Selenium Distribution and Speciation in the Hyperaccumulator *Astragalus bisulcatus* and Associated Ecological Partners

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The goal of this study was to investigate how plant selenium (Se) hyperaccumulation may affect ecological interactions and whether associated partners may affect Se hyperaccumulation. The Se hyperaccumulator *Astragalus bisulcatus* was collected in its natural seleniferous habitat, and x-ray fluorescence mapping and x-ray absorption near-edge structure spectroscopy were used to characterize Se distribution and speciation in all organs as well as in encountered microbial symbionts and herbivores. Se was present at high levels (704–4,661 mg kg⁻¹ dry weight) in all organs, mainly as organic C-Se-C compounds (i.e. Se bonded to two carbon atoms, e.g. methylselenocysteine). In nodule, root, and stem, up to 34% of Se was found as elemental Se, which was potentially due to microbial activity. In addition to a nitrogen-fixing symbiont, the plants harbored an endophytic fungus that produced elemental Se. Furthermore, two Se-resistant herbivorous moths were discovered on *A. bisulcatus*, one of which was parasitized by a wasp. Adult moths, larvae, and wasps all accumulated predominantly C-Se-C compounds. In conclusion, hyperaccumulators live in association with a variety of Se-resistant ecological partners. Among these partners, microbial endosymbionts may affect Se speciation in hyperaccumulators. Hyperaccumulators have been shown earlier to negatively affect Se-sensitive ecological partners while apparently offering a niche for Se-resistant partners. Through their positive and negative effects on different ecological partners, hyperaccumulators may influence species composition and Se cycling in seleniferous ecosystems.

Selenium (Se) hyperaccumulation in plants is an evolutionary adaptation that facilitates the accumulation of Se to levels in excess of 0.1% plant dry weight or more than 1,000 mg Se kg⁻¹ dry weight (Beath et al., 1939a, 1939b). An extreme example is *Astragalus bisulcatus* (two-grooved milkvetch), which is capable of accumulating Se to levels up to 15,000 mg Se kg⁻¹ dry weight (Galeas et al., 2007). Like other hyperaccumulators, populations of *A. bisulcatus* are almost exclusively found on seleniferous soils, where Se levels may range from 1 to 100 mg Se kg⁻¹ soil (Beath et al., 1939a, 1939b).

Se is thought to be taken up by plants mainly as selenate (SeO₄²⁻), the predominant form of bioavailable Se in soils, via sulfate transporters and reduced and incorporated into selenoamino acids by the sulfur (S) assimilation pathway (for review, see Sors et al., 2005; Pilon-Smits and Quinn, 2010). Selenate is reduced to selenite (SeO₃⁻) and then to selenide (Se²⁻), which is incorporated into selenocysteine (SeCys). The nonspecific incorporation of SeCys into proteins is presumed to be toxic (Stadtman, 1990). Se hyperaccumulators avoid Se toxicity by methylating SeCys to methylselenocysteine (MeSeCys) via a unique enzyme, SeCys methyltransferase, effectively circumventing the misincorporation of SeCys into protein (Neuhierl and Böck, 1996).

Microfocused x-ray fluorescence (μXRF) mapping and x-ray absorption near-edge structure (μXANES) have previously been utilized to investigate the spatial distribution and chemical speciation of Se in hyperaccumulator leaves (Freeman et al., 2006b). Whereas nonhyperaccumulator plants were found to accumulate Se primarily in the leaves as selenate (de Souza et al., 1998), Se hyperaccumulators accumulate Se predominantly in
the leaf epidermis in organic forms with a C-Se-C configuration (i.e. Se bonded to two carbon atoms; Freeman et al., 2006b). A. bisulcatus showed specific Se sequestration in the leaf trichomes, in the form of C-Se-C compounds that were identified as MeSeCys and $\gamma$-glutamyl-MeSeCys, using liquid chromatography-mass spectroscopy (LC-MS; Freeman et al., 2006b).

Se hyperaccumulators exhibit variability in Se hyperaccumulation throughout the growing season and between different organs. Seasonal fluctuations in Se hyperaccumulation were observed in a field study of A. bisulcatus (Galeas et al., 2007). During the spring, Se appeared to be translocated from the root to the emerging leaves, reaching maximum levels in mid-spring. Leaf Se levels then dropped gradually during summer and fall and rose in reproductive organs and roots, as Se was presumably remobilized from the leaves to reproductive organs and back into the roots during shoot senescence. The form of Se that is remobilized may be organic, as A. bisulcatus was shown to accumulate mostly organic Se in young leaves and roots, whereas older leaves hyperaccumulated mainly inorganic selenate and at much lower concentrations (Pickering et al., 2000).

The functional significance of Se hyperaccumulation and the ecological implications of the extreme Se levels have been a topic of recent interest. Several studies suggest that Se hyperaccumulation evