal-Mag, The Scotts Co., Marysville, OH), and were supplied twice a week with one of 5 Se concentrations: 0 µM, 20 µM, 40 µM, 60 µM, and 80 µM Na_2SeO_4. The shoots of *B. juncea* plants not used for cross-pollinations were harvested and separated into vegetative (shoots and leaves) and reproductive (flowers, siliques, and seeds) structures. The tissues were dried for 3 days at 60°C, weighed for dry biomass and analyzed for Se concentration as described below. Other plants from the same treatments were used for cross-pollination experiments and to collect pollen for pollen germination experiments, as described below.

Pollen Germination

Semi-solid pollen germination media (PGM) was prepared (18% sucrose, 0.01% boric acid, 1mM $CaCl_2$, 1mM $Ca(NO_3)_2$, 1mM $MgSO_4$ and 5% agar) (Carlson *et. al* 2009) in 3 Se concentrations: 0 mg L^{-1}, 100 mg L^{-1}, and 1000 mg L^{-1} Na_2SeO_4 to simulate *in planta*

carpel Se concentrations. Anthers from newly opened *B. juncea* flowers from plants treated with 0, 20, 40, 60, or 80 µM Na_2SeO_4 and *S. pinnata* flowers from plants treated with 0 or 80 µM Na_2SeO_4 were collected and placed on all three PGM Se treatment plates. The pollen grains were allowed to germinate on the plates in a moist environment for either 3 hours (for *B. juncea*) or 24 hours (for *S. pinnata*), photographed under a compound light microscope, then the number of pollen tubes that broke the pollen coat were counted and the percentage of pollen germination calculated.

Cross-Pollination Experiments

Flowering *B. juncea* plants used as the maternal plant were emasculated, and unopened flowers were removed. Anthers with visibly dehiscing pollen were removed from the paternal plant and rubbed on the stigma of the maternal flowers. Most Se treatments were used as both maternal and paternal plants for a total of 23 cross-pollinations for every possible combination of Se treatments (e.g. the 40 µM X 60 µM cross was conducted 23 different times). 80 µM plants did not make enough flowers to complete all crosses, resulting in lower numbers of crosses. Glassine pollination bags were placed over the cross-pollinated inflorescences (Ward *et al.*2009). When the siliques began dehiscing and the seeds were dark brown, the seeds were harvested and weighed. The average individual seed weight was calculated.

For seed germination studies, seeds from all crosses were placed on sterile filter paper moistened with ddH_2O inside culture plates (Lifeline Sciences, Frederick, CO). The dishes were closed and sealed with Parafilm® (Pechiney Plastic Science, Menasha,

WI) and placed under fluorescent lights (24°C, 12h L/12h D). Every day the number of

germinated seeds was recorded and the percentage of seed germination was calculated.

To quantify fecundity, 10 *B. juncea* plants from each Se treatment were set aside

from cross-pollinations to set seed. The total number of seeds produced by each plant

was counted and the total seed biomass weighed.

Pollinator Studies

Plant-pollinator experiments were conducted with colonies of the European honey bee

(*Apis mellifera*) at a non-seleniferous field site in central Fort Collins. The honey bees

were provided with a choice between flowering high-Se and low-Se *Stanleya pinnata*

plants pre-treated with 80 or 0 μM Na_2SeO_4, respectively. One high-Se and one low-Se

plant were placed 10 m from each other and 10 m from a group of 4 beehives (Naug and

Arathi 2007, Quinn *et al, unpublished data*). Plants were observed (by 2 observers, one at

each position) over 45-minute time periods. Potential pollinator preference was

determined by calculating the percentage of total potential pollinators (flying insects) and

the percentage of total honey bees that visited either high- or low-Se plants in the 45

minute observation time. In addition, the total number of bees that visited the 80 μM and

0 μM Na_2SeO_4 treated plants was recorded. This experiment was repeated 22 times. The

plant-pollinator experiments were performed between 9:00 am and 12:00 pm from June 5

to August 30, 2010. For each observation period one person collected data from high-Se

treated plants and another individual from low-Se treated plants. To reduce the possibility

of bees habituating to the location of the plants, the groups of plants were removed from

the site after the 45 minute observation period and returned to the field site 15 minutes

later, but with the high- and low-Se positions switched. Individuals observing remained in the same location after plants were switched. Four researchers were used as observers to prevent potential bias during data collection.

Honey and hive samples were obtain either from commercial sources of from local beekeepers. The European honeys were purchased in the Netherlands; one originates from central Spain, and the others from unspecified European sources. Most Colorado samples were local beekeepers; except for the Lyons sample which was purchased and originates from unspecified Colorado locations.

Elemental Analysis

For all treatments of *B. juncea* and *S. pinnata* leaves, flowers, and produced seeds, 0.1 g of dried samples were acid-digested in nitric acid as described by Zarcinas *et al.* (1987) and analyzed for Se analysis via Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) as described by Fassel (1978). The same was done with comb honey samples collected from a variety of seleniferous and non-seleniferous sites across Northern Colorado and Europe, to be analyzed for a variety of element concentrations. The honey was not dried before digestion and contained an estimated 80%DW and 20% water.

Statistical Analysis

All statistical analyses were performed using JMP-IN (version 3.2.6, SAS Institute, Cary, NC) or SAS software (9.2, SAS Institute, Cary, NC). One-way ANOVAs were used to compare several means, multiple linear regression ANCOVA models were used to

analyze the effect of several variables and the interactions, Chi-square tests were done to analyze percentages, and Student's t-tests were used to compare two means. All ANOVAs and ANCOVAs were *post-hoc* evaluated using Tukey-Kramer tests for significance at $\alpha = 0.05$.

RESULTS

Plant Material

In *B. juncea,* treatment with increasing Se concentration resulted in significantly increased plant Se concentration in both leaf and reproductive tissues ($P<0.0001$, Fig 2.1a, and $P<0.0001$, Fig 2.1d). Also in *S. pinnata,* the leaf and reproductive tissues of the high-Se treatment were significantly higher than the low-Se treatment ($P<0.0001$, Fig 2.1c, and $P=0.0017$, Fig 2.1f). Treatment with increasing Se concentrations decreased the dry biomass of *B. juncea* in both vegetative and reproductive tissues ($P<0.0001$, Fig. 2.1b, and $P<0.0001$, Fig. 2.1e). The effect, however, was not significant until the 60 μM SeO_4 treatment. The *S. pinnata* plants used in the pollinator studies and pollen germination experiments therefore were not harvested and dry biomass data not collected.

Pollen Germination

With respect to pollen germination in *B. juncea*, the Se level in the plant and the Se level in the media interacted and had different effects on the pollen germination ($F= 2.93$, $p=0.0035$, fig2.2). On pollen germination medium containing no Se, pollen collected from plants that had received the 60 and 80 μM Na_2SeO_4 treatment germinated less often (ratios of 0.30 and 0.29, respectively) than pollen from plants that received other Se

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treatments (0.40, 0.45, and 0.40, p=0.0480, Fig 2.2a). On the 100 mg L^{-1} Na_2SeO_4 pollen germination medium, plants with increasing Se levels showed significantly decreasing pollen germination, with germination ratios ranging from 0.45 in the no Se treatment to 0.0 in the 80 μM Se treatment (P=0.0035, Fig. 2.2b). On the 1,000 mg L^{-1} Na_2SeO_4 pollen germination medium, there were no significant differences in pollen germination between increasing Se treatments (Fig 2.2c). The Se concentration in the media had a significant effect on the pollen germination with the average ratio in the no Se treatment being the highest at 0.37 compared to the 100 mg L^{-1} Se treatment, which was the lowest at 0.27 (F=7.47, p=0.0007). The 1000 mg L^{-1} media treatment was not significantly different from either the no Se or moderate Se media treatments.

S. pinnata showed different results than B. juncea. At each of the three Se concentrations in the medium, there was no significant difference between plant Se treatments (P = 0.1549, P = 0.3096, P = 0.7103, Fig 2.2d-f). There was a significant effect of Se in the media, and S. pinnata pollen germinated better on media containing Se than they did on media without Se. The average germination ratio for the no Se treatment was 0.13, which was significantly lower compared to the high Se media treatment at 0.19 and the moderate Se media treatment at 0.27 (F = 9.82, p <0.0001, Fig. 2.2d-f).

Cross Pollinations

Cross-pollinations were performed between B. juncea plants grown at different Se concentrations. Seeds from each of the crosses were weighed to obtain the individual seed weight to measure if there is any effect on health of the seed with increasing Se concentration. The paternal Se treatment had no effect on the individual seed weight

(P=0.9538, Fig 2.3a-e). The maternal Se treatment did have a significant effect on seed weight, especially at leaf concentrations above 1000 mg Se kg^{-1} (P < 0.0001, Fig 2.3f, Fig 2.1a). The 20, 40 and 60 μM Se maternal treatments resulted in a 20% reduction in seed weight compared to the 0 Se treatment, and the 80 μM treated plants even showed a 90% reduction in seed weight. However, the heaviest seeds were obtained from the 40 μM X 60 μM Se cross with the weight of 3.31 mg compared to the 0 μM X 0 μM Se cross with the weight of 2.98 mg (Fig. 2.3c).

Seeds from each of the cross-pollinations were collected and analyzed for their Se concentration. Due to the high number of seeds required for Se analysis and the necessity of seeds for seed germination studies, only one replicate for each cross was obtained and analyzed. In some cases, there were not enough seeds to run the Se analysis; their Se concentration is not shown. For each maternal Se concentration, the paternal Se concentration did not affect the seed Se concentration (P = 0.9966, Fig 2.4a-d). The increasing maternal treatment concentration, however, significantly increased seed Se concentration (P < 0.0001, Fig 2.4e).

In the seed germination study, all cross-pollinations done with an 80 μM Na$_2$SeO$_4$ plant as the maternal or paternal plant did not produce enough seeds for adequate statistical power and are not included in the statistical analysis. The study was terminated after 7 days because germination rates of most treatments were beginning to plateau (Fig. 2.5f). Chi-square analyses were done on the frequency of germination at the end of the 7 day germination period for an estimate of total percentage of seed germination. All p-values are reported in the text, and chi-square values and p-values are reported on Fig 2.5. When considering both maternal and paternal Se treatments together, the combination of

treatments significantly affected the total seed germination (P <0.0001, Fig 2.5). When the maternal Se treatment was analyzed across all paternal Se treatments, the maternal Se treatment significantly affected the total percentage of seed germination (P <0.0001, Fig 2.5f). The seed germination ratio decreased with increasing levels of maternal Se treatment. The paternal Se treatment, when analyzed across all maternal Se treatments also had a significant effect on the total percentage of seed germination (P = 0.0100), but there was no consistent relationship between increasing paternal Se treatment and seed germination. When the data were plotted, the shape of the germination curve appeared to be affected by the paternal Se treatment more so than the maternal treatment. To further investigate these differences, the percentage of seed germination on the 5th day was analyzed.

When the interaction of the maternal and paternal Se treatments were analyzed, the Se treatment showed a significant effect on the percentage of seed germination by day 5 (P <0.0001). The maternal and paternal Se treatment effects were also analyzed separately, and both treatments yielded a significant effect on seed germination (P< 0.0001, and P < 0.0001). Chi-square values and probabilities within each maternal treatment show the significance of effect of the paternal Se concentration (Fig 2.4a-d). Increasing paternal Se concentrations had a significant negative effect when the maternal Se treatment was 0 μM Na_2SeO_4 (p = 0.0052, Fig 2.4a). When the maternal Se treatment was 20 and 40 μM Na_2SeO_4, the paternal Se treatment did not have a significant effect (p = 0.2380, p = 0.5193 Fig 2.5b-c). Then, at the maternal Se treatment of 60 μM Na_2SeO_4, the increasing Se treatment of the paternal plant significantly increased seed germination

(p = 0.0013, Fig 2.5f). Thus, the effect of the paternal Se treatment on seed germination

depended on the Se level of the mother plant.

The total weight of seeds produced from each plant not used in cross pollination

experiments was significantly decreased with increased seed Se concentration

(F=15.7589, p < 0.0001, Fig. 2.6a). Plants treated with 20 µM Se had on average 20%

lower total seed weight, 40 µM Se treatment resulted in 60% lower total seed weight, and

plants treated with 60 or 80 µM Se even produced no seeds at all.   Similarly, when the

total number of seeds was counted, there was a significant effect of increasing Se

concentration (F = 17.9588, p < 0.0001, Fig 2.6b). Plants not treated with Se produced

more seeds than plants treated with increasing Se. This effect became significant above

20 µM Na_2SeO_4, corresponding to a leaf and flower Se concentration above 500 mg Se

kg^{-1} DW (Fig 2.1 a,d).

