DISSERTATION

ECOLOGICAL EFFECTS OF SELENIUM HYPERACCUMULATION ON PLANT COMMUNITY STRUCTURE AND POTENTIAL IMPLICATIONS FOR SELENIUM CYCLING

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2019

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ABSTRACT

ECOLOGICAL EFFECTS OF SELENIUM HYPERACCUMULATION ON PLANT COMMUNITY STRUCTURE AND POTENTIAL IMPLICATIONS FOR SELENIUM CYCLING

Areas of high selenium (Se) in soils of the Western United States harbor plant species with the capacity to tolerate and then benefit from Se at very high concentrations (1,000-15,000 mg Se/kg dry weight (DW)). The main form of Se in these soils is SeO_4^{2-} (selenate). Its solubility and chemical similarity to sulfate (a plant nutrient) lead plants to take up and assimilate selenate via sulfate transporters and assimilatory enzymes. Selenium is not essential for plants, but can be beneficial at tissue levels below 5 mg Se/kg DW, by reducing oxidative stress. At elevated tissue concentration, Se accumulation can lead to detrimental effects. The levels at which plants begin to experience negative effects from Se is species-dependent and extremely variable, even between and within populations.

The fact that hyperaccumulators contain high levels of a potentially toxic element may have a large effect on their interactions with their community. The central questions in my research were: How does the presence of hyperaccumulators, as repositories and cyclers of Se, affect their plant community, and how may hyperaccumulators affect Se cycling in their local environment and at larger scales? Understanding how Se hyperaccumulators affect their ecological partners and local Se cycling may serve as a model for how other types of hyperaccumulators are affecting community assembly and elemental cycling in their ecosystems. Selenium hyperaccumulators may be a relatively

important component in Se cycling, and their study may help in our understanding the general role of vegetation in Se cycling on regional scales.

This dissertation starts with a literature review that serves as an overall introduction (Chapter 1), followed by two experimental chapters. The specific questions addressed in the first part of my studies (field seasons 1 and 2), as described in Chapter 2, were: Does the presence of Se hyperaccumulators affect the distribution and concentration of Se in soil? Do hyperaccumulators affect overall vegetation properties and species composition, and do some plant species positively or negatively co-occur with Se hyperaccumulators? In the first field season, plant survey and soil Se mapping were performed at three different sites in paired plots. Plots with and without hyperaccumulators were compared for: 1) bare ground, canopy cover and species richness, 2) relative species abundance, 3) soil Se distribution and concentration. Plots with hyperaccumulators showed an overall trend of higher bare ground, lower canopy cover, higher species richness, and 2-3-fold higher soil Se levels (in 2 of 3 sites). These trends that areas with hyperaccumulators tend to have higher and more heterogeneous Se levels, less and more variable vegetation, and altered species composition were not consistently significant across all sites, and it was hypothesized that the effect of hyperaccumulators may have been diluted by their low abundance, and the relatively large area of survey.

In the second field season, a new design was implemented, focusing on areas 3 m in diameter around hyperaccumulators versus non-accumulators, in 44 paired plots on one site. Highly significant results were obtained, showing higher bare ground, lower canopy cover and higher species richness in plots with hyperaccumulators; soil Se

concentration was also higher in plots with hyperaccumulators. Thus, hyperaccumulators seem to have the described effects on their local soil and vegetation, which are highly significant across at least 3 m diameter (~4x their canopy). This may be disproportionately large relative to their abundance.

In the third field season, as described in Chapter 3, the focus was on properties of plant species that showed positive or negative co-occurrence with Se hyperaccumulators. Main questions addressed were: which species are most abundant directly adjacent to hyperaccumulators? How do Se accumulation and tolerance compare in species found to positively or negatively co-occur with hyperaccumulators? Soil and leaf samples were taken from the five nearest species growing next to 54 hyperaccumulators and compared to an overall vegetation survey. X-ray microprobe analysis was performed on positively and negatively co-occurring species, to determine localization and forms of Se in their leaves. The field survey was followed with a lab Se tolerance and accumulation experiment using species found to positively or negatively co-occur. The x-ray microprobe analysis revealed more organic Se for the positively co-occurring species, and several of them showed high Se accumulation capability (up to 900 mg Se/kg dry weight).

Overall, the results from these studies support the hypothesis that hyperaccumulators affect local soil Se distribution, creating more heterogeneity (“hot spots”). This increase in environmental heterogeneity is associated with lower vegetative cover, but increased plant biodiversity. Plant species composition around hyperaccumulators is different, which is measurable at a scale of at least four times the canopy diameter. Some plant species show positive, others negative co-occurrence

with hyperaccumulators, which may be related with their degree of Se tolerance. Neighbors of Se hyperaccumulators had a higher tissue Se concentration, as compared to when the same species grew elsewhere in the area.

Through these positive and negative effects, facilitating Se-tolerant plant community members but lowering the fitness of Se-sensitive members, Se hyperaccumulators may have a disproportionality large effect on their plant community. This, in turn is expected to affect other trophic levels, as well as overall Se cycling in their local ecosystem. This will be interesting to investigate in future research.

ACKNOWLEDGEMENTS

I would like to acknowledge the work of those that have preceded me, at this university, in Se research, and science in general. And, more importantly, the countless unknown others that have made possible the context in which I find myself in all the aspects of my life. It is clearly beyond my ability to understand their efforts, but I appreciate them nonetheless.

More immediately, my wife Lisa has inspired and encouraged me in ways that have been indispensable in allowing me to accomplish much of what I have been able. My son Emmett in his quiet and direct dedication in seeking to understand has reminded me of my roots in this path of inquiry in the world. He has given me the impetus to move on to the next new thing.

In my undergraduate studies at C.S.U., Colin Quinn helped me design and implement my first experiments, and the many conversations with Jen Cappa helped give direction to my future studies. After I started graduate school, I had amazing undergraduates to work with. I want to mention in particular Jake Heiner and Kelsey Craigo. I can’t overstate how much they helped. All of my past and current lab mates have been amazing and have offered tremendous help over the years.

My committee has been great, I chose well. My meetings were not only highly informative and helpful for honing my studies, because of the members, there was an atmosphere that led to some extremely lively and interesting discussions for us all.

Lianne has always treated me with steady patience. She has a marvelous and flexible mind and I will miss our many theoretical conversations about plants, selenium

and the many other topics we touched. Her nearly immediate email replies and almost continuous availability have helped make this path to PhD possible. She and her husband Rien have provided an amazing environment in which to learn.

I am humbled by all that has been given to me.

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[1]CHAPTER 1: SELENIUM HYPERACCUMULATORS AND SELENIUM CYCLING

1. Background

Selenium (Se) is chemically similar to sulfur (S). Most plants non-specifically take up Se via S transporters, and the natural variation in plant Se accumulation is directly correlated with their S accumulation. However, certain plant species called Se hyperaccumulators have evolved the ability to distinguish Se from S. They concentrate and tolerate Se in their tissues at levels greater than 1,000 mg/kg DW, and in some individuals as high as 15,000 mg/kg DW (Knight & Beath, 1937). TheSe levels in Se hyperaccumulator species vary greatly within and between populations of the same species growing in seleniferous soils (Feist & Parker, 2001; Zhang *et al.*, 2006; Cappa *et al.*, 2014; El Mehdawi *et al.*, 2015b). The types of soils where Se hyperaccumulators are found can vary greatly as well (Beath & Gilbert, 1936; Beath *et al.*, 1937). Some of the earliest accounts of Se hyperaccumulators were by a chemist named O.A. Beath working at the Agricultural Experimental Station outside Laramie, Wyoming, USA. Beath's early work in documenting livestock poisoning led him to Se hyperaccumulators (Agricultural & Station, 1921). Beath extensively studied Se hyperaccumulators and their habitat in Wyoming (Hamilton & Beath, 1964). He coined several terms describing plants that accumulate differing levels of Se: he termed hyperaccumulators "indicator plants" because their presence "indicated" seleniferous soils. Tissue concentrations of 1,000 mg/kg DW found in field-collected plant tissues are commonly considered

[1] Chapter 1 adapted from Reynolds & Pilon-Smits, 2018 (used with permission)

hyperaccumulator levels (White, 2016). Other characteristics hyperaccumulators have in common will be discussed in depth in section 3.2 below.

There are around 50 known Se hyperaccumulator taxa that span 7 families, but most are within 3 families: Fabaceae, Asteraceae and Brassicaceae (Pilon-smits *et al.*, 2017). Judged from its occurrence within different clades, Se hyperaccumulation appears to be an evolutionarily derived trait, and non-accumulation the ancestral condition (Cappa & Pilon-Smits, 2014). Given the seeming convergent evolution of Se hyperaccumulation across these families, what are some potential selection pressures that lead to hyperaccumulation? There are a few proposed hypotheses (Boyd & Martens, 1992; Salt *et al.*, 1998). By far the most widely studied, as reviewed in section 5.3, has been the elemental defense hypothesis, which states that plant accumulation of toxic trace elements like Se offers a defensive benefit against herbivory; other hypotheses are that it benefits plants *via* allelopathy (as reviewed in 5.1), causing toxicity to other plants, and by enhancing drought resistance (as mentioned in 3.1, 5.1) (Boyd, 2007). In addition to selection pressures favoring Se accumulation in plants, there may be selection pressures against the Se hyperaccumulation syndrome, for instance if hyperaccumulation is associated with reduced growth and, thus, fitness. Alternatively, while Se hyperaccumulation may give certain advantages on seleniferous soils, it may lead to reduced fitness on non-seleniferous soils. Such constraints may explain the rarity of Se hyperaccumulators in non-seleniferous areas and be a contributing factor for their low abundance in seleniferous areas (less than 5% of the vegetation)The ecological impact of Se hyperaccumulation on the local environment is another fascinating area of inquiry. Se in soils is generally low as compared to the

dramatically concentrated Se in the root and shoot of Se hyperaccumulators (Galeas *et al.*, 2007). Concentration of Se to toxic levels, coupled with the relocation of the Se to locations accessible to more organisms (aboveground, in tissues, at soil surfaces) may have striking influences on the biotic environment. The presence of Se at toxic concentrations in Se hyperaccumulator tissues may dramatically affect the biotic interactions these plants have with herbivores, pollinators, other plants and microbes (both within the plant and in its external area of influence). These biotic interactions may have a powerful influence on the ecosystems where hyperaccumulators grow, altering species composition, favoring Se tolerant taxa. The redistribution of Se by hyperaccumulators also likely creates a more heterogeneous environment. Environmental heterogeneity has long been thought to increase species richness (Hooper *et al.*, 2005; Katayama *et al.*, 2014). The importance of Se hyperaccumulators' influence on local Se cycling as well as impacts on broader Se cycling is an area lacking study. I will review the current literature on ecological aspects of plant Se accumulation and discuss a potential role of plant Se hyperaccumulators in Se cycling, both locally and more broadly. In addition to concentrating Se by 2-3 orders of magnitude, Se hyperaccumulators completely transform inorganic environmental selenate to methyl-selenocysteine (methyl-SeCys, different from non-hyperaccumulator vegetation (see sections 3.1, 3.2). This change in Se speciation likely changes its bioavailability and movement in the food chain (Fernández-Martínez & Charlet, 2009). The overall change in spatial Se distribution, concentration and forms of Se due to the presence of Se hyperaccumulators may have positive or negative effects on local biota, depending on particular species' tolerance/sensitivity and utilization of Se. These collective effects

may constitute selection pressures on species, influencing species composition and distribution in seleniferous areas and possibly more broadly. I will conclude this review by discussing the potential importance of Se hyperaccumulators on local community composition and possible influences further away.

2. Overview of Se distribution and cycling through the abiotic and biotic realm

2.1 Global Se cycling

There is relatively little known about the contribution of terrestrial vegetation in Se cycling, nor of the relative contribution of hyperaccumulators. Here I will summarize current knowledge about global Se cycling; for in-depth coverage of the subject please refer to recent reviews by Sharma (Sharma *et al.*, 2014) and Winkel (Winkel *et al.*, 2015).

The origin of Se in soils varies greatly. Dependent on the redox condition of the location Se is found in four different oxidation states: selenate Se(VI), selenite Se(IV), elemental selenium Se(0), and selenide Se(-II). Se(-II) can occur as organic selenium compounds, usually proteins containing the amino acids selenocysteine and selenomethionine, or as metal selenide mineral, typically with aluminum and iron sediments and rocks. Primary forms found in waters are selenate and selenite as they are soluble, but all of the other forms have been found associated with particulates in some waters as well (Plant *et al.*, 2013). Atmospheric Se originates from natural and anthropogenic sources; it is estimated that around 40% of total atmospheric Se is of anthropogenic origins (Wen & Carignan, 2007). Mining and refining of metals, and coal combustion account for 70-90% of anthropogenic Se emissions (Wen & Carignan,

2007). Natural sources of Se include particulate matter from sea spray and dust and volatilization from organisms; ocean microorganisms are considered the primary emitters of volatile Se (Wen & Carignan, 2007). Airborne inorganic Se can be either from natural or anthropogenic sources and can be H_2Se, Se and SeO_2, but these are thought to quickly become particulate matter in the atmosphere (Wen & Carignan, 2007). For an in-depth review of current knowledge of atmospheric Se, see Wen and Cargnan (2007).

Parent rock material, as opposed to deposition, is thought to be the main source of Se in soils worldwide but in certain areas like Western UK and South East China deposition from the atmosphere can be a major factor as well (Winkel *et al.*, 2015). Soil Se concentration range from 0.01 to 100 µg/g. Se may be released to soils by both natural weathering from parent rock material, biogenic processes, and human activity (Dhillon & Dhillon, 2003). Certain minerals have higher Se concentrations, including sulfide minerals where Se readily substitutes for sulfur (S), iron oxides of sediments that strongly bind Se, and phosphatic minerals (Lakin, 1972). Selenium concentrations are generally greater in shales than in other rock types and particularly in black shales (carboniferous), which can reach 600 µg Se/g (Plant *et al.*, 2013). Shales are the main source of soil Se in Ireland, China and the Western USA (Winkel *et al.*, 2015).

Weathering of minerals and run-off from irrigation are the main contributors of Se to fresh water. Soil Se levels are strongly correlated to soil parent material, and if the geology of an area is diverse, soil Se distribution can be highly variable (Plant *et al.*, 2013). Anthropogenic sources of Se contribute a significant amount of Se to soils. The use of phosphatic minerals in agriculture may be particularly relevant to Se cycling;

these minerals are mined for agricultural applications, and so applied directly to crop plants (Dhillon & Dhillon, 2003). Mining, refining and burning of fossil fuels are other anthropogenic sources of Se (Winkel *et al.*, 2015). For an overview of Se cycling between earth and atmosphere, and contributions of different processes, see Figure 1.1.

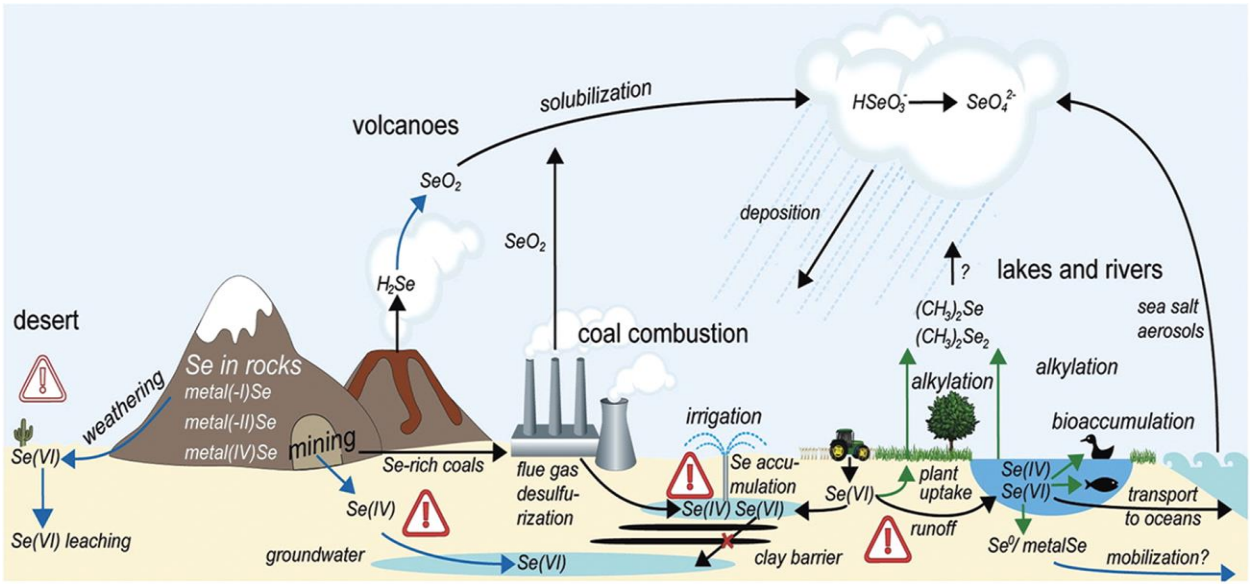

Figure 1.1. Conceptual model for global selenium cycling (from Winkel et al., 2012, with permission).

2.2. Selenium in Western US habitats: from the soil to the plant interface

In this section, an overview is given of the origin and cycling of Se in soils in the Western United States, where the vast majority of reported Se hyperaccumulator plant species occur (White, 2016). For a more extensive review of processes governing the forms of Se in soils in general, please refer to Fernández-Martínez & Charlet (2009).

Much of the work documenting Se in soils of the Western United States started in connection to livestock poisoning caused by vegetation growing on seleniferous soils (Beath et al. 1935; Trelease & Martin 1936). The primary source of Se in soils of the

Western United States is soil parent material (rocks), from which Se is released via weathering and leaching, becoming more bioavailable in the process (Dhillon & Dhillon, 2003). Selenium in parent material in the Western U.S. mainly originates from volcanic activity during the Cretaceous period (145-66 MYA). Therefore, seleniferous soils in the Western US were present long before the evolution of plant Se hyperaccumulation. For instance, the genus *Stanleya*, which contains the hyperaccumulator *Stanleya pinnata*, likely arose less than 5 MYA (Edger *et al.*, 2015).

Because Se adsorbs firmly to clay, some of the highest Se levels are found in sedimentary rocks high in clay content such as the Pierre and Niobrara formations, both sedimentary shales of the Cretaceous period. (Byers *et al.*, 1936; Fernández-Martínez & Charlet, 2009). These formations are the main Se containing formations found throughout the Western United States (Dhillon & Dhillon, 2003). There are other, earlier formations deposited by volcanic activity during the Permian and Triassic era that also contain high levels of Se, but they are much less common; the important commonality is that Se was deposited due to volcanic activity (Byers *et al.*, 1936; Beath *et al.*, 1937, 1939).

Shales are heterogeneous in nature (Matamoros-Veloza Adriana *et al.*, 2011) and Se concentrations in shales are likewise heterogeneous (Tuttle *et al.*, 2014). Erosion and leaching are key processes affecting Se distribution in seleniferous soils, and the efficiency of these processes is subject to features of the local landscapes. Variability in erosion and leaching further contributes to heterogeneity in Se distribution. This uneven distribution may be important for biodiversity on these landscapes (Katayama *et al.*, 2014).

In addition to heterogeneity in spatial Se distribution, forms of Se vary as well, which is very important for bioavailability. Bioavailable forms of Se in soil are typically selenate, selenite and seleno-amino acids. Abiotic conditions, particularly redox potential and pH affect the relative abundance of these forms. Most soils are oxic and therefore inorganic Se in soil is mostly present in the most oxidized form, selenate. Under reducing, anoxic conditions, selenite, selenide and elemental Se are more prevalent (Fordyce, 2007; Fernández-Martínez & Charlet, 2009). The most abundant form of Se weathered from Mancos shale (Niobrara formation) is selenate. Selenate leached from Mancos shale in one study was found to increase with soil depth; the authors hypothesized this was due to leaching from surface layers and a lack of water in deeper soil horizons due to the arid environment (Mast *et al.*, 2014). Selenate is water soluble and highly bioavailable (Schiavon & Pilon-Smits, 2017). Selenite, another bioavailable form of Se, is not abundant in Western soils due to the higher pH of soils (Mast *et al.*, 2014). Selenium can also be strongly bound to organic components in these shales (Mast *et al.*, 2014; Tuttle *et al.*, 2014). This binding also occurs with organic matter in soils, reducing availability of Se to plants (Li *et al.*, 2017). The release of Se from soil organic matter is facilitated by microbes, which are often very tolerant to Se and in some cases utilize Se as an energy source (Stolz *et al.*, 2006). In addition to soluble inorganic selenate and selenite, Se can be found in soils bound to aluminum and iron oxides, making it unavailable to plants and microbes (Plant *et al.*, 2013).

Other organic forms of Se found in soils are biogenic in origin. Some of this organic Se originates from soil microbes, which can assimilate inorganic forms of Se into organic forms like amino acids; they can also produce elemental Se and volatile

dimethylselenide (DMSe) and dimethyldiselenide (DMDSe). Microbial Se metabolism is complex: different taxonomic groups have different pathways, some of which function in essential Se metabolism, some in energy production and some in Se detoxification. For an extensive review, see (Stolz *et al.*, 2006). Microbial Se volatilization removes Se from soil and thus from the local Se pool, but contributes to Se cycling over large distances. Microbial reduction of seleno-oxyanions to mostly insoluble elemental Se lowers Se bioavailability to other biota, including plants.

3. Plant Se uptake, translocation, transformations, and metabolic responses

3.1. Selenium metabolic responses and transformations - benefits and toxicity

Plants in terrestrial settings mainly encounter bioavailable Se in the form of selenate, which they take up using sulfate transporters. In more reducing environments like wetlands, selenite may be more abundant, which plants can take up via phosphate transporters. Selenite-derived Se typically accumulates mainly in the root, while selenate is readily translocated from root to shoot via sulfate transporters. For comprehensive reviews of Se uptake and transformation by plants, see e.g. Terry et al. (2000), Sors et al. (2005), and Schiavon and Pilon-Smits (2017). A summary follows below.

Selenate can be reduced enzymatically to selenite in a two-step process via ATP sulfurylase and APS reductase. Reduction of selenate to selenite appears to be a rate-limiting step for Se assimilation to organic forms (Pilon-Smits *et al.*, 1999). When selenate or selenite accumulate in plants, it can cause oxidative stress (Van Hoewyk, 2013). Plants can quickly reduce selenite to selenide, likely non-enzymatically involving

glutathione, and selenide can be coupled to O-acetylserine to form the seleno-aminoacid selenocysteine (SeCys). Higher plants are not known to have any selenoproteins that involve specific incorporation of SeCys. However, SeCys can be non-specifically incorporated into proteins instead of Cys. This can disrupt protein function and thus result in another form of Se toxicity (Van Hoewyk, 2013). Plants can avoid SeCys from accumulating by further converting it to selenomethionine (SeMet) in a 3-step enzymatic process. SeMet may be further converted to volatile dimethylselenide (DMSe) (N. Terry, A.M. Zayed, M.P. de Souza, 2000). Alternatively, plants may break down SeCys into alanine and elemental Se, which is insoluble and not toxic. Some plants also methylate SeCys into methyl-SeCys via SeCys methyltransferase (SMT). This mechanism of Se tolerance has been found particularly in Se hyperaccumulator species, but also in some non-hyperaccumulators like broccoli and garlic (Neuhierl & Böck, 1996; Lyi *et al.*, 2005). Organic forms of Se may end up in soil when organisms that have accumulated them are recycled. Plant roots readily take up organic forms of Se like SeMet, and translocate and volatilize them at much higher rates than inorganic forms of Se (Zayed *et al.*, 1998).

As mentioned above, Se can have toxic effects on plant physiology due to oxidative stress or to non-specific incorporation of Se into proteins. Depending on the plant species, these toxic effects may occur at tissue levels upwards of 100 mg kg^{-1} DW for non-hyperaccumulators like *Arabidopsis thaliana* or 500 mg kg^{-1} DW for secondary Se accumulators like *Brassica juncea* (Prins *et al.*, 2011). Plant mechanisms that avoid incorporation of SeCys into proteins have been listed above. In addition, plants may avoid oxidative stress via production of enhanced levels of antioxidant metabolites (e.g.

ascorbate, glutathione) and antioxidant enzymes. Low, sub-toxic tissue levels of inorganic Se (10-50 mg kg^{-1} DW) can induce these processes, leading to higher capacity to scavenge reactive oxygen species (ROS). This can explain why low levels of Se can promote photosynthetic efficiency and enhance plant growth, both under unstressed conditions or when challenged to a variety of –mainly abiotic- stresses (Hartikainen, 2005; Schiavon *et al.*, 2017). For this reason, Se is considered a beneficial element for plants.

3.2. Special attributes of selenium hyperaccumulator species

Selenium hyperaccumulator species across different plant families share a number of characteristic physiological and biochemical traits that together offer plants extreme Se accumulation and tolerance. Plants also experience beneficial effects of Se, but do not appear to require Se for their growth. Below I will summarize ways in which Se hyperaccumulators differ from other species.

In selenate:sulfate competition experiments, related Brassicaceae *B. juncea* (non-hyperaccumulator) and *S. pinnata* (Se hyperaccumulator) showed remarkably different Se:S interactions. While 5 mM sulfate supply almost completely abolished selenate uptake (from 20 µM) in the non-hyperaccumulator, it had relatively little effect on selenate uptake in the hyperaccumulator (Harris *et al.*, 2014; Schiavon *et al.*, 2015). Tissue Se/S ratio is higher in hyperaccumulators than other vegetation grown under similar conditions (Cappa *et al.*, 2014), and they experience less inhibition of selenate uptake by sulfate (White *et al.*, 2007; Harris *et al.*, 2014; El Mehdawi *et al.*, 2018). And indeed, Se/S ratio in *B. juncea* tissues largely reflected the Se/S ratio in the media, while *S. pinnata* was found to enrich itself with Se relative to S, as compared to the

media (Schiavon *et al.*, 2015). A similar result was found in Se hyperaccumulator *Astragalus racemosus*, which enriched itself with Se, as evidenced from a higher Se/S ratio compared to the supplied media (DeTar *et al.*, 2015). Hyperaccumulators have higher selenate uptake rates, associated with elevated expression levels of different sulfate transporter genes (Schiavon *et al.*, 2015; El Mehdawi *et al.*, 2018). The underlying mechanisms for these phenomena remain to be elucidated, but the key to the apparent ability of Se hyperaccumulators to discriminate between selenate and sulfate and preferentially take up selenate, may be a mutation in a sulfate/selenate transporter. The sulfate transporter family (SULTR) is a large ubiquitous family of transport proteins. In *Arabidopsis thaliana* two high-affinity sulfate transporters *SULTR1;1* and *SULTR1;2* mediate sulfate uptake into roots, SULTR1;2 being the predominant transporter (Barberon *et al.*, 2008). Schiavon et al. (2015) found *S. pinnata* to have much higher transcript levels of a *Sultr1;2*-like gene, relative to *B. juncea*. This is in agreement with an earlier macroarray study comparing transcript abundance between *S. pinnata* and *S. albescens* (a secondary accumulator) by Freeman et al. (2010), which reported *S. pinnata* to have constitutively elevated transcript abundance for *Sultr1;2*. Similarly, when Se hyperaccumulators *A. bisculatus* and *A. racemosus* were compared to non-hyperaccumulator *Astragalus* species, it was shown that under regular S status hyperaccumulators had elevated transcript levels for sulfate transporters (Cabannes *et al.*, 2011). The increased *SULTR1;2* transcript abundance in these hyperaccumulator species may be caused by differential regulation (e.g. higher expression of a transcription factor), by mutations in the promoter region of the *Sultr1;2* gene, by gene duplication events leading to increased gene copy number,

or higher transcript stability. In either case, increased transcript levels would be expected to be associated with higher transporter protein abundance and uptake capacity. In addition, mutations in the coding region may have given rise to altered substrate specificity, favoring selenate over sulfate. Given that Se hyperaccumulation is a convergent trait among eudicots, the underlying molecular mechanisms may differ and be lineage-specific. For other hyperaccumulated elements, an increase in copy number has been the most reported cause of increased transporter abundance (Lanz *et al.*, 2008; Ueno *et al.*, 2011; Craciun *et al.*, 2012).

Furthermore, hyperaccumulators show higher translocation from root to shoot via xylem, and from mature leaves to young leaves and reproductive organs (especially pollen and ovules) via phloem (Quinn *et al.*, 2011; Cappa *et al.*, 2014), probably due to higher expression levels of one or more xylem loading sulfate or amino acid transporters. The predominant form of Se accumulated in all organs of hyperaccumulators is the amino acid methyl-SeCys, with minor fractions of γ-glutamyl-methyl-SeCys or selenocystathionine (Freeman *et al.*, 2006b; Cappa *et al.*, 2014). In contrast, many non-hyperaccumulators accumulate mainly inorganic Se (Pilon-Smits *et al.*, 1999). The large fraction (>90%) of organic Se in hyperaccumulators is due to higher rate of conversion from inorganic to organic forms, because of overexpressed enzymes in the S assimilation pathway, in combination with SeCys methyl transferase (Freeman *et al.*, 2010; Schiavon *et al.*, 2015). This Se assimilation may take place in part in roots, judged from high root expression levels of Se assimilation enzymes (Schiavon *et al.*, 2015; El Mehdawi *et al.*, 2018) and the presence of methyl-SeCys in xylem fluid (Freeman *et al.*, 2006b). Because methyl-SeCys is not incorporated into

proteins, it can be sequestered safely. As a further potential Se tolerance mechanism, hyperaccumulators sequester methyl-SeCys in particular tissues where it is kept away from sensitive metabolic processes, particularly in vacuoles of the epidermis (Freeman *et al.*, 2006b, 2010). In addition, hyperaccumulators have higher levels of antioxidants, that likely help prevent Se-associated oxidative stress (Freeman *et al.*, 2010). Higher Se volatilization rates have also been measured from hyperaccumulators, in the form dimethyldiselenide, which is derived from methyl-SeCys (Bañuelos *et al.*, 2015). Interestingly, Se hyperaccumulators not only show extreme Se tolerance, but actually grow increasingly better with rising tissue Se concentration, up to 2-fold more than in the absence of Se (Trelease & Martin, 1936; El Mehdawi *et al.*, 2012). The underlying mechanism for this extreme positive growth response is as yet unknown.

4. Possible effects of hyperaccumulators on local Se cycling and transformation

Plants can affect soil Se concentration, spatial distribution and chemical speciation, by accumulating inorganic Se via their extensive root system, translocating it to other plant parts, metabolizing it from inorganic selenate to a variety of organic seleno-compounds, volatilizing it in part and redepositing it back to the soil via litter deposition and root exudation (Fig. 1.2). Hyperaccumulators hold a special place among vegetation because of their greater propensity to take up and accumulate Se, translocate Se to shoots, volatilize it (as DMDSe rather than DMSe like other plants) and transform it into specific organic forms (methyl-SeCys, particularly). Hyperaccumulators in the Western US are generally fairly large perennialswith a canopy reaching 1.5 m diameter (Alford *et*

al., 2012). The Se concentration in their adjacent soil has been found to

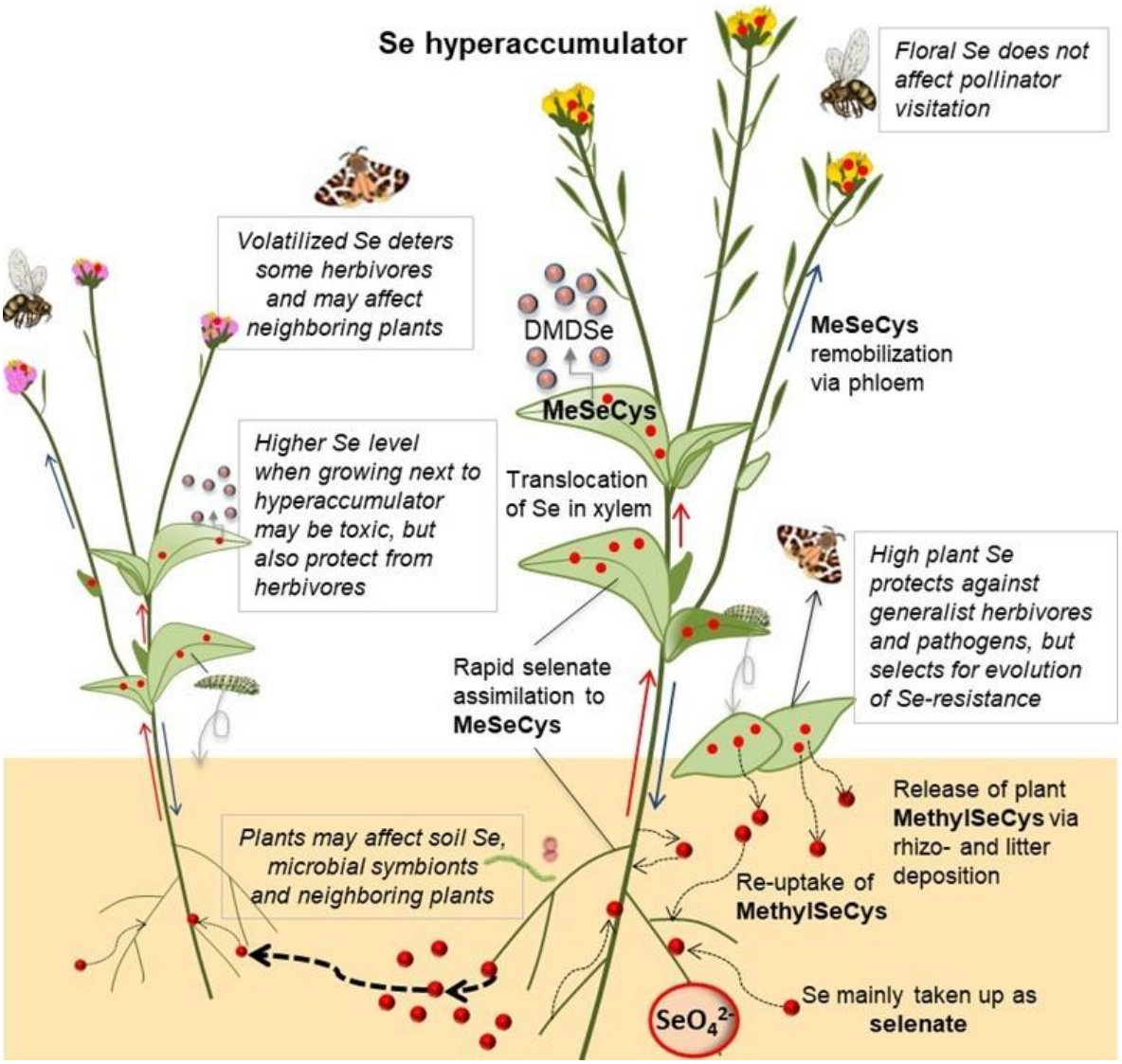

Figure 1.2. Overview of Se fluxes and transformations in Se hyperaccumulator, and ecological implications (modified from Schiavon and Pilon-Smits., 2017)

be around ten times higher (El Mehdawi *et al.*, 2011a) as compared to soil further away, forming a local Se “hot spot”. It cannot be excluded that these hot spots already existed before they were colonized by hyperaccumulators – this is difficult to determine-, but it is also feasible that they are the result of hyperaccumulator activity. If so, hyperaccumulators may be instrumental in enhancing soil heterogeneity. Bio-

concentration of Se inside and around Se hyperaccumulators, and Se transformation to volatile or organic, more bioavailable forms may be expected to affect local ecology and Se movement in the food web. Observed effects to date of plant-accumulated Se on ecological interactions are reviewed in the next section.

5. Biotic ecological interactions of Se hyperaccumulators

Plants have many types of ecological interactions, both belowground and aboveground. The high Se levels found in all hyperaccumulator organs as well as in their surrounding soil may affect all of these direct interactions, and may reverberate at higher trophic levels, if they form a portal for Se into the food chain. In this section, I will discuss current knowledge on effects of Se on the various interactions of plants with ecological partners. These collective effects are also depicted in Figure 1.2.

5.1 Plants

Selenium hyperaccumulators alter their local environment through Se accumulation, making it inhospitable to some plants and beneficial to others. This influence on other plants through chemical means is termed allelopathy. Allelopathy has more typically been applied to the use by plants of secondary compounds, but this concept can extend to elements taken up and subsequently released by the plant (Wilson & Agnew, 1992; Boyd & Martens, 1998). Elemental allelopathy is an understudied and fascinating area for future exploration. For a review of studies on elemental allelopathy see Morris et al (Morris *et al.*, 2009). Elemental allelopathy can be above ground; through volatilization of chemicals, decomposition of leaf tissue on the

soil, or below ground through root turnover and root exudation below the surface. Below I will present evidence for elemental allelopathy with Se hyperaccumulators.

Negative allelopathy: In a field study on a seleniferous site, soil Se levels were found to be 7-13 times higher near Se hyperaccumulators versus soil around non-hyperaccumulator plants in the same area (El Mehdawi *et al.*, 2011a). Selenium release through hyperaccumulator root and shoot turnover may be expected to cause these elevated Se levels. There is some evidence that the soil Se levels around hyperaccumulators can be toxic to neighboring vegetation. El Mehdawi et al. found a reduction in vegetative ground cover near hyperaccumulators, relative to non-hyperaccumulators (El Mehdawi *et al.*, 2011a). In addition, a lab study was conducted testing germination of a Se-sensitive plant species, *Arabidopsis thaliana* on soil + litter or soil collected from around hyperaccumulators versus non-hyperaccumulators in the same area (El Mehdawi *et al.*, 2011a). Only half as many seeds germinated on the soil + litter collected near hyperaccumulators. When tested with soil alone the reduction was 6-7 times (El Mehdawi *et al.*, 2011a). The plants that did germinate on the hyperaccumulator-associated soil were reduced in size and had elevated Se levels.In another study, some evidence was found for soil Se enrichment from hyperaccumulator leaf litter. A litter decomposition study found increased Se levels in soil under hyperaccumulator leaf litter after 12 months (Quinn *et al.*, 2010b).

Positive allelopathy: At low levels, Se has a positive effects on plants. The tissue concentration range where beneficial effects of Se are observed in plants differs dramatically, from as low as 3 mg/kg dry weight Se to as much as 100 mg/kg dry weight Se (Rani *et al.*, 2005). The benefit (increased biomass) seems to be through an

increase in antioxidant capacity (Yao *et al.*, 2009; Hasanuzzaman & Fujita, 2011; Hasanuzzaman *et al.*, 2011; Han *et al.*, 2013; Nawaz *et al.*, 2015; Ahmad *et al.*, 2016). This can offer an advantage via drought protection and even under unstressed conditions, due to more efficient photosynthesis.

The Se around hyperaccumulators likely consists of organic Se compounds, considering the high proportion of organic Se in hyperaccumulator tissues (Freeman *et al.*, 2006b). Indeed, rhizosphere soil Se speciation from hyperaccumulator *Astragalus bisulcatus* indicated 70% organic Se with C-Se-C configuration, similar to the methyl-SeCys found in the hyperaccumulator (El Mehdawi *et al.*, 2015b). Being that organic Se is taken up more easily by most plants relative to inorganic forms (Zayed *et al.*, 1998), this may contribute to the increased Se levels found in plants growing near hyperaccumulators.

In a study that looked at plant-plant interactions of Se hyperaccumulators in their natural environment, two plants species were found to have 3-7 times higher Se levels in their tissues when growing adjacent to hyperaccumulators than when growing further away from them. Not only did they have higher Se levels, but both species had around twice the biomass of plants of the same species growing away from hyperaccumulators, and showed less herbivory damage (El Mehdawi *et al.*, 2011b). This observation implies a strong positive selection pressure for those plants in the hyperaccumulators' community that are capable of tolerating increased Se.

There is wide variation in Se levels within Se hyperaccumulator species and populations (Feist & Parker, 2001; Cappa *et al.*, 2015). This accumulation variability could be due to genetic or environmental factors, but is likely a combination of both. The

variable Se levels introduced by different hyperaccumulator individuals is a further level of heterogeneity introduced, giving the opportunity to a range of Se tolerant/sensitive organisms.

Known Se hyperaccumulators in the Western U.S. are all perennial plant species, and over time have likely altered the abiotic environment, specifically concerning Se levels and Se forms in soil. These changes in the abiotic environment are potentially new niches created through the action of Se hyperaccumulators. These "Se niches" have been shown to affect vegetation (El Mehdawi, Quinn & E. A. H. Pilon-Smits 2011; El Mehdawi, Quinn & E. A. H. Pilon-Smits 2011) and over time it can be envisioned that they have an influence on the overall plant community and possibly higher trophic levels.

5.2. Microbes and Se hyperaccumulators

Nearly all plants have associations with microbes: fungal, bacterial and microscopic eukaryotes (Turner *et al.*, 2013). The interactions of Se hyperaccumulators and their associated microbial community has received increased attention in the last decade. Selenium appears to play a role in both positive and negative microbial associations of Se hyperaccumulators. Interactions between fungi, Se and hyperaccumulators have been studied in three contexts, based on the nature of the associations: pathogens, decomposers, and symbionts (rhizosphere or endophytic).

Fungi are very important to most plants, and in fact there is evidence that fungal associations may have been a key for plants to colonize land (Heckman, *et al.*). While many plant-associated fungi benefit their host, some fungi are plant pathogens and

some can be either depending on the status of the plant and environmental conditions. Early studies found Se to have a protective role against two fungal pathogens. Selenium pre-treated *B. juncea* plants (a secondary Se accumulator) showed reduced infection and more biomass production than control plants when infected with leaf and pathogen *Alternaria brassicicola* and stem/root pathogen *Fusarium sp*. (Hanson *et al.*, 2003). These results not only shed light on a possible ecological function of Se hyperaccumulation in pathogen protection, but may also be applicable in agriculture to help battle fungal pathogens. More studies are needed to better understand which Se concentrations in plant tissues are required to protect plants from different pathogens, and the relative efficiency of different seleno-compounds in this respect.

Fungal communities can adapt to high-Se environments, as demonstrated in a large study where rhizosphere fungi from seleniferous and non-seleniferous areas were isolated and characterized, and those from seleniferous areas were found to be much more tolerant to Se (Wangeline *et al.*, 2011). The study also contrasted rhizosphere fungi associated with Se hyperaccumulators to those from non-hyperaccumulator plants in the same seleniferous areas, and found that the site where they were collected correlated better with Se tolerance than the Se concentration of the plant they were associated with. This may be an indication that the Se at the site drove the adaptation and not the Se hyperaccumulator. However, the Se concentration used in this study to test for Se resistance was rather low, and it cannot be excluded that a difference in Se resistance would have been apparent at a higher Se concentration. The authors also found that overall fungal diversity between seleniferous and non-seleniferous sites was not significantly different (Wangeline *et al.*, 2011), indicating that high-Se habitats do not

have less fungal biodiversity. Seleniferous sites all had similar fungal groups associated with them, even though they were at least 60 miles apart, which is possible evidence for convergent evolution in the fungal communities in response to a similar selection pressure (Wangeline *et al.*, 2011).

The contribution of fungi and bacteria to plant uptake and metabolism of Se, by altering the form of Se in the rhizosphere or within the plant, is another interesting area of study. In a 2012 study (Valdez Barillas *et al.*, 2012) the Se composition was investigated in various plant tissues of the Se hyperaccumulator *Astragalus bisulcatus* and found to include up to 31% Se^0 (elemental Se) in stem cortex tissues as well as in root nodules and seeds (Valdez Barillas *et al.*, 2012). It has been shown that many bacteria readily produce elemental Se from selenite under oxic conditions, and some also from selenate under anoxic conditions (Stolz *et al.*, 2006). Although plants also contain enzyme activity that can produce elemental Se (SeCys lyase), elemental Se has only been found in rare cases in plants, namely in Se hyperaccumulator individuals growing in the field. Several findings point to a microbial source of the elemental Se in these hyperaccumulators (Valdez Barillas *et al.*, 2012; El Mehdawi *et al.*, 2014). Firstly, the finding that nodules contained elemental Se but the adjacent root proper did not points to the *Rhizobium* symbiont as the source of elemental Se in nodules. In the same study, elemental Se was found in seeds of *A. bisulcatus* that harbored an endophytic *Alternaria* species, but not in uninfected seeds, pointing to the fungus as source of the Se^0 (Valdez Barillas *et al.*, 2012; Lindblom *et al.*, 2013b) Furthermore, while hyperaccumulators in the field sometimes showed Se^0 in roots or stems, individuals from the same species grown in the greenhouse did not. It is feasible that specific

endophytic microbes colonized plants in the field and produced Se^0, while those in the greenhouse were not exposed to such microbes and not colonized (Valdez Barillas *et al.*, 2012; Lindblom *et al.*, 2013b).

There have been a number of studies investigating the effect of microbes collected from the rhizosphere or endosphere of Se hyperaccumulators or other seleniferous habitats on plant Se metabolism. These studies used a variety of approaches, but often found similar effects. A common response to inoculation was improved plant biomass, and often there was also increased Se uptake. Some studies used individual bacterial or fungal isolates and some used an inoculum made from diluted rhizosphere soil (de Souza MP *et al.*, 1999; Shahabivand *et al.*, 2012; Lindblom *et al.*, 2013a, 2014, Durán *et al.*, 2013, 2015; El Mehdawi *et al.*, 2015b; Nawaz *et al.*, 2015; Sura-de Jong *et al.*, 2015a; Yasin *et al.*, 2015). Often the mechanisms underlying these effects were not investigated. However, de Souza et al. (1999) elucidated the mechanism in one study where higher Se accumulation and volatilization was observed in *B. juncea* after inoculation with environmental bacteria from a seleniferous area. Along with the increased accumulation and volatilization, the authors observed enhanced root hair abundance, probably due to plant growth regulators produced by bacteria, and higher levels of serine in the rhizosphere, which likely upregulated plant sulfate uptake and assimilation. These two factors combined were likely causal for the increased accumulation and volatilization of Se (de Souza MP *et al.*, 1999).

Selenium does not appear to impede litter decomposition in seleniferous habitats. In fact, the opposite was found: when leaf litter from hyperaccumulators and related non-hyperaccumulator plants were placed on the soil surface of a seleniferous

area where Se tolerant decomposers would be expected to occur, leaf litter from hyperaccumulators decomposed faster, and contained more cultivable fungi and bacteria (Quinn *et al.*, 2010b). Seleniferous litter is a habitat that may give the opportunity for reduced competition due to the requirement of Se tolerance to utilize the litter as a food source (Quinn *et al.*, 2010b). Presence of high Se in litter creates a unique niche where tolerant detritivores have a competitive edge.

High plant Se accumulation does not appear to negatively impact colonization by bacterial endophytes. Based on Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis, endophyte species diversity was comparable between hyperaccumulator and non-hyperaccumulator species from the same seleniferous area (Sura-de Jong *et al.*, 2015b). Around fifty isolates from *A. bisulcatus* and *S. pinnata* were characterized and found to be highly Se resistant; all could produce elemental Se from selenite (Staicu *et al.*, 2015; Sura-de Jong *et al.*, 2015a). Some of the isolated bacteria were tested for, and found to have, properties known to promote growth in plants, and indeed were found to promote plant growth when inoculated to individuals of related plant species (Sura-de Jong *et al.*, 2015a). Judged from T-RFLP analysis, endophytic bacterial species composition was more similar for individuals of the same species, regardless of Se content or how close they grew to one another (Sura-de Jong *et al.*, 2015a). Thus, each Se hyperaccumulator may be associated with a distinct community of bacterial endosphere microbes (Sura-de Jong *et al.*, 2015a).

In another study (Bauer *et al.*, 2018) microbiome sequencing was used to compare the rhizosphere microbiome of three Se hyperaccumulator species and related non-hyperaccumulator species from the same seleniferous area. There was higher

alpha diversity in the hyperaccumulator microbiomes, indicating the Se hyperaccumulation does not negatively affect rhizosphere bacterial colonization but rather may offer more habitat diversity through variation in Se concentration, resulting in more species diversity. Principle component analysis (PCA) revealed a significant difference in species composition between rhizomicrobiomes of Se hyperaccumulators and related non-hyperaccumulators from the same seleniferous site. Thus, interestingly, Se compounds in the rhizosphere of different unrelated hyperaccumulator species appear to commonly affect rhizomicrobiome species composition. Around 400 strains of rhizospheric bacteria from hyperaccumulators and non-hyperaccumulators from seleniferous and non-seleniferous sites were isolated and characterized for Se resistance (Cochran and Pilon-Smits, unpublished results). Almost all isolates were highly Se-resistant, thriving while growing on plates containing up to 200 mM selenate or selenite. Therefore, it does not appear that observed difference in rhizosphere bacterial species composition was due to Se toxicity. More likely, presence of Se altered competition between taxa, offering an advantage to those that utilized Se best. These microbial communities and their host species likely are evolving together. As with microbial associations of other organisms, these associated microbes may have aided Se hyperaccumulators in their history of adaptations to ever-changing conditions (Zilber-Rosenberg & Rosenberg, 2008).

5.3. Herbivores and Se

There have been a large number of studies on the effects of plant Se on plant-herbivore interactions. The overall finding is in alignment with the elemental defense hypothesis: increasing plant Se concentration increasingly protects plants from

herbivory by a wide variety of invertebrate and vertebrate herbivores (for a review, see El Mehdawi & Pilon-Smits 2012). This protective effect was owing to a combination of deterrence and toxicity. Toxic effects on generalist herbivores after ingestion of high-Se plant material were found for both Se hyperaccumulators and non-hyperaccumulators, indicating that different forms and levels of Se can be protective. Selenium protected against herbivores with different feeding modes (phloem feeders, cell disrupters, leaf chewers), indicating that Se is present and protective in different plant parts. Even volatile Se can protect plants via deterrence: aphids (phloem feeding herbivores) avoided feeding on plants that contained Se even before tasting the plant (Hanson *et al.*, 2004). This type of avoidance may also happen for adult diamondback moths (*Plutella xylostella*), which avoided laying eggs on plants containing high Se levels (Freeman *et al.*, 2006a). Selenium may also deter continued feeding after feeding is initiated. In choice feeding studies, larvae of Lepidoptera (cabbage white butterfly, diamondback moth), as well as crickets, grasshoppers, and prairie dogs opted to move to low-Se plants after their initial taste of Se-rich plants (Freeman *et al.*, 2006b, 2007). In non-choice feeding studies, a wide variety of invertebrate herbivores that were forced to feed on high-Se plant material suffered toxicity and died, with the exception of a snail species (Hanson *et al.*, 2003).

In addition to these aforementioned studies that were all controlled choice- or non-choice feeding studies, several field studies were done. One field survey by Galeas et al (Galeas *et al.*, 2008) of invertebrate abundance and composition in a seleniferous area found that two hyperaccumulator species harbored fewer arthropods, as well as fewer arthropod species and different species composition compared to two similar non-

hyperaccumulator species on the same site. Hyperaccumulators also exhibited less herbivory damage. Another field survey found Se hyperaccumulator *A. bisulcatus* to be one of few species to thrive on heavily grazed prairie dog towns (Quinn *et al.*, 2008). Furthermore, two manipulative field studies were done, involving grasshoppers (Freeman *et al.*, 2007) and prairie dogs (Freeman *et al.*, 2009) respectively. Individuals of the hyperaccumulator species *S. pinnata* were pre-treated with or without Se and then planted on a seleniferous field site where these herbivores were abundant. In both cases, the Se-treated plants were eaten significantly less and survived better than plants not pre-treated with Se.

While Se apparently functions well as a form of elemental defense, protecting plants from a wide variety of herbivores, as with all plant defenses there is evidence that some herbivores have overcome this defense. During field investigations on a seleniferous site, it was noticed that there were certain herbivore species commonly found on hyperaccumulator plants containing Se levels around 2,000 mg/kg DW and caused substantial leaf or seed herbivory (Freeman *et al.*, 2006a, 2012; Valdez Barillas *et al.*, 2012). These herbivores were collected and analyzed for their Se concentration and speciation. Two leaf herbivores (both moth larvae) appeared to be Se tolerant, as they were found to contain Se levels around 250 mg/kg DW, which is ten-fold higher than those found to be lethal for generalist Lepidoptera larva (Hanson *et al.*, 2003; Freeman *et al.*, 2006a). Three seed herbivore species, on the other hand, contained Se levels below 20 mg/kg DW (i.e. 100-fold lower than the seed Se concentration), but produced high-Se excrement, suggesting that Se exclusion was their Se resistance mechanism. One of the Se-tolerant moths, a diamondback moth population from a

seleniferous area, was further investigated using choice and non-choice feeding studies in comparison with a diamondback moth population from a non-seleniferous area, in a lab setting (Freeman *et al.*, 2006a). There were striking differences between both populations. In choice studies, animals from the non-seleniferous population were deterred by high Se plants, both with respect to oviposition and larval feeding, while the seleniferous population showed no preference. In non-choice studies, animals from the non-seleniferous population showed toxicity and high mortality when forced to feed on Se-rich hyperaccumulators; they grew well on the same species when it was not supplied with Se, indicating that Se was the toxic agent. In contrast, animals from the seleniferous population thrived on high-Se plant material. The key to this tolerance appears to be that the form accumulated was methyl-SeCys, the same form found in the host plant. The Se sensitive population accumulated SeCys, which readily can enter proteins, replacing Cys, rendering the protein less functional (Freeman *et al.*, 2006a). Thus, the Se tolerant moth appears to have lost its capacity to demethylate methyl-SeCys. It is interesting to note that this diamondback moth is both an invasive species that has been in the Western US less than 100 years, and is notorious for developing resistance to natural and man-made toxins (Talekar & Shelton, 1993). In conclusion, high-Se plants seem to offer a niche for Se-resistant herbivores. As mentioned in the previous section, high-Se litter may also provide a niche for Se-resistant detritivores; not only microbes, but also micro-arthropods were found in higher numbers on high-Se than low-Se leaf litter (Quinn et al. 2010).

5.4. Pollinators and Se

Considering the finding that protection from insect herbivores is a benefit of Se hyperaccumulation, similar deterrence of pollinating insects might be expected to decrease plant reproduction and thus fitness. However, several lines of study indicate that pollinator visitation and foraging is not affected by floral Se content, even though hyperaccumulators often have particularly high Se levels in their floral parts, including nectar and pollen (Quinn *et al.*, 2011; Hladun *et al.*, 2016). When *S. pinnata* or *B. juncea* plants were pretreated with/without Se and placed in a field site near an apiary, visitation was not affected by Se content. Visitors actively collected high-Se pollen and nectar: honeybees and bumblebees collected after visitation were found to carry Se-rich pollen in their pollen baskets, and to contain Se in their tissues (Quinn *et al.*, 2011). Interestingly, the non-native honeybees had 10-fold lower tissue Se levels (<25 mg/kg DW) than the native bumblebees (250 mg/kg DW). These bumblebee levels were similar to those found in Se-tolerant insect herbivores. This may indicate that this native pollinator has (co-) evolved Se tolerance and utilizes Se hyperaccumulator species as a food source, in return offering the plants pollinating services. The presence of such a Se-tolerant pollinator is vital for the reproductive fitness of these hyperaccumulators, which are insect-pollinated, self-incompatible and do not reproduce vegetatively. Conclusions from this field study are in agreement with results from a lab study with individual bees, which found that honeybees did not have a preference to feed on sugar water with or without various seleno-compounds (Hladun *et al.*, 2016). In non-choice feeding studies, these seleno-compounds were found to be toxic to honeybees at elevated levels.

5.5. Selenium in higher trophic levels

Given the fact that there are Se-tolerant herbivores and pollinators that feed on Se hyperaccumulators and that accumulate substantial Se levels in their tissues, it is likely that these carry the plant-derived Se further up into the food chain. Indeed, several species of parasitic wasps have been found to complete their life cycle in Se-rich moth larvae that feed on hyperaccumulators; these wasps contained similar Se levels as their host and in the same forms (Freeman *et al.*, 2006a; Valdez Barillas *et al.*, 2012). Thus, the plant, herbivore and parasites all appear to share similar Se tolerance mechanisms: they accumulate MeSeCys, thus avoiding Se incorporation into proteins. These findings show that Se hyperaccumulators form a portal for Se into the food chain, via Se-tolerant herbivores and higher trophic levels.

In a field survey, arthropods collected from Se-hyperaccumulating plants were found to contain 3- to 10-fold higher Se concentrations than arthropods from the same species found on non-hyperaccumulating species on the same site, indicating that Se is indeed moving into the food chain; several of these species were predatory arthropods, others were herbivores (Galeas *et al.*, 2008). Interestingly, nineteen arthropod species were collected only from hyperaccumulating plants, including 11 herbivores, four predators, three omnivores and one parasite. The Se levels found in several of these arthropod species collected from hyperaccumulators are indicative of Se tolerance (50-200 mg kg^{-1} DW). This suggests there may be hyperaccumulator- specialist species at multiple trophic levels. It is important to note that the arthropods still had Se levels 10-fold lower than those found in their plant host, and thus do not appear to biomagnify the Se in the food chain.

6. Integrative summary of effects of plant Se on ecological interactions

The overall trend from ecological studies so far is that high Se levels accumulated inside and around Se hyperaccumulators negatively affect Se-sensitive ecological partners, while offering a niche and having positive effects on Se-resistant partners. Negative effects, for instance, were found for many generalist herbivores, sensitive plant species, and sensitive fungal pathogens. Evidence for Se resistance (and often tolerance) was found for several leaf and seed herbivores, neighboring vegetation, endophytic fungi and likely certain pollinators. Bacteria hold a special place in that they do not seem to suffer toxicity from the Se levels commonly found associated with Se hyperaccumulators; however, plant Se does appear to affect microbial competition, since it can affect the plant microbiome.

Selenium-resistant ecological partners may be expected to benefit from their association with Se hyperaccumulators in different ways. First, they can utilize them as a habitat and a potential food source, taking advantage of this niche that is unavailable to Se-sensitive competitors. Moreover, since Se is an essential element for many invertebrates as well as mammals, elevated Se levels may have a positive physiological effect on ecological partners. Selenium-tolerant herbivores and their predators may also benefit ecologically from accumulated Se if it protects them from predation via deterrence or toxicity. This remains to be investigated.

The overall ecological effects of Se hyperaccumulation for the hyperaccumulator itself are clearly beneficial: the Se protects them from herbivory and pathogen attack and gives them competitive advantage over other plants via elemental allelopathy, while having no apparent consequences for some mutualist partners: their rhizosphere and endosphere microbiome diversity is not compromised, nor is visitation by pollinators.

These ecological exchanges are fulfilled by Se-resistant species, which may have co-evolved with the hyperaccumulators, or developed Se tolerance for their own ecological benefits. However, there is also evidence that Se-resistance has evolved in certain herbivores; some may even specialize in feeding on hyperaccumulators. Selenium hyperaccumulation likely has evolved incrementally under continuous selection pressure from herbivores, and this evolutionary “arms race” appears to still be ongoing, as with other types of plant defenses.

It is interesting to consider the potential effects of Se hyperaccumulators on species composition and structure in their local environment, which may extend to all trophic levels, and is likely to be even more widespread, yet the magnitude of effect diminished. Furthermore, hyperaccumulators may affect Se movement in the food chain and Se cycling. Hyperaccumulators concentrate Se and transform it to more bioavailable, organic forms that are readily taken up by other organisms. They also volatilize Se at high rates, releasing it into the atmosphere from where it can be redeposited elsewhere.

Selenium hyperaccumulators represent a type of biotic and abiotic (through deposition of Se) environmental filter that seems to improve the fitness of some ecological partners while reducing the fitness of others.

The complexity of their ecological effect is due to altering concentration, and form of Se and the myriad of responses that the biotic and abiotic environment reacts. The influence exerted by hyperaccumulators, as discussed above, is horizontal within the plant community and vertical at higher trophic levels and when it affects microbial processes. Changes in fitness will affect the local species pool by increasing or

decreasing species richness, relative species abundance and community composition. These local effects will then have an influence on regional species pools of which they are a part. Through a cyclical interaction over time between local and regional ecological processes, Se hyperaccumulators may have a disproportionately large effect on their local ecosystem, both spatially and temporally, due to the strong selection pressures they represent. The effects of Se hyperaccumulators on Se redistribution and forms may create unique niches, increase landscape heterogeneity and lead to increased species richness.

7. Evolutionary aspects of Se hyperaccumulation

Some interesting evolutionary questions related to hyperaccumulation are: What may be the physiological and ecological benefits and constraints of Se hyperaccumulation? What sequence of events led to Se hyperaccumulation? Did tolerance and accumulation evolve simultaneously or sequentially, and what were the physiological, biochemical and genetic steps involved? These steps can be surmised by looking at current variability within non-accumulator taxa (e.g. *Arabidopsis*), secondary accumulator taxa (e.g. *Brassica*) and hyperaccumulator taxa (e.g. *Stanleya*) in a family. Ideally, closely related non-accumulator, accumulator and hyperaccumulator taxa can be further analyzed using powerful genomic approaches, to fully elucidate the evolutionary patterns and mechanisms associated with Se hyperaccumulation. Based on collective evidence so far, a model for the evolution of hyperaccumulation may be hypothesized to involve the following series of events. Likely, the first stage involves genetic variation within non-accumulator plant populations with respect to Se accumulation, caused by differential expression of genes involved in S/Se uptake.

Perhaps there also was already variation in Se tolerance at that initial stage, e.g. via variation in levels of antioxidant compounds and certain species having a kind of preadaptiaton, or particular phenotypes that helped with tolerance. In seleniferous areas, higher Se accumulation likely led to increased fitness due to physiological benefits and limited ecological benefits (some herbivory protection). Over time, these selection pressures favored incremental increases in Se accumulation and tolerance, until a tolerance ceiling was reached, at which novel mechanisms needed to be evolved. This was when true hyperaccumulation emerged, characterized by qualitative changes in Se metabolism and sequestration. The molecular mechanisms involved may include evolution of Se-specific transporters and enzymes, and altered expression patterns with respect to tissue-specificity. Convergent evolution of Se hyperaccumulation in different clades may have involved different molecular evolutionary pathways, but appear to also involve many shared mechanisms.

8. Scope of this Ph.D project

The central question to this work is how Se hyperaccumulators might be affecting their local vegetation. I became interested in this question as an undergraduate student, when observing particular vegetation patterns at a high-Se site at Pine Ridge Natural Area, CO: the species *Symphyotrichum ericoides* grew in large monocultures around hyperaccumulators, but not in the landscape as near as 50 m away (El Mehdawi *et al.*, 2014). The goal of my Ph. D. project was to test the hypothesis that Se hyperaccumulators have broad ecological effects on their local vegetation, through their effects on soil Se distribution and the combined negative and positive effects on Se-sensitive and Se-tolerant plant species, respectively. I also wanted to get some insight

into how hyperaccumulators may be altering local Se cycling through their unique ability to assimilate and concentrate Se. My approach, in broad terms, was to compare species composition between similar areas with and without hyperaccumulators.

In the first field season of my Ph.D work (2013 study), I mapped soil Se levels over an area that contained hyperaccumulators and a similar area nearby that didn't, across three sites. In addition, I surveyed the species contained on these plots, and their relative contributions to the overall vegetation. I hypothesized that the area with hyperaccumulators would have higher Se levels and would differ in overall vegetation patterns and species composition. Based on the results from the first field season, in the next year (2014 study) I altered the experimental design to focus in on the possible influence of hyperaccumulators. I compared the immediate vegetation (3 m diameter) around hyperaccumulators to vegetation around non-hyperaccumulators. The dosage effect of hyperaccumulator Se concentration on surrounding vegetation was also investigated in this study. Finally, in 2015 I focused more on vegetation around hyperaccumulators (the nearest five species) growing around hyperaccumulators, hypothesizing that certain species would be more frequently associated with hyperaccumulators. This field survey was followed by a controlled laboratory study comparing Se tolerance, accumulation, and Se tissue distribution and speciation of selected species that stood out for their positive or negative association with hyperaccumulators.

The effects of Se hyperaccumulation on community assembly are relevant in the context of local Se cycling, and invite further investigations into the contribution of Se hyperaccumulators and general terrestrial vegetation to global Se cycling. In a broader

sense, these impacts of Se abundance and distribution in soils and of Se hyperaccumulators may serve as a case study for how trace elements and the organisms that hyperaccumulate them uniquely influence ecological processes.

CHAPTER 2: A COMPARISION OF SOIL SELENIUM AND VEGETATION IN THE PRESENCE VS ABSENCE OF HYPERACCUMULATORS

Introduction

Selenium is widely and unevenly distributed in soils of the Western United States (Dhillon & Dhillon, 2003). Release of Se from parent material can be through natural weathering, biogenic activity and human activity (Dhillon & Dhillon, 2003). Variability in parent rock material, as well as uneven release rates due to local abiotic and biotic conditions leads to a heterogeneous Se distribution in soil (Lakin, 1972); geological differences further contribute to this heterogeneity (Plant *et al.*, 2013). The form of Se in soils is largely dependent on redox conditions and biotic interactions. In general, bioavailable Se in soils is primarily SeO_4^{2-} (selenate), but Se can also be found as SeO_3^{2-} (selenite), and both of these forms are highly soluble in water, contributing to their bioavailability (Plant *et al.*, 2013). Livestock poisoning caused by vegetation growing on seleniferous soils (> 0.1 µg Se/g soil) was an important initial motivation for work documenting Se in soils of the Western United States (Agricultural & Station, 1921). Although Se is an essential nutrient for most animals, the concentration range difference between sufficient and toxic is quite narrow (Fordyce, 2013).

Selenium is not essential for plants. Uptake of Se for plants is inadvertent: due to the chemical similarity between Se and the plant macronutrient sulfur (S), sulfate transporters provide the main means of entry (Schiavon & Pilon-Smits, 2017). The subsequent accumulation of Se is directly connected to the way plants translocate and assimilate S. Because Se enters through the S assimilation pathway, the concentration

of Se in a plant is directly related to sulfate uptake and assimilation capacity, and the relative concentrations of selenate and sulfate in soils (Sors *et al.*, 2005). One of the ways Se is toxic to plants is the incorporation of Se into cysteine in place of S (becoming seleno-cysteine); replacement of cysteine by seleno-cysteine can disrupt protein function and thus result in Se toxicity (Van Hoewyk, 2013). The other mechanism by which Se becomes toxic to plants is due to oxidative stress from inorganic selenate and selenite (Van Hoewyk, 2013).

At low tissue levels (1-5 mg Se/kg DW (dry weight)) Se can provide a benefit to plants; the mechanism of these benefits is still largely unknown, but conferring tolerance to oxidative stress has been widely shown (Vesk & Reichman, 2009; Feng *et al.*, 2013; Khan *et al.*, 2015; Kaur *et al.*, 2016; Ahmad *et al.*, 2016). Above these relatively low tissue levels, for most plants, the benefit quickly turns to toxicity, due to oxidative damage and to protein malfunction if Se displaces S in proteins (Van Hoewyk, 2013). Tissue concentration where plants start to experience toxicity varies by species, from very low levels of around 5 mg Se/kg DW for Se-sensitive species to as high as 500 mg Se/kg DW (Prins *et al.*, 2011). Even within a species there may be dramatic differences in Se tolerance (Zhang *et al.*, 2006). At the extreme end of Se tolerance are hyperaccumulators of Se, which have very high tissue Se concentrations and rather than experiencing toxicity, they seem to benefit from it (Schiavon & Pilon-Smits, 2017). The most widely accepted threshold above which a plant is considered a hyperaccumulator is 1,000 mg Se/kg DW while growing in naturally seleniferous habitats (Boyd & Martens, 1992; Schiavon & Pilon-Smits, 2017). For instance, *Astragalus bisulcatus* can have as much as 14,000 mg Se/kg DW, and *Stanleya pinnata*

4,000 mg Se/kg DW Se DW (Galeas *et al.*, 2007; White, 2016). Not only do different species of Se hyperaccumulators have a wide range of Se concentrations, but also within a species Se levels can vary greatly between and within populations (Feist & Parker, 2001; Zhang *et al.*, 2006; Cappa *et al.*, 2014; El Mehdawi *et al.*, 2015b).

Hyperaccumulators are different from other species not only in levels of Se they can tolerate in their tissues: they also have higher Se to sulfur (S) ratios, more organic Se (particularly methyl-selenocysteine), preferentially take up Se over S, and sequester Se in specific tissues (leaf epidermis and margins) and organs (reproductive organs) (Freeman *et al.*, 2006b; Quinn *et al.*, 2011). The change in form of Se in soils may have particular ecological consequences. Organic Se is more bio-available than inorganic Se (Fordyce, 2013) and may be less toxic to plants.

Investigations into why Se hyperaccumulation evolved, have yielded the bulk of the evidence in support of the "elemental defense hypothesis" (Boyd & Martens, 1992): Se has been shown to protect plants from generalist herbivores as well as some fungal pathogens (Boyd & Martens, 1992; Hanson *et al.*, 2003, 2004, Freeman *et al.*, 2007, 2009; Quinn *et al.*, 2010a). Furthermore, concentration of Se around hyperaccumulators may result in "elemental allelopathy" to other plant species (El Mehdawi et al., 2011a). The term allelopathy, often used for plant-plant interactions, is the capacity of plants to produce a positive or negative effect on other organisms through some chemical means, e.g. the production of secondary plant compounds as a protection from herbivory (Fraenkel, 1959). In the case of "elemental allelopathy" by hyperaccumulators it is the concentration of an element from the soil in plant tissues and its deposition in certain areas that offers the plant benefits. Plants around hyperaccumulators may be

positively or negatively affected by the Se that is concentrated by hyperaccumulators. Proximity to hyperaccumulators is associated with higher Se accumulation in neighboring plants; if these are able to tolerate the Se they are encountering, positive allelopathy may result from physiological benefits (enhanced growth) as well as protection from herbivory (El Mehdawi *et al.*, 2011b, 2014)). However, if they cannot tolerate the Se, they may suffer toxicity (El Mehdawi *et al.*, 2011a).

Hyperaccumulators are changing the distribution and form of Se in soils through assimilation of Se into organic forms and turnover of high-Se root and shoot tissues. Through these processes, they may affect the fitness of other plant species, favoring Se-tolerant individuals and exerting a negative effect on Se-sensitive individuals. Through these various processes, hyperaccumulators may have a disproportionately large effect on their community (relative to their abundance), leading to differences in vegetation patterns relative to communities without hyperaccumulators. The study described here explores this hypothesis. The specific questions addressed are: how do areas with and without hyperaccumulators compare with respect to: 1) soil Se concentration and distribution, 2) overall vegetation properties, and 3) plant species composition? The results from this study give insight into the contribution of Se hyperaccumulators to soil heterogeneity and Se cycling, and the scale of their effects on local plant communities.

Methods

2013

Field data collection

Data collection started on July 16th 2013. There were three sites where data were collected: Pine Ridge Natural Area (PR), Cathy Fromme Prairie Natural Area (CF) and Coyote Ridge Natural Area (CR) (Fig. 2.1-2.4). All sites are northwest of Fort Collins Colorado.

Table. 2.1. GPS coordinates for Pine Ridge Natural Area (PR), Cathy Fromme Prairie Natural Area (CF) and Coyote Ridge Natural Area (CR). For plots "with" hyperaccumulators and those "without" hyperaccumulators.

Site	Latitude	Longitude
PR with	40.545496	-105.133213
PR without	40.542182	-105.132026
CF with	40.519566	-105.124796
CF without	40.519108	-105.124536
CR with	40.480898	-105.125547
CR without	40.481271	-105.125571

Within each of the three sites, there were two plots, one with hyperaccumulators (*Astragalus bisulcatus* and *Stanleya pinnata*) and one without.

In choosing the plot containing hyperaccumulators, the inclusion of the most hyperaccumulators within the plot was the main criterion. In this way the rough size and orientation of the plot were determined. For PR a 90 x 20 m area was laid out and data were collected along five transects (Fig.2.1 far right diagram). Point intercept method (Elzinga *et al.*, 1998) was used for all three sites for data collection at 1m intervals along each of the five transects. Data collection was started for all plots (3 with hyperaccumulators and 3 without), on all sites, in the southwest corner of the plot. Plant species of the first plant canopy "hit" and then the basal "hit" were recorded for each point. The basal hit could have been a plant where it met the soil or bare soil, leaf litter

or a rock. These three were combined in the estimates for bare ground. The paired plot on the same site without hyperaccumulators was selected by two main criteria: 1) It must lack hyperaccumulators within its area. 2) It must have the same size, aspect, slope and orientation. These paired plots without hyperaccumulators are located within each of three sites. The shape of the plot for CF and CR were different: they were 60 m x 20 m (same area) due to geographic limitations. All sites and their relative position are shown in Figures 2.1-2.4. A census of all hyperaccumulator plants was done for all of the three plots with GPS coordinates obtained using a Trimble GPS unit (GEO XH 6000). The GPS data were corrected using Trimble GPS Pathfinder software, and accuracy was found to be within 250 cm for all points. All hyperaccumulator GPS data were entered into ArcGIS (ver.10.5.1) (Figs. 2.1-2.4). In addition to vegetation data, soil samples were collected at each of the six plots. The surface organic matter was pushed aside and a 15 ml tube filled with soil. This soil was then sieved with a 1 mm screen and homogenized. Seventy-two soil samples per plot (both with and without hyperaccumulators) were taken, each within a different 5-x-5 m square within the overall plot. These soil samples were taken at a randomized location within each 5-x-5m square. (Fig. 2.1 far right diagram).

Sample preparation and elemental analysis

Approximately 2 g portions were separated from the sieved and homogenized soil and dried at 50^{o}C for 48 hours. From this 2 g of soil, 400 mg was weighed, placed in a digestion tube (25 mm diameter, 200 mm long) and 2 ml of concentrated ultra-trace grade nitric acid was added to each sample. After addition of the acid, the tubes containing the samples were placed on a heating block and digested at 50^{o}C for 2 hours

followed by 6 hours at 125°C. After digestion was complete, the samples were diluted with distilled water to 10 ml (for a 25 x dilution) transferred to 15 ml conical tubes and sealed until analysis.

Inductively coupled plasma mass spectrometry (ICP-MS) was performed using a Perkin-Elmer Elan DRCII instrument (detection limit for Se is approximately 0.01 ppb) according to the manufacturer's instructions. A standard curve for Se was created using seven concentrations of an ICP-MS Se standard (NIST) with known Se concentrations. A quality control solution was created using 1ml from each of the digested soil, and run every five samples. These quality control measurements were used to correct for sensor drift over the duration of the run (Haugen *et al.*, 2000).

Calculation of vegetation attributes and statistics

After data were compiled, four main vegetative properties were calculated: bare ground (BG), foliar cover (FC), species richness (SR) and relative species abundance (RSA). These were calculated based on the methods in "Measuring and Monitoring Plant Populations" (Elzinga *et al.*, 1998). Statistical analyses were done using R (ver. X64 3.32). Paired t-tests ($\alpha=0.05$) were performed for each of the above vegetative properties comparing plots with hyperaccumulators to those without (Fig. 2.6B-D for BG,FC and SR; Tables 2.2-2.4 for RSA). In addition, a t-test was performed comparing all three plots with hyperaccumulators to the three without ($\alpha=0.05$) (Fig. 2.6A). Soil Se concentration was compared using paired t-tests ($\alpha=0.05$), performed between plots with hyperaccumulators vs. those without (Fig. 2.5).

Soil Se data were entered into ArcGIS (ver. 10.5.1) and the empirical Bayesian kriging tool was used to produce six soil Se interpolation maps (Fig. 2.2-2.4). The interpolation was run with default settings except the number of simulations was increased to 10,000.

2014

Field data collection

A follow-up study was performed the next year beginning July 21st, 2014. This study was performed in three different areas in Pine Ridge Natural Area (Fig. 2.7). In this study, hyperaccumulators were located and a 3 m diameter ring was placed around them. An estimate for canopy cover, bare ground and species richness were recorded by the same person in each plot. A census of all species found in the 3 m ring was performed giving 10 minutes at each plot to equalize the census depth. The youngest mature leaves were sampled from each of the 22 hyperaccumulator plants for Se concentration analysis. The leaf Se concentration can vary in different developmental stages, and the youngest mature leaves are most easily distinguished. In addition to vegetation data, soil samples were taken from the base of each of the central plants as described above. These procedures were carried out in 22 plots with hyperaccumulators at the center and 22 plots with a similarly sized non-hyperaccumulator at the center.

Sample preparation and elemental analysis

Soil samples were prepared and analyzed with ICP-MS using the same procedures as described above for the 2013 soil samples.

In addition to soil, leaf samples were prepared by drying, homogenizing and then weighing 100 mg. This 100 mg sample was placed into a digestion tube with 1 ml of concentrated ultra-trace grade nitric acid. The leaf material was digested as described above for the soil samples. After cooling, the samples were diluted with distilled water to 10 ml. The samples were then analyzed using Inductively coupled plasma optical emission spectrometry (ICP-OES) on the Perkin Elmer Optima 7300 Dual View ICP-OES. The detection limit is 10 ppb; appropriate Se standards were used, and a quality control every 20 samples (Fig. 2.9B).

Calculation of vegetation attributes and statistics

After data were compiled, three main vegetative properties were calculated: bare ground (BG), foliar cover (FC) and species richness (SR). These were calculated based on the methods in “Measuring and Monitoring Plant Populations” (Elzinga *et al.*, 1998). Statistical analyses were done using R (ver. X64 3.32).

Paired t-tests (α=0.05) were performed for each of the three vegetative properties, separating hyperaccumulator plots into *Astragalus bisulcatus* plots and *Stanleya pinnata* plots (Fig. 2.8).Soil Se data were analyzed by performing paired t-tests (with hyperaccumulators vs. without) on the three areas, both separately and combined (Fig. 2.9A). T-tests were performed comparing Areas 1, 2 and 3 (Fig. 2.7) as well as *A. bisulcatus* and *S. pinnata* (α=0.05). Finally, correlation analysis was done for BG, CC and SR relative to Se in soil and Se in the hyperaccumulator (α=0.05) (Figs. 2.10 and 2.11).

2013 Field Survey

The experimental approach to investigate the effects of Se hyperaccumulators on local Se distribution and surrounding vegetation was paired comparison of plots with and without hyperaccumulators in the same area. The 2013 field season focused on three sites: Pine Ridge Natural Area (Pine Ridge), Coyote Ridge Natural Area (Coyote Ridge) and Cathy Fromme Prairie Natural Area (Cathy Fromme). On each of the three sites a plot was selected that contained *A. bisulcatus* and *S. pinnata* hyperaccumulators, and a same-size plot nearby that did not. Figure 2.1 shows an overview map in the far left panel giving an indication of the relative positions of the sites. All are located on the eastern edge of the foothills of the Rocky Mountains near Fort Collins, Colorado, along a North-South stretching seleniferous Pierre shale formation (https://ngmdb. usgs.gov/Prodesc/proddesc_68589.htm). In the middle panel of Figure 2.1, each of the three sites can be seen more closely, showing the relative position of the “with” and “without” plots within each site. The far right panel of Figure 2.1 shows a diagram of an individual plot (could be “with” or “without”), illustrating how vegetation and soil was surveyed and sampled. On the far left of the diagram, indicated by the arrow at the bottom, is an example of a transect. This transect has hash marks representing the frequency (every 1 m) at which a canopy and soil surface plant species observational data were taken. The other vertical lines represent the other transects where data were collected in the same way. The filled black circles represent the random locations in each 5x5 m quadrant where soil samples were taken.

Soil elemental analysis data that were collected from these locations were used to create extrapolated maps of Se distribution in each plot, using ArcGIS (version 10.5.1).

Results

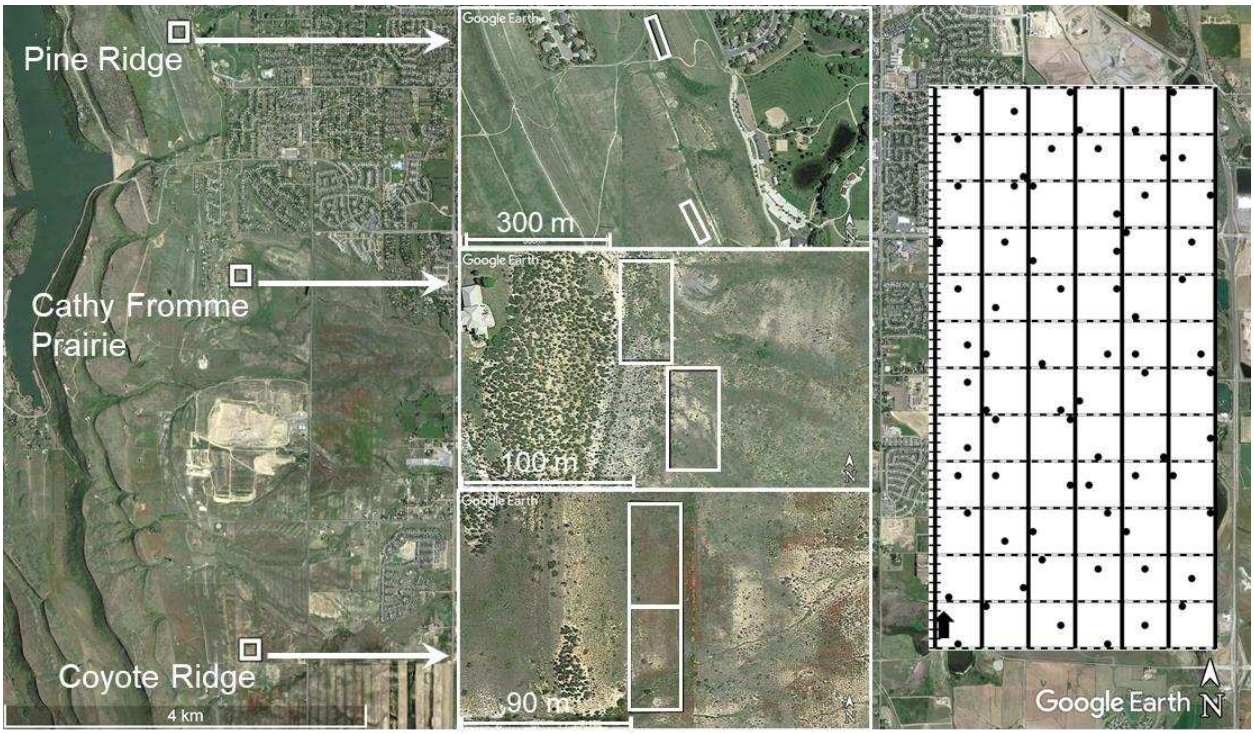

Fig. 2.1. Overview of 2013 survey. *Left*: Overview of the three field sites west and southwest of Fort Collins, Colorado. *Middle*: Closer view of the three sites individually with the rectangles representing the field sites. The northern most rectangle, in all three cases, is the site with hyperaccumulators. *Right*: A diagram of the field site each square of the grid is 5 x 5 m. The black dots within each of the squares is the random location where a soil sample was taken. The arrow on the southwest corner represents the starting point at which the line point sampling survey began, and continued at 1 m intervals south to north along each north to south line.

At Pine Ridge (Fig. 2.2), the soil Se levels ranged from 2.0 - 23.4 µg/g in the plot with hyperaccumulators (A) and 1.33 - 2.80 µg/g in the plot without hyperaccumulators (B). All these Se levels are indicative of seleniferous soil (Kabata-Pendias, 2011). However, the plot containing hyperaccumulators had higher Se concentration overall, and the observed range in soil Se concentration was larger in the plot with hyperaccumulators than in the one without, indicating more soil Se heterogeneity. The majority of hyperaccumulators were located in the intermediate range of the extrapolated soil Se levels (Fig. 2.2A), with a few exceptions: One *S. pinnata* (middle

right) was growing in the higher Se area (darker red color), and the four northernmost individuals of *S. pinnata* were growing in a lower-Se area (indicated in blue).

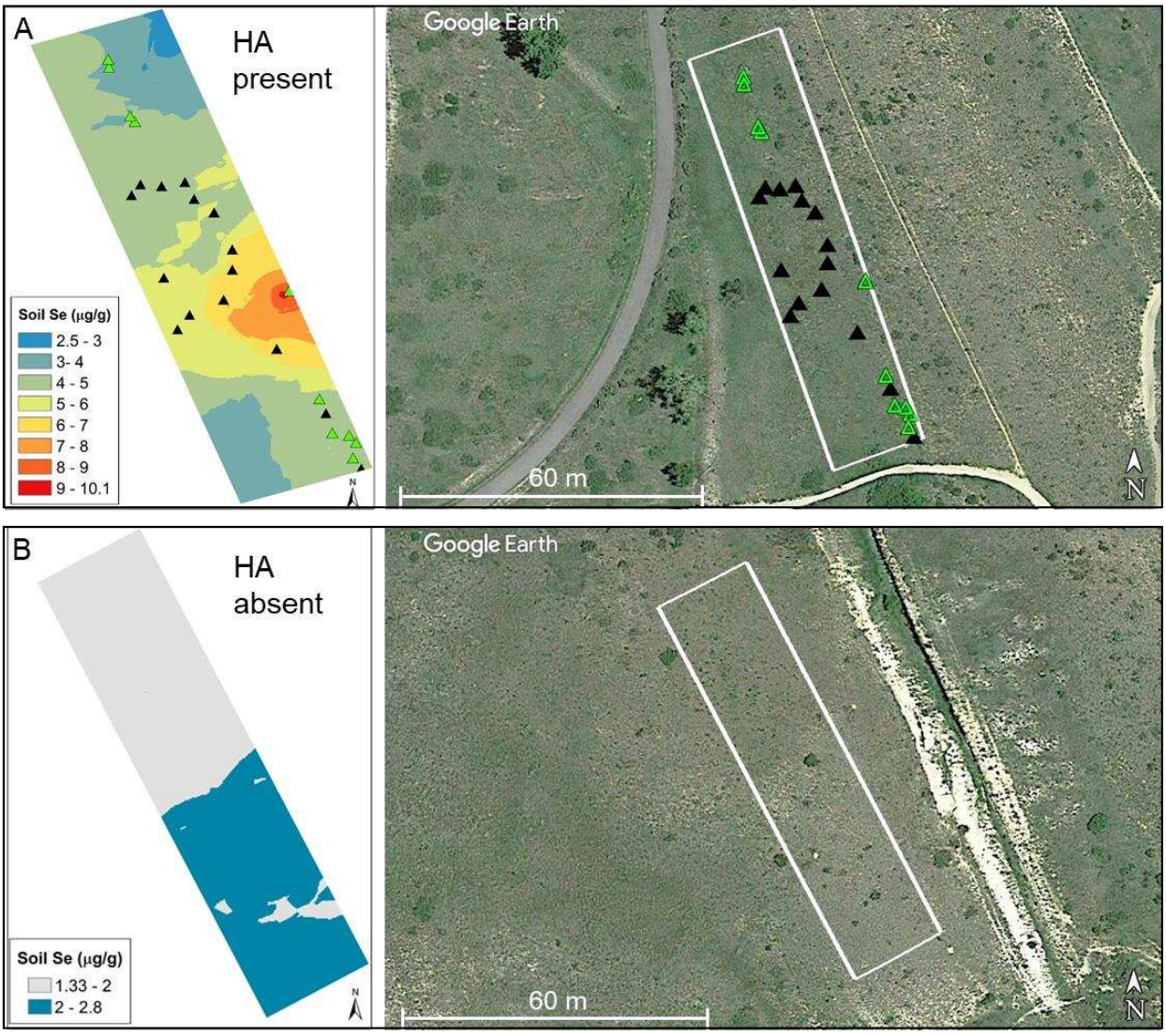

Fig. 2.2. 2013 survey of soil Se concentration and occurrence of Se hyperaccumulators on Pine Ridge Natural Area. A: Plot where hyperaccumulators occur, as indicated by triangles (black: *A. bisulcatus,* green: *S. pinnata)*. *B*: Plot without hyperaccumulators. *Left:* Extrapolated soil Se concentration maps; r*ight:* Satellite image of the plot locations.

At the second site, Cathy Fromme Prairie, the majority of the hyperaccumulators from both species occurred in the intermediate range of soil Se concentrations (Fig. 2.3A); a single *S. pinnata* was found in the higher Se range (farthest east) and seven of

the 18 *S. pinnata* were in the lower Se range. There was a greater range in soil Se concentration in the Cathy Fromme plot with hyperaccumulators, ranging from 3.6 - 23.8 µg/g (Fig. 2.3A), than in the plot without hyperaccumulators (1.6 - 11.7 µg/g, Fig. 2.3B), again indicating greater heterogeneity in soil Se levels in the plot with hyperaccumulators. Both plots are seleniferous, judged from these data. At Coyote Ridge, the third site, the majority of the hyperaccumulators were found in the middle to high end of the soil Se range for that plot, with a single plant (the northernmost) found in the lower Se area of the plot (Fig. 2.4A). The soil Se range for the plot with hyperaccumulators was 0.9 - 2.2 µg/g (Fig. 2.4A) and the range for the plot without was 3.5 - 6.4 µg/g (Fig. 2.4B). Thus, at Coyote Ridge soil Se levels were not as high compared to the other two sites (Fig. 2.2, 2.3). Furthermore, Coyote Ridge soil Se levels were lower and showed a smaller range in the plot with hyperaccumulators than in the plot without (Fig. 2.4A, B), opposite to the findings from Pine Ridge and Cathy Fromme. Also, while the hyperaccumulators at Pine Ridge and Cathy Fromme were most often found in areas with soil Se ranging from 4.5 - 6.5 µg/g, at Coyote Ridge they were absent in the plot that had soil in this Se range, but rather occurred on soil with Se levels between 0.9 -2.2 µg/g. On each of the three sites, average soil Se levels were significantly ($P < 0.05$) different between the plot containing hyperaccumulators (A) and the plot without (B) (Fig. 2.5). At Pine Ridge, soil Se concentration in the plot with hyperaccumulators was 2.7-fold higher compared to the corresponding plot without

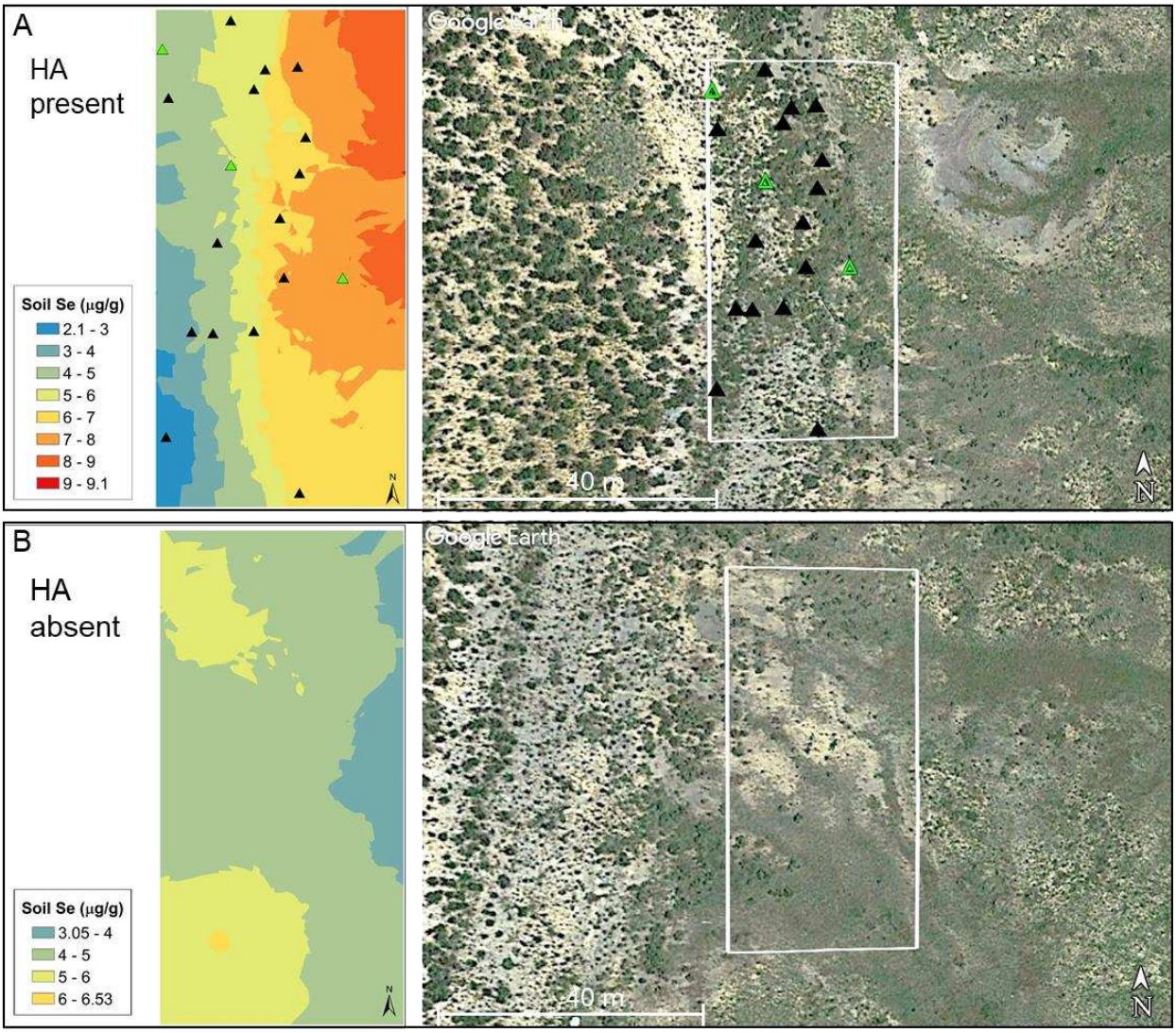

Fig. 2.3. 2013 survey of soil Se concentration and occurrence of Se hyperaccumulators on Cathy Fromme Prairie Natural Area. A: Plot where hyperaccumulators occur, as indicated by triangles (black: *A. bisulcatus,* green: *S. pinnata)*. *B*: Plot without hyperaccumulators. *Left:* Extrapolated soil Se concentration maps; r*ight:* Satellite image of the plot locations.

hyperaccumulators (4.8 vs. 1.8 µg/g), and at Cathy Fromme soil Se was 1.5-fold higher in the plot with hyperaccumulators than the one without (6.9 vs. 4.6 µg/g). At CoyoteRidge the difference in soil Se levels was 3-fold, with the hyperaccumulator-

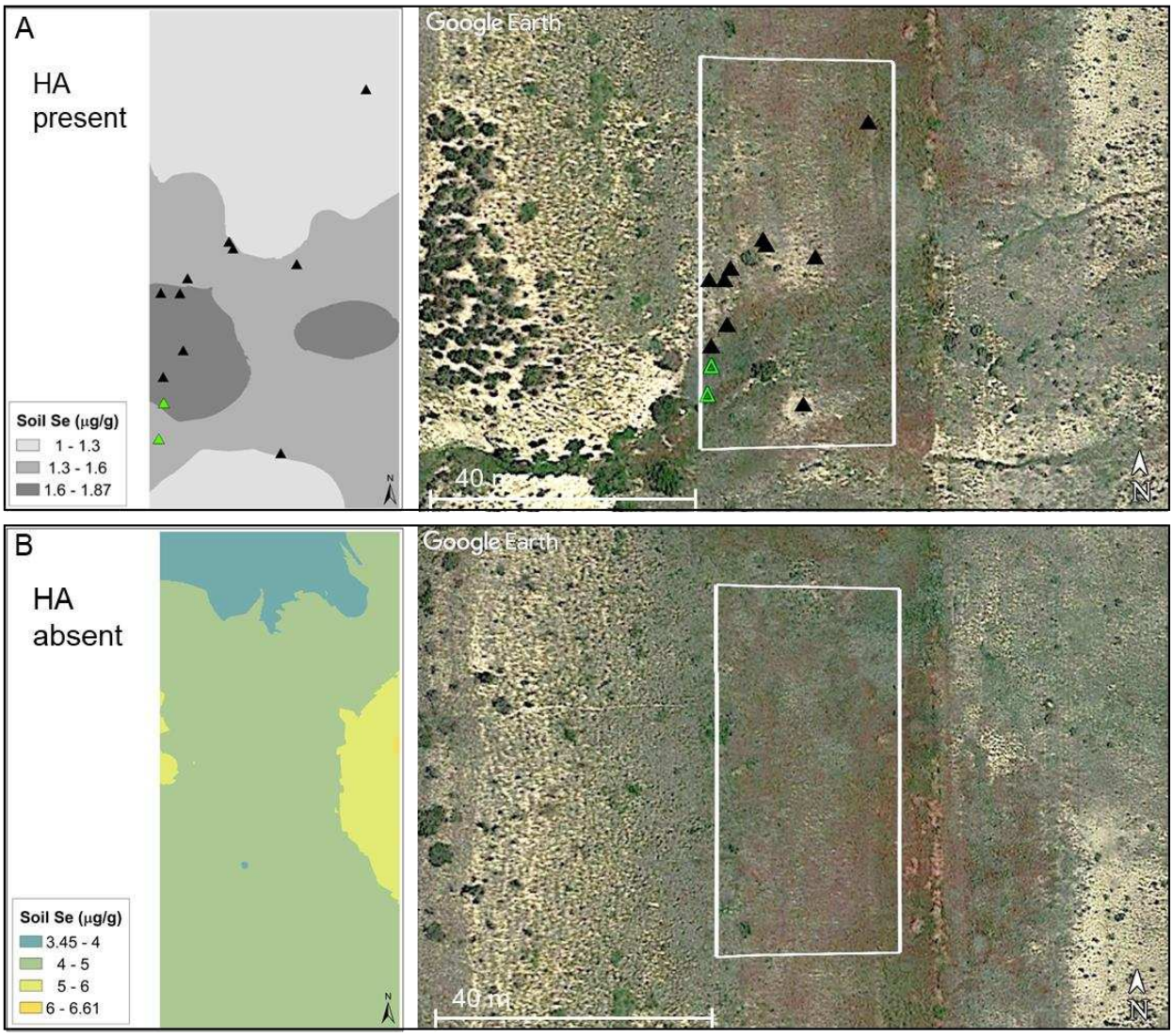

Fig. 2.4. 2013 survey of soil Se concentration and occurrence of Se hyperaccumulators on Coyote Ridge Natural Area. A: Plot where hyperaccumulators occur, as indicated by triangles (black: *A. bisulcatus,* green: *S. pinnata)*. *B*: Plot without hyperaccumulators. *Left:* Extrapolated soil Se concentration maps; *right:* Satellite image of the plot locations.

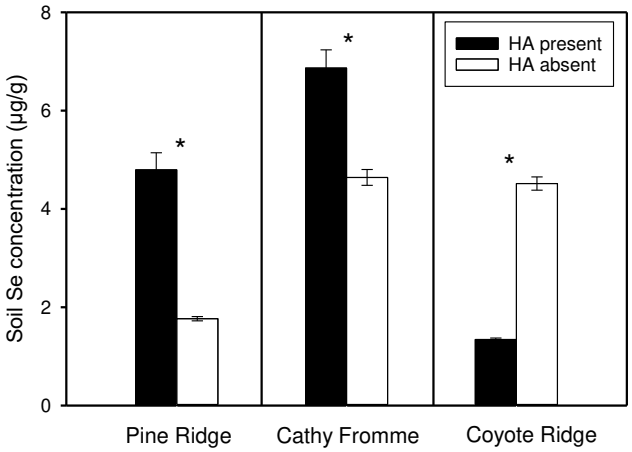

Fig. 2.5. 2013 data for average soil Se concentrations for the three sites. Asterisks indicate statistically significant differences between plot with and without hyperaccumulators ($P<5*10^{-8}$).

containing plot on averaging 1.3 µg/g and the one without hyperaccumulators 4.5 µg/g (Fig. 2.5).

Next, the potential effects of hyperaccumulators on vegetation properties were investigated. Transect data were used to calculate percentage of bare ground and foliar cover as well as species richness for each of the six plots (with or without hyperaccumulators on three sites). When averaged across all three sites (Fig. 2.6A), the apparent (but non-significant) trends were relatively more bare ground (12%) in plots with hyperaccumulators than in the plots without (7%), and less foliar cover (62%

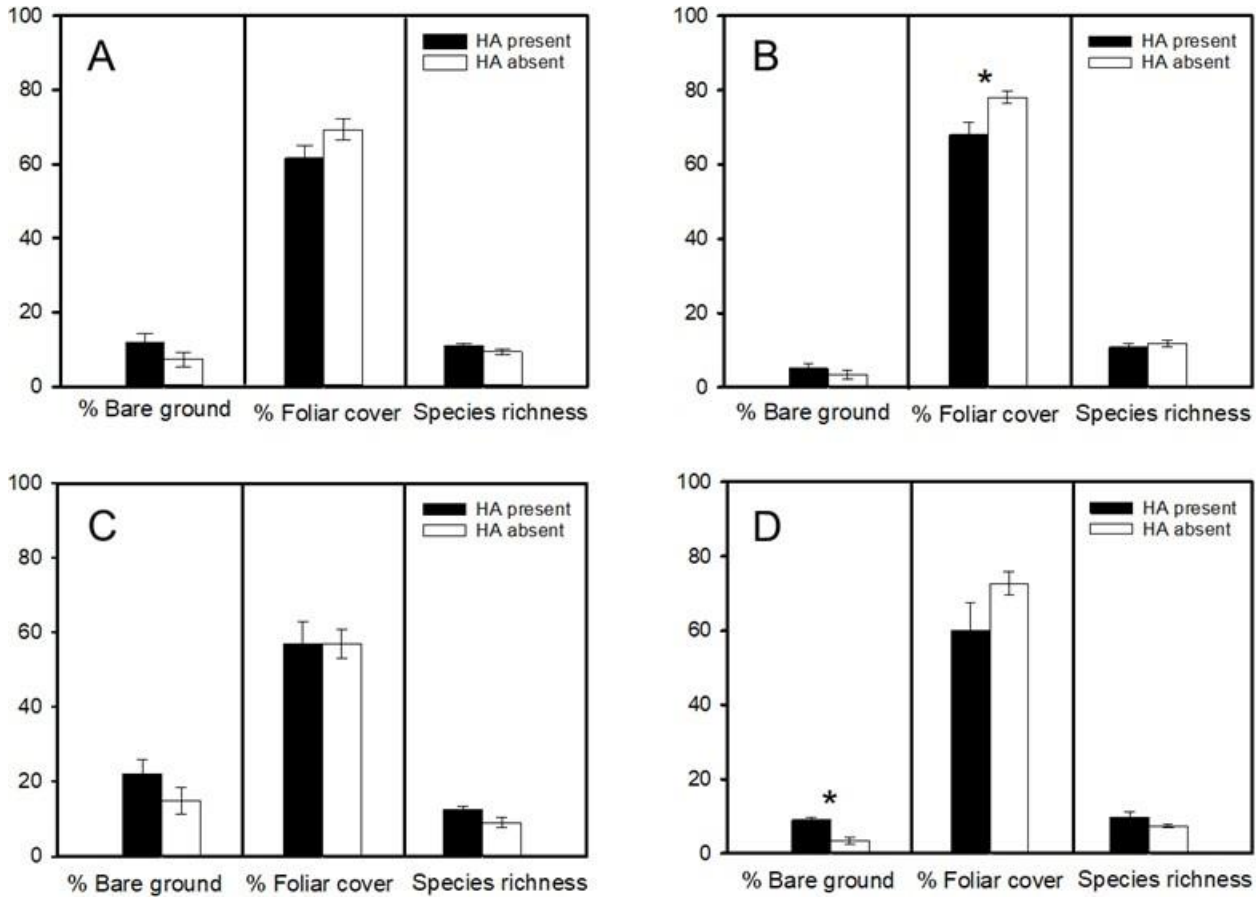

Fig. 2.6. 2013 vegetation survey data (bare ground, foliar cover, species richness) on three sites, each consisting of two plots, one with hyperaccumulators occurring, and one without. *A*: All three sites combined; *B*: Pine Ridge Natural Area; *C*: Cathy Fromme Prairie Natural Area; *D*: Coyote Ridge Natural Area. Asterisk indicates statistically significant differences between plot with and without hyperaccumulators ($P<0.05$), otherwise not significant.

vs. 69%). Also, average species richness tended to be higher in the plot with (11) than in the plots without (9.4) hyperaccumulators (Fig 2.6A, NS). For each of the parameters, two out of three sites supported these trends (Fig. 2.6B-D), but they were only significant for foliar cover at Pine Ridge (Fig. 6B), and percentage bare ground at Coyote Ridge (Fig. 2.6D).

Relative species abundance of all plant species in the vegetation were compared between the respective with and without hyperaccumulator plots for all three sites (Tables 2.2-2.4). At each of the three sites, the presence of hyperaccumulators

coincided with a difference in abundance for several species; some species showed positive co-occurrence and others negative co-occurrence with hyperaccumulators. At Pine Ridge, five species (*Hesperostipa comata*, *Bouteloua curtipendula*, *Senecio sp*., *Eriogonum divaricatum* and *Tragia ramosa*) stood out for being found more frequently in plots without hyperaccumulators than in plots with (negative co-occurrence), and four species (*Symphyotrichum ericoides, Artemisia ludoviciana, Pascopyrum smithii, Nassella viridula*) because they were found more frequently in plots with hyperaccumulators than in plots without (positive co-occurrence) (Table 2.2). At Cathy Fromme, there were four species (*Pascopyrum smithii*, *Tragopogon dubius*, *Convolvulus arvensis, and Alyssum alyssoides*) found more frequently in plots without than in plots with hyperaccumulators, and five species (*Comandra umbellata*, *Helianthus pumilus*, *Achnatherum hymenoides*, *Hesperostipa comata*, and *Astragalus bisulcatus*) found more frequently in plots with hyperaccumulators than in plots without (Table 2.4). At Coyote Ridge, one species (*Pascopyrum smithii*) was found more frequently in plots without hyperaccumulators than in plots with, and two species (*Tragopogon dubius* and *Lathyrus eucosmus*) were found more frequently in plots with hyperaccumulators than in plots without (Table 2.4).

Table 2.2. Pine Ridge Natural Area 2013 comparisons of relative species abundance (RSA), for species in plots with vs. plots without hyperaccumulators. P values were obtained from a t-test comparing average raw occurrence counts between the plot with hyperaccumulators to the one without (n=5 transects).

Species	Average times found per transect (relative species abundance, RSA)		RSA Ratio	P=
	HA present	**HA** absent	**+HA/-HA**	
A. Less abundant on HA plot				
Hesperostipa comata	14.4 ± 4.6 (23.5)	43.8 ± 1.6 (65.8)	0.4	**0.0003**
Bouteloua curtipendula	0.2 ± 0.2 (0.3)	2.2 ± 0.5 (3.3)	0.1	**0.0054**
Eriogonum divaricatum	0.0 ± 0.0 (0.0)	0.8 ± 0.4 (1.2)	0.0	**0.0650**
Tragia ramosa	0.0 ± 0.0 (0.0)	0.8 ± 0.4 (1.2)	0.0	**0.0650**
Evolvulus nuttallianus	0.0 ± 0.0 (0.0)	0.6 ± 0.4 (0.9)	0.0	0.1720
Alyssum alyssoides	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.3)	0.0	0.3466
Bromus arvensis	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.3)	0.0	0.3466
Liatris punctata	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.3)	0.0	0.3466
Mentzelia decapetala	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.3)	0.0	0.3466
Psoralidium tenuiflorum	0.0 ± 0.0 (0.0)	0.4 ± 0.4 (0.6)	0.0	0.3466
Schizachyrium scoparium	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.3)	0.0	0.3466
Pseudoroegneria spicata	2.4 ± 1.2 (3.9)	3.2 ± 1.4 (4.8)	0.8	0.6713
Yucca glauca	2.6 ± 1.7 (4.2)	3.0 ± 1.0 (4.5)	0.9	0.8457
Helianthus pumilus	2.4 ± 0.6 (3.9)	2.6 ± 0.9 (3.9)	1.0	0.8608
B. More abundant on HA plot				
Symphyotrichum ericoides	2.8 ± 0.9 (4.6)	0.0 ± 0.0 (0.0)	N/A	**0.0116**
Artemisia ludoviciana	0.6 ± 0.2 (1.0)	0.0 ± 0.0 (0.0)	N/A	**0.0400**
Pascopyrum smithii	5.8 ± 2.1 (9.5)	1.2 ± 0.4 (1.8)	5.3	**0.0662**
Nassella viridula	11.6 ± 4.0 (19)	3.4 ± 1.2 (5.1)	3.7	**0.0873**
Astragalus bisulcatus	0.4 ± 0.2 (0.7)	0.0 ± 0.0 (0.0)	N/A	0.1411
Achnatherum hymenoides	1.0 ± 0.6 (1.6)	0.0 ± 0.0 (0.0)	N/A	0.1525
Bromus inermis	12.6 ± 7.9 (20.6)	0.4 ± 0.2 (0.6)	34.3	0.1599
Asclepias pumila	0.2 ± 0.2 (0.3)	0.0 ± 0.0 (0.0)	N/A	0.3466
Convolvulus arvensis	0.2 ± 0.2 (0.3)	0.0 ± 0.0 (0.0)	N/A	0.3466
Oenothera suffrutescens	0.2 ± 0.2 (0.3)	0.0 ± 0.0 (0.0)	N/A	0.3466
Opuntia polyacantha	0.2 ± 0.2 (0.3)	0.0 ± 0.0 (0.0)	N/A	0.3466
Tragopogon dubius	0.2 ± 0.2 (0.3)	0.0 ± 0.0 (0.0)	N/A	0.3466
Bouteloua gracilis	0.8 ± 0.6 (1.3)	0.4 ± 0.2 (0.6)	2.2	0.5447
Bouteloua dactyloides	0.6 ± 0.4 (1.0)	0.4 ± 0.2 (0.6)	1.6	0.6811
Rhus trilobata	1.8 ± 0.9 (2.9)	1.6 ± 0.8 (2.4)	1.2	0.8743
Artemisia frigida	0.2 ± 0.2 (0.3)	0.2 ± 0.2 (0.3)	1.1	1.0000

Table 2.3. Cathy Fromme Prairie Natural Area 2013 comparisons of relative species abundance (RSA), for species in plot with vs. plot without hyperaccumulators. P values were obtained from a t-test comparing average raw occurrence counts between the plot with hyperaccumulators to the one without (n=5 transects).

Species	Average times found per transect (relative species abundance, RSA)		RSA Ratio	P=
	HA present	**HA** absent	+HA/-HA	
A. Less abundant on HA plot				
Pascopyrum smithii	3.4 ± 2.5 (10)	19.8 ± 1.9 (57.9)	0.2	**0.0008**
Tragopogon dubius	0.0 ± 0.0 (0.0)	0.8 ± 0.4 (2.3)	0.0	**0.0650**
Convolvulus arvensis	0.2 ± 0.2 (0.6)	1.0 ± 0.3 (2.9)	0.2	**0.0650**
Rosa woodsii	0.0 ± 0.0 (0.0)	0.4 ± 0.2 (1.2)	0.0	0.1411
Nassella viridula	0.4 ± 0.4 (1.2)	1.6 ± 0.8 (4.7)	0.3	0.2217
Bouteloua dactyloides	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.6)	0.0	0.3466
Dalea purpurea	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.6)	0.0	0.3466
Sphaeralcea coccinea	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.6)	0.0	0.3466
Sporobolus cryptandrus	0.0 ± 0.0 (0.0)	0.6 ± 0.6 (1.8)	0.0	0.3466
B. More abundant on HA plot				
Comandra umbellata	1.8 ± 0.6 (5.3)	0.2 ± 0.2 (0.6)	9.1	**0.0318**
Helianthus pumilus	2.0 ± 0.7 (5.9)	0.2 ± 0.2 (0.6)	10.1	**0.0400**
Achnatherum hymenoides	3.0 ± 0.7 (8.8)	1.0 ± 0.5 (2.9)	3.0	**0.0558**
Hesperostipa comata	1.6 ± 0.8 (4.7)	0.0 ± 0.0 (0.0)	N/A	**0.0844**
Astragalus bisulcatus	1.8 ± 0.9 (5.3)	0.0 ± 0.0 (0.0)	N/A	**0.0851**
Astragalus tenellus	1.8 ± 1.0 (5.3)	0.0 ± 0.0 (0.0)	N/A	0.1005
Cercocarpus montanus	0.6 ± 0.4 (1.8)	0.0 ± 0.0 (0.0)	N/A	0.1720
Symphyotrichum ericoides	1.6 ± 0.9 (4.7)	0.2 ± 0.2 (0.6)	8.0	0.1783
Ericameria nauseosa	8.4 ± 2.9 (24.7)	3.4 ± 2.2 (9.9)	2.5	0.2032
Rhus trilobata	0.8 ± 0.5 (2.4)	0.2 ± 0.2 (0.6)	4.0	0.2897
Atriplex canescens	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.3466
Bromus arvensis	0.8 ± 0.8 (2.4)	0.0 ± 0.0 (0.0)	N/A	0.3466
Elymus elymoides	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.3466
Gutierrezia sarothrae	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.3466
Liatris punctata	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.3466
Mentzelia decapetala	0.4 ± 0.4 (1.2)	0.0 ± 0.0 (0.0)	N/A	0.3466
Solidago missouriensis	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.3466
Lathyrus eucosmus	0.6 ± 0.4 (1.8)	0.2 ± 0.2 (0.6)	3.0	0.3972
Aristida purpurea	1.4 ± 0.2 (4.1)	1.0 ± 0.4 (2.9)	1.4	0.4554
Bromus inermis	1.0 ± 0.4 (2.9)	0.6 ± 0.4 (1.8)	1.7	0.5237
Alyssum simplex	0.4 ± 0.2 (1.2)	0.2 ± 0.2 (0.6)	2.0	0.5447

Table 2.4. Coyote Ridge Natural Area 2013 comparisons of relative species abundance (RSA), for species in plot with vs. plot without hyperaccumulators. P values were obtained from a t-test comparing average raw occurrence counts between the plot with hyperaccumulators to the one without (n=5 transects).

Species	Average times found per transect (relative species abundance, RSA)		RSA Ratio	P=
	HA present	**HA** absent	+HA/-HA	
A. Less abundant on HA plot				
Pascopyrum smithii	2.2 ± 0.7 (6.1)	5.8 ± 1.7 (13.4)	0.5	**0.09**
Artemisia frigida	0.4 ± 0.2 (1.1)	1.8 ± 0.9 (4.1)	0.3	0.16
Bromus arvensis	1.4 ± 0.5 (3.9)	4.4 ± 2.5 (10.1)	0.4	0.27
Nassella viridula	19.0 ± 3.8 (52.8)	23.8 ± 2.8 (54.8)	1.0	0.34
Achnatherum robustum	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.5)	0.0	0.35
Convolvulus arvensis	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.5)	0.0	0.35
Ericameria nauseosa	0.0 ± 0.0 (0.0)	0.2 ± 0.2 (0.5)	0.0	0.35
Rhus trilobata	0.2 ± 0.2 (0.6)	1.0 ± 1.0 (2.3)	0.2	0.46
Psoralidium tenuiflorum	0.8 ± 0.4 (2.2)	1.2 ± 0.5 (2.8)	0.8	0.53
Pseudoroegneria spicata	0.2 ± 0.2 (0.6)	0.4 ± 0.2 (0.9)	0.6	0.54
Bouteloua curtipendula	0.2 ± 0.2 (0.6)	0.4 ± 0.4 (0.9)	0.6	0.67
B. More abundant on HA plot				
Tragopogon dubius	0.6 ± 0.2 (1.7)	0.0 ± 0.0 (0.0)	N/A	**0.04**
Lathyrus eucosmus	1.4 ± 0.7 (3.9)	0.0 ± 0.0 (0.0)	N/A	**0.10**
Oenothera suffrutescens	0.4 ± 0.2 (1.1)	0.0 ± 0.0 (0.0)	N/A	0.14
Achnatherum hymenoides	0.4 ± 0.4 (1.1)	0.0 ± 0.0 (0.0)	N/A	0.35
Alyssum simplex	0.4 ± 0.4 (1.1)	0.0 ± 0.0 (0.0)	N/A	0.35
Bromus inermis	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.35
Evolvulus nuttallianus	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.35
Gutierrezia sarothrae	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.35
Mentzelia decapetala	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.35
Stanleya pinnata	0.2 ± 0.2 (0.6)	0.0 ± 0.0 (0.0)	N/A	0.35
Bouteloua dactyloides	2.2 ± 1.4 (6.1)	0.8 ± 0.6 (1.8)	3.3	0.39
Symphyotrichum ericoides	0.4 ± 0.2 (1.1)	0.2 ± 0.2 (0.5)	2.4	0.54
Hesperostipa comata	4.2 ± 2.5 (11.7)	2.4 ± 2.4 (5.5)	2.1	0.61
Aristida purpurea	0.2 ± 0.2 (0.6)	0.2 ± 0.2 (0.5)	1.2	1.00
Helianthus pumilus	0.2 ± 0.2 (0.6)	0.2 ± 0.2 (0.5)	1.2	1.00
Rosa woodsii	0.2 ± 0.2 (0.6)	0.2 ± 0.2 (0.5)	1.2	1.00

2014 Field Survey

The results from the 2013 study revealed some interesting patterns, but these were seldom significant. This was perhaps due to the experimental setup, studying very large plots with sparse hyperaccumulator vegetation. To explore the effects of hyperaccumulators on local Se distribution and vegetation at a smaller spatial scale, in 2014 a follow-up study was conducted at one of the previously studied sites, Pine Ridge Natural Area, focusing this time on areas immediately surrounding 22 hyperaccumulator plants, comparing them with nearby similar areas surrounding 22 non-hyperaccumulators (3 m diameter paired plots).

Soil Se concentration was determined at the center of the plots, and used to create an extrapolated soil Se concentration map for the entire region over which the samples were taken (Fig. 2.7). This revealed roughly three areas (1, 2 and 3 in Fig. 2.7). Area 2, which was the Pine Ridge site used in 2013 had higher Se levels than 1 and 3 (Fig. 2.7, 2.8A). There were clear hyperaccumulator-associated differences in soil Se levels.

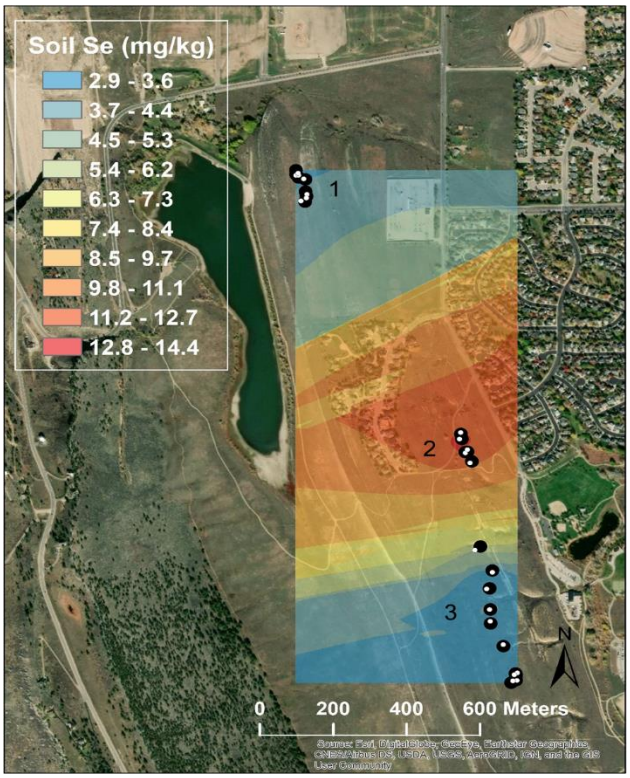

Fig. 2.7: Overview of 2014 field site, Pine Ridge Natural Area, just west of Fort Collins, Colorado, with sub-areas 1,2 and 3 (sub-area 2 was also surveyed in 2013). Color overlay shows an extrapolation of the soil selenium levels using 2014 data. Each black circle represents a 3m diameter circle containing *A. bisulcatus* or *S. pinnata* (hyperaccumulators). The smaller white circle represents the location of the paired 3m diameter circle without a hyperaccumulator present (which was adjacent, not overlapping).

Across the entire sampling area, soil Se ranged from 1.7 - 34.7 µg/g in plots with hyperaccumulators and from 0.8 - 16.1 µg/g in plots without hyperaccumulators, and on average the Se levels were ~2-fold higher in soil close to hyperaccumulators than that close to the paired control plant (Fig. 2.7, 2.8A). This hyperaccumulator effect was significant across the entire site (areas 1, 2 and 3 combined) as well as for individual areas 1 and 3; area 2 showed more variance, but the same trend (Fig. 2.8A, asterisks). Leaf Se concentration in the hyperaccumulator species revealed a geographic pattern that mimicked that in the soil, with plants in area 2 having 2-3 fold higher Se concentration than those in areas 1 and 3 (Fig 2.8B).

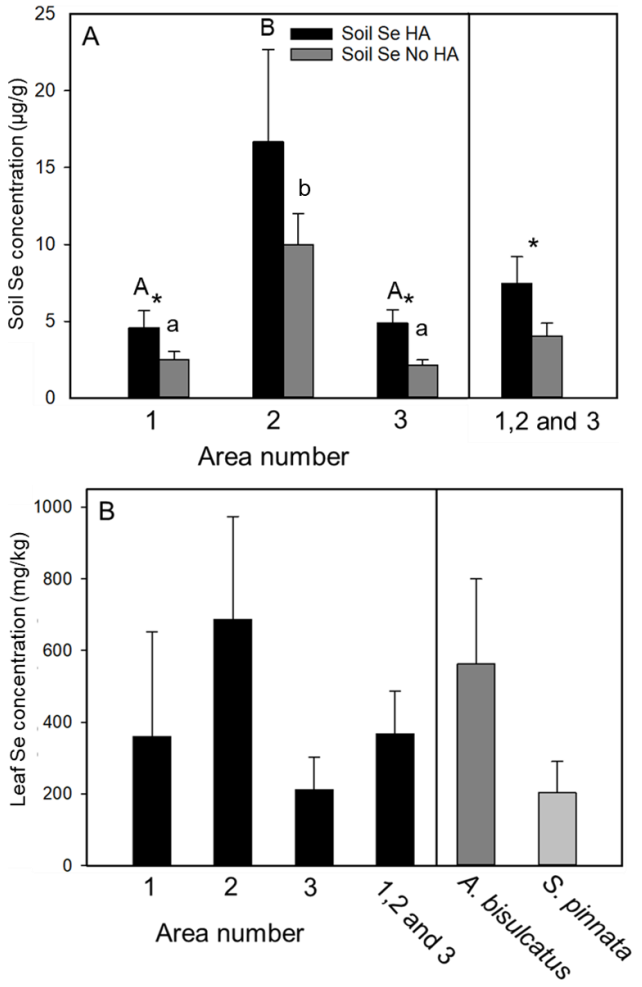

Fig. 2.8. 2014 survey of soil and hyperaccumulator Se concentrations at Pine Ridge Natural Area. *A:* Soil Se concentration for areas 1, 2 and 3 (indicated on Fig. 7 map) or all combined. Asterisks indicate significant ($P<0.05$) difference between plots with hyperaccumulators (HA) and plots without. Letters indicate statistically significant differences between areas 1, 2 and 3 for plots with hyperaccumulators (upper case) or without (lower case). *B:* Leaf Se concentration for Se hyperaccumulators *A. bisulcatus* and *S. pinnata* in the 3 different areas and combined.

Leaf Se concentration in hyperaccumulator species revealed a geographic pattern that mimicked that in soil, with plants in area 2 having 2-3 fold higher Se concentration than those in areas 1 and 3 (Fig 2.8B). However, correlation analysis showed no significant correlation between Se concentration in soil and plant across the site (not shown). The two hyperaccumulator species differed in Se accumulation: *Astragalus bisulcatus* had on average 3-fold higher leaf Se levels than *Stanleya pinnata* (Fig. 2.8B).

Next, the vegetation parameters percentage bare ground, foliar cover and species richness were determined and compared between plots with hyperaccumulators and those without hyperaccumulators (Fig. 2.9). For plots containing either *A. bisulcatus* (Fig. 2.9A) or *S. pinnata* (Fig. 2.9B), the results revealed the same statistically significant difference: there was relatively more bare ground, less canopy cover, and higher species richness in the area around hyperaccumulators than around non-hyperaccumulator plants. No correlations with soil Se or plant Se concentration were found for any of the three vegetation parameters (Fig. 2.10 and 2.11).

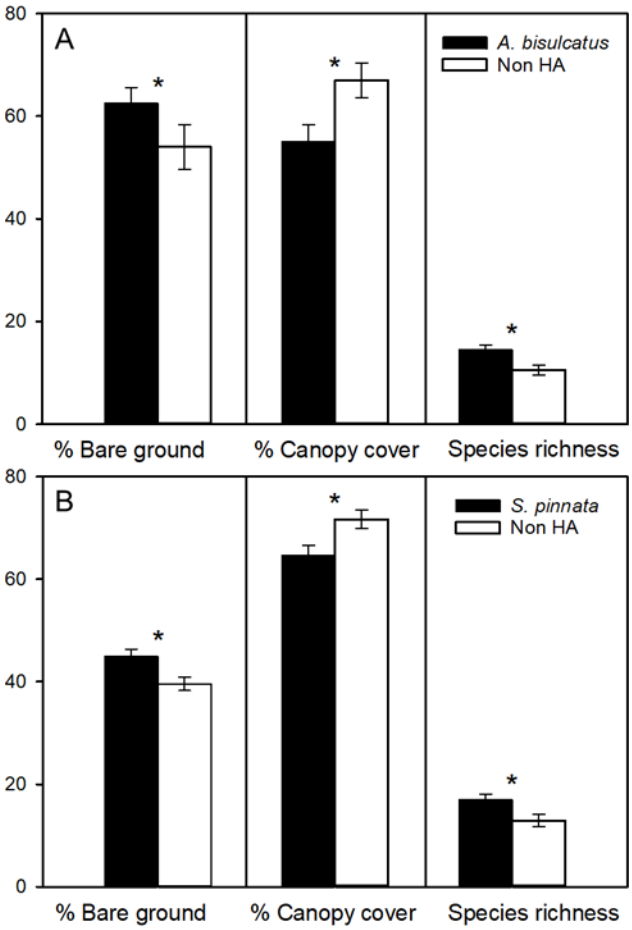

Fig. 2.9: 2014 vegetation survey data (% bare ground, % canopy cover and species richness) at Pine Ridge Natural Area, comparing pairs of 3 m diameter plots with a hyperaccumulator (HA) in its center or a non-hyperaccumulator (n=22 pairs of plots). *A*: plots with *A. bisulcatus* as the central hyperaccumulator. *B*: plots with *S. pinnata* as the central plant. Asterisk indicates statistically significant ($P<0.05$) differences between plots with hyperaccumulators at the center (black bar) and those with a non-hyperaccumulator in the center (white bar).

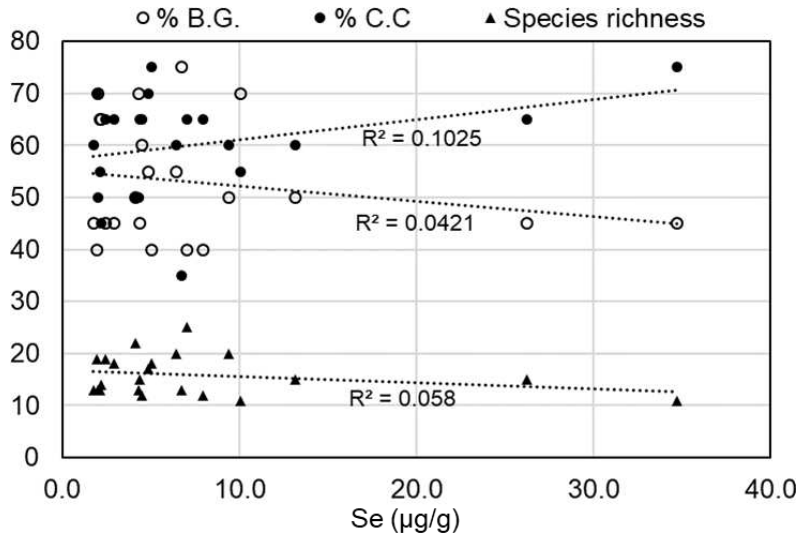

Fig. 2.10. Correlation of soil Se concentration to % bare ground (white circles), % canopy cover (black circles) and species richness (black triangles) for plots with hyperaccumulators (NS).

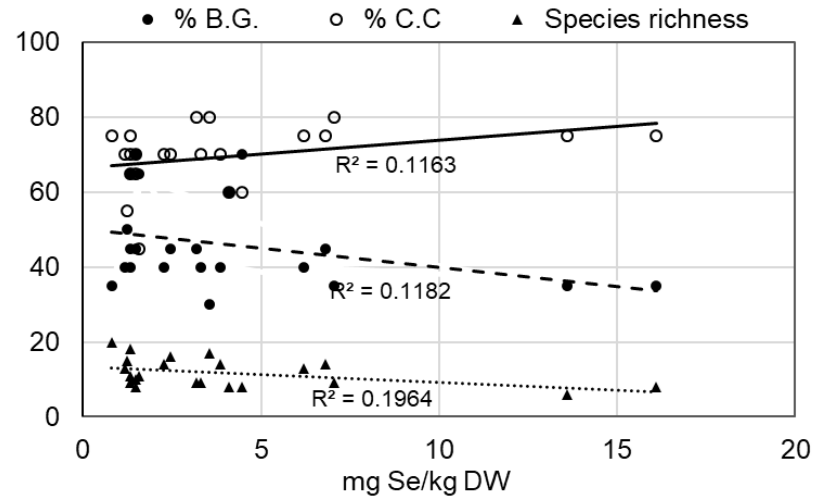

Fig. 2.11. Correlation of plant Se concentration to % bare ground (white circles), % canopy cover (black circles) and species richness (black triangles) for plots with hyperaccumulators (NS).

Discussion

Past ecological studies in our lab have found that Se in hyperaccumulators can protect them from herbivory, through deterrence and toxicity, and elevated Se levels around hyperaccumulators (presumably from high-Se litter) can have allelopathic

effects, both positive and negative, on surrounding plants. In particular, a lab study showed that germination of *Arabidopsis thaliana* seeds was inhibited by soil from around hyperaccumulators as opposed to soil from around non-hyperaccumulators from the same area (El Mehdawi et al., 2011a). At the same time, there was evidence that Se-resistant herbivores and neighboring plants may benefit from their association with Se hyperaccumulators, being able to exclusively utilize the special niches they offer (El Mehdawi *et al*., 2011d). The implication drawn from those studies is that hyperaccumulators may have a disproportionately large impact on their local ecology (relative to non-hyperaccumulators), negatively affecting Se-sensitive ecological partners while favoring Se-resistant ones. This hypothesis is the primary focus of this work. Rather than focusing on single species ecological interactions, this work studied plant interactions at the community level. Hyperaccumulators were hypothesized to influence vegetative cover qualitatively and quantitatively, leading to a community that has relatively more Se-resistant species. The sphere of influence of individual hyperaccumulator plants was unknown, and investigated in the process.

Two observational studies (2013 and 2014) were performed to investigate the effects of Se hyperaccumulators on local Se distribution and surrounding vegetation. Both methods used comparison of paired plots with and without hyperaccumulators in the same area. The scale of the 2013 study was much larger than that of the second year, which focused on the immediate surroundings of the hyperaccumulator. Data on vegetative characteristics from both years showed similar trends, which were significant only in the second season. The area around hyperaccumulators had relatively more bare ground, less canopy cover and more vegetative species richness. The soil around

hyperaccumulators was found to have elevated levels of Se in both studies, as compared to soil further away from them. This may in part be responsible for the observed differences in the local vegetation. Therefore, these findings regarding overall plant community parameters agreed well with earlier studies of interactions between hyperaccumulators and individual neighbor plants, and generally agreed with the hypothesis. The finding that vegetative differences between areas with and without hyperaccumulators were more pronounced in the 2014 field season, when focusing on a smaller sphere of influence, i.e. the vegetation in a 3 m diameter circle around the hyperaccumulator, suggests that the influence of hyperaccumulators decreases with distance. The relative abundance of the hyperaccumulators (2013 study) on the sites that contain hyperaccumulators was low: Pine Ridge, 0.5%; Cathy Fromme, 5.2% and Coyote Ridge, 0.6%. This low abundance of the hyperaccumulator may limit the possible effect they have on their local plant community, even if such effect would be disproportionally big compared to other species. Additionally, the Se concentrations in the soil are extremely heterogeneous, this heterogeneity could mean that a plant very sensitive to Se may be growing within centimeters of soil that it could not tolerate; thus, the possible effect of hyperaccumulators or high Se soil is likely a relatively local phenomenon.

The increase in % bare ground in areas with hyperaccumulators may be related to the previously observed negative allelopathic effects seleniferous soils have on the germination and growth of Se-sensitive species in the plant community. Interestingly, this negative effect could then open up space for the germination and growth of species that either can exclude the Se or are tolerant to it. A decrease in canopy cover could be

directly related to a decrease in the number of plants growing in areas with hyperaccumulators, or less vigorous growth. The observed increase in species richness may be due to increased heterogeneity in the environment, with respect to soil Se properties. This increased heterogeneity was observed in this study, and likely is brought about through the redistribution of Se from deeper soil layers to the soil surface, via turnover of shoot and root material. This seasonal enrichment of soils with Se also adds temporal variation, in addition to spatial variation, a further dimension of heterogeneity. A positive correlation between plant species richness and environmental heterogeneity has been widely observed in other studies (Stein *et al.*, 2014; Stein & Kreft, 2015), including a study comparing serpentine soils (which have high levels of nickel and chromium) with control soils (Baker, 1987). It is hypothesized that with more environmental heterogeneity, in this case particularly in regards to toxic (to some species) levels of elements in the soil, there is an increase in possible niches for more diverse species to occupy.

Not only were general vegetation patterns different near hyperaccumulators, but species composition was also different. It should be noted that the relative over- or under-abundance of plant species in the vicinity of hyperaccumulators do not necessarily indicate an effect caused by the hyperaccumulator. There clearly are other factors at play affecting plant species composition, besides Se in soil or presence of hyperaccumulators: even in plots without hyperaccumulators the common and rare species often were not the same. Nevertheless, several species on each site could be identified that occurred relatively more frequently around hyperaccumulators when contrasted with adjacent areas without hyperaccumulators (positive co-occurrence with

Se hyperaccumulators) (Table 2.2-2.4). Of note is *Symphyotrichum ericoides*, which was more abundant in all three plots with hyperaccumulators relative to those without (Table 2.2-2.4). This was in agreement with our previous work: it grew better and suffered less herbivory when growing near hyperaccumulators (El Mehdawi *et al*., 2011a). *S. ericoides* was found to even reach hyperaccumulator Se levels in the field (>1000 mg Se/kg DW). Further characterization in a lab setting revealed that this species' growth was significantly enhanced by Se (El Mehdawi *et al*., 2014). *Tragopogon dubius* and *Helianthus pumilus* were also found in hyperaccumulator plots more frequently Species can also possibly avoid Se by having a shallow root system or actively growing during times of the year when there is lower Se in the soils.

Some species were less abundant in plots with hyperaccumulators, and thus showed negative co-occurrence with Se hyperaccumulators. Of note for Pine Ridge was *Hesperostipa comata*. It is a dominant species on this site, but it was found 3-fold more frequently in plots without hyperaccumulators (p=0.0003) (Table 2.2). Of note also is *Pascopyrum smithii*, which was found less frequently on sites with hyperaccumulators for both Cathy Fromme (p=0.0008) and Coyote Ridge (p=0.09) but it was found more frequently with hyperaccumulators on Pine Ridge (p=.07) (Table 2.2 and 2.3). This is likely indicative of other factors involved in community assembly.

Soil Se concentration in plots containing hyperaccumulators was much higher and more heterogeneous for both Pine Ridge (2.5-fold higher) and Cathy Fromme (2-fold higher) than in their respective control plots without hyperaccumulators (Fig. 2.5). This increased heterogeneity in an area with hyperaccumulators could be due to the concentration of Se in tissues of hyperaccumulators followed by turnover of these

tissues whereby Se is deposited in a more condensed soil area relative to the area from which the hyperaccumulator foraged. The Se is also brought from deeper soil layers to the surface, and converted from inorganic to organic forms, making it more available to other organisms. The hyperaccumulators at these two sites primarily occurred in soils in the mid-range of Se for those sites (Fig. 2.1 and 2.2).

Unexpectedly, the Coyote Ridge site showed the opposite: there was a greater than 2-fold lower soil Se concentration in the plot with hyperaccumulators than in the plot without (Fig. 2.5). Based on available information, it is not clear why this site is different in this respect. Another way in which Coyote Ridge differed from the other two sites is that the hyperaccumulators there were found in the upper range of the occurring soil Se, while at Pine Ridge and Cathy Fromme they were found in the mid-range of soil Se concentrations (Fig. 2.1-2.3). So, although soil Se on the plot with hyperaccumulators at Coyote Ridge was lower, the hyperaccumulators were found in similar soil Se ranges, implying that hyperaccumulators have a range of preference for soil Se. It is also important to note that all of these are high-Se sites: the lowest soil levels found on all sites, with or without hyperaccumulators, were 3 fold greater than average soil Se levels (Kabata-Pendias, 2011).

In 2014, in agreement with the 2013 soil data (except for Coyote Ridge), plots with hyperaccumulators generally had higher Se than those without (Fig. 2.9A). Even the 3 m plots without hyperaccumulators had at minimum approximately 2.5 µg/g Se. which is still higher than the Coyote Ridge plot with hyperaccumulators from 2013. This higher soil Se at site 2 (in both with and without plots) was correlated with higher leaf Se found in the hyperaccumulators from that area, while sites 1 and 3 had 3 fold less soil

Se (for both with and without) which also correlated to less leaf Se in the plants growing there(Fig. 2.9). A census was taken of the species present in these 3 m plots and the composition compared between plots with hyperaccumulators and those without: *Guttierezia sarothrae* occurred 6-fold more frequently in hyperaccumulator plots (found in 12 of 22 hyperaccumulator plots as compared to 2 of 22 in plots without). We found *G. sarothrae* Se levels above 3000 mg Se/kg DW at Pine Ridge, in a very healthy looking plant (unpublished result), as did Beath et al (Beath *et al.*, 1939), so this species is clearly tolerant to high Se levels.

These data showed that the plots containing hyperaccumulators had more bare ground, less canopy cover and more species richness. It seems it would be related to Se either in hyperaccumulator tissue or the surrounding soils, but surprisingly when a correlation between the above vegetative characteristics and Se in the soil or plant was tested none was found (Fig. 2.10 and 2.11). It seems likely that the witnessed effect is not linear with Se levels but more likely there is a threshold above which the effect is found. An additional possibility is the temporal variability in Se levels in both plant and soil, as well as the spatial heterogeneity in soil Se in general (Plant *et al.*, 2013), but particularly where there are hyperaccumulators (Figs. 2.2, 2.3 and 2.8).

Presence of Se hyperaccumulators corresponded to changes in vegetative characteristics but this effect was not completely clear until the vegetation within 3 m was surveyed. This implies that the range of influence for hyperaccumulators is somewhere between 0-3 m but dramatic enough to be seen at 3 m. Changes in the temporal and spatial distribution of Se in the landscapes where Se hyperaccumulators are found, and their effects on vegetation has implications for Se cycling in these local

systems: affecting other trophic levels through increased bioavailability of Se through changing the form from inorganic to organic forms as well as bringing the Se from lower soil horizons to the surface.

CHAPTER 3: POSITIVE AND NEGATIVE CO-OCCURENCE OF SELENIUM HYPERACCUMULATORS WITH PLANT SPECIES OF THEIR COMMUNITY.

Introduction

This group of studies builds upon the main findings from the 2013 and 2014 work. General vegetation properties in areas that contain hyperaccumulators differed from comparable areas without hyperaccumulators: there was less vegetation cover, more bare soil and higher species richness. These findings were highly significant at a scale of 3 m diameter, and the same patterns were observed at a large scale, in 90-x-20 m plots. Hyperaccumulator abundance in these plots varied from less than 1% of vegetative cover (2 sites) to 5% (1 site). Therefore, the results supported the hypothesis that hyperaccumulators have a disproportionally large effect on their local vegetation. The hypothesized underlying mechanism for this hyperaccumulator effect on their plant community was positive and negative selection pressures on Se-tolerant and Se-sensitive neighbor species, respectively. To address this hypothesis of positive and negative selection effects, further studies focused on characterizing the Se-related properties of plant species in the community that showed the clearest positive or negative co-occurrence effect of hyperaccumulators.

In the 2015 study, the specific questions addressed were: 1) How does soil Se concentration relate to distance from Se hyperaccumulators? 2) Is Se concentration in a hyperaccumulator correlated with that in its neighboring plants? 3) Which species are found in the near vicinity of hyperaccumulators (<50 cm, under the canopy), and do they occur more frequently there, as compared to the overall landscape? 4) How do Se

tolerance and accumulation properties compare between species that positively or negatively co-occur with hyperaccumulators, and what are the patterns of Se tissue distribution and chemical speciation in these species?

In these studies we started with a newly designed field survey approach to pinpoint species of interest, followed by a controlled lab Se tolerance and accumulation experiment using selected species. Furthermore, x-ray microprobe analysis was done to image the distribution of Se and other elements in intact frozen field-collected samples from species of interest, as well as forms of Se accumulated (Se speciation).

Methods

Field sampling

On June 28th 2015, data collection started in Pine Ridge Natural Area Fort Collins, CO, latitude: 40.545496, longitude: -105.133213. A total of fifty-four hyperaccumulator plants were located, and the five plant species growing nearest to the hyperaccumulator were identified and recorded. In addition, a soil sample was taken at the base of the stem for each hyperaccumulator as well as at the base of each of the five nearest plant species. Soil samples were taken by brushing aside all organic matter at the surface and taking a 4 gram sample. In addition to soil samples, the youngest mature leaf was collected from each hyperaccumulator and each of the five nearest species. For the reference a 50m baseline was used and six 25m transects were located along it, three East and three West alternating. Along each of these east and west transects a Daubenmire quadrat was used at each meter alternating sides (north

and south) each time (Coulloudon *et al.*, 1999). The data from the Daubenmire plots was then used to estimate canopy cover (Coulloudon *et al.*, 1999).

Lab studies

A follow-up lab study was performed using seeds collected in the areas described above. A total of 15 species were sown on Pro-Mix brand potting soil in the lab. Of these 15 species, five species, *Tragopogon dubius, Bromus inermis, Bromus tectorum, Artemisia ludoviciana, and Artemisia frigida,* had at least six individuals germinate. The seedlings from these five species were split into two groups: control given ¼ strength Hoagland's solution (Hoagland & Arnon, 1938) only, and the treatment group given the same solution with 20 µM sodium selenate added. All species were grown for five weeks with 14:10 L:D photoperiod. They were then harvested, washed, roots and shoots separated and dried in a 50°C drying oven for 24 hours. After drying they were weighed and total biomass recorded for root and shoot separately. The Se accumulated in the root and shoot material was analyzed as described below.

Elemental analysis

Soil samples were dried at 50°C for 2 days. Each sample was homogenized and sieved through a 1 mm screen. Approximately 400 mg of each sample was weighed and placed in a 25 mm x 200 mm acid digestion tube. Two ml of ultra-trace grade 70% nitric acid was added to each sample. After addition of acid, each sample was heated to 50°C for two hours and then 125°C for 6 hours. After cooling, each sample was diluted to 10 ml with distilled water. Inductively coupled plasma mass spectrometry (ICP-MS) was performed using a Perkin-Elmer Elan DRCII instrument (detection limit for Se is

approximately 0.01 ppb) according to the manufacturer's instructions and including appropriate standards and controls.

The leaf samples were dried at 50ºC for 24 hours. After drying, each of the leaf samples was crushed and homogenized. Approximately 100 mg was weighed and placed in a 25 mm x 200 mm acid digestion tube and 1 ml of ultra-trace grade 70% nitric acid was added to each. The tubes were then placed on a heating block and heated to 50ºC for 2 hours and then 125ºC for 6 hours. After cooling, the samples were diluted to 10 ml with distilled water. All leaf samples were then analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), model Perkin-Elmer 7300 DV, according to the manufacturer's instructions, including appropriate standards and controls.

X-ray microprobe analyses

Selenium localization and speciation were analyzed using X-ray microprobe imaging, as described by Quinn et al. (2011). These analyses were performed at beamline 10.3.2 (hard X-Ray microprobe) of the Advanced Light Source (ALS), at the Lawrence Berkeley National Laboratory (Berkeley, CA, USA). Localization of Se (and Ca and K) was determined on frozen intact leaves, using micro-focused X-ray fluorescence (μXRF) mapping. Micro X-ray absorption near-edge structure spectroscopy (μXANES) was used to analyze for Se speciation in selected spots as indicated with circles in the XRF figures. These were selected in areas with high Se concentration, according to the μXRF data. The fitting of the μXANES spots was performed using a library of characterized ~50 standard selenocompounds. Se speciation and μXRF maps were recorded in fluorescence mode using a Ge solid state

detector. Spectra were calibrated using a red amorphous Se standard, with the main peak set at 12660 eV.

Statistical analyses

Statistical analyses were done using R (ver. X64 3.32). T-tests were performed comparing the root and shoot biomass for the lab study (α=0.05) (fig. 3.7A,B). One-way Anova was used to compare all of the different species in the lab experiment (α=0.05) (fig. 3.8).

Results

Fifty-four hyperaccumulator individuals (*S. pinnata* or *A. bisulcatus*) were selected, and the five species growing nearest to the hyperaccumulators recorded and counted ("Near 5" in Table 3.1 and 3.2).

Table 3.1. Plant species occurrence as influenced by the vicinity of hyperaccumulators at PR locations 1 (table 3.1A) and 2 (table 3.1B). First two columns show USDA abbreviations (as in Fig. 3.1) and the species to which they refer. Near 5 counts refers to the number of times that species was observed growing as one of the nearest 5 species to a hyperaccumulator. The Near 5 RSA is the relative species abundance, i.e. fraction of the total of all 5 nearest species to all hyperaccumulators (5 x 54 = 270 counts).The Daub RSA is the relative species abundance for that species based on Daubenmire plot canopy cover data. Last column is the average of all collected leaves for plants found as one of the 5 nearest to hyperaccumulators ± SEM. ND means there was no data for that species. If there are no values for hyperaccumulators (ASBI2 and STPI) they were not encountered as one of the nearest five to the other hyperaccumulator or they were not surveyed in the Daubenmire plots.

A.

Species code	Species name	Near 5 Counts	Near 5 RSA	Daub % Canopy	Daub RSA	Avg. mg Se/kg DW
ALSI	*Alyssum simplex*	4	9.1	3.1	5.8	10±1
AMPS	*Ambrosia psilostachya*	1	2.3	0.1	0.2	18
ASBI2	*Astragalus bisulcatus*	0	0	0.6	1.1	335±40
BOCU	*Bouteloua curtipendula*	3	6.8	6.9	13.1	3±1
BODA2	*Bouteloua dactyloides*	5	11.4	8.2	15.4	4±1
BOGR2	*Bouteloua gracilis*	1	2.3	0	0	12
BRIN2	*Bromus inermis*	1	2.3	0.2	0.4	5
COAR4	*Convolvulus arvensis*	7	15.9	2.4	4.5	10±1
MEOF	*Melilotus officinalis*	6	13.6	2.4	4.5	17±3
NAVI	*Nassella viridula*	2	4.6	0.3	0.6	10±0.3
OESU3	*Oenothera suffrutescens*	2	4.6	0.6	1.2	9±5
PASM	*Pascopyrum smithii*	8	18.2	11.8	22.2	9±1
TAOF	*Taraxacum officinale*	1	2.3	0	0	ND
TRDU	*Tragopogon dubius*	3	6.8	0.3	0.6	20±10

B.

Species code	Species name	Near 5 Counts	Near 5 RSA	Daub % Canopy	Daub RSA	avg mg Se/kg DW Near	Far
ALSI	*Alyssum simplex*	3	4.7	4.1	2.6	337±189	0
ARLU	*Artemisia ludoviciana*	6	3.5	2.9	2.3	183±28	0
ASBI2	*Astragalus bisulcatus*	0	0	1.5	2.4	8284±992	n/a
ASPU	*Asclepias pumila*	1	0.4	4.0	6.3	39±0	ND
BRTE	*Bromus tectorum*	1	4.7	2.0	1.5	452±0	ND
BRIN2	*Bromus inermis*	3	1.6	22.9	18.1	369±73	ND
ERNA10	*Ericameria nauseosa*	1	0.8	1.7	1.5	223±0	ND
HEAN3	*Helianthus annuus*	2	3.1	6.6	4.4	219±106	3.4
OESU3	*Oenothera suffrutescens*	1	2.0	1.0	0.7	397±0	ND
OPPO	*Opuntia polyacantha*	1	0.4	0.2	0.3	95±0	ND
PASM	*Pascopyrum smithii*	4	6.7	18.0	10.8	503±78	13.8
PSSPS	*Pseudoroegneria spicata*	2	6.7	3.0	3.3	54±19	ND
RHTR	*Rhus trilobata*	1	1.2	2.5	1.7	41±0	6.7
SYER	*Symphyotrichum ericoides*	3	1.6	3.0	2.0	3838±309	0.4
STPI	*Stanleya pinnata*	0	0	0	0	1459±196	n/a
TRDU	*Tragopogon dubius*	3	1.6	1.9	1.5	300±70	64.7
YUGL	*Yucca glauca*	1	6.7	2.0	1.2	39±0	ND

Table 3.2. Plant species occurrence as influenced by the vicinity of hyperaccumulators at PR locations 1 (table 2.1A) and 2 (table 3.1B). First two columns show USDA abbreviations (as in Fig. 3.1) and the species to which they refer. Near 5 counts refers to the number of times that species was observed growing as one of the nearest 5 species to a hyperaccumulator. The Near 5 RSA is the relative species abundance, i.e. fraction of the total of all 5 nearest species to all hyperaccumulators (5 x 54 = 270 counts). The Daub RSA is the relative species abundance for that species based on Daubenmire plot canopy cover data. Last column is the average of all collected leaves for plants found as one of the 5 nearest to hyperaccumulators ± SEM. ND means there was no data for that species. If there are no values for hyperaccumulators (ASBI2 and STPI) they were not encountered as one of the nearest five to the other hyperaccumulator or they were not surveyed in the Daubenmire plots.

Species code	Species	Near 5 Counts	Near 5 RSA	Daub % Canopy	Daub RSA	Avg. (mg Se/kg DW
ACHY	*Achnatherum hymenoides*	6	2.4	1.36	1	28±6
ALSI	*Alyssum simplex*	12	4.7	4.10	2.6	4±1
AMPS	*Ambrosia psilostachya*	2	0.8	0.10	0.2	10
ARDR4	*Artemisia dracunculus*	2	1.1	0	0	30±0.3
ARFR4	*Artemisia frigida*	1	0.4	0.37	0.2	50
ARLU	*Artemisia ludoviciana*	9	3.5	2.85	2.3	20±9
ARPU9	*Aristida purpurea*	2	0.8	2.69	2.7	8±4
ASBI2	*Astragalus bisulcatus*	0	0	0.14	35	1458±94
ASMO7	*Astragalus mollissimus*	1	0.6	0	0	219
ASTE5	*Astragalus tenellus*	4	1.6	0.74	1.8	45±12
BOCU	*Bouteloua curtipendula*	10	3.9	7.33	4.6	57±10
BRTE	*Bromus tectorum*	12	4.7	2.00	1.5	43±4
COUM	*Comandra umbellata*	2	0.8	1.01	0.8	213±12
ELEL5	*Elymus elymoides*	1	0.4	0.52	0.5	71
ERNA10	*Ericameria nauseosa*	2	0.8	1.65	1.5	6
EUBR	*Euphorbia brachycera*	1	0.4	0.69	1.7	68
EVNU	*Evolvulus nuttallianus*	3	1.2	1.75	1.2	152±66
GUSA2	*Gutierrezia sarothrae*	3	1.2	1.86	1.9	597±222
HEAN3	*Helianthus annuus*	8	3.1	6.64	4.4	28±6
HECO26	*Hesperostipa comata*	20	7.9	22.84	16.3	59±4
LASE	*Lactuca serriola*	9	3.5	0.05	0.1	89±12
LIDA	*Linaria dalmatica*	2	0.8	1.26	1.2	188±101
LILE3	*Linum lewisii*	5	2	2.09	1.5	46±15
MEDE	*Mentzelia decapetala*	1	0.4	0.31	0.8	27
NAVI	*Nassella viridula*	7	2.8	4.73	2.6	48±10
OESU3	*Oenothera suffrutescens*	5	2	1.03	0.7	31±10
OEVI	*Oenothera villosa*	2	0.8	0.84	0.7	96±64
PASM	*Pascopyrum smithii*	17	6.7	18.00	10.8	70±23
PHBE2	*Physaria bellii*	5	2	0.17	0.4	375±69
POPR	*Poa pratensis*	2	0.8	0.24	0.4	156±108
PSSPS	*Pseudoroegneria spicata*	17	6.7	3.03	3.3	62±5
PSTE5	*Psoralidium tenuiflorum*	3	1.2	2.53	1.7	8±1
RACO3	*Ratibida columnifera*	1	0.4	0.31	0.8	16
RHTR	*Rhus trilobata*	4	1.6	3.01	2	5±2
ROWO	*Rosa woodsii*	1	0.4	1.15	1.2	6
STPI	*Stanleya pinnata*	1	0.4	0.57	1.4	1107±28
SYAS3	*Symphyotrichum ascendens*	3	1.2	0.95	2.3	17±8
SYER	*Symphyotrichum ericoides*	4	1.6	1.88	1.5	382
SYFE	*Symphyotrichum fendleri*	3	1.7	0	0	1129±126
THME	*Thelesperma megapotamicum*	2	0.8	0.33	0.3	150±78
TOHO	*Townsendia hookeri*	3	1.2	0.24	0.6	394±85
TRDU	*Tragopogon dubius*	17	6.7	2.01	1.2	53±7
TRRA5	*Tragia ramosa*	5	2	2.79	3.3	31±6
VETH	*Verbascum thapsus*	2	0.8	0.05	0	19±7

Tables 3.1 and 3.2 show the species name associated with the USDA symbols indicated in figures 3.1,3.2 and 3.7. The relative species abundance (RSA, Table 3.1 and 3.2) data were used to calculate the ratios used in figs. 3.1 and 3.2. “Near 5 counts” is the number of times that species was found as one of the 5 species growing closest to a hyperaccumulator for that site. Plant species found relatively more frequently near hyperaccumulators than expected based on Daubenmire surveys have ratios >1 and can be seen on the left side of Figs. 3.1 and 3.2. In contrast, the species on the right in Figs. 3.1 and 3.2 (ratios <1) are those that were found less frequently as one of the nearest five species of hyperaccumulators, compared to Daubenmire surveys.

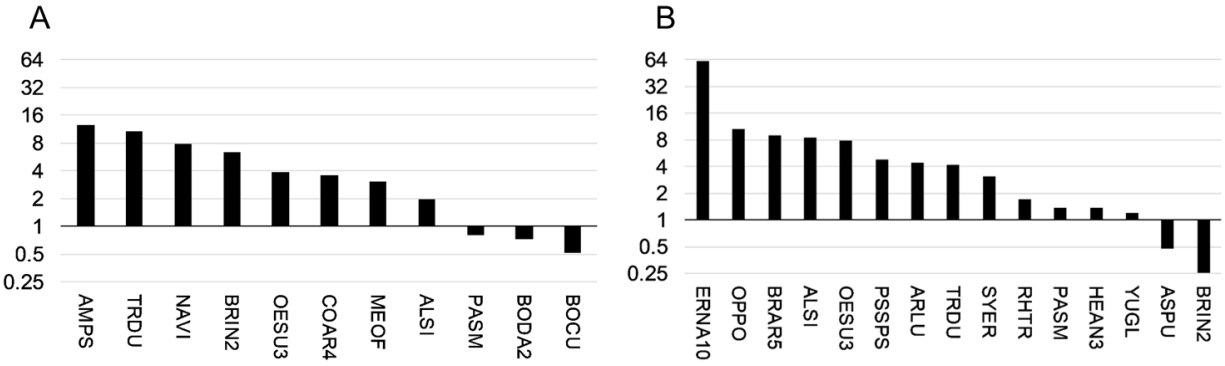

Fig. 3.1. Positive and negative co-occurrence of different plant species with Se hyperaccumulator species at Pine Ridge, locations 1 (A, lower in Se) and 2 (B, higher in Se). Table 3.1 shows the full species names, and the observation data used to calculate the ratios shown here, which represent the fold difference in the frequency the species as one of the nearest five to hyperaccumulators relative to the frequency of the same species in the overall area (from Daubenmire plot data).

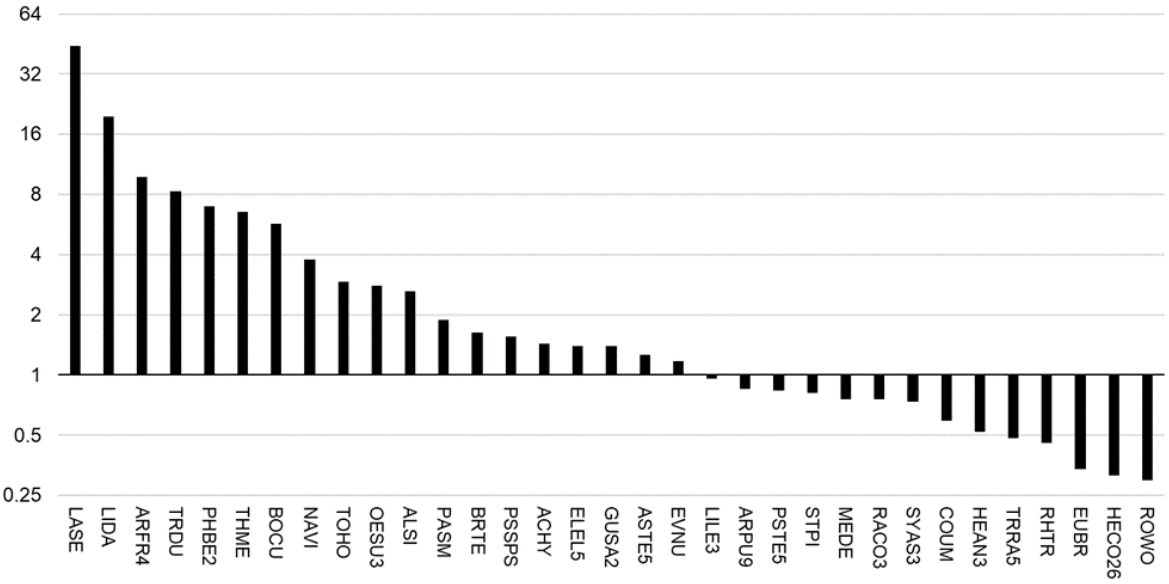

Fig. 3.2. Positive and negative co-occurrence of different plant species with Se hyperaccumulator species at Pine Ridge, locations 1 (A, lower in Se) and 2 (B, higher in Se). Table 3.1 shows the full species names, and the observation data used to calculate the ratios shown here, which represent the fold difference in the frequency the species as one of the nearest five to hyperaccumulators relative to the frequency of the same species in the overall area (from Daubenmire plot data).

Species found more frequently growing near hyperaccumulators at all three sites are: *Alyssum simplex*, *Oenothera suffrutescens* and *Tragopogon dubius*, and two species were found to positively co-occur in two of the three sites: *Bromus tectorum* and *Nasella viridula*. The species with the highest Near 5/Daubenmire ratio was *Ericameria nauseosa* in the Pine Ridge 2 site. In regards to species less abundant near hyperaccumulators than expected, there were three that stood out: *Hesperostipa comata*, *Euphorbia brachycera* and *Rosa woodsii*; each of these species were at least four-fold less abundant near hyperaccumulators than expected based on Daubenmire data. It should be noted that only species that occurred in both the Daubenmire plots and as one of the nearest five species are included in figs. 3.1 and 3.2.

The average leaf Se concentration in those plants found as one of the five nearest species to the hyperaccumulator ranged from 4.3 mg Se/kg dry weight for

Bouteloua dactyloides to 2,974 mg Se/kg DW for *Symphyotrichum ericoides*. The hyperaccumulator *S. pinnata* averaged 1,459 ± 196 mg Se/kg dry weight for Pine Ridge site 1 and 1,107 ± 28 mg Se/kg dry weight for Coyote Ridge; there was no *S. pinnata* located at Pine Ridge site 2. The hyperaccumulator *A. bisulcatus* averaged 8,284 ± 992 mg Se/kg dry weight for Pine Ridge site 1 and 13,927 ± 253 mg Se/kg dry weight for Pine Ridge site 2; there was no *A. bisulcatus* located at Coyote Ridge. After *S. ericoides* (hyperaccumulator co-occurrence pattern: +), the species next highest in Se were *Gutierrezia sarothrae* (+), *Townsendia hookeri* (+), *Physaria bellii* (+), and *B. inermis* (-/+, depending on site). To investigate the effect of the hyperaccumulator's proximity, the leaf Se concentration was also determined of field plants of the same species, collected at a minimum of 50 m from a hyperaccumulator (Table 3.1B). The Se concentration was lower at this longer distance for all species surveyed.

The Se concentration in soil was analyzed at 0.5 meter successive intervals from the stem of five hyperaccumulator plants (0 m) to 2 m from the plant. Se concentration in soil significantly decreased after one and a half meter from the hyperaccumulator (Fig. 3.3).

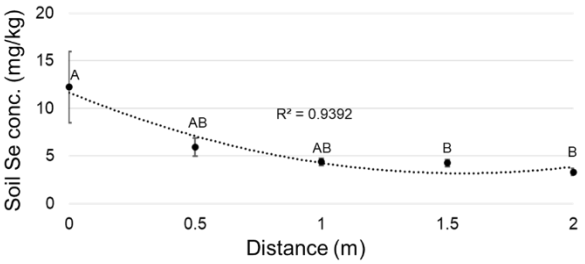

Fig. 3.3. Soil Se concentration measured at half meter successive intervals from hyperaccumulators (n=5). Shown data represent mean and Standard Error of the Mean. Letters above graph indicate which means were significantly different (ANOVA and Tukey Kramer, P=.004).

Selected species that differed in their co-occurrence properties were further investigated for their Se speciation and localization, as well as their Se tolerance. *Bromus inermis* was selected as a negatively co-occurring species (Fig. 3.1B); as positively co-occurring species *Alyssum simplex* (Fig. 3.1, 3.2) and *Artemisia frigida* (Fig. 3.2) were chosen. Leaves from these three species were collected in the field and analyzed by x-ray microprobe analysis for Se localization (x-ray fluorescence, XRF) and chemical speciation (x-ray absorption near-edge structure, XANES). Selenium was distributed throughout the leaf of *B. inermis* (Fig. 3.4A, B), with a concentration in a pattern of straight parallel lines, likely the vasculature. The chemical speciation of Se was determined in the *B. inermis* leaf locations indicated (Fig. 3.4B). Around half of the Se appeared to be organic and the other half inorganic (Fig. 3.4C). The organic forms were fitted as 40% C-Se-C compounds (possibly selenomethionine or methyl-selenocysteine) and 9% seleno-diglutathione ($SeGSH_2$, C-S-Se-S-C). Inorganic Se was fitted as elemental Se (26% Se0), selenite (17% SeIV) and selenate (% SeVI).

The leaf of A. simplex showed diffuse Se distribution throughout the leaf, with increased concentration in the central vein and slight concentration in the other vasculature and in the stellate trichomes (Fig. 3.5A-C). Calcium was very concentrated

in the trichomes (much more so than Se), while potassium was present throughout the leaf (Fig. 3.5B). The form of Se was investigated in the A. simplex leaf at the positions indicated (Fig. 3.5B), and found to be three-quarter organic (73%), consisting of 70% C-Se-C compounds and 3% SeGSH2 (Fig. 3.5D). The inorganic Se fraction was comprised of three forms: 16% Se(VI), 6% Se(IV) and 4% Se(0).

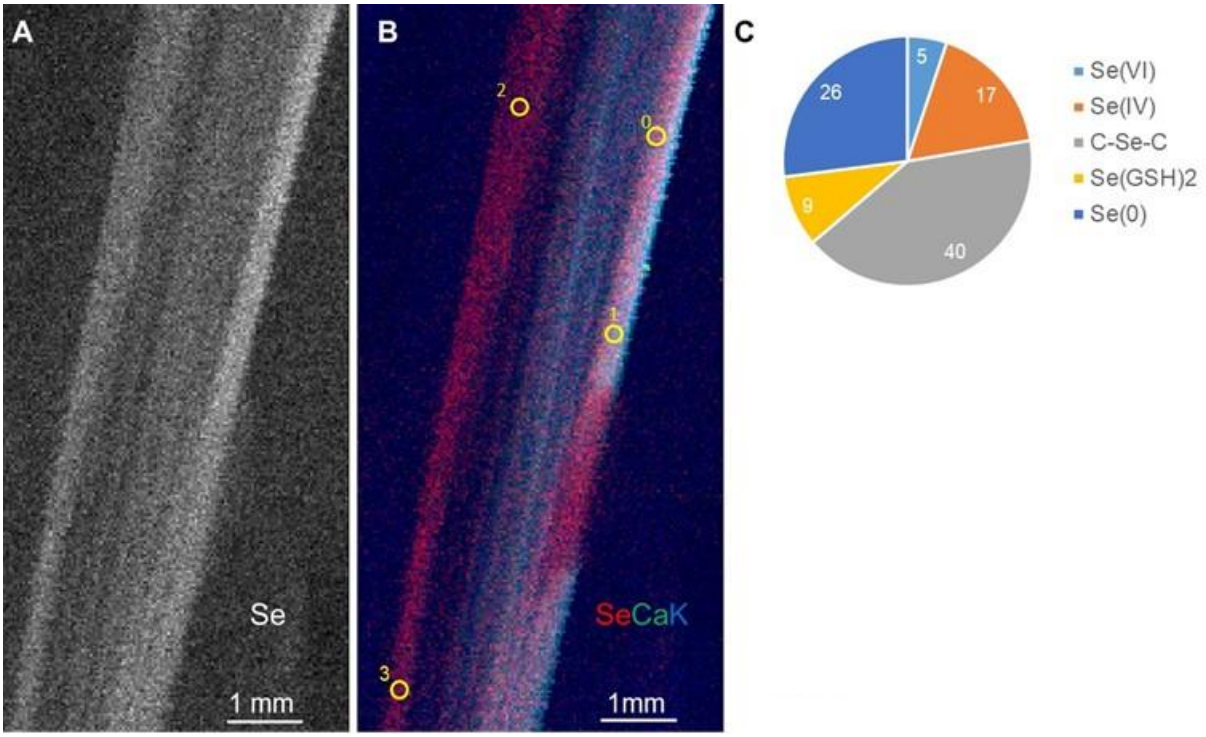

Figure 3.4. 1Selenium localization and speciation in Bromus inermis leaf collected at Pine Ridge. A, B:XRF maps of Se (A) or of Se, Ca and K (B); yellow circles show locations of XANES collection; C: average fit of Xanes spectra (average NSS = 4.55 10-3.

