Do selenium hyperaccumulators affect selenium speciation in neighboring plants and soil? An X-Ray Microprobe Analysis


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Do Selenium Hyperaccumulators Affect Selenium Speciation in Neighboring Plants and Soil? An X-ray Microprobe Analysis

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Neighbors of Se hyperaccumulators *Stanleya pinnata* and *Astragalus bisulcatus* were found earlier to have elevated Se levels. Here we investigate whether Se hyperaccumulators affect Se localization and speciation in surrounding soil and neighboring plants. X-ray fluorescence mapping and X-ray absorption near-edge structure spectroscopy were used to analyze Se localization and speciation in leaves of *Artemisia ludoviciana*, *Symphyotrichum ericoides* and *Chenopodium album* growing next to Se hyperaccumulators or non-accumulators at a seleniferous site. Regardless of neighbors, *A. ludoviciana*, *S. ericoides* and *C. album* accumulated predominantly (73–92%) reduced selenocompounds with XANES spectra similar to the C-Se-C compounds selenomethionine and methyl-selenocysteine. Preliminary data indicate that the largest Se fraction (65–75%), both in soil next to hyperaccumulator *S. pinnata* and next to nonaccumulator species was reduced Se with spectra similar to C-Se-C standards. These same C-Se-C forms are found in hyperaccumulators. Thus, hyperaccumulator litter may be a source of organic soil Se, but soil microorganisms may also contribute. These findings are relevant for phytoremediation and biofortification since organic Se is more readily accumulated by plants, and more effective for dietary Se supplementation.

Keywords: *Artemisia ludoviciana*, *Chenopodium album*, *Symphyotrichum ericoides*, X-ray absorption near-edge structure spectroscopy, X-ray fluorescence mapping

Introduction

Selenium (Se) naturally occurs in soils, particularly Cretaceous shale and seleniferous rocks (Rosenfeld and Beath 1964; Beath 1982; Kabata-Pendias 1998). Selenium is essential for many organisms including mammals, many prokaryotes and certain algae (Zhang and Gladyshev 2009) for the production of redox-active selenoproteins that function in scavenging free radicals (Hatfield et al. 2014). Hence, having sufficient dietary Se has been reported to reduce the risk of cancers, HIV infection and heart disease (Goldhaber 2003; Shin et al. 2007; Kato et al. 2010; Hatfield et al. 2014). There is no evidence that Se is essential for higher plants, although Se can positively affect plant growth and antioxidant capacity (Pilon-Smits et al. 2009). Selenium is toxic at higher levels, because of its similarity to sulfur (S). The seleno-amino acids selenocysteine (SeCys) and selenomethionine (SeMet) can be non-specifically incorporated into proteins instead of cysteine or methionine, causing toxicity (Brown and Shrift 1982; Stadtman 1990, 1996; Smith et al. 1995). There is a narrow range between Se deficiency and toxicity, and both are problems for humans and livestock worldwide (Chen et al. 1980; Hoffmann and Berry 2008; Li et al. 2009; Quinn et al. 2011a). Selenium accumulating plants have been used for cleaning up areas that have dangerously high levels of Se (phytoremediation). The Se-enriched plant material may be used to supplement human or animal diets, to prevent Se deficiency (biofortification) (Bañuelos et al. 2011). In areas low in soil Se, crop plants like broccoli, garlic, onion, rice or wheat may also be fortified by adding Se to the fertilizer (Zhu et al. 2009; Fairweather-Tait et al. 2011).

Selenium enters the food chain through plants, which take-up Se via S transporters and metabolize Se through S transporters and enzymes (Terry et al. 2000; Sors et al. 2005). Plant species vary with respect to Se accumulation and tolerance. Most species contain less than 100 mg Se kg\(^{-1}\) DW and are considered nonaccumulators of Se. Other species can accumulate 100–1,000 mg Se kg\(^{-1}\) DW when growing on seleniferous soil and are termed Se accumulators. There is a small group of so-called Se hyperaccumulating plants that accumulate more than 1,000 and up to 15,000 mg kg\(^{-1}\) DW (1.5%) of their DW.
as Se without toxicity (Beath et al. 1939; Galeas et al. 2007; White et al. 2007). The genus Astragalus contains the majority of plant species that are Se hyperaccumulators. For instance Astragalus bisulcatus is a well-studied Se hyperaccumulator (Beath et al. 1939; Rosenfeld and Beath 1964; Neuhierl and Böck 1996; Pickering et al. 2003; Freeman et al. 2006b; Galeas et al. 2007). The genus Stanleya also contains at least one Se hyperaccumulating species, Stanleya pinnata (Beath et al. 1939; Feist and Parker 2001).

Selenium hyperaccumulators differ from accumulators and nonaccumulators with respect to their spatial distribution and chemical speciation of Se, as revealed by micro-focused X-ray fluorescence (μXRF) mapping and Se K-edge X-ray absorption near-edge structure (μXANES) spectroscopy studies and liquid- or gas chromatography – mass spectrometry (LCMS/GCMS). Hyperaccumulators accumulate Se predominantly in the form of organic Se with a C-Se-C configuration (Pickering et al. 2000, 2003). One of the main forms is methyl-SeCys, which is produced by the enzyme SeCys methyltransferase, SMT (Neuhierl and Böck 1996; Freeman et al. 2006a). Methyl-SeCys does not get incorporated into proteins, and can therefore be accumulated safely (Neuhierl and Böck 1996). Other forms of C-Se-C found in hyperaccumulators are γ-glutamyl-methyl-SeCys in A. bisulcatus and seleno-cystathionine in S. pinnata, as determined by liquid chromatography – mass spectrometry (Freeman et al. 2006a). In several Se accumulator and non-accumulator species (Brassica juncea, Arabidopsis thaliana, hybrid poplar), the majority of Se has been shown to remain as inorganic selenate or selenite which are toxic because they can cause oxidative stress and get incorporated into proteins (de Souza et al. 1998; Pilon-Smits et al. 1998; Van Hoevell et al. 2005; Grant et al. 2011). This difference in Se speciation between hyperaccumulators and non-hyperaccumulators may lead to different Se sequestration patterns: hyperaccumulators store Se mostly in the leaf epidermis (sometimes in leaf hairs) and in reproductive tissues, particularly pollen, ovules and seeds (Freeman et al. 2006a; Quinn et al. 2011b). In non-hyperaccumulators, Se can be found throughout the leaf, often accumulated in vascular tissues, and typically Se levels are higher in leaves than in flowers (Quinn et al. 2011b).

Since hyperaccumulators are found predominantly on seleniferous soils (Beath et al. 1934), they appear to require Se in order to successfully compete with other plant species. Selenium may provide hyperaccumulators with a large physiological growth benefit or with an ecological benefit, and there is evidence for both. Selenium significantly (2–3 fold) improved growth of hyperaccumulators A. bisulcatus and S. pinnata (El Mehdawi et al. 2012). There is also convincing evidence for ecological benefits of Se hyperaccumulation (for a review see El Mehdawi and Pilon-Smits 2012). Selenium hyperaccumulation offers the plant enhanced resistance to a variety of Se-sensitive herbivores and pathogens (Vickerman et al. 2002; Hanson et al. 2003, 2004; Freeman et al. 2006b, 2007, 2009; Quinn et al. 2007, 2008, 2010). Hence, Se hyperaccumulation may be considered a form of elemental defense (Boyd and Martens 1992; Boyd 2007, 2010). Selenium may also be used as a form of elemental allelopathy: soil around hyperaccumulators was found to be 7–9 fold enriched in Se, and Se-sensitive plants grown on this soil showed reduced germination and growth, corresponding with elevated Se levels (El Mehdawi et al. 2011a). A caveat for this elemental allelopathy hypothesis is that it is very hard to determine whether the observed Se “hot spots” around hyperaccumulators are caused by the plants concentrating Se on their surrounding soil surface, or rather are naturally occurring in seleniferous soils and favor the establishment of hyperaccumulators. Interestingly, the high-Se areas associated with Se hyperaccumulators were found to have a positive effect on the growth of plant neighbors (an effect known as facilitation), if these are resistant to the associated elevated Se levels (El Mehdawi et al. 2011b). Symphyotrichum ericoides and Artemisia ludoviciana accumulated up to 20-fold more Se, were two-fold bigger, and harbored fewer herbivores when growing in Se-rich areas around hyperaccumulators than when they were growing next to non-accumulators. Based on these results, we hypothesize that hyperaccumulators enrich their surrounding soil with Se, and this can positively or negatively affect neighboring plants, depending on their sensitivity to Se.

If indeed hyperaccumulators enrich their surrounding soil with Se, the mechanism of this phytoenrichment may be a combination of litter deposition and root exudation. An earlier study by Quinn et al. (2011a) showed that high-Se leaf litter from A. bisulcatus decomposed readily in a seleniferous habitat, harbored more microbial and micro-arthropod decomposers than low-Se litter, and led to enrichment of the underlying soil. There is also evidence of rhizodeposition of Se by hyperaccumulators. Roots of hyperaccumulators A. bisulcatus and S. pinnata were shown to contain mainly C-Se-C, both when growing in the field and when treated with selenate in a controlled greenhouse study (Lindblom et al. 2012). Roots of A. bisulcatus treated with selenate were found to exclude fairly high levels of Se in the form of C-Se-C compounds (El Mehdawi et al. 2012). In the same study by (El Mehdawi et al. 2012), in which pairs of plants were co-cultivated in different species combinations and supplied with selenate, the non-accumulator species Stanleya elata was found to contain a significantly higher fraction of C-Se-C when growing next to S. pinnata than when growing next to another S. elata.

In the field study described here we investigated whether hyperaccumulator plants affect Se localization and speciation in neighboring plants, and whether they affect Se speciation in nearby soil. To address this question, μXRF mapping and Se K-edge μXANES were used to investigate Se distribution and speciation in leaves of A. ludoviciana, S. ericoides and Chenopodium album growing next to hyperaccumulators or next to non-hyperaccumulators. Selenium speciation was also analyzed in soil adjacent to hyperaccumulators and non-accumulators.

Materials and Methods

Study Area and Sampling

The field site for this study is Pine Ridge Natural Area in Fort Collins, CO, USA. The naturally seleniferous soil (shale) and
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Fig. 1. Leaf Se and S concentration of (A,B) Artemisia ludoviciana (C,D) Symphyotrichum ericoides (E,F) Chenopodium album collected from around hyperaccumulator species (S. pinnata and A. bisulcatus) and from around non hyperaccumulator vegetation in seleniferous habitat (Fort Collins, Colorado, USA). Values shown are represent means ± SEM (n = 9), different lower case letters above bars indicate significantly different means (ANOVA, P < 0.05).

vegetation properties of this area were described in detail in a previous study (El Mehdawi et al. 2011a). For this study, we sampled three naturally occurring plant species on the site: A. ludoviciana (white sage; Asteraceae), S. ericoides (white heath aster; Asteraceae) and C. album (lambsquarters; Chenopodiaceae). On Pine Ridge Natural Area these three species are often found in the vicinity of Se hyperaccumulating species A. bisulcatus (two-grooved milkvetch, Fabaceae) and S. pinnata (prince’s plume, Brassicaceae).

To investigate the effect of Se hyperaccumulator plants on soil Se speciation using XANES, soil samples were collected from around the stem of Se hyperaccumulators A. bisulcatus and S. pinnata, as well as soil located >4 m away from any hyperaccumulator (bulk soil) at Pine Ridge Natural Area. The A. bisulcatus and S. pinnata plants growing on the site are 15–40 cm in canopy radius and 40–70 cm tall. For each plant, a composite soil sample was collected from the top 2 cm of the soil and <5 cm from the taproot. If any litter was present, this was removed before sampling. Three replicate soil samples were collected per treatment group, each from around a different plant. Soil samples were sieved using mesh with 1 mm² holes and then stored frozen at -80°C until X-ray microprobe analysis and Se and S analysis.

To determine whether proximity to Se-hyperaccumulating plants affects Se and S concentration in neighboring plants, youngest mature leaves were collected for elemental analysis from A. ludoviciana, S. ericoides and C. album, either growing in close proximity (< 1 m) to the hyperaccumulator species A. bisulcatus or S. pinnata or away (> 4 m) from any hyperaccumulator (n = 3). Since C. album was only found next
to *S. pinnata* but not next to *A. bisulcatus* it was sampled in two locations instead of three. To investigate the effect of Se hyperaccumulator plants on Se localization and speciation in neighboring plant species, one of the *A. ludoviciana, S. ericoides* and *C. album* leaves collected next to *A. bisulcatus*, next to *S. pinnata* or >4 m away from any hyperaccumulator were rinsed with distilled water and then flash-frozen in liquid nitrogen and stored at –80°C until X-ray microprobe analysis.

**Statistical Analysis**

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

**Results**

There was a pronounced difference in leaf Se concentration in *A. ludoviciana* plants depending on their proximity to hyperaccumulators: leaf Se levels were significantly higher when they were growing next to hyperaccumulators *S. pinnata* or *A. bisulcatus* as compared to when they were growing next to non-hyperaccumulator species (Fig. 1A). In fact, when growing next to either of these hyperaccumulators, *A. ludoviciana* reached hyperaccumulator levels itself (>1,000 mg kg⁻¹ DW). Leaf Se levels in *S. ericoides* plants were 7–8 fold higher when they were growing next to hyperaccumulators *S. pinnata* and *A. bisulcatus* as compared to when they were growing next to non-hyperaccumulators (Fig. 1C). The leaf Se concentration in *C. album* was 2-fold higher when they were growing next to hyperaccumulator *S. pinnata* as compared to when they were growing next to other species (Fig. 1E, *P* = 0.06). As reported earlier, *A. ludoviciana* and *S. ericoides* were 2–3 fold bigger in size when growing next to the hyperaccumulators *A. bisulcatus* and *S. pinnata* than when growing next to non-hyperaccumulators ([El Mehdawi et al. 2011b], and *C. album* showed a similar positive growth effect when in proximity to *S. pinnata* (it was 2-fold bigger when growing next to *S. pinnata* than when growing next to non-hyperaccumulators, results not shown). As a result of their higher Se levels and higher biomass, the total amount of Se accumulated per plant (concentration x biomass) was 8–14 fold higher for *A. ludoviciana* and *S. ericoides*, and 4-fold higher for *C. album* when growing next to hyperaccumulators (*P* < 0.05). Since Se hyperaccumulators are known to contain not only higher Se

**Fig. 2.** Selenium (A) and S (B) concentration in soil collected from around hyperaccumulator species (*S. pinnata* and *A. bisulcatus*) and from around non-hyperaccumulator vegetation in seleniferous habitat (Fort Collins, Colorado (USA)). Values shown represent means ± SEM (*n* = 12); different lower case letters above bars indicate significantly different means (*P* < 0.05).

**Elemental and X-ray Analyses**

For Se and S elemental analyses, the plant samples were dried at 50°C for 72 h and 100 mg DW of each sample was digested in nitric acid, as described by Zarcinas et al. (1987). Soil samples were extracted and analyzed as described by El Mehdawi et al. (2012); the soil was air-dried at room temperature for 3 d, and then sieved through a 1 mm mesh. Five gram of soil was extracted with 10 mL of a solution containing 1 M ammonium bicarbonate and 5 mM diethylenetriaminepentaacetate (AB-DTPA), shaking for 2h at room temperature. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used as described by Fassel (1978) to determine Se and S concentration in the acid plant digests and the soil extracts.

X-ray microprobe analyses were performed on intact frozen leaf material from *A. ludoviciana, S. ericoides* and *C. album* sampled as described above. The material was kept frozen and mounted on a Peltier stage kept at –30°C during X-ray microprobe analysis. Tissue distributions of Se, Ca and Fe were then determined using μXRF mapping, and chemical speciation of Se was determined using Se K-edge μXANES spectroscopy in areas of interest, followed by least-squares linear combination fitting (LCF) of experimental XANES spectra in the range of 12,630 to 12,850 eV using a library of nine standard selenocompounds, all as described by Banuelos et al. (2011) and Quinn et al. (2011b). The best LCF was obtained by minimizing the normalized sum of squares residuals [NSS = 100×∑(μexp − μfit)²/∑(μexp)²], where μ is the normalized absorbance (NSS = 0 = perfect fit). The error margin for the reported fraction for each selenocompound is ±10%.
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Fig. 3. X-ray fluorescence elemental mapping of leaves of (A) Artemisia ludoviciana grown next to hyperaccumulator S. pinnata. (B) Artemisia ludoviciana grown next to hyperaccumulator A. bisulcatus. (C) Artemisia ludoviciana grown next to non hyperaccumulator vegetation. Selenium is shown in red, calcium in green, and manganese in blue. For each species the bottom right panel shows a tricolor overlay of Se, Ca and Fe. (Continued)

levels but also higher S levels than other vegetation on seleniferous soils (Galeas et al. 2007; El Mehdawi et al. 2011a), we also compared the S levels of the A. ludoviciana, S. ericoides and C. album plants under study. Leaf S levels in A. ludoviciana were not significantly affected by neighboring species (Fig. 1B). In S. ericoides the S levels were 35% higher when growing next to S. pinnata compared to other plant species (Fig. 1D, $P < 0.05$), and in C. album the S levels were 50% higher when growing next to hyperaccumulator S. pinnata (Fig. 1F, $P < 0.05$).

As an estimation of bioavailable elemental concentrations, AB-DTPA extractable Se and S were analyzed in soils next to hyperaccumulators and non-accumulators. The soil Se levels were 2.5–3 fold higher next to the hyperaccumulators as compared to next to non-hyperaccumulators in the same area (Fig. 2A). The bioavailable S levels were also somewhat elevated (17%) in soil next to S. pinnata, as compared to non-hyperaccumulator soil; A. bisulcatus soil was intermediate in S level and not significant from either other soil type (Fig. 2B).
μ-XRF mapping was used to compare leaf Se distribution in the three species when growing next to hyperaccumulators or non-hyperaccumulators (Fig. 3). The Se signal was more intense in *A. ludoviciana* collected next to *S. pinnata* (7,500 counts) and next to *A. bisulcatus* (7,000 counts) than when collected next to non-hyperaccumulators (2,500 counts), which is in agreement with the ICP-AES results shown in Figure 1. In *A. ludoviciana* leaves from all three locations, the Se signal was observed throughout the leaf, with a highest intensity in the mid-vein (Fig. 3A-C); this may in part be explained by increased leaf thickness at the vein. There was also a tendency for the Se signal to be higher along the leaf margins; this was more pronounced in the leaf collected from the plant that grew next to non-hyperaccumulators (Fig. 3C) than in leaves collected next to *S. pinnata* (Fig. 3A) or *A. bisulcatus* (Fig. 3B). The leaf edges were not visibly thickened or rolled up, so leaf thickness does not appear to contribute to the higher Se signal.

XANES indicated that the Se in *A. ludoviciana* leaves grown next to *S. pinnata* consisted primarily (61-80%) of reduced selenocompound(s), indistinguishable from the C-Se-C standard compounds methyl-selenocysteine and selenomethionine; the remainder was modeled as red elemental Se (15%) and selenite (SeO$_3^{2-}$, 10%). The predominant form of Se in *A. ludoviciana* growing next to *A. bisulcatus* was also a reduced selenocompound similar to C-Se-C standards (75-92%), and most of the remainder was again modeled as selenite (7.5%). Selenium in *A. ludoviciana* growing away from hyperaccumulators was modeled primarily (56-94%) as reduced C-Se-C-like compounds as well, with smaller fractions of red elemental Se (12%) and selenite (8%). There were no significant differences in Se speciation between leaves from the three locations (Table 1; note that the error margin on XANES LCF was ±10%).

The leaf distribution of other elements was also analyzed. The localization of Ca and Fe were chosen to be co-visualized in the XRF figures since they help visualize leaf structures and, with that, Se localization patterns. In *A. ludoviciana* growing next to *A. bisulcatus* or *S. pinnata*, Ca was concentrated in the mid-vein and leaf margins (Fig. 3A, B), but in the leaf collected from *A. ludoviciana* growing next to non-hyperaccumulators the Ca was evenly distributed throughout the leaf (Fig. 3C). The Fe in *A. ludoviciana* leaves was highly concentrated in discrete locations all across the leaf that may correspond with leaf hairs.

Selenium distribution in leaves of *S. ericoides* was similar in leaves collected from plants growing next to hyperaccumulators (Fig. 4A, B) or non-hyperaccumulators (Fig. 4C), although the intensity of the Se signal was higher from leaves collected next to *S. pinnata* and *A. bisulcatus* (8,000 and 4,000 counts, respectively) than from leaves collected next to non-hyperaccumulators (2,000 counts). The Se was in all cases distributed fairly evenly throughout the leaf, but appeared less concentrated in the mid-vein and the extreme leaf edges. Selenium was also detected in some of the small leaf hairs. Calcium and Fe were clearly concentrated in the leaf hairs, but appear to be in different hairs (Fig. 4B, C).

Selenium speciation in *S. ericoides* leaves grown next to *S. pinnata* indicated Se was modeled to be present mainly in the form of reduced compounds (63-84%) similar to organic C-Se-C form, with smaller fractions of selenite (9%), selenodiglutathione (SeGSH$_2$, 8%), and selenate (SeO$_4^{2-}$, 3%). In *S. ericoides* growing next to *A. bisulcatus* the leaf Se was modeled to consist primarily (41-96%) of reduced compounds similar to organic C-Se-C; the remainder was SeGSH$_2$ (13%), selenite (7%), and red Se (4%). Selenium speciation in leaves of *S. ericoides* growing next to non-hyperaccumulators could not be determined, as the Se
Effects of Se Hyperaccumulators on Speciation in Neighboring Plants

Table 1. Selenium speciation in plant leaf material of *S. ericoides*, *A. ludoviciana* and *C. album* determined from XANES

<table>
<thead>
<tr>
<th>Plant Configuration</th>
<th>NSS %</th>
<th>C-Se-C %</th>
<th>SeGSH₂ %</th>
<th>SeO₃²⁻ %</th>
<th>SeO₄²⁻ %</th>
<th>Red Se</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. ludoviciana</em> grown next to <em>S. pinnata</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>3.9 × 10⁻⁴</td>
<td>80</td>
<td>ND</td>
<td>9</td>
<td>ND</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>4.7 × 10⁻⁴</td>
<td>73</td>
<td>ND</td>
<td>10</td>
<td>ND</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>9.6 × 10⁻⁴</td>
<td>80</td>
<td>ND</td>
<td>10</td>
<td>ND</td>
<td>11</td>
</tr>
<tr>
<td>4</td>
<td>7.6 × 10⁻⁴</td>
<td>61</td>
<td>ND</td>
<td>12</td>
<td>ND</td>
<td>20</td>
</tr>
<tr>
<td>Average ± SE</td>
<td>74 ± 4</td>
<td>ND</td>
<td>10 ± 1</td>
<td>ND</td>
<td>15 ± 2</td>
<td></td>
</tr>
<tr>
<td><em>A. ludoviciana</em> grown next to <em>A. bisulcatus</em></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.9 × 10⁻⁴</td>
<td>75</td>
<td>ND</td>
<td>10</td>
<td>ND</td>
<td>0.3</td>
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<tr>
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<td>ND</td>
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<tr>
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<td>3.1 × 10⁻⁴</td>
<td>92</td>
<td>ND</td>
<td>6</td>
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<td>2.7 × 10⁻⁴</td>
<td>87</td>
<td>ND</td>
<td>6</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Average ± SE</td>
<td>87 ± 4</td>
<td>ND</td>
<td>7 ± 1</td>
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<td>0.08</td>
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<tr>
<td><em>A. ludoviciana</em> grown away from hyperaccumulators</td>
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<tr>
<td>1</td>
<td>1.9 × 10⁻³</td>
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<td>ND</td>
<td>11</td>
<td>ND</td>
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<td>2.9 × 10⁻³</td>
<td>94</td>
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<tr>
<td>Average</td>
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<td>ND</td>
<td>8</td>
<td>ND</td>
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<td><em>S. ericoides</em> grown to <em>S. pinnata</em></td>
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<td></td>
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<tr>
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<td>6.1 × 10⁻⁴</td>
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<td>12</td>
<td>7</td>
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<td>ND</td>
<td>8</td>
<td>4</td>
<td>2</td>
</tr>
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<td>84</td>
<td>ND</td>
<td>5</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Average ± SE</td>
<td>77 ± 5</td>
<td>8 ± 5</td>
<td>9 ± 2</td>
<td>3 ± 0.6</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td><em>S. ericoides</em> grown to <em>A. bisulcatus</em></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>3.2 × 10⁻³</td>
<td>96</td>
<td>ND</td>
<td>6</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>1.1 × 10⁻³</td>
<td>83</td>
<td>ND</td>
<td>9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>6.1 × 10⁻⁴</td>
<td>41</td>
<td>39</td>
<td>8</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>Average ± SE</td>
<td>73 ± 16</td>
<td>13</td>
<td>8 ± 0.9</td>
<td>0.9±0.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><em>S. ericoides</em> grown away from hyperaccumulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>8.5 × 10⁻³</td>
<td>ND</td>
<td>82</td>
<td>20</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td><em>C. album</em> grown to <em>S. pinnata</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.1 × 10⁻⁴</td>
<td>89</td>
<td>ND</td>
<td>4</td>
<td>3</td>
<td>ND</td>
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<tr>
<td>2</td>
<td>2.4 × 10⁻⁴</td>
<td>94</td>
<td>ND</td>
<td>3</td>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>4.0 × 10⁻⁴</td>
<td>93</td>
<td>ND</td>
<td>3</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>Average ± SE</td>
<td>92 ± 1</td>
<td>ND</td>
<td>4 ± 0.3</td>
<td>6 ± 1</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Plants were growing in the field next to hyperaccumulators *S. pinnata* and *A. bisulcatus* or away from hyperaccumulators. Values shown for each form of Se represent% of total Se. NSS: Normalized Sum of Squares (measure for quality of fit); ND: not detectable.

signal was too low to obtain a reliable spectrum that could be fitted.

The XRF Se signal in the *C. album* leaf collected next to *S. pinnata* was 8-fold higher (20,000 counts, Fig. 5A) than the Se signal in the *C. album* leaf collected next to non-accumulators (2,500 counts, Fig. 5B). In leaves from both locations, Se was distributed uniformly, but with somewhat higher concentration in the vasculature (Fig. 5A, B). The Se distribution appears somewhat mottled, with Se-richer areas that may correspond with trichomes (Fig 5B). Calcium was clearly concentrated in the star-shaped trichomes (Fig. 5A, B). Iron was highly concentrated in discrete locations all across the leaf, which may correspond with trichomes but if so the Fe is not present in the entire trichome and does not clearly co-localize with Ca or Se (Fig. 5A, B).

XANES analysis showed that the Se in leaves of *C. album* grown next to *S. pinnata* consisted primarily (89–93%) of reduced forms with spectra similar to C-Se-C compounds (Table 1); the remainder was modeled as selenate (6%) and selenite (4%). Due to the low Se signal, no reliable XANES spectrum could be obtained from the *C. album* leaf collected next to non-accumulators.

Comparative Se speciation analysis (XANES) was also performed on soil collected next to hyperaccumulators and non-hyperaccumulators, to investigate the form of Se available to *A. ludoviciana*, *S. ericoides* and *C. album* in each location (Fig. 6). The Se signals for the soil samples were very low, particularly for soil collected next to *A. bisulcatus*, where only one XANES spectrum could be obtained, and it was of insufficient quality to be informative (sum...
Fig. 4. X-ray fluorescence elemental mapping of leaves of (A) *Symphyotrichum ericoides* grown next to hyperaccumulator *S. pinnata*. (B) *Symphyotrichum ericoides* grown next to hyperaccumulator *A. bisulcatus*. (C) *Symphyotrichum ericoides* grown next to non-hyperaccumulator vegetation. Selenium is shown in red, calcium in green, and manganese in blue. For each species the bottom right panel shows a tricolor overlay of Se, Ca and Fe. (Continued)

of squares >5.10^{-3}). Soil around non-hyperaccumulators and around hyperaccumulator *S. pinnata* did yield three usable XANES spectra each that showed consistent fits (Table 2). In the soil collected next to non-hyperaccumulators, 71–80% of the Se was modeled as reduced selenocompounds with spectra indistinguishable from the organic C-Se-C compounds methyl-selenocysteine and selenomethionine, and the remaining 20–28% as selenite (Table 2). Similarly, the spectra from soil next to hyperaccumulator *S. pinnata* were fitted as primarily (58–71%) reduced selenocompounds, the remainder (29–38%) being selenite (Table 2).

**Discussion**

In the field study described here we investigated whether hyperaccumulator plants affect Se localization or speciation in neighboring plants, and whether they affect Se speciation in nearby soil. We found no significant difference in Se speciation in *A. ludoviciana*, *S. ericoides* and *C. album* leaves collected at different proximity to Se hyperaccumulators. The main form of Se in all three species was reduced Se with a XANES spectrum similar to organic selenocompounds with a C-Se-C configuration. This could correspond with
MeSeCys, Se-cystathionine, γ-Glu-MeSeCys or SeMet; the XANES spectra for C-Se-C compounds are indistinguishable. We were somewhat surprised to find such a high fraction of C-Se-C in these species, since C-Se-C is typically found in Se hyperaccumulators (Freeman et al. 2006a), while many non-hyperaccumulators tend to accumulate more inorganic Se when supplied with selenate (de Souza et al. 1998; Van Hoewyk et al. 2005). However, we recently found that S. ericoides is able to produce C-Se-C when supplied with selenate (El Mehdawi et al. 2014). We do not know whether A. ludoviciana and C. album have the same capability. The organic Se in the three plant species may also simply be a reflection of the form of Se present in the soil. The predominant form of Se (65–75%) in the soil adjacent to hyperaccumulator S. pinnata and in soil collected next to non-accumulators was also modeled to be reduced Se with high similarity to C-Se-C compounds. It cannot be excluded that some other reduced form of Se has a similar XANES spectrum to C-Se-C; e.g. no metal selenides were included as standards in this study. However, judged from Rhyser et al. (2005) who showed spectra from a wide variety of soil selenocompounds, none appear to have spectra identical to organic C-Se-C compounds (Ryser et al. 2005). If indeed this seleniferous soil contains a high percentage of organic Se it is intriguing, as it suggests that a large fraction of soil Se in this area is of biological origin. This is in agreement with a report by Beath et al. (1946), which showed that the bioavailable Se in a seleniferous shale soil from Utah where no hyperaccumulators were reported consisted of exclusively selenate, whereas in shale from Niobrara County, Wyoming, in a location where Se hyperaccumulators occurred, most of the bioavailable Se was organic Se, and the remainder selenate. Beath et al. found a relatively higher fraction of organic Se in the upper soil layer (70% organic Se in soil at 0–50 cm depth) than in deeper soil (26% organic Se in soil at 50–100 cm depth). The authors concluded that the abundance of the organic Se likely reflected the activity of the locally occurring hyperaccumulator A. racemosus, which contained 14,920 mg Se kg\(^{-1}\) DW. In analogy, since the hyperaccumulators on Pine Ridge soil accumulate C-Se-C (Freeman et al. 2006a), and are quite abundant on the site, the large fraction

Table 2. Selenium speciation in soil collected next to hyperaccumulator S. pinnata and next to non-hyperaccumulators, as determined from XANES

<table>
<thead>
<tr>
<th></th>
<th>NSS %</th>
<th>C-Se-C %</th>
<th>SeO₃²⁻ %</th>
<th>SeO₄²⁻ %</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil next to non-hyperaccumulators</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.6 × 10⁻³</td>
<td>80</td>
<td>20</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>2.1 × 10⁻³</td>
<td>71</td>
<td>28</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>3</td>
<td>1.8 × 10⁻³</td>
<td>74</td>
<td>26</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Average ± SE</td>
<td>75 ± 4</td>
<td>25 ± 4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil next to hyperaccumulator S. pinnata</td>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>3.2 × 10⁻³</td>
<td>64</td>
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<tr>
<td>2</td>
<td>2.9 × 10⁻³</td>
<td>71</td>
<td>29</td>
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<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>4.0 × 10⁻³</td>
<td>58</td>
<td>38</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Average ± SE</td>
<td>65 ± 6</td>
<td>34 ± 5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each replicate soil sample was collected from around a different plant. Values shown for each form of Se represent % of total Se. NSS: Normalized Sum of Squares (measure for quality of fit); ND: not detectable. C-Se-C: MeSeCys/SeMet/SeCystathionine (indistinguishable). Forms of Se that were not detected in any of the samples and therefore not tabulated: SeO, Se-cysteine, Se-cystine, Se(GSH)₂.
Fig. 5. X-ray fluorescence elemental mapping of leaves of (A) Chenopodium album grown next to hyperaccumulator S. pinnata. (B) Chenopodium album grown next to non hyperaccumulator vegetation. Selenium is shown in red, calcium in green, and manganese in blue. For each species the bottom right panel shows a tricolor overlay of Se, Ca and Fe.

of reduced Se in the soil at each of the locations sampled may have been deposited by the Se hyperaccumulators, e.g. via litter and root deposition. The roots of perennial prairie forbs including Astragalus spp. have been reported to reach 2 m in depth and 1 m in width (Weaver 1958), so can scavenge a large soil volume. The canopy of individual hyperaccumulators at Pine Ridge Natural Area reaches up to 0.8 m in diameter, and the plants often occur in clusters. Their litter may be spread by wind, and Se ingested from hyperaccumulators (live or litter) may be spread by various detrivores and herbivores (Quinn et al. 2011a; Freeman et al. 2006a; Valdez Barillas et al. 2012). The soil sample collected next to non-hyperaccumulators was on average 10 m away from Se hyperaccumulators. It is feasible that the Se hyperaccumulators, given enough time, can influence Se speciation in surrounding soil in a radius of 10 m or more. Microbes may additionally affect soil Se speciation by converting inorganic soil Se to organic forms, and by converting organic Se deposited by hyperaccumulators into inorganic forms (Lindblom et al. 2012). It has been reported for Symphyotrichum eatonii growing in Se-containing reclaimed mine soil, that rhizosphere soil and plant roots contained relatively more selenate (+6), while bulk soil contained more reduced Se (-2, 0) (Oram et al. 2011). The Se in the rhizosphere was more bioavailable than the bulk soil Se, leading the authors to hypothesize that oxidation of reduced soil Se to more bioavailable selenate in the rhizosphere facilitated Se.
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Fig. 6. Selenium Se K-edge μXANES spectra obtained from soil next to hyperaccumulators (S. pinnata and A. bisulcatus) and from soil next to non-hyperaccumulators (bulk soil). For comparison, four standard spectra are also shown, from methyl-SeCys (a C-Se-C compound), SeMet (a C-Se-C compound), selenite (SeO$_3^{2-}$) and elemental Se (Se(0)). The Se speciation results deduced from these spectra are shown in Table 2.

uptake by the plant. In an X-ray microprobe study by Ryser et al. (2005, 2006) a mining byproduct called middle waste shale in Idaho, U.S.A. was shown to contain Se in four oxidation states: selenide (-2), elemental Se (0) / selenite (+4) and selenate (+6). The authors concluded that the more reduced forms elemental Se and selenide from the parent material oxidized over time to the more mobile selenite and selenate forms.

One of the three species, A. ludoviciana, showed some evidence that its leaf Se distribution was affected by Se hyperaccumulator neighbors. There was relatively more Se concentrated along the leaf margins when A. ludoviciana was growing next to non-hyperaccumulators than when it was growing next to hyperaccumulators. This may be related to the lower leaf Se concentration next to non-hyperaccumulators: the overall lower Se abundance may make it easier to see the elevated Se concentration in the leaf margins. It is also possible that as leaf Se concentration increases, the plant first fills up the leaf margin to capacity and then stores additional incoming Se throughout the rest of the leaf. A similar sequestration pattern has been observed in S. pinnata (Freeman et al. 2006a). Preferential sequestration of Se in the margins may function to keep Se away from sensitive leaf processes in the mesophyll (photosynthesis), and it may also effectively protect leaves from leaf-chewing herbivores, which often attack at the margins first. Indeed, the Se in A. ludoviciana has been shown to protect it from herbivory. High-Se A. ludoviciana leaves collected next to hyperaccumulators better deterred grasshoppers in laboratory choice experiments and were more toxic to them in non-choice experiments, as compared to low-Se leaves from the same species collected next to non-hyperaccumulators (El Mehdawi et al. 2011b). Similarly, in the field the high-Se A. ludoviciana plants harbored fewer herbivores and showed less herbivory damage than their low-Se counterparts.

Among the other elements whose leaf distribution was mapped in A. ludoviciana, S. ericoides and C. album, Ca was concentrated in the leaf hairs in C. album and to a lesser extent also in S. ericoides; in A. ludoviciana Co-localized with Se and was most concentrated in the mid-vein and leaf margins. Iron was highly concentrated in discrete locations all across the leaf, in all three species. In S. ericoides the Fe-rich locations corresponded to leaf hairs, and these were different hairs than the ones that concentrated Ca; apparently the leaves had at least two different trichome types, perhaps with different functions. In the other two species the high-Fe specks may have corresponded with leaf hairs as well, but this is hard to judge from the images. The Fe specks in C. album leaves did not have the same shape as the trichomes, but may have been at the base of the Ca-filled leaf hairs, as was found for Mn in Brassica juncea (Freeman et al. 2006a) and Alyssum murale (Tapper 2008).

In conclusion, this study did not find direct evidence that hyperaccumulators affect the Se speciation in neighboring vegetation: the three plant species tested showed the same Se speciation regardless of the proximity of hyperaccumulators. A surprising finding was that the predominant forms of Se in neighboring vegetation as well as in soil was C-Se-C, even in locations >10 m from hyperaccumulators. It may be hypothesized that hyperaccumulators influence Se speciation on a larger scale than previously thought. Alternatively, the C-Se-C found throughout the area may be of other biological origin, e.g. microbial. This may be addressed in future studies. In earlier studies, soil around Se hyperaccumulators contained up to 7-13 fold higher (total) Se levels compared to soil further away (El Mehdawi et al. 2011a, b). This is in agreement with this study, where bioavailable Se levels were found to be 2.5-3 fold higher in soil adjacent to Se hyperaccumulator plants compared to soil adjacent to other vegetation. These Se “hot spots” around hyperaccumulators may be due to phytoenrichment by hyperaccumulators via litter deposition (Quinn et al. 2011a) and/or root exudation (El Mehdawi et al. 2012b). Alternatively, they may be naturally occurring geological phenomena that favor the establishment of hyperaccumulators; this is very hard to distinguish. If hyperaccumulators affect soil Se speciation as well as Se distribution, one possible hypothesis to interpret our collective findings, then
hyperaccumulators may profoundly influence Se cycling in seleniferous areas.

This study has relevance for Se phytoremediation and biofortification, for several reasons. First, the finding that the major form of Se in these plant species (and apparently also in soil) is organic Se is of significance since organic Se is more readily taken up by plants and more suitable for biofortification than inorganic Se. Furthermore, as demonstrated here, x-ray microprobe analysis offers a powerful tool to study chemical speciation, not only in soil or water but also in vivo in intact organisms. As is the case for Se, the chemical speciation of toxic elements often affects their solubility and toxicity, and thus their risk to society and the environment. This is relevant for regulators and site managers. With limited remediation funds, regulators may use chemical speciation data to prioritize the cleanup of sites containing more toxic forms of elements. Site managers may benefit from determination of the chemical speciation of toxic elements in polluted sites, both in the soil/water as well as in the plants, animals and microorganisms that inhabit the site, as it can give important insight into biotransformation processes through the food chain. Hyperaccumulator species such as the ones studied here are particularly interesting in this respect, as they vastly bioaccumulate toxic elements and can also biotransform them into different chemical species.

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References