Pollinator Studies

S. pinnata plants treated with 80 µM Na_2SeO_4 had significantly higher Se concentration

in the floral tissue compared to *S. pinnata* plants not receiving Se treatments (p < 0.0001,

Fig 2.7a). When *A. mellifera* were offered a choice to forage on *S. pinnata* plants that

had received Se or plants treated without Se, they showed no preference toward either

plant group. In a 45 minute period, the percentage of visits to a flower containing Se was

not significantly different from the percentage of visits to a flower not containing Se (Fig

2.7c). The same result was seen for honey bees visiting the flowers (Fig 2.7d) and for

other potential pollinators visiting the flowers in the 45 minute time period (Fig 2.7b).

When honey bee products obtained from Colorado bee keepers were analyzed for Se and other elements, the honey samples contained up to 2 mg Se kg^{-1} Se fresh weight in the honey, and hive and comb pieces contained up to 7 mg Se kg^{-1} Se fresh weight (Table 1). Elements like aluminum, copper, iron, etc. were examined and compared between honey collected in Colorado (seleniferous) and honey collected in Europe (largely non-seleniferous). An exhaustive list of elements examined can be found in Table 2. None of the examined elements differed significantly between the Colorado and European honeys (Table 2).

DISCUSSION

The purpose of the studies described in this chapter was to analyze the effect of increasing plant Se accumulation on reproduction. Plants were treated with increasing Se concentrations and biomass, pollen germination, individual seed weight, seed Se concentration, seed germination, and total seed production were measured. The effect of floral Se on visitation by pollinators was also examined.

The accumulator *B. juncea* showed inhibited growth and reproduction with floral Se concentration in excess of 500 mg Se kg^{-1} DW when examining DW biomass, pollen germination, seed germination, and seed production. The hyperaccumulator *S. pinnata*, however, showed no effect of Se even at floral concentrations of approximately 4,000 mg Se kg^{-1} DW. In the greenhouse trials, *B. juncea* receiving 60 μm Na_2SeO_4 had floral concentrations in excess of 1,500 mg Se kg^{-1} DW and vegetative concentrations above 1,000 mg Se kg^{-1} DW (Fig. 2.1a), and showed a significant decrease in biomass. In

contrast, the hyperaccumulator *S. pinnata* had floral concentrations close to 4,000 mg Se kg^{-1} but this had no significant effect on growth.

When examining pollen germination in *B. juncea*, the 60 μM and 80μM Se treatments led to decreased pollen germination rates. Furthermore, increasing Se concentration in the media decreased pollen germination. Pollen germination in *S. pinnata*, however, was not affected by high Se concentration. In fact, *S. pinnata* pollen germinated better on media containing Se. An interesting observation in these studies was that pollen from Se-rich *B. juncea* plants appeared to be better able to fertilize Se-rich maternal plants, perhaps indicative of a physiological predisposition to cope with the toxic effects of the encountered Se. In an earlier study conducted by Searcy and Macnair (1990), Cu-tolerant plants fertilized with Cu-sensitive pollen showed decreased levels of fertilization. The authors hypothesized that the pollen tubes from Cu-sensitive plants did not survive and grow as well as the pollen from Cu-tolerant plants in the high-Cu environment of the style. In this study, moderate levels of Se treatments in the paternal plants improved seed germination and seed weight of Se-rich maternal plants. It is possible that plants treated with no or low Se produce pollen tubes that are inhibited by the high concentrations of Se in the style and ovule of a plant treated with higher Se concentrations. Pollen from a plant receiving high Se levels may not be as vulnerable in a style with high Se concentrations. It would be interesting to further investigate this Se-dependent pollen-pistil interaction and identify the underlying physiological mechanisms.

In general, the Se concentration of the maternal plant had the most pronounced effect on the parameters of reproduction. The individual seed weight, seed germination, and seed production were all detrimentally affected by increasing maternal Se levels,

with significant effects happening above the 40 or 60 µM Na_2SeO_4 treatment (which corresponds to 500-1,000 mg Se kg^{-1}DW in tissue Se concentration). The maternal plant appears to contribute more resources to seed production than the paternal plant, and thus it is to be expected that increasing Se concentration in the maternal plant would have a stronger effect on seed production. One exception is that the paternal Se concentration appeared to additionally affect total percentage of seed germination within the same maternal Se treatment. Upon further examining reproductive parameters in *B. juncea*, there was in general a negative effect of increasing Se concentration; however there were several instances where interaction of similar Se concentrations was actually beneficial. For example, when maternal Se concentrations were paired with similar paternal Se concentrations, the seeds had a higher germination rate compared to dissimilar paternal Se concentrations. This could have implications for crops produced from agricultural fields containing Se. For example, seeds produced by *Brassica napus* grown on high Se soils are made into biodiesel (Bañuelos 2006).

The results obtained suggest a physiological difference between the accumulator *B. juncea* and hyperaccumulator *S. pinnata* (Chapter 1, Table 1). Overall, increasing levels of Se in maternal tissues negatively affected reproduction in *B. juncea*, but only significantly at levels already showing toxicity in these accumulators. The defining ceiling Se concentration of accumulators is 1,000 mg Se kg^{-1} DW, while hyperaccumulators can have Se concentrations up to 10,000 mg Se kg^{-1}. Indeed, Se toxicity in the accumulator is apparent from decreased biomass, decreased pollen germination, decreased seed weight, decreased seed germination, and decreased number of seeds produced. In contrast, increasing levels of Se in hyperaccumulators, sometimes

up to 10,000 mg Se kg^{-1} DW, did not show any significant effect on reproduction when considering pollen germination. These data suggest that 1,000 mg Se kg^{-1} DW is an appropriate defining limit for Se accumulators. It is important to note in this context that common levels of Se in accumulators found in the field do not reach Se concentrations close to 1,000 mg Se kg^{-1}DW and instead are more commonly around 50 mg Se kg^{-1} DW Se, although greenhouse levels of Se-supplied plants can be 100-1000 mg Se kg^{-1} DW (Bañuelos 2005). Thus, accumulators have the ability to accumulate Se in greenhouse environments where Se is readily provided, but certain physiological tolerance processes are lacking when compared to hyperaccumulators. This lack of tolerance mechanisms does not allow tissue concentrations higher than 1,000 mg Se kg^{-1} DW without detrimental effects on growth and reproduction.

Hyperaccumulators seem to have evolved physiological mechanisms to both tolerate and accumulate Se at extremely high levels. Research so far indicates that the hyperaccumulators store more Se in organic form, particularly methyl-selenocysteine (MeSeCys), which does not get incorporated into protein and therefore is relatively non-toxic. This likely is one reason for their extreme Se tolerance (Neuhierl and Böck, 1992). Moreover, hyperaccumulators appear to store more Se in specialized structures in the epidermis, protecting vital metabolic processes from the toxic effects of Se (Freeman et al., 2006, 2009). More detailed follow-up studies should be conducted in accumulators and hyperaccumulators to further investigate the physiological mechanisms responsible for their difference in tolerance to Se; this may provide insight into the evolutionary path to hyperaccumulation.

The findings of this study have potential consequences for pollinators of hyperaccumulators, since the pollinators observed seemed to have no preference or aversion to forage on plants with high Se concentrations in the flowers. Neither total pollinators nor honey bees observed in the field showed any aversion to forage on Se-rich *S. pinnata* or *B. juncea*. Indeed, both native (i.e. *Bombus sp.*) and introduced (i.e. *A. mellifera*) pollinators showed significant Se levels in their tissues, particularly the bumble bees (*Bombus sp.*) (Quinn *et al, unpublished data*). Honey bees were found to have up to 15 mg Se kg^{-1} DW in/on their bodies, and likely will bring pollen and nectar containing Se back to their hive, feed it to their young and incorporate it into their honey and wax. When honey was examined from hives in seleniferous areas, there were detectable levels of Se in honey, averaging approximately 1 mg Se kg^{-1}DW. These concentrations, at the suggested serving size for humans (1 tablespoon honey/day) will not be sufficient to induce Se toxicity, and would provide around 30% of the daily recommended Se intake (which is 50 µg). Beekeepers, especially in seleniferous areas could have their honey tested and potentially market their honey as Se-enriched. Because of the health benefits Se can confer, like antioxidant and anti-carcinogenic activity and protection from viral infections, Se-fortified foods like honey have potential to be marketed as a health supplement (Ellis and Salt, 2003).

In the past it has been shown that low levels of Se in both accumulators and hyperaccumulators can act as a plant defense against a wide variety of herbivores as well as some fungi. Freeman *et al* (2007) fed grasshoppers and crickets (*Orthoptera*) plants with Se concentrations as low as 145 mg Se kg^{-1} DW and still saw effective defense against these herbivores. Selenium was even more effective against aphids, where 10 mg

Se kg^{-1} DW already protected the plants from phloem-feeding *Myzus persicae* (Hanson *et al*. 2004)*.* Caterpillar (*Pieris rapae*) herbivory was deterred by 600 mg Se kg^{-1} DW (Hanson *et al*. 2003). In field plants, a Se range of 50-750 mg Se kg^{-1}Se was sufficient for deterring prairie dogs (*Cynomys ludovicianus*). However, some herbivores have evolved tolerance to these levels of Se and can feed freely on accumulators. Freeman *et al* (2006) describes diamondback moth larvae (*Plutella xylostella*) feeding without ill effects on hyperaccumulators with concentrations of 2,000 mg Se kg^{-1}. Together, these studies paint an interesting picture of the evolution of Se accumulation. Plants probably began to accumulate low levels of Se nonspecifically through the S assimilation pathway, and even those low levels of Se aided in defense against herbivory. Continuous selection pressure in the form of herbivory may have favored higher and higher plant Se accumulation, up to the point where plants experienced toxicity. It appears that for accumulators this level is between 500 and 1,000 mg Se kg^{-1}DW, when physiological processes such as pollen germination and seed production become affected by the Se, resulting in an evolutionary disadvantage for further Se accumulation. In seleniferous habitats, some herbivores evolve tolerance to Se, leading to an increase in herbivory and a need for the plant to evolve even higher Se levels (Freeman *et al.* 2006). Here, additional tolerance mechanisms and true hyperaccumulation may arise, leading to plants that experience no ill effect of Se on reproductive processes, and even more protection from herbivores. Along with the evolution of hyperaccumulation, insects with positive interactions with hyperaccumulators, such as native pollinators, may have evolved tolerance to Se and forage on hyperaccumulator plants without suffering toxicity. Finally,

even at true hyperaccumulator levels, some herbivores have disarmed the elemental defense of Se (Freeman *et al.* 2006).

The studies conducted here are the first to examine reproductive parameters for Se hyperaccumulators and accumulators, and one of the first for hyperaccumulators of any element. These studies provide further insight into positive and negative selective pressures in the evolution of Se hyperaccumulation and hyper-tolerance. In addition to increasing evolutionary insight, these findings can have important applications for the cultivation of accumulators and hyperaccumulators in agriculture or for phytoremediation.

Better knowledge of the effects of Se on reproduction/ seed production in *B. juncea* may benefit Se-fortified agriculture and phytoremediation. Seed production of crops grown on seleniferous sites has the potential to be optimized according to Se concentration, and fields of accumulators can be used for phytoremediation without deterring pollinators. Since pollinators do not avoid high-Se plants and readily store Se in their tissues and honey, the effect of the Se on pollinator health warrants further research. The Se in honey collected around seleniferous sites remains under the levels of toxicity and may actually have health benefits to the consumer.

FIGURE LEGENDS

Figure 2.1. Biomass and tissue Se concentration of *B. juncea* and *S. pinnata* treated with different Se concentrations. A) Se concentration (mg Se kg^{-1} DW) in *B. juncea* leaf (n=8). B) Dry weight (g) of *B. juncea* vegetative tissues (n= 16). C) Se concentration (mg Se kg^{-1}DW) of *S. pinnata* leaf tissues (n=15). D) Se concentration (mg Se kg^{-1} DW) and E) dry weight (g) of *B. juncea* reproductive tissues (n=8, n= 16). F) Se concentration (mg Se kg^{-1} DW) of *S. pinnata* reproductive tissues. Values are means +/- SE; different letters above bars represent a significant difference (α = 0.05).

Figure 2.2. Pollen germination ratios for *B. juncea* and *S. pinnata* treated with different Se concentrations. A) *B. juncea* Pollen germination ratio on germination medium without Se (n=30 plants). B) *B. juncea* Pollen germination on medium containing 100 mg L^{-1} Na$_2$SeO$_4$ (n=12 plants). C) *B. juncea* Pollen germination on medium containing 1000 mg L^{-1} Na$_2$SeO$_4$ (n=12 plants). D-F) *S. pinnata* pollen germination ratios on medium containing 0, 100, and 1000 mg L^{-1} Na$_2$SeO$_4$, respectively (n=10 plants). Values are means +/- SE, a different letter above bars represents a significant difference (α = 0.05).

Figure 2.3. Average individual seed weight from crosses of *B. juncea* parents treated with different Se concentrations. A) Average weight (mg) of an individual seed produced from a cross-pollination of paternal plants treated with different Se concentrations and maternal plants not treated with Se (numbers of seeds weighed were 316 for 0 μM, 266 for 20 μM, 179 for 40 μM, and 220 for 60 μM). B-E) Average weight (mg) of an

individual seed from maternal plants treated with 20 (B, numbers of seeds weighed were

503 for 0 µM, 434 for 20 µM, 313 for 40 µM, and 171 for 60 µM), 40 (C, numbers of

seeds weighed were 297 for 0 µM, 270 for 20 µM, 298 for 40 µM, 286 for 60 µM, and 30

for 80 µM), 60 (D, numbers of seeds weighed were 299 for 0 µM, 223 for 20 µM, 225 for

40 µM, and 315 for 60 µM) and 80 (E, numbers of seeds weighed were 3 for 0 µM, 2 for

20 µM, and 9 for4 0 µM) µM Na_2SeO_4 treatments. The Se treatment of the paternal plant

(µM Na_2SeO_4) is shown on the x-axis in all panels. F) Average weight (mg) of an

individual seed from maternal plants; paternal Se treatments are pooled. Values are

means +/- SE, a different letter above bars represents a significant difference ($\alpha = 0.05$).

Figure 2.4.   Selenium concentration in the seed produced from cross-pollination of

parents treated with different Se concentrations. A) Se concentrations in seeds from all

crosses with 0 µM Na_2SeO_4 maternal plants. Paternal plant Se treatments are shown

along the x-axis. B) Se concentrations with 20 µM SeO_4 maternal plants. C) Se

concentrations with 40 µM Na_2SeO_4 maternal plants. D) Se concentrations with 60 µM

Na_2SeO_4 maternal plants. Values are actual values. Not enough seed material was

obtained for multiple reps for statistical purposes. E) Se concentrations in seeds with all

different Se treatments of the maternal plants; paternal Se treatments are pooled (n=4).

Values are means +/- SE, a different letter above bars represents a significant difference

($\alpha = 0.05$).

Figure 2.5. Progressive seed germination rates (seeds germinated/total seeds sown) over a

period of 7 days of seeds obtained from crosses of parents treated with different Se

concentrations. A) Seeds produced from cross-pollinations with 0 µM Na_2SeO_4 treated

maternal plants. B) Seeds from maternal plants treated with 20 µM Na_2SeO_4. C) Seeds

from maternal plants treated with 40 μM Na_2SeO_4. D) Seeds from maternal plants treated with 60 μM Na_2SeO_4. E) Seeds from maternal plants treated with 80μM Na_2SeO_4. In each of the graphs different lines correspond to different paternal Se treatments (μM Na_2SeO_4) as labeled in the legend. F) Combination of paternal treatments within maternal Se treatments. Lines correspond to the maternal Se treatment (μM Na_2SeO_4), as described in the legend. N= at least 150 seeds except in the 80μM treatments, which had less than 10. Values are the means +/- SE. All χ^2 and p-values reported were calculated using SAS statistical software.

Figure 2.6. Fecundity parameters of plants of all Se treatments not used in cross-pollinations. A) The total seed weight (g) per plant (n=16 plants). B) The total number of seeds per plant (n=16 plants). Values are means +/- SE; different letters above bars represent a significant difference ($\alpha = 0.05$).

Figure 2.7: Pollinator observations. A) Selenium concentration in flowers of high- and low-Se *S. pinnata* used for pollinator visitation studies. B) The percent of potential pollinators visiting the high- or low-Se plant. C) The percent of honey bees visiting the high- or low-Se plant. D) The number of individual honey bee visits per plant during 45 minute observational periods. Observations were conducted 22 different days. Values are means +/- SE, an asterisk between bars represents a significant difference ($\alpha = 0.05$).
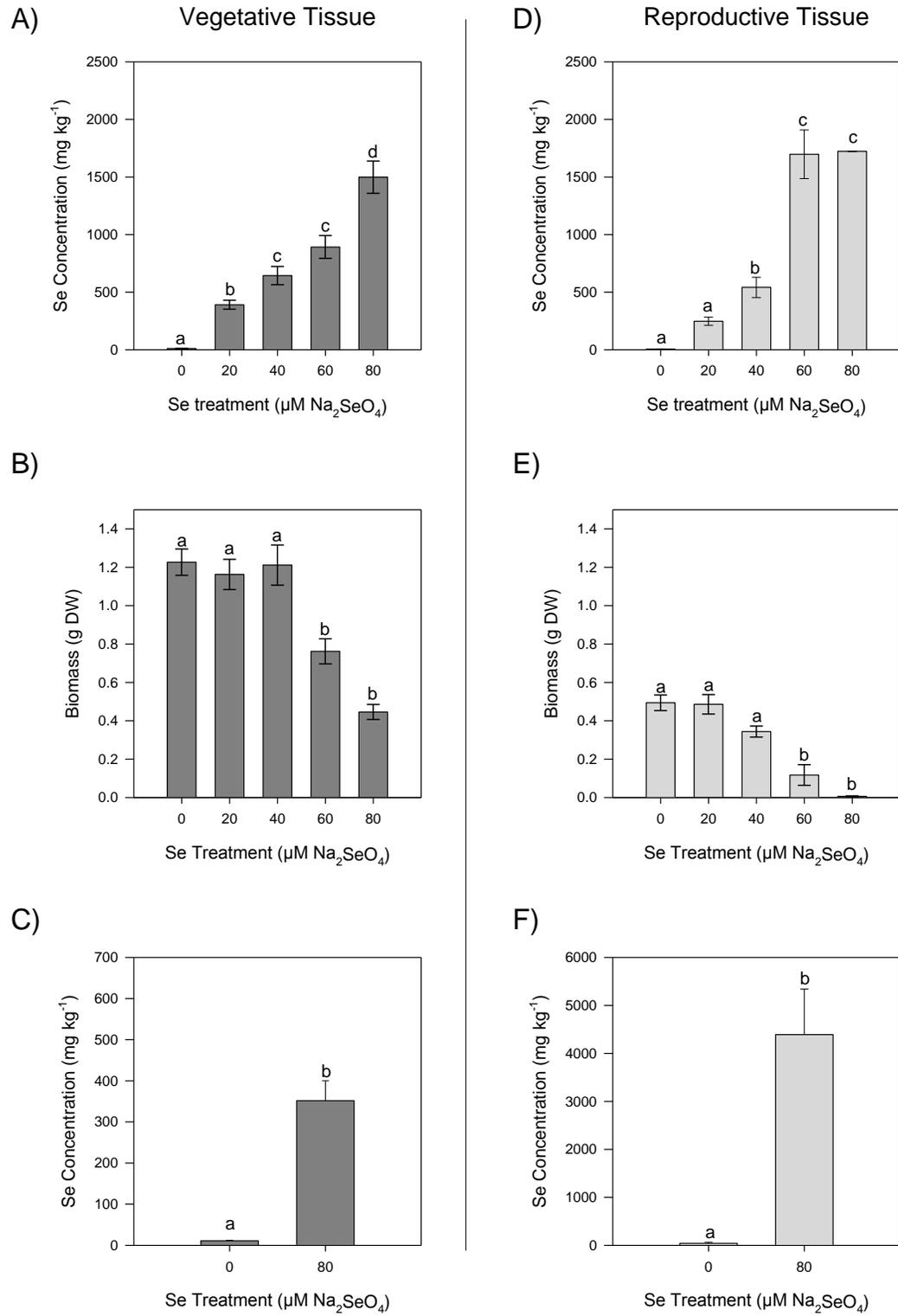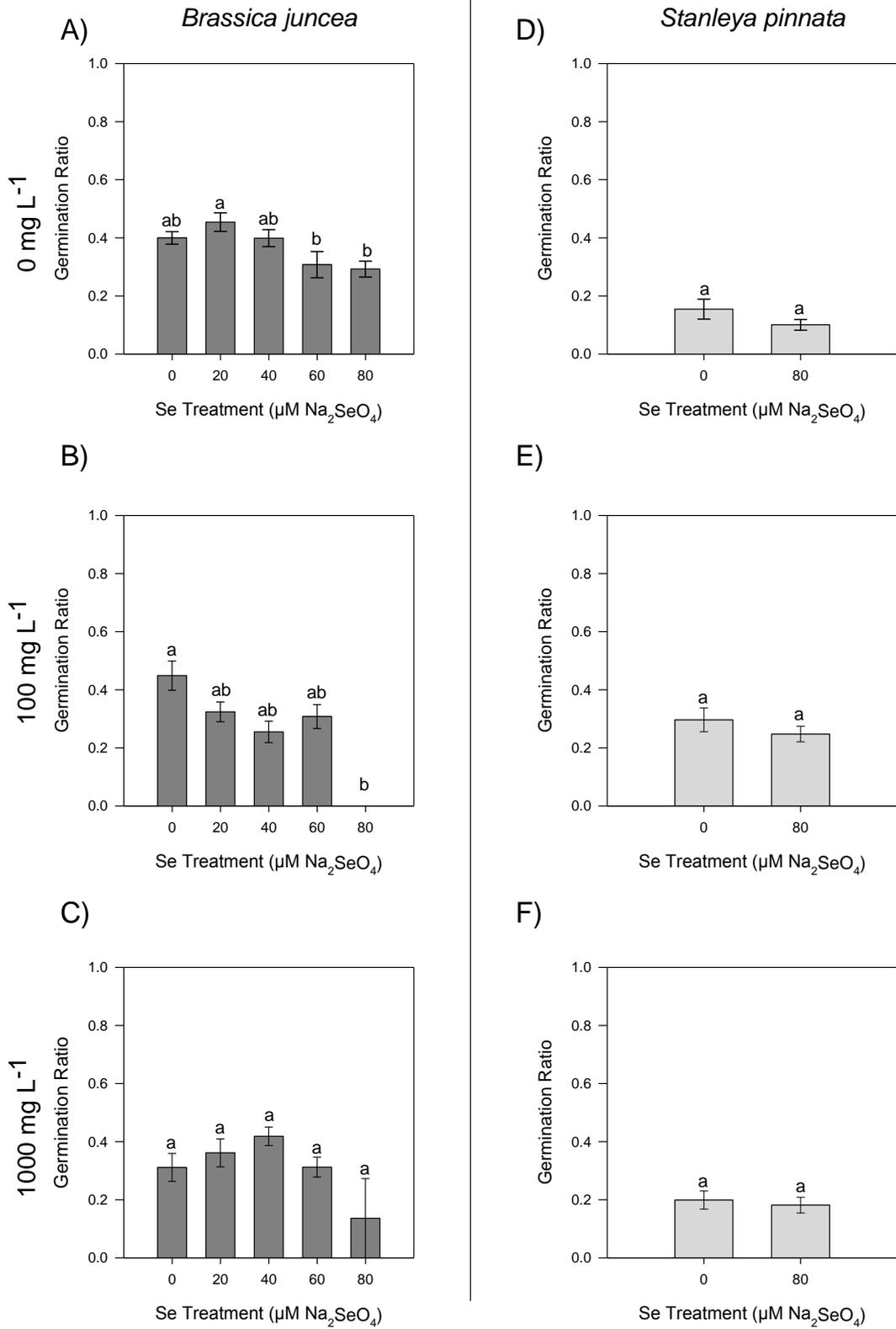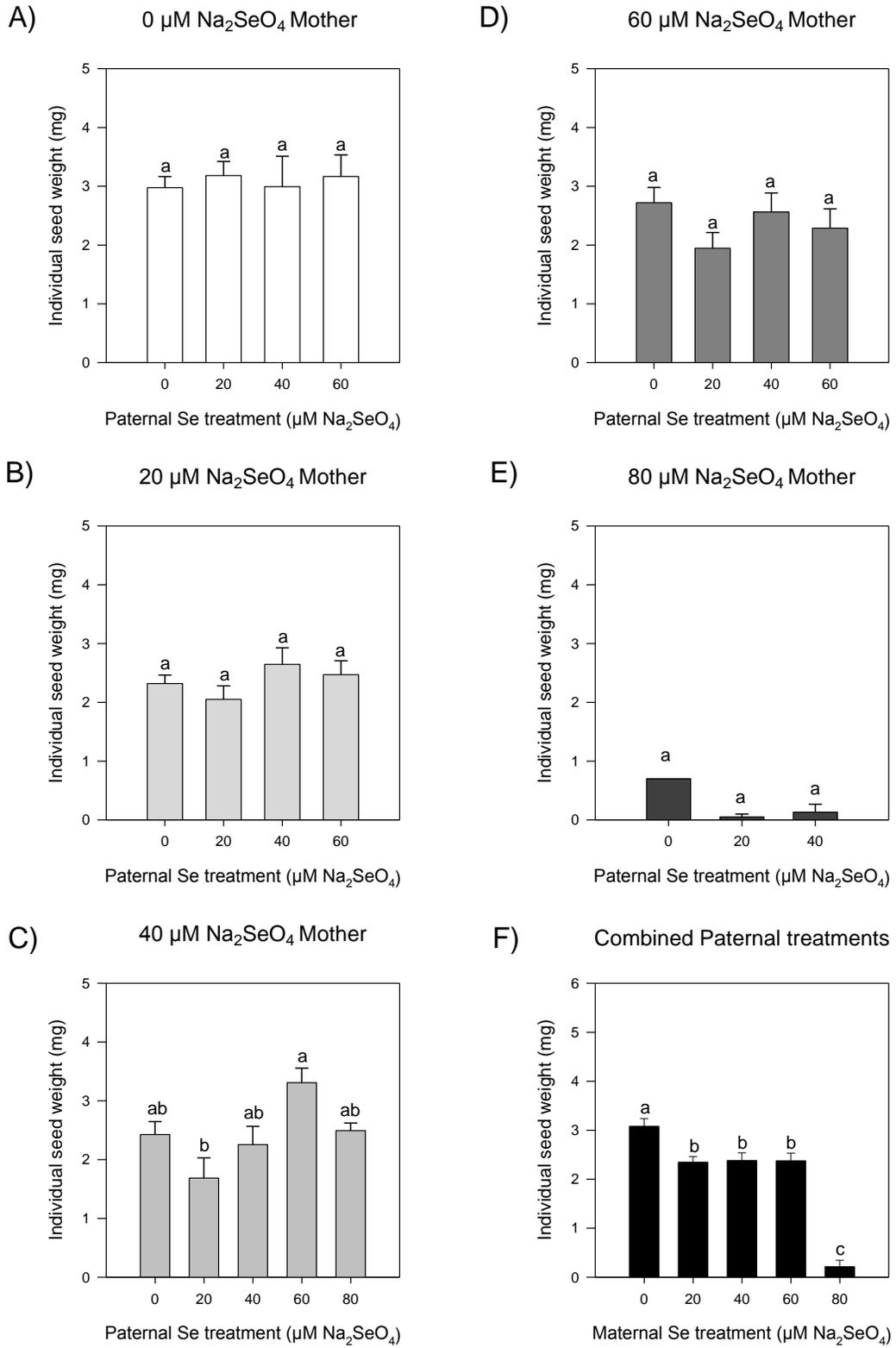*Note: Fig 2.7 is also in Quinn *et al*, unpublished data
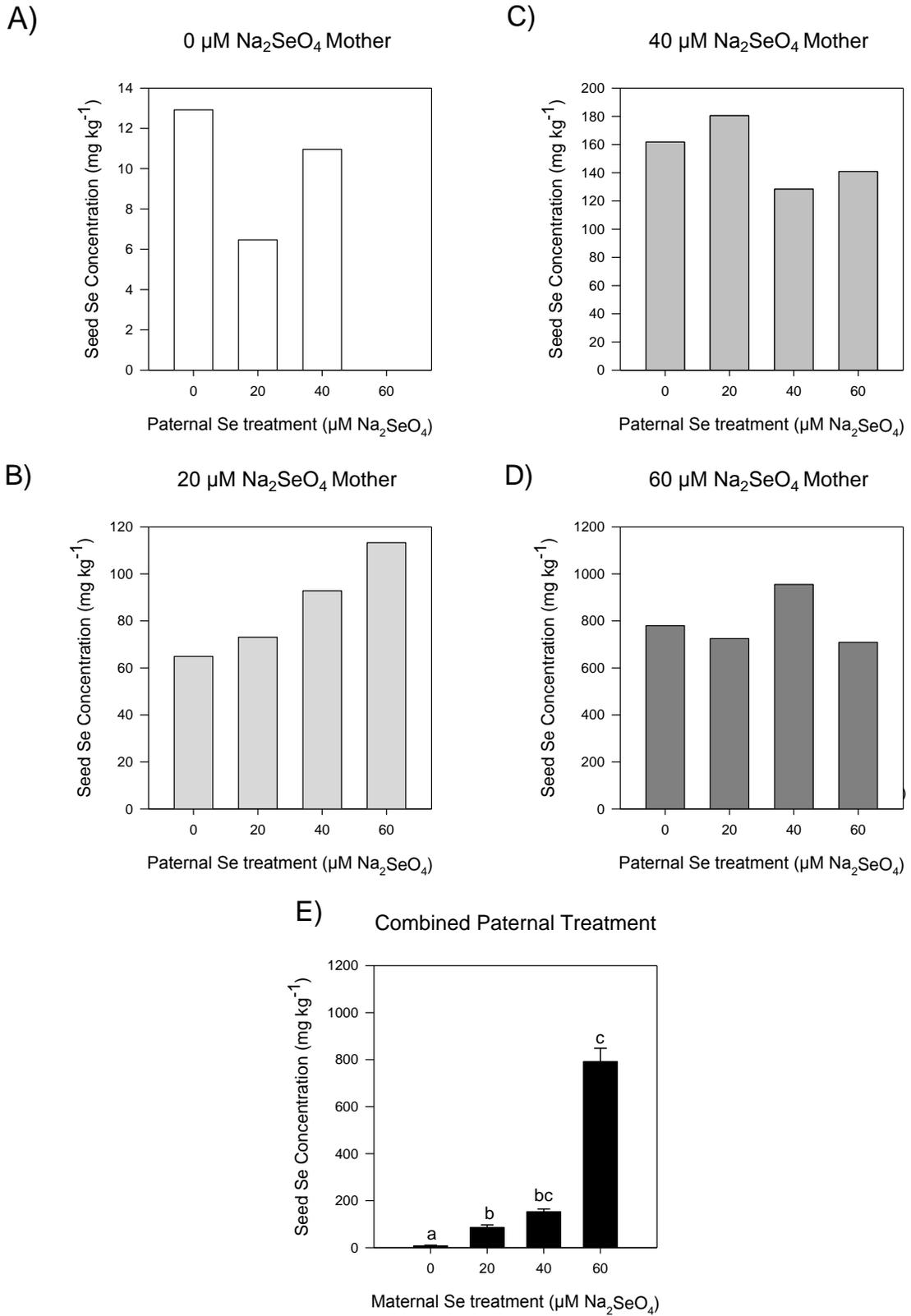
Fig. 2.1

Fig. 2.2

Fig. 2.3

Fig. 2.4

Fig. 2.5

A) 0 µM Na$_2$SeO$_4$ Mother

$\chi^2 = 12.75$
p = 0.0052

D) 60 µM Na$_2$SeO$_4$ Mother

$\chi^2 = 15.63$
p = 0.0013

B) 20 µM Na$_2$SeO$_4$ Mother

$\chi^2 = 4.22$
p = 0.2380

E) 80 µM Na$_2$SeO$_4$ Mother

0 µM Na$_2$SeO$_4$
20 µM Na$_2$SeO$_4$
40 µM Na$_2$SeO$_4$
60 µM Na$_2$SeO$_4$
80 µM Na$_2$SeO$_4$

C) 40 µM Na$_2$SeO$_4$ Mother

$\chi^2 = 2.26$
p = 0.5193

F) Combined Paternal Treatments

$\chi^2 = 40.28$
p < 0.0001

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Fig. 2.6

A)

Weight of Total Seed Production (mg) vs Se Treatment (μM Na$_2$SeO$_4$)

B)

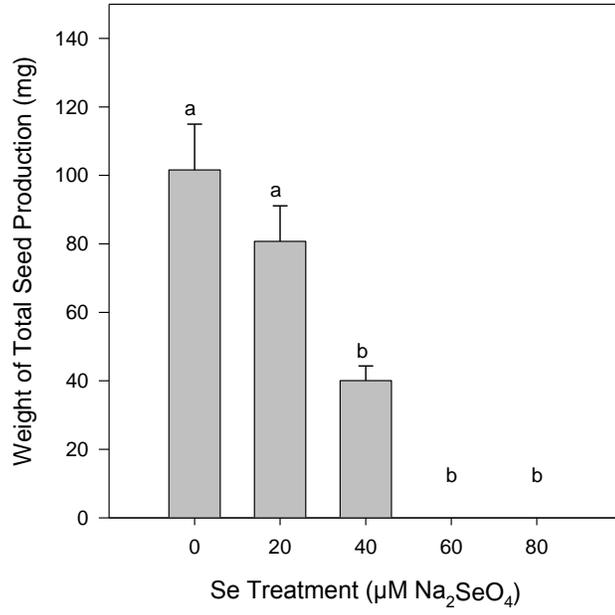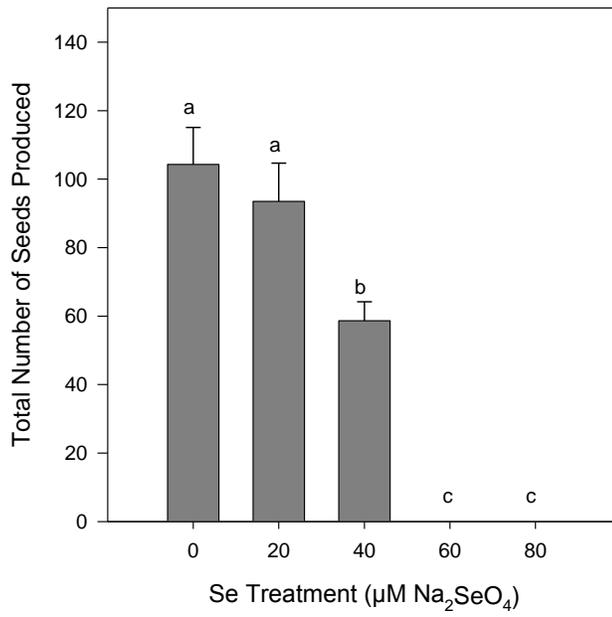Total Number of Seeds Produced vs Se Treatment (μM Na$_2$SeO$_4$)
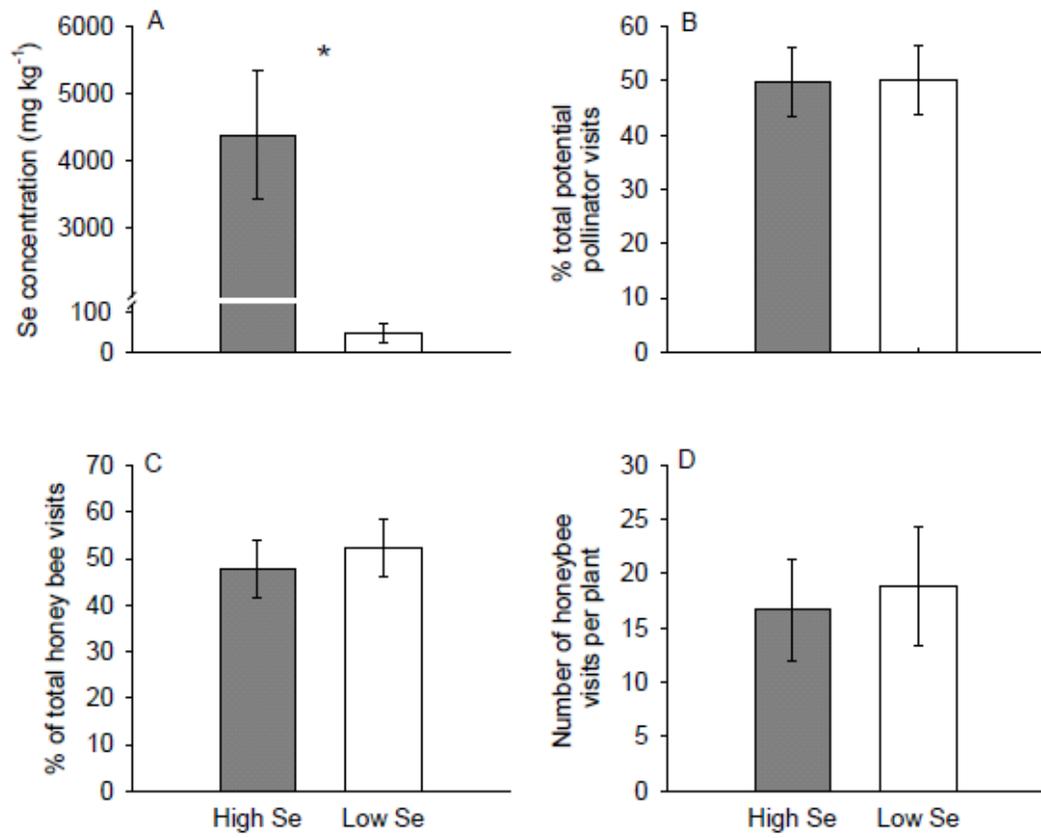
Fig 2.7

Table 1: Selenium concentrations from honey collected from Colorado. Concentrations (mg Se kg^{-1} FW) were calculated from ICP-AES. Data represents the average of subsamples of honey collected ± SE (n=3).	
Sample Location	Se
Lyons alfalfa honey	1.09 ± 0.122
Lyons wildflower honey	0.94 ± 0.20
Lyons clover honey	1.28 ± 0.21
Thornton honey	1.00 ± 0.07
Thornton hive bits	5.15 ± 0.36
Thornton comb bits	4.74 ± 1.01
Fort Collins 1 honey	0.90 ± 0.30
Fort Collins 2 honey	0.75 ± 0.16
Fort Collins 2 comb bits	0.82 ± 0.54
Fort Collins 3 honey	0.41 ± 0.22
Laporte honey	0.78 ± 0.21
Significance tests were not run on subsamples	

Table 2: Elemental concentrations of honey collected from Colorado and Europe

Se concentration (mg Se kg^{-1}FW) was calculated from ICP-AES. Data represent the average concentration of total Se ± SE. Honey from each site was pooled (Colorado n=9, Europe n=3)

Element	Colorado	Europe
Al	21.16 ± 15.52	12.75 ± 8.5
As	0.04 ± 0.05	0.07 ± 0.07
Cu	3.02 ± 0.51	3.82 ± 1.19
Fe	5.32 ± 0.96	7.04 ± 0.78
Li	0.68 ± 0.11	0.89 ± 0.17
Mg	19.21 ± 2.59	25.12 ± 3.26
Na	103.25 ± 29.90	86.31± 23.58
P	45.70 ± 8.42	49.59 ± 7.11
Pb	0.61 ± 0.11	0.71 ± 0.16
S	34.70 ± 7.07	22.7 ± 3.81
Se	0.94 ± 0.15	0.98 ± 0.24
Zn	1.49 ± 0.33	2.6 ± 0.8

There are no significant differences in element concentrations between Colorado and Europe

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CHAPTER 3

INTERACTIONS BETWEEN ROOT NEMATODES AND SELENIUM
HYPERACCUMULATORS

INTRODUCTION

Selenium (Se) is a trace element naturally occurring in seleniferous soils, such as shale

from the Cretaceous and early Tertiary periods (White *et al*. 2004). Many areas in the

Western United States have elevated levels of Se in soils, sometimes in excess of 10 mg

Se kg^{-1}, while places like the Northeastern United States have little or no Se in soils

(Reeves and Baker 2000). Se is necessary for animal and human health, but even

moderate quantities can lead to symptoms such as weakened immune systems and heart

disease (Rayman 2000, Brown and Arthur 2001, Goldhaber 2003).

Selenium is toxic to most plant species at tissue concentrations above 100 mg Se

kg^{-1}, and for crop plants the toxicity threshold can be as low as 10 mg Se kg^{-1} DW (Burau

1982, Terry *et al.* 2000). However, there are some hyperaccumulator plant species that

can accumulate Se to concentrations as high as 10,000 mg Se kg^{-1} DW, or 1% of the

plant's dry weight (Quinn *et al.* 2008). Shoot Se concentrations of hyperaccumulators in

the field are commonly found to be between 1,000-5,000 mg Se kg^{-1} DW in the shoot

(Galeas *et al* 2007).

All plants can assimilate Se through S transporters and the S assimilation pathway

(Terry *et al* 2000). Hyperaccumulators are different from other plants in that they take

up Se preferentially over sulfur (S) and translocate relatively more Se to their shoot. In non-hyperaccumulators Se is toxic because it becomes incorporated into the amino acids selenomethionine (SeMet) and selenocysteine (SeCys). These Se-containing amino acids then get non-specifically incorporated into proteins and cause improper folding and function (Brown and Shrift 1981). The predominant form of Se in hyperaccumulators is methyl-selenocysteine (MeSeCys), which is not incorporated into proteins and therefore relatively non-toxic. Freeman *et al* (2006a) investigated the distribution and speciation of Se in hyperaccumulators and found Se focused in high concentrations in the leaf margins or leaf hairs. De Souza and coworkers (1998) saw a different pattern of Se distribution and speciation in the Se accumulator *Brassica juncea*: when plants were fed inorganic selenate (the most common form of Se in soils (Galeas *et al.* 2007), selenate was the predominant form of Se accumulated. Thus, conversion of Se to MeSeCys and specific sequestration in leaf margins may both contribute to Se hyperaccumulation and hypertolerance.

Selenium hyperaccumulator species are perennials, and the Se distribution *in planta* fluctuates with the season. Galeas and colleagues (2007) found that Se is translocated from the roots to the young leaves in the spring and early summer, from old to young leaves and reproductive structures in the late summer, and back to the roots in the fall. The seasonal fluctuation patterns in leaf Se concentration differed between hyperaccumulators and non-accumulator species living in the same habitat. Hyperaccumulator Se levels peaked in spring while those of non-accumulators peaked in summer; leaf S levels peaked in summer for both species. So far no information is

available about Se speciation and distribution in the root of hyperaccumulators and non-accumulators.

One proposed hypothesis for the functional significance of hyperaccumulation is basic elemental defense (Boyd, 2007). Selenium is toxic to most animals at concentrations well below those found in hyperaccumulators. Indeed, plants with elevated shoot Se concentrations were toxic to, and deterred, a wide variety of above-ground herbivores including grasshoppers, crickets, and aphids (Hanson *et al.* 2003, 2004; Freeman *et al.* 2007, 2009; Quinn et al., 2008, 2010). The specialized high-Se pockets found by Freeman *et al.* (2006a) along hyperaccumulator leaf margins are most likely the first tissues encountered by herbivores and may function to protect the plant from these herbivores. Some herbivores such as a Colorado population of the diamondback moth larvae, however, have evolved tolerance to the high levels of Se in hyperaccumulators and are no longer deterred by Se (Freeman *et al* 2006b).

There has been considerable study of the above-ground interactions of hyperaccumulators and their Se-tolerant and -susceptible herbivores. The numerous below-ground interactions of hyperaccumulators remain largely unexplored. Most of the hyperaccumulator rhizosphere analyses to date focus on root exudates and on tolerance of the fungi and bacteria living in the root zone (Boyd and Martens 1998; de Souza *et al* 1999; Alford *et al* 2010). One facet of hyperaccumulator-herbivore interactions that has not been explored for any hyperaccumulator is the interaction with plant-parasitic nematodes. Nematodes are associated with the roots of plants in several different ways. Some nematodes use a stylet to pierce the epidermis of the root and draw out nutrients and sugars. Other nematodes actually bore through the epidermis and live inside the root

cortex (Bird and Bird 2001). Plant-parasitic root nematodes are a common pest in subsistence and organic farming, especially in the tropics, and can cause damaging knots and root rot (Bridge 1988). Common Se concentrations in hyperaccumulator roots are between 100 and 1,500 mg Se kg^{-1} DW, depending on the season, which are expected to be toxic to most root herbivores (Galeas *et al* 2007). Therefore, increasing root Se concentrations are expected to lead to decreasing numbers of nematodes. However, when examining the shoot herbivores, there were some organisms with developed resistance to high Se levels, so it is possible some nematodes have evolved tolerance to Se and can successfully forage on hyperaccumulator roots.

In this study we describe Se speciation and distribution in the roots of hyperaccumulators and survey field hyperaccumulator roots in fall and spring, for Se as well as numbers of root-associated nematodes. The study has four objectives: 1) Gain insight into Se distribution and speciation in hyperaccumulator roots. 2) Analyze the seasonal flow of Se between roots and shoots of plants growing in their natural seleniferous habitat. 3) Investigate the effect of root Se concentration on number of nematodes associated with those roots in the field. 4) Investigate the success of re-inoculation of collected nematodes on greenhouse-grown high- and low-Se hyperaccumulators plants. This study aims to provide insight into the role of Se hyperaccumulation in protecting plants from root herbivory.

MATERIALS AND METHODS

Field Nematode Collections

Pine Ridge Natural Area in Fort Collins, Colorado is a naturally seleniferous site that is home to two Se hyperaccumulator species: *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae). Entire plants including taproots were collected from this field location, 5 individuals per species in the fall of 2009 and 5 individuals per species in the spring of 2010. Plants harvested were small, mature plants more than one year of age in order to harvest a large, intact large taproot. Because of the limited number of plants from each species growing on the field site and the slow reestablishment rate of both species, 5 samples was the maximum number of plants that could be extracted from the field without hindering reproductive success. Plants were harvested from varying locations within the study site to obtain a Se gradient, and were not collected closer than 10 m from each other.

Root sections, leaf, and reproductive plant tissues were dried and digested for Se analysis via ICP-AES (Fassel 1978, Zarcinas *et al.* 1987). The taproots and large lateral roots were used in nematode extractions, as described below, and after nematode extractions the root was dried and the dry weight obtained to calculate the number of nematodes per g DW.

Nematode Inoculation Studies

A 1/3 fraction of nematodes collected from hyperaccumulators harvested from the field were used to inoculate *S. pinnata* plants grown in the greenhouse. The plants had been grown in a greenhouse for 3 years (24/20°C day/night,16-h photoperiod, 300 µmol m^{-2}

sec[-1] photosynthetic photon flux) on Pro mix BX (Premier Horticulture, Quakertown, PA) and Turface (MVP, Premier Products LLC, Buffalo Grove, IL). The plants were watered three times a week; once a week fertilizer was supplied at 1g per liter of water Miracle-Gro Excel (15:5:15 Cal-Mag, The Scotts Co., Marysville, OH). Furthermore, half of the plants received 20 μM Na_2SeO_4 twice a week, while the other half received just water.

Each Se treatment was further divided into nematode inoculation treatments: no nematodes, nematodes extracted from *S. pinnata* from the field, and nematodes extracted from *A. bisulcatus* from the field. Extractions containing approximately 20 nematodes were kept in 0.5 ml of dH_2O and delivered to the plant directly on the soil next to the root. There were 3 plants per inoculation in each Se treatment, for a total of 18 plants. 24 weeks after nematode inoculation, root and leaf material from each plant was harvested and used for nematode extraction and Se analysis. Nematodes were extracted from fresh roots as described below. For Se analysis root and shoot material was dried, acid-digested and elemental concentrations determined via ICP-AES as described by Pilon-Smits *et al.* (1999).

Nematode Extraction and Quantification

All nematode extractions were carried out using the taproot and large lateral roots, using the Baermann Funnel extraction procedure (Hooper 1990). In this method live nematodes are given time to crawl out of the root into water where they are collected in water by three consecutive 15 ml fractions were collected in 24 hour increments, for a total extract of 45 ml. The nematodes were allowed to settle at the bottom of the vial at room temperature for another 24 hours, after which the volume was reduced by removing the top 40 ml, to a final volume of 5 ml. To this extract, 5 ml of 90% formaldehyde at

90°C was added to preserve the nematodes (Coleman *et al.* 1999) with the exception of nematodes used for inoculations.

From the total extract, the bottom 2 ml was removed and examined under an inverted compound light microscope. The total number of nematodes in the extract was calculated and divided by root dry weight to obtain the total number of nematodes/g dry weight.

Selenium Distribution and Speciation

Se distribution in hyperaccumulator roots was determined by x-ray absorption spectroscopy (XAS), using a combination of micro-focused X-ray fluorescence (μXRF) mapping and γ X-ray Analysis of Near-Edge Spectra (γXANES).

Field-collected taproots n of *A. bisulcatus* and *S. pinnata* were sliced into ~0.5 mm cross-sections with a frozen razor blade over dry ice. The sections were kept frozen and shipped on dry ice to beamline 10.3.2 at the Advanced Light Source at the Lawrence Berkeley National Lab in Berkeley, CA. The frozen cross-sections were mounted onto a Peltier stage kept at -33°C to reduce potential beam radiation damage. μXRF elemental maps imaged Se distribution, and following the mapping, 3 spots in the cortex, wood, and epidermis layer were further examined for Se speciation by XANES. The XANES spectra from the root tissues were compared to a range of standard Se compounds to determine the identity and relative abundance of selenocompounds in the tissues (Pickering *et al.* 1999).

Spectra were dead-time corrected, pre-edge background subtracted, and post-edge normalized (Kelly *et al.* 2008). A Red selenium standard was used to calibrate the

spectra. Least square linear combination (LSQ) fitting of Se XANES spectra was performed in the 12630-12850 eV range, using a library of standard selenocompounds. The error on the percentages of species present is estimated to be ± 10%. All data processing and analyses were performed with custom LabVIEW (National Instruments) programs provided at the beamline.

RESULTS

Se Concentration, Speciation, and Distribution

There was substantial variation in root Se concentration in hyperaccumulators *S. pinnata* and *A. bisulcatus* growing in their natural seleniferous habitat. In both species independent of season, Se concentrations were higher in the cortex (peripheral part of the root) than in the central wood tissue (P < 0.0001, Fig 3.1a-d). The magnitude of this difference of Se concentration increases in the spring: On average, *S. pinnata* root cortex had a 3.7 times higher Se concentration than the corresponding wood in the spring compared to 2.2 times higher Se level than wood in the fall. Similarly, *A. bisulcatus* root cortex had a 7.6 times higher Se level than the wood in the spring compared to 1.5 times higher Se concentration than the wood in the fall.   For *A. bisulcatus* the Se concentration in the root was higher compared to that in the leaves in the fall (P = 0.0377, Fig 1a); the reverse was seen in the spring when the Se concentration was higher in the leaves compared to the root (P = 0.0004, Fig. 3.1c, d). The Se concentrations of *S. pinnata* leaves were higher than root Se concentrations in the spring (P < 0.0001 Fig. 3.1d).   In the fall, there was not sufficient *S. pinnata* leaf material to collect for ICP; therefore there

is not enough information to compare allocation in spring and fall for *S. pinnata* (Fig. 3.1b).

In *S. pinnata* taproot cross-sections, the Se signal was more concentrated in the outside section of the root compared to the middle (Fig. 3.2a,b). The Se in both cortex and wood was mostly organic Se (C-Se-C, indistinguishable from the MeSeCys standard), with a small percentage as inorganic selenite (SeO_3^{2-}) (~5%, Fig. 3.2c,d). In soil/epidermis XANES spots, there was also predominately organic Se, but a relatively larger percentage of selenite compared to the root cortex or wood (~30%, Fig. 3.2e). The same pattern is seen in *A. bisulcatus* root cross-sections, where the Se signal was highest in the cortex, and low in the wood (Fig. 3.3a,b). The speciation of the Se was also similar to *S. pinnata* with more selenite found in the soil/epidermis (30%) compared to cortex or wood (15%). In *A. bisulcatus*, however, there was relatively more selenite in cortex and wood compared to *S. pinnata* (Fig.3.3c-e).

Field Nematode Counts

All of the hyperaccumulator taproots from the field yielded nematodes in both fall and spring, even those with the highest Se concentrations (up to 1,300 mg Se kg^{-1} DW, Fig 3.4a-c). The number of plants available for this study does not allow for rigorous statistical analysis, but based on the data available there is no apparent correlation between nematode root colonization and root Se concentration in either the fall or the spring. Root nematode abundance was overall higher in spring than in fall (Figure 3.4a,b). When both seasons and plants were pooled, there was a downward trend in the number of nematodes with increasing Se concentrations, but not supported by a

significant negative correlation (Fig 3.4c). More sampling is needed to confirm such a trend; in any case, there were still nematodes found even at high Se concentrations (Fig 3.4).

Greenhouse Nematode Inoculations

The *S. pinnata* greenhouse plants receiving 20 μM Na_2SeO_4 had on average a ~8 fold higher Se concentration in the leaves than plants not treated with Se, and on average a ~10 fold higher Se concentration in the roots (P = 0.0392, P < 0.0001, Fig. 3.5a,b). Within the 80 μM Na_2SeO_4 treatment, both nematode inoculations resulted in significantly lower leaf Se levels (Fig. 3.5a). Nematode inoculation also affected root Se concentration (P = 0.0196, Fig.3.5b). Within the Se treatment, the plants inoculated with nematodes extracted from *S. pinnata* showed significantly reduced root Se concentration compared to plants inoculated with nematodes from *A. bisulcatus*. The plant group with no inoculation was not significantly different from either inoculation treatment (P = 0.0209, Fig. 3.5b).

When the nematodes were counted 24 weeks after inoculation, there were significantly (6.8-fold) more nematodes found in *S. pinnata* not receiving Se treatment compared to *S. pinnata* plants receiving a Se treatment (P = 0.0425, Fig. 3.6). There was no difference in nematode abundance between the inoculation treatments.

DISCUSSION

X-ray absorption spectroscopy studies on the roots of hyperaccumulators from seleniferous soils showed that the Se was predominantly located in the cortex of the root, with smaller amounts in the wood. XANES showed that the primary species of Se was

the non-toxic methylated organic Se, in both the cortex and wood. Using these same

roots, the Se-dependent interaction with root-associated nematodes was examined, which

showed that increasing Se concentrations in the root yielded a somewhat lower, but non-

significant, number of nematodes (fig 3.4). Moreover, in greenhouse-grown

hyperaccumulators, the Se-treated plants showed significantly lower numbers of

nematodes in their roots. These results are novel and significant because the distribution

and speciation of Se in hyperaccumulator roots has never been reported, nor has the

effect of Se on root herbivores been studied. The finding that Se is accumulated to high

levels in the cortex, and that high-Se roots harbor fewer nematodes suggests that Se

protects hyperaccumualtors from these root herbivores. At the same time, finding that

even the highest-Se roots harbored some nematodes suggests the presence of at least

some Se-tolerant nematodes in seleniferous habitats that can successfully forage on

hyperaccumulators. These patterns are similar to those observed in earlier studies above-

ground, which showed that Se accumulation protects plants from generalist herbivores

but can give rise to the evolution of specialist, Se-tolerant herbivores. As such, the results

from both the above- and below-ground studies lend support to the elemental defense

hypothesis which states that hyperaccumulation serves as protection from herbivory, and

shed light on selection pressures that have driven the evolution of hyperaccumulation.

As also observed by Galeas *et al.* (2007), there appears to be a seasonal flow of Se

between hyperaccumulator roots and shoots. The Se levels in root tissues were highest in

the fall, and there was more Se in young leaves in the spring. One possible function for

this reallocation of Se to root tissues in the fall could be to serve as a defense against

potential root herbivores. Also, it may be advantageous for the plant to transfer the

ecologically valuable Se from shoot, leaf, and floral tissues as they senesce at the end of the growing season to the root and the seeds, which persist throughout the winter. Instead of shedding Se in the senescing leaves, hyperaccumulators perhaps reallocate Se to the root, both for added protection through the winter and as a temporary storage of Se to reallocate to new shoot growth the next spring.

According to ICP-AES and XAS data, the Se in the root is allocated primarily to the cortex. The speciation of Se in the cortex is similar to that in the wood. XANES data showed primarily C-Se-C, likely MeSeCys, with a small amount of selenite. In leaves of hyperaccumulators MeSeCys was also the primary Se species, but selenate made up the remainder of the Se (Freeman *et al.* 2006). Selenium is thought to be taken up as selenate by the roots and translocated to the shoot to be metabolized to MeSeCys, which is the primary form found in hyperaccumulators (Terry *et al.* 2000). The finding that the root also accumulates mainly MeSeCys may either suggest that the root is capable of selenate assimilation itself, or that the plant metabolizes Se in the shoot, and then reallocates MeSeCys back to the roots.

The observed Se distribution in hyperaccumulator roots has interesting implications for root-associated nematodes and other root herbivores in seleniferous areas. Hyperaccumulators preferentially allocate Se to the outer section of the root, which is where herbivores first interact with root tissues. This increased concentration of Se could deter the herbivore from the root tissue and/or have a toxic effect on the herbivore, and *via* these mechanisms, protect the vascular tissue inside the wood without needing to accumulate Se in wood tissues. In leaves of hyperaccumulators, Freeman and colleagues (2006) also observed preferential allocation of Se to the margins and

specialized epidermal structures. They hypothesized this distribution would protect the plant from leaf herbivores because the margin is the first part of the leaf the herbivores encounter.

In this study showing varying root Se concentrations in hyperaccumulators harvested from the seleniferous site, there was a slight, though non-significant, decrease in the number of nematodes with increasing root Se concentration. While nematode abundance decreased with root Se concentration, it is important to note that there still were nematodes found associated with the roots of hyperaccumulators with high levels of Se, concentrations that would normally be toxic to most organisms (Freeman *et* al 2007). This suggests that some nematodes living at or around these concentrations may have evolved Se tolerance and may be Se specialists, found only in environments with high Se concentrations. In one study by Ellis and colleagues (2002), they reported a decrease in nematode populations on a site heavily contaminated with metals compared to a site with low levels of metals, so nematodes appear to be generally sensitive to other metals as well. However, some nematodes are reported to have elevated tolerance to other metals such as cadmium and arsenic (Broeks *et al.* 1996). In one study, heavy copper (Cu) pollution selected for higher Cu tolerance in native populations of nematodes compared to nematodes on non-polluted sites (Millward and Grant 2000). Perhaps nematodes living in seleniferous soils have evolved a tolerance to Se compared to a non-seleniferous. Lower levels of root nematodes were observed when greenhouse-grown plants were inoculated with nematodes and treated with Se as compared to controls not treated with Se, which suggests that Se concentration had a significant negative effect on the number of nematodes extracted from the plant roots. Regardless of the source of

nematodes used in the inoculation, Se-treated plants harbored fewer root nematodes compared to plants not receiving Se. Even no-Se plants not receiving nematodes had higher numbers of nematodes compared to high-Se plants receiving nematodes. This suggests that Se fertilizers have the potential to knock down existing levels of nematodes and reduce the colonization by new nematodes. Nematodes are one of the many pests faced by farmers, and particularly in subsistence and sustainable agriculture systems, pesticides may not be an option (Bridge 1996). Instead of treating crops with organic pesticides, perhaps Se fertilization could be used to fortify roots with Se and reduce nematode levels. The levels of Se used in this greenhouse experiment (20 μM Na_2SeO_4) may be undesirable for most crop situations, therefore follow-up experiments should be done to observe which Se concentration that is acceptable for agriculture is still effective in nematode protection. The work presented in this study also provides a foundation for further evolutionary and ecologically important investigations into the rhizosphere interactions of Se hyperaccumulators.

FIGURE LEGENDS

Fig. 3.1: Selenium concentrations from field plants, in fall and spring. A) Se concentrations (mg Se kg^{-1}DW) of entire root sections, cortex and wood sections, and shoots from *A. bisulcatus* collected from the field in the fall (A, n=5) and spring (C, n=5). B) Se concentrations (mgSe kg^{-1}DW) of entire root sections, cortex and wood sections, and shoots from *S. pinnata* collected from the fall (B, n=5) and spring (D, n=5). Each plant part was dissected into 3 parts and each part was analyzed for Se concentration. Values are those means +/- SE.

Fig. 3.2: XAS analysis of Se distribution and speciation of *S. pinnata*. A) XRF map of *S. pinnata* root cross-section (n=1). Selenium is shown in red and Ca in green. B) XRF map with Se in white showing XANES spots. C-E) Speciation percentages of Se at select spots from the Cortex (C), Wood (D), and Soil/epidermis layer (E). Pie chart percentages are the averages of 3 XANES spots located on the cross-section.

Fig. 3.3 XAS analysis of Se distribution and speciation of *A. bisulcatus*. A) XRF map of root cross-section (n=1). Selenium is shown in red, Ca in green, and Fe in blue. B) XRF map with Se in white showing XANES spots. C-E) Speciation percentages of Se at select spots from the Cortex (C), Wood (D), and soil/epidermis layer (E). Pie chart percentages are the averages of 3 XANES spots located on the cross-section.

Fig. 3.4: Nematode counts/g DW from field plants, fall and spring. A) Number of nematodes (per g DW) extracted from *A. bisulcatus* and *S. pinnata* taproots harvested

from the field in the fall (n=10). B) Number of nematodes (per g DW) extracted from *A. bisulcatus* and S. *pinnata* taproots harvested from the field in the spring (n=10). C) All nematode counts pooled for both *A. bisulactus* and *S. pinnata* over both seasons (n = 20).

Fig. 3.5: Selenium concentrations in greenhouse plants used for nematode inoculations. A) Leaf Se concentrations (mg Se kg^{-1}DW) from *S. pinnata* treated with 0 or 80 µM Na_2SeO_4 and inoculated with water, nematodes extracted from *S. pinnata,* or nematodes extracted from *A. bisulcatus* (n=3). B) Root Se concentrations (mg Se kg^{-1}DW) from *S. pinnata* according to Se treatment and nematode inoculation (n=3). Values are means +/- SE, and a different letter above the bars represents a significant difference ($\alpha = 0.05$).

Fig 3.6: Nematode counts/g DW from *S. pinnata* plants grown in the greenhouse with various nematode inoculations. A) The inoculation treatments are with no nematodes, *S. pinnata* nematodes, and *A. bisulcatus* nematodes, as denoted by different patterns. The *S. pinnata* plants (n=3) received 0 and 80 µM Na_2SeO_4. The number of nematodes counted/ g dry weight are counted from a 5ml extract. B) Number of nematodes counted/g dry weight in plants not receiving Se treatments compared to plants receiving Se treatments. Values are means +/- SE, and a different letter above the bars represents a significant difference ($\alpha = 0.05$).