The *A. frigida* leaf also showed diffuse Se distribution, but with clearly higher levels in what appears to be the vasculature (Fig. 3.6A, B). XANES revealed that three-quarters of the Se in the *A. frigida* leaf at the locations indicated (Fig. 3.6B) consisted of organic Se with C-Se-C structure (78%); the remainder was inorganic: 18% Se(IV), 4% Se(VI) and 2% Se(0) (Fig. 3.6C). Thus, Se localization was similar across the three species,

showing diffuse distribution throughout the leaf with concentration in the vasculature. *Alyssum simplex* additionally appeared to store some Se in its trichomes.

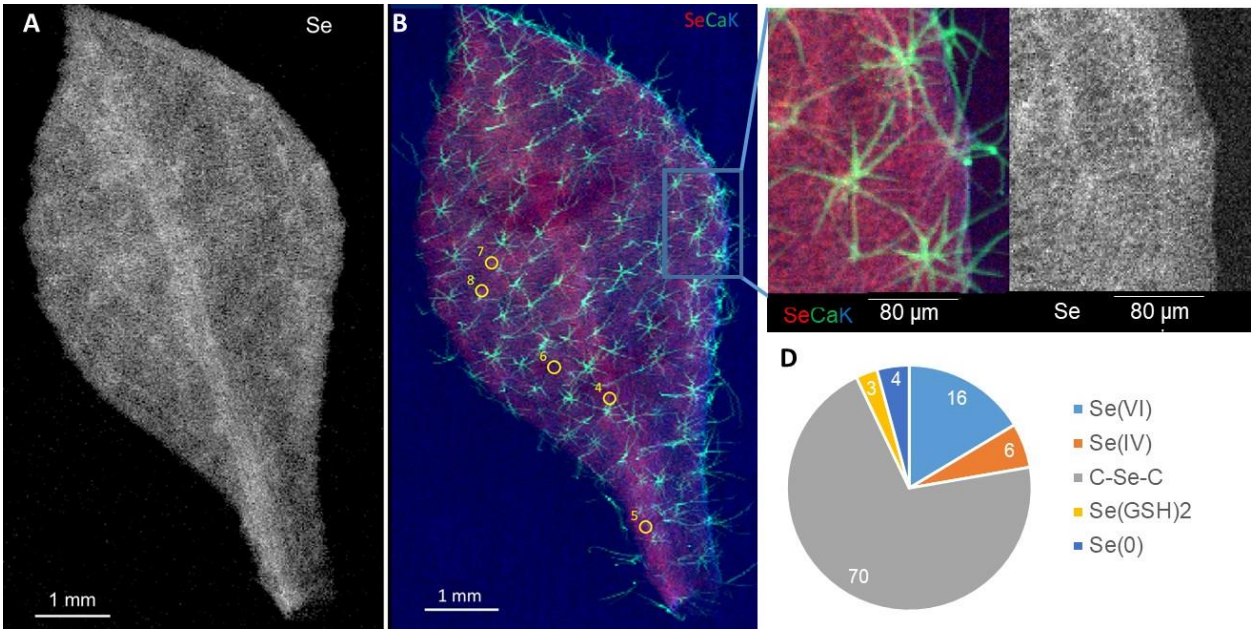

Figure 3.5. Selenium localization and speciation in Alyssum simplex leaf collected at Pine Ridge. A, B: XRF maps of Se (A) or of Se, Ca and K (B); yellow circles show locations of XANES collection; C: average fit of XANES spectra (average NSS = 2.1 10-3).

The Se speciation differed between negative co-occurring species *B. inermis* and the two positively co-occurring species, in that *B. inermis* had relatively more inorganic and less organic Se, and had a larger fraction of elemental Se.

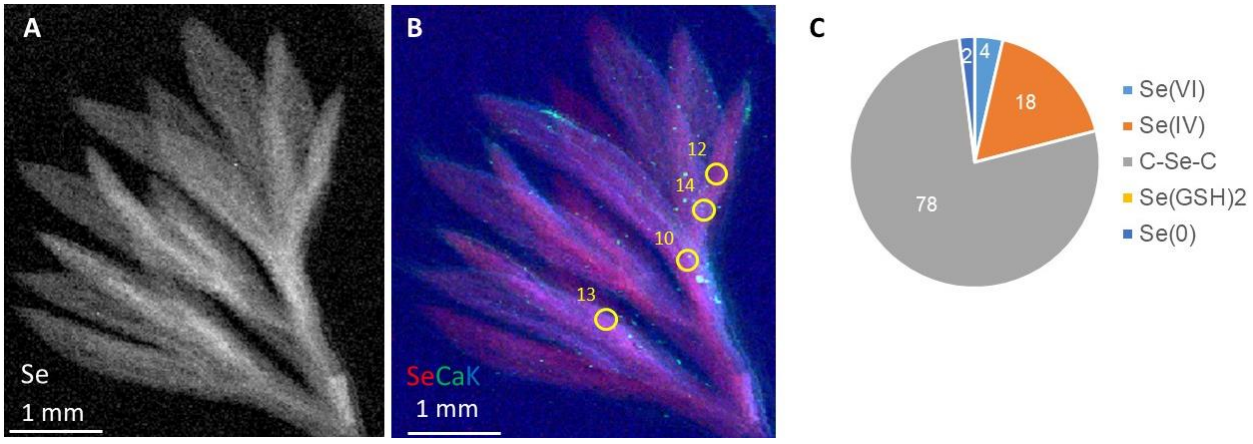

Figure 3.6. Selenium localization and speciation in Artemisia frigida leaf collected at Pine Ridge. A, B: XRF maps of Se (A) or of Se, Ca and K (B); yellow circles show locations of XANES collection; C: average fit of XANES spectra (average NSS = 1.76 10-3).

A follow-up lab Se tolerance study was carried out, using seeds from species that differed in co-occurrence (+, - or neutral) with hyperaccumulators, collected from the areas where the 2015 field study was performed. Five species had sufficient germination to provide meaningful results: *Artemisia ludoviciana* (+)(ARLU), *Bromus inermis* (-) (BRIN2), *Bromus tectorum* (+) (BRTE), *Artemisia frigida* (+) (ARFR4) and *Nasella viridula* (~0) (NAVI) (Figs. 3.1-3.2). The average biomass for plants of these five species was compared between a 20 mM selenate treatment and a control treatment (Fig. 3.7). For *A. ludoviciana*, *B. inermis* and *A. frigida*, the root and shoot biomass were on average lower for plants treated with selenate than for the control treatment, but this was only statistically significant for roots of *A. frigida* (Fig. 3.7). For *N. viridula* root biomass was unaffected, but shoot biomass less for plants treated with selenate (Fig. 3.7). Exceptionally, for *B. tectorum* the root biomass was significantly greater with selenate than without (P=0.011), and the shoot biomass was very slightly higher (N.S.).

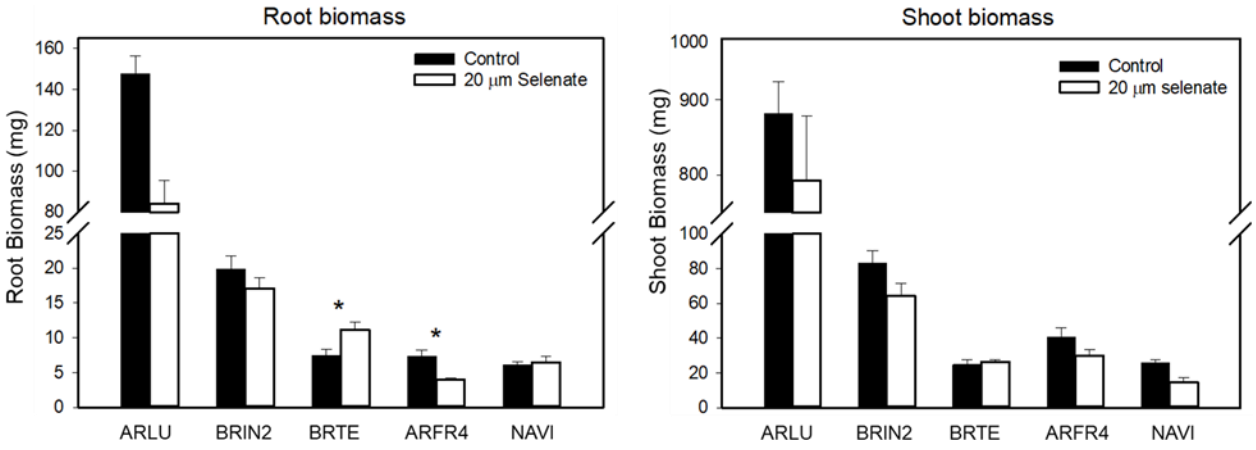

Fig. 3.7. Root and shoot biomass of five species grown in controlled lab conditions with 20 µm sodium selenate or without selenate (control). Full names of the species can be found in Tables 3.1 and 3.2. Asterisks denote significant difference between means for +/- Se treatment of a species (P<0.05).

When the selenate-supplied plants were analyzed for their tissue Se concentration, the two *Artemisia* species had by far the highest levels (Fig 3.8). In *A. ludoviciana,* shoot Se was ~800 mg/kg DW and *A. frigida* contained ~700 mg Se/kg DW. When compared to the next highest shoot concentration in *B. inermis, A. ludoviciana* was around five-fold higher. For roots, *A. ludoviciana* had ~700 mg Se/kg DW while *B. inermis* (the next highest) had 10-fold lower level. The Se concentration was generally higher in shoot than in root for all species, but this was much less pronounced for *A. ludoviciana* and *N. viridula*.

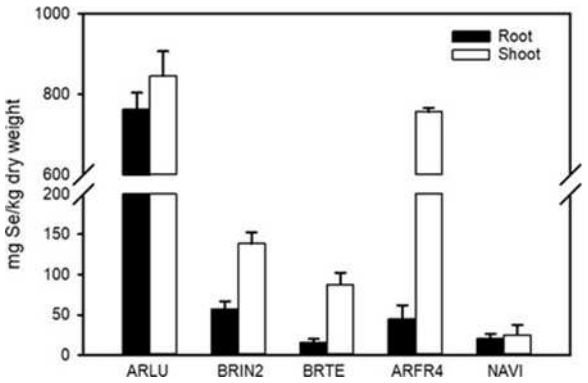

Fig. 3.8. Selenium concentration in the roots and shoots of species treated with or without 20 µm sodium selenate in controlled lab conditions. Full names of the species can be found in Tables 3.1 and 2. In cases where Se was not detectable in one or more of the replicates, half of the detection limit was used, and the resulting values thus are estimates (see methods) This is the case for BRAR5, ARFR4 and NAVI root as well as NAVI shoot.

Discussion

The goal of this study was to attempt to address a significant question in regard to the ecology of Se hyperaccumulators: Does the presence of Se hyperaccumulators correspond with differences in their local plant communities, particularly with respect to the presence or absence of other plant species near hyperaccumulators? The

hypothesis was that some species would be disproportionally more or less abundant around hyperaccumulators (*A. bisulcatus* and *S. pinnata*), depending on their Se sensitivity: less sensitive species showing positive co-occurrence, and more Se-sensitive species negative co-occurrence. Candidate species, found to occur more or less frequently near hyperaccumulators than in the overall landscape, were then further characterized by lab uptake and tolerance studies and x-ray microprobe analysis.

Twenty-two plant species were found at least three-fold more frequently near hyperaccumulators than could be expected based on their overall local abundance, and thus can be said to show positive co-occurrence with Se hyperaccumulators (Fig. 3.1 and 3.2). The species of hyperaccumulator, *A. bisulcatus* or *S. pinnata*, did not matter in this respect, so the effect may be more related to a higher Se content experienced by neighbors rather than any other species-specific effect like nitrogen fixing capability (data not shown). The average Se concentrations for all of these positively co-occurring individuals exceeded levels that begin to show toxicity for most plant species,(Brown & Shrift, 1982; Kaur *et al.*, 2016) and in some cases were at or near Se hyperaccumulator levels ($\geq$ 1,000 mg Se/kg DW, (Boyd & Martens, 1992). Se levels in plants growing near hyperaccumulators were higher than when growing further from hyperaccumulators. Moreover, the soil Se concentration under the canopy of hyperaccumulators was found to be elevated. Thus, it appears that hyperaccumulators are surrounded by a Se “hot spot”, and plants growing in this area experience elevated tissue Se levels. Some species that were found to be relatively more abundant in zones surrounding hyperaccumulators may benefit from these elevated Se levels while other species may experience a negative effect from growing there, perhaps due to Se toxicity, as

hypothesized. Positive effects of Se on plants may be physiological or ecological. Selenium at tissue levels ≤5 mg Se/kg DW can benefit plant physiology by increasing antioxidant capacity (Rani *et al.*, 2005). At higher levels (≥5 mg Se /kg DW), Se increasingly protects plants from herbivory (Freeman *et al.*, 2007; El Mehdawi *et al.*, 2011c,d). Of course, to enjoy these positive ecological effects, plants first have to tolerate the tissue Se levels they experience.

Tragopogon dubius was found in all three sites to be at least four-fold over-abundant near hyperaccumulators. *Tragopogon dubius* is an annual (or biennial) that is often found on disturbed ground (http://swbiodiversity.org); perhaps its overabundance is related to its ability to take advantage of bare ground found in more abundance near hyperaccumulators, and indeed more bare ground was found in the studies from Chapter 2 of this manuscript as well an earlier study in our lab (El Mehdawi *et al.*, 2011a). The average Se levels in *T. dubius* were 91 ± 30 mg/kg DW (Table 3.1 and 3.2) across all three sites, with a high of 488 mg Se/kg DW at Pine Ridge 2. Thus, it may be rather Se-tolerant. Interesting to note in this respect is that in the lab Se tolerance study, the single *T. dubius* plant obtained accumulated 882 mg Se/kg DW in its shoot when fed 20 µM Se, with no apparent toxicity symptoms (not shown in results section due to lack of replication).

Oenothera suffectescens was also found on all three sites to positively co-occur with hyperaccumulators. The Se levels found in this species in the field indicate a capacity to accumulate relatively high levels of Se (up to 397 mg Se/kg DW), coupled with Se tolerance. Similar to *T. dubius*, this species is often found in disturbed areas and has low water needs (http://swbiodiversity.org).

The third species found to positively co-occur with hyperaccumulators on all three sites was *Alyssum simplex*. It had high Se levels growing in the field (up to 603 mg Se/kg DW), indicating high Se tolerance. Indeed, the two seedlings obtained in the lab study each accumulated above 1,000 mg Se/kg DW in the shoot after feeding with 20 µM selenate, without toxicity symptoms (not included in results section for lack of sufficient replication). Similar to the two species discussed above, *A. simplex* is also common in disturbed areas, and is an annual (http://swbiodiversity.org). As a shallow rooting winter annual, it may indeed be well suited to life near a hyperaccumulator: Se levels in the soil near a hyperaccumulator have been found to be lowest in spring (Galeas *et al.*, 2007) likely due to leaching; competition for light is minimal, as the hyperaccumulator has not yet produced canopy; Se at the surface has been found to be lower relative to deeper horizons (El Mehdawi *et al.*, 2011a). This seasonal and spatial Se difference may have given the opportunity to develop the tolerance shown by the field and lab plants sampled. *Bromus tectorum* (field brome) was found to positively co-occur with hyperaccumulators on two of the three sites. It is a winter annual grass species (Beck, 2009) that inhabits disturbed sites. *B. tectorum* had similar field levels of Se (up to 452 mg/kg DW) to the above species. When grown from seeds collected from the Pine Ridge 2 site, and treated with or without Se, the root biomass of the Se-treated plants was actually higher compared to the control treatment and the shoot biomass was nearly equal. The shoot Se levels in lab conditions reached nearly 150 mg Se/kg DW, indicating tolerance to Se and perhaps a positive growth effect. In a previous study, *S. ericoides* and *A. ludoviciana* were found to be facilitated by growing next to hyperaccumulators, showing higher Se levels and reduced herbivory (El Mehdawi *et al.*,

2011c). In the current lab study there were no herbivores, so the positive growth response must have been entirely due to physiological factors.

The list of species found less often near hyperaccumulators than expected is much smaller, because many species found in the Daubenmire plots were not at all found as one of the top five species near hyperaccumulators, and thus not counted. Only the species found in both plots could be compared and, of course, those species that negatively co-occur, by definition, should be absent more often as one of the nearest five species. Nevertheless, a few species were found with high relative abundance in the landscape overall, but infrequently near hyperaccumulators, perhaps because they are sensitive to Se. The two that stand out in this regard are *B. inermis* less at PR2 site and *Hesperostipa comata* at CR both are ~23% of the Daubenmire canopy cover and both are ~4-fold less abundant near hyperaccumulators. If Se hyperaccumulators, via the Se in their tissues and surrounding soil, affect neighbor species positively or negatively, is this effect Se concentration-dependent, and how large is their sphere of influence? When looking at correlation of Se in the hyperaccumulator and the Se content in the surrounding plants, a significant correlation was found for quite a few positive co-occurring species species (with enough replication), both as individual species and as a group (notably, *T. dubius*, *A. simplex*, *O. suffrutescens* and *B. tectorum*). A correlation between soil Se levels at the base of the hyperaccumulator and the leaf levels in the nearest five plants was not found; this could be due to fluctuating soil Se levels and forms, the limited differences in distance (most were under the canopy) and other chemical properties in the soil such as sulfur form and levels. In addition, no correlation was found between the surface soil Se

concentration beneath the hyperaccumulator directly next to the stem and the leaf Se levels in the hyperaccumulator. Still, neighboring plant species contained higher Se levels when growing next to hyperaccumulators than when growing far away (Table 3.1B).

There was a pronounced local increase in Se concentration in soil around hyperaccumulators close to the plant, but beyond the canopy edge (>50 cm), soil Se decreased substantially (Fig. 3.3.) Therefore, the sphere of influence of HA on surrounding vegetation due to higher local soil Se concentration is likely highest under their canopy, and may be limited to that area. The reason for the higher Se under the canopy is likely leaf litter deposition and subsequent decomposition, but may also be root exudation. (Quinn *et al.*, 2010a; El Mehdawi *et al.*, 2011c, 2015a). The forms of Se in hyperaccumulator species has been found to be more organic relative to non-hyperaccumulator species, particular in the form of non-proteinogenic aminoacids. This is hypothesized to be a possible tolerance mechanism, as it prevents oxidative stress from inorganic Se forms as well as toxic effects of non-specific incorporation of Se-aminoacids into proteins (Freeman *et al.*, 2006b, 2010; El Mehdawi *et al.*, 2015a). Thus, hyperaccumulator litter decomposition and root deposition processes locally release organic Se into the soil, which is more readily accumulated by plants than inorganic Se (Zayed *et al.*, 1998). Therefore, even slight increases in local soil Se concentration, in organic forms, likely already elevate Se levels in neighboring vegetation, and the Se accumulated might be expected to be enriched in organic forms.

When the forms of Se accumulated in species with different co-occurrence patterns were investigated using X-ray microprobe analysis, the negatively co-occurring

species *B. inermis* had relatively more inorganic and less organic Se (Fig. 3.4), compared to the two positively co-occurring species *A. simplex* (Fig. 3.5) and *A. frigida* (Fig. 3.6).

As mentioned, species with lower Se tolerance typically accumulate relatively more inorganic Se; the negative co-occurrence of *B. inermis* may be related to lower Se tolerance to the elevated soil Se levels around hyperaccumulators. In the lab Se tolerance study, *B. inermis* did not stand out for being more Se sensitive than positively co-occurring species. However, it is noteworthy that its tissue Se levels were much lower than that of the positively co-occurring species in the lab experiment. Also interesting to note is that the field leaf Se levels for *B. inermis* in the area where it showed negative co-occurrence (PR area 2) were over 2.5-fold higher than those obtained in the controlled study (370 vs. 140 mg Se/kg DW, respectively). Thus, it may well be that *B. inermis* would show more negative effects at higher accumulation levels. Interestingly, in PR area 1, where overall Se levels in the soils and vegetation were much lower (only 5 mg/kg for *B. inermis*), *B. inermis* actually showed positive co-occurrence with hyperaccumulators. Thus, it is possible that the mode of interaction between a certain neighboring plant species and a hyperaccumulator can be both positive and negative, depending on the experienced Se concentration in the neighbor. As stated above, low tissue Se levels often confer positive growth effects and higher anti-oxidant production. The threshold tissue Se levels between Se benefit and Se toxicity will be species-dependent.

In the lab Se tolerance and accumulation study, the two positively co-occurring species *A. ludoviciana* and *A. frigida* accumulated much higher Se levels than *B.*

inermis and the other species tested (Fig. 3.7 and 3.8). In the field they also contained relatively high Se levels compared to other species, but not as high as they did in the lab study. The levels obtained in the lab (~800 mg/kg DW) appeared to be toxic, considering the reduction in biomass, albeit only significant for ARFR root. However, at the Se levels experienced in the field (183 and 50 mg/kg DW), they may have experienced benefits, considering their positive co-occurrence with hyperaccumulators.

In the x-ray microprobe analysis, *A. frigida* and *A. simplex* had a relative large fraction (70-78%) of organic Se, relative to *B. inermis* (50%). As mentioned, Se (hyper) accumulation and tolerance often is characterized by an ability to better convert inorganic to organic Se. It is also possible that plants in the field are exposed to some extent to organic Se (leaching from hyperaccumulator litter), as opposed to selenate which was used in the lab. Incidentally, in *B. inermis* a large fraction of elemental Se was found; this may be due to endophytes or soil microbes: many bacterial and fungal endophytes from hyperaccumulators growing at the Pine Ridge site have been found to produce elemental Se (Lindblom *et al.*, 2013a, 2014; Sura-de Jong *et al.*, 2015b).

The specific questions for this 2015 study can now be re-addressed: 1) How does soil Se concentration relate to distance from Se hyperaccumulators? It was found that soil Se concentration is higher adjacent to hyperaccumulators, but quickly diminishes after 50 cm, which is very close to the average canopy diameter (56 cm) we found for hyperaccumulators in this study. This provides evidence that hyperaccumulators do indeed change the Se concentration in the area and likely they influence the form of Se as well (Schiavon & Pilon-Smits, 2017). The average distance of the nearest five species was ~20 cm, so well within the area of influence where

hyperaccumulators affect Se form and concentration. Therefore, it is possible hyperaccumulators are providing a selection pressure to these plants. Indeed, the concentration in the hyperaccumulator correlated to the Se concentration in multiple species that showed positive co-occurrence. How this local influence of hyperaccumulators is projected into the greater landscape and to higher and lower trophic levels, and the possible cumulative effects over time will be an interesting area for further study.

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APPENDIX I: P67UBLICATIONS ([1]SHARED FIRST AUTHOR)

Quinn CF, Freeman JL, Reynolds RJB, Cappa JJ, Fakra SC, Marcus MA, Lindblom SD, Quinn EK, Bennett LE, Pilon-Smits EAH. **2010**. Selenium hyperaccumulation offers protection from cell disruptor herbivores. *BMC ecology* **10**: 19.

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