Fig 3.7: Photos of plant parasitic nematodes extracted from hyperaccumulator roots. A) Plant parasitic nematode from *S. pinnata*, extracted in the fall. B) Plant parasitic nematode from *A. bisulcatus*, extracted in the fall.
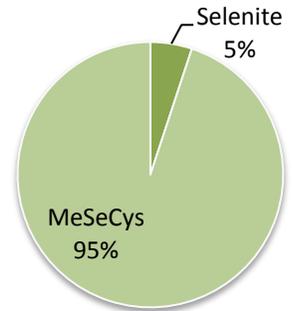
FIGURES

Fig. 3.1
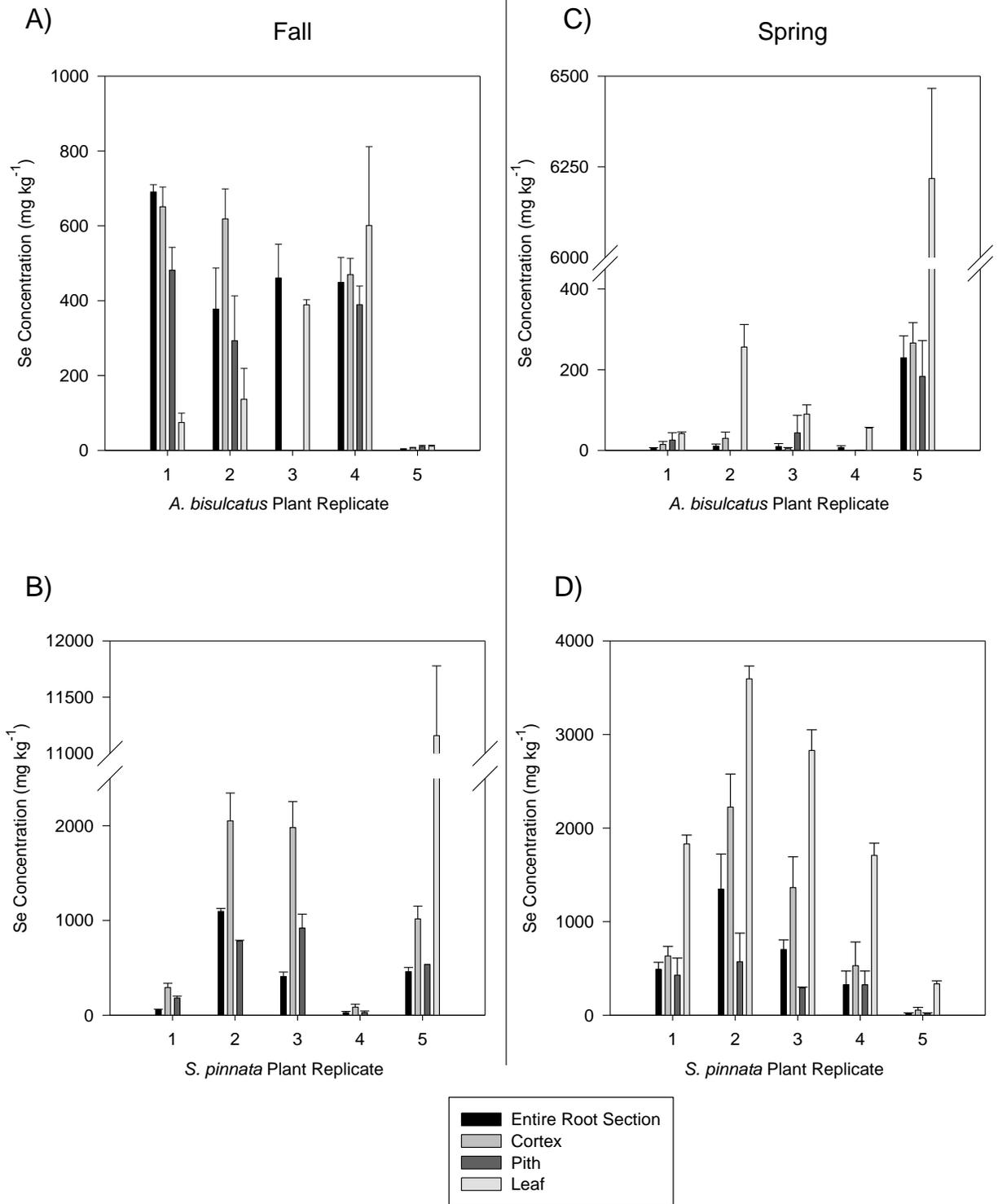
Fig. 3.2 **X-ray absorption spectroscopy (XAS) analysis of Se distribution and speciation in *S. pinnata*.**
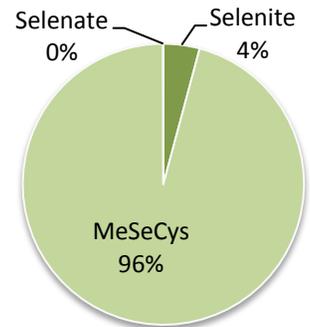
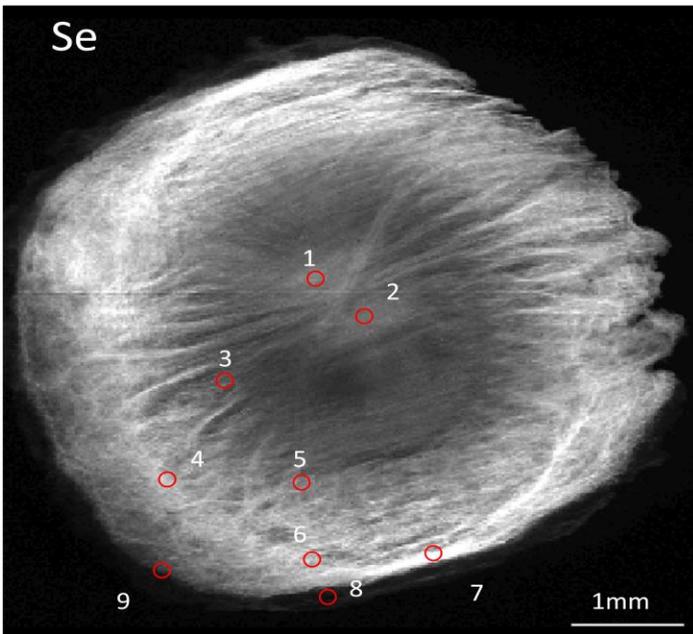**A)** XRF map (Se in red, Ca in green)
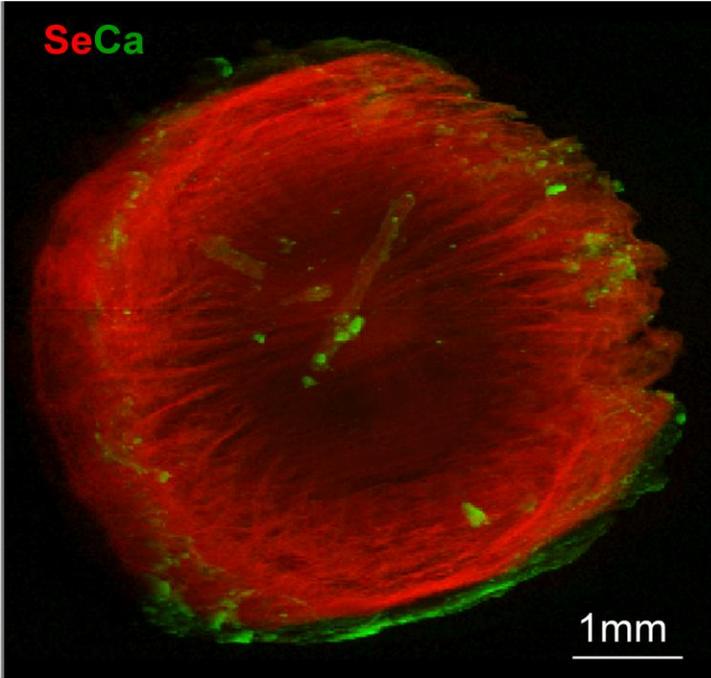
C) XANES Se speciation – Cortex (panel B, locations 4-6)

D) XANES Se speciation – Wood (panel B, locations 1-3)

B) XRF map (Se in white; XANES spots indicated)

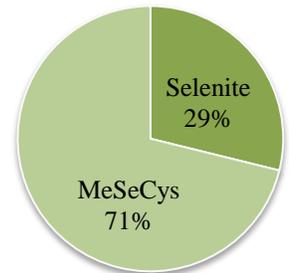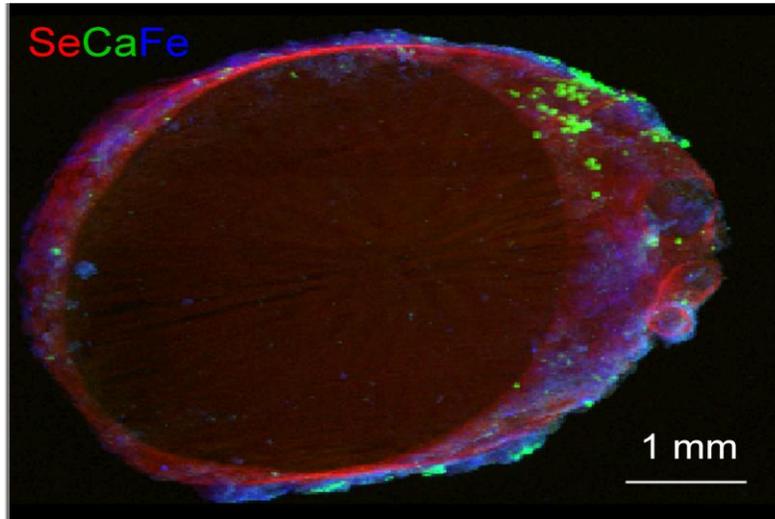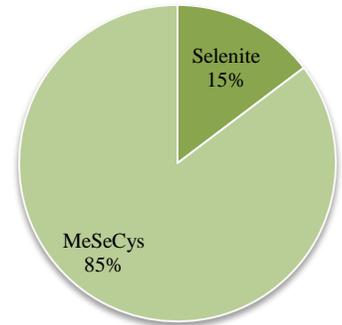**E)** XANES Se speciation - Soil/Epidermis (panel B, locations 7-9)

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Fig. 3.3 **X-ray absorption spectroscopy (XAS) analysis of Se distribution and speciation in *A. bisulcatus***

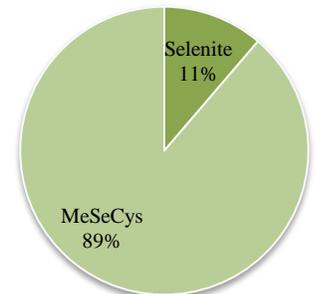**A)** XRF Map (Se in red, Ca in green, Fe in blue)

C) XANES Se speciation – Cortex (panel B, locations 3,6, and 8)

B) XRF map (Se in white; XANES spots indicated)
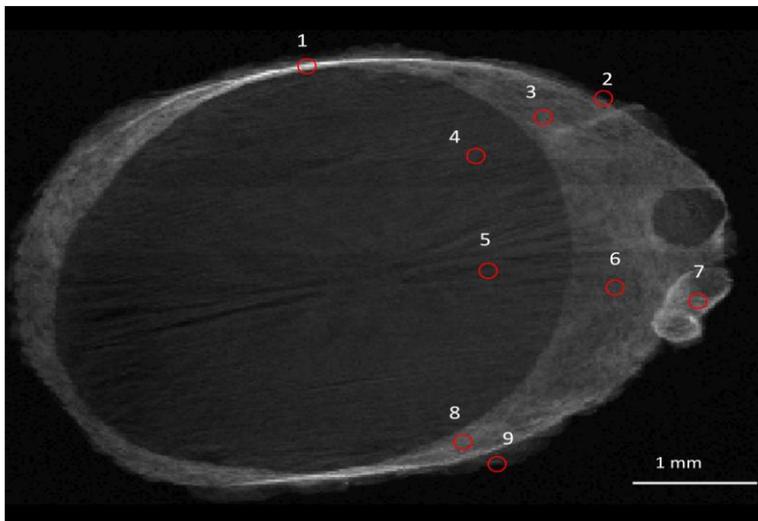
D) XANES Se speciation – Pith (panel B, locations 4,5)

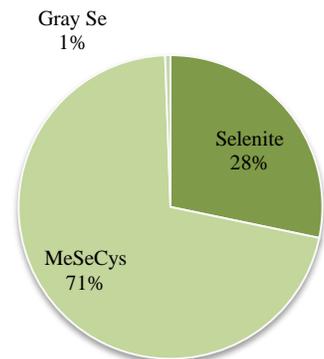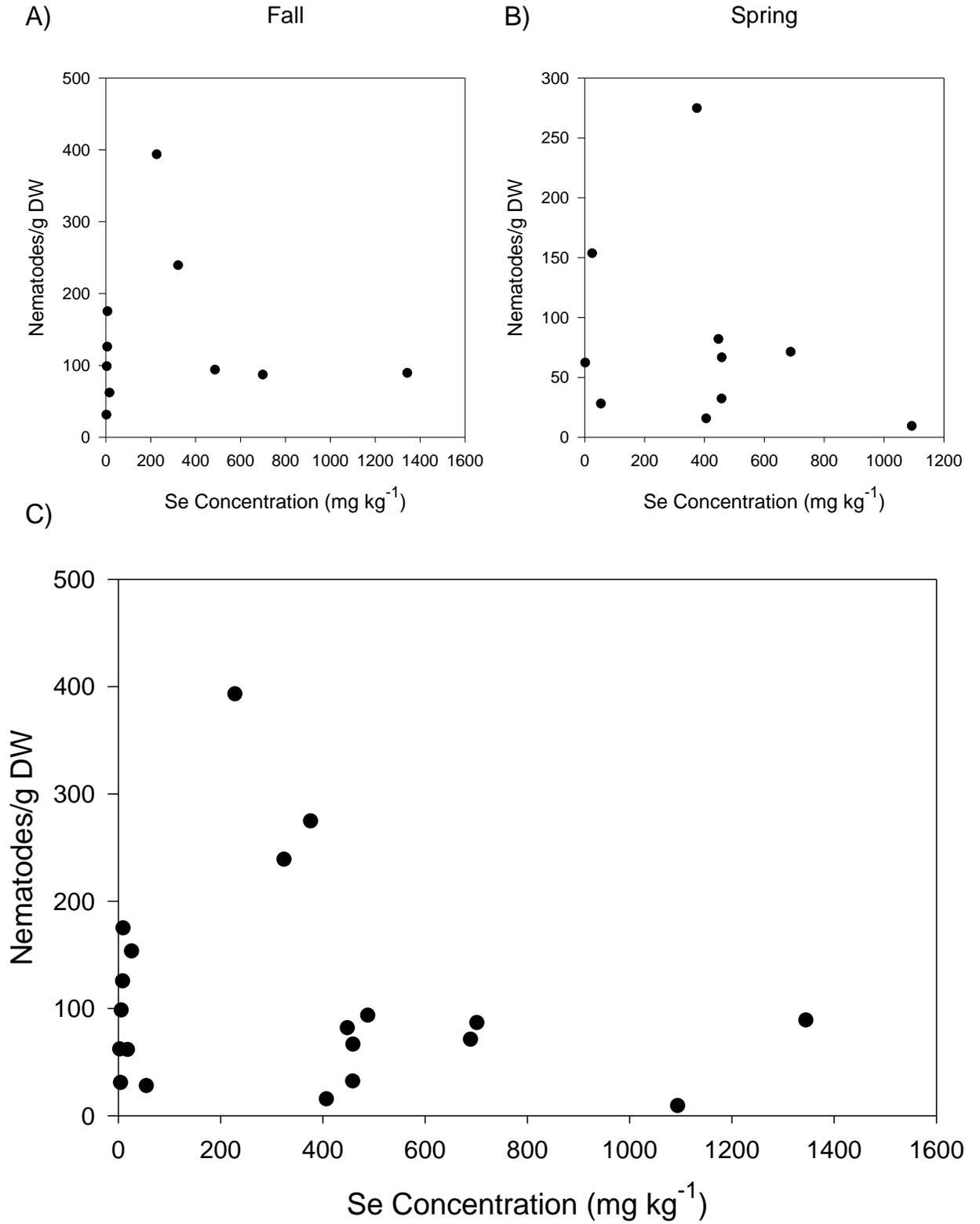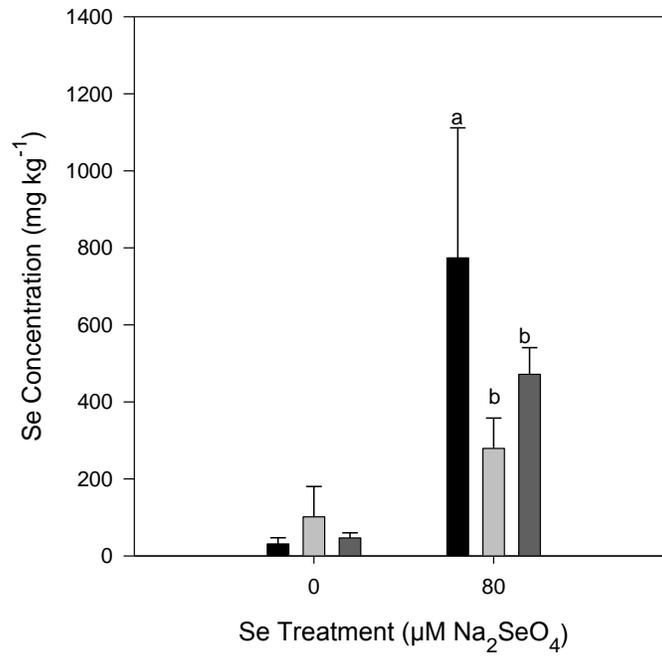**E)** XANES Se speciation - Soil/Epidermis (panel B, locations 1,2,7,and 9)

Fig. 3.4

A) Fall

B) Spring

C)

75

Fig. 3.5

A)

Leaf Se Concentration

B)

Root Se Concentration

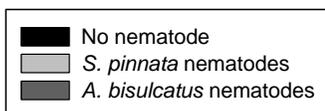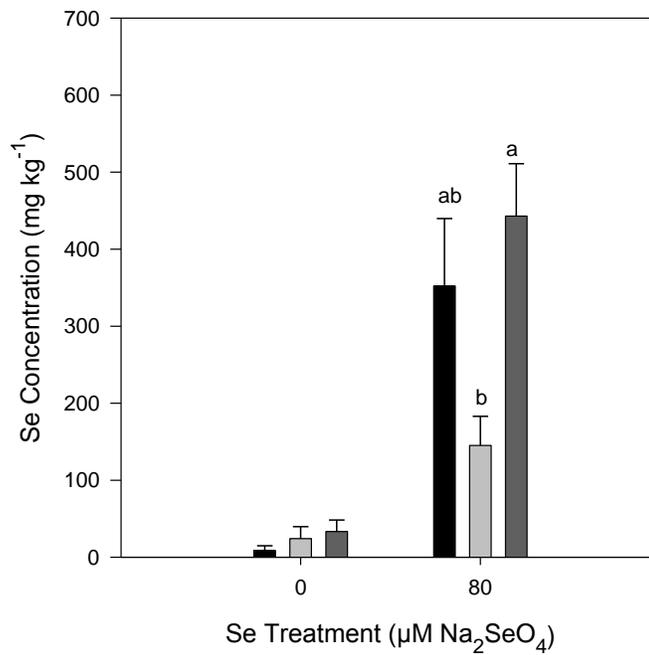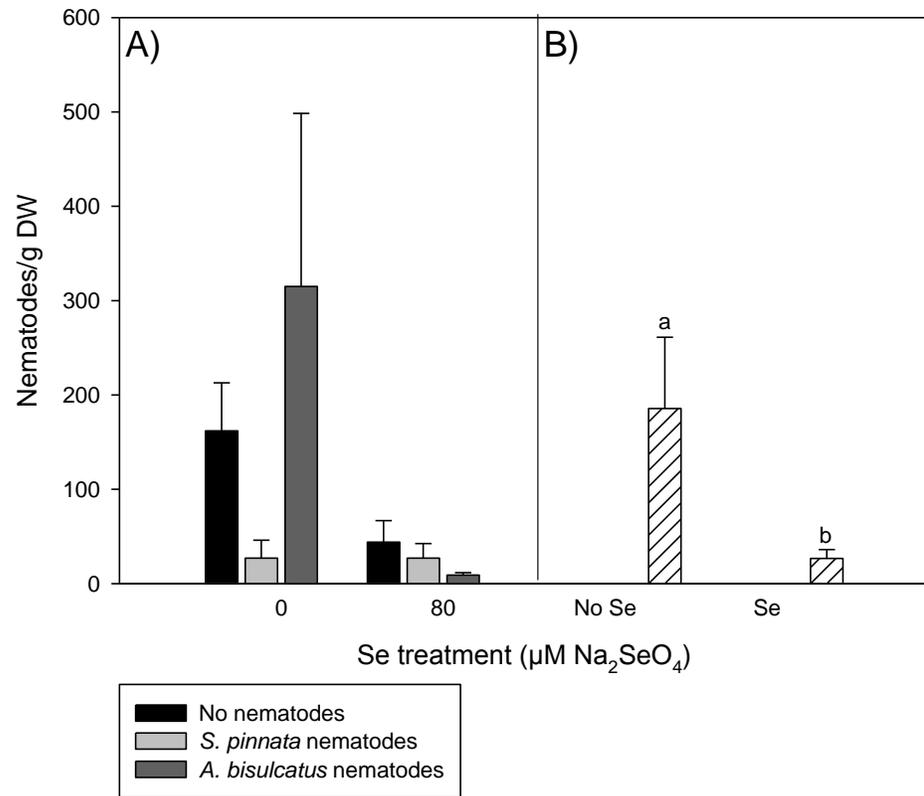Fig 3.6

Fig 3.7

A)

B)

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SUMMARIZING DISCUSSION

The presence of Se in soils and particularly the presence of hyperaccumulator plants on these seleniferous soils have many implications for the local ecological interactions. Earlier studies already showed Se accumulation to protect plants from a wide variety of leaf herbivores due to deterrence and toxicity, but also evidence of Se-tolerant herbivores that can occupy the niche provided by hyperaccumulators. Another advantage of Se accumulation shown in earlier studies is protection from pathogenic fungi. Furthermore, the germination and growth of Se-sensitive plants were shown to be significantly inhibited on soil around hyperaccumulators, suggesting a role for Se in elemental allelopathy. An apparent additional advantage of Se accumulation, as shown in the studies described in this thesis, is protection from root nematodes. In the same study information was collected for the first time about the form and distribution of Se in hyperaccumulator roots.

Another important type of ecological interaction that has been largely unexplored until recently is between hyperaccumulators and pollinators. As shown in this thesis, even the extremely high levels of Se in the floral tissues of hyperaccumulators do not deter honey bees and other potential pollinators. While the potential effects of the ingested Se on pollinator health remain to be investigated, there was no indication that Se levels in honey reach toxic levels; on the contrary, the Se-enriched honey could be

considered nutritionally fortified. The finding that pollinators readily foraged on high-Se

plants indicate that hyperaccumulator plants do not pay a reproductive cost in terms of

pollinator visitation. This is particularly significant because hyperaccumulator *S. pinnata*

is insect-pollinated and self-incompatible.

In addition to the potential ecological cost of Se accumulation for reproduction

via effects on plant-pollinator interactions, there may be a physiological cost via effects

on pollen germination, fertilization and seed germination. The results described in this

thesis indicate that indeed there is such a cost, but only in accumulators (and presumably

in non-accumulators), but that hyperaccumulators have evolved physiological Se

tolerance mechanisms and do not suffer any negative effects of Se on reproductive

functions.

Considering these various evolutionary aspects of Se accumulation, there seem to

be more positive selective pressures towards Se accumulation than negative selection

pressures away from it. Se accumulating plants keep neighboring plants at a distance and

are protected from pathogens and herbivores, yet retain their pollinators. Some

herbivores, however, are co-evolving with Se accumulators and when these species

evolve tolerance to Se they can feed freely on hyperaccumulator plants. As described in

this thesis, root nematodes were found even in roots with extremely high Se

concentrations, suggesting they are Se tolerant, perhaps even Se specialists. It will be

interesting for further studies to investigate to what extent hyperaccumulators form a

portal for Se into the local food chain via such Se-tolerant herbivores as well as via the

non-discriminating pollinators.

The findings from these studies also have broader impacts for agriculture and human and animal health. Pollinators are not deterred by Se-containing plants, so Se-containing crops grown in seleniferous areas should still be pollinated. Bee health may be either positively or negatively affected by Se ingestion from such crops (Se is a nutrient for insects, but toxic at higher levels). Furthermore, honey containing Se from bees foraging on Se-rich flowers may be marketed as a health supplement. Plants growing on seleniferous soils likely require reduced levels of pesticides, or Se may even have the potential to completely replace pesticides and protect plants against a wide variety of above- and below-ground herbivores and pathogens. This would be especially useful in organic and subsistence farming.

The findings from these studies provide a framework for further in-depth studies of plant-herbivore and plant-pollinator interactions in agricultural field settings, and may help optimize agricultural practices in phytoremediation and in the production of Se-fortified crops. The findings from these studies also shed further light on the factors that drive the evolution of hyperaccumulation and the ecological implications of this fascinating phenomenon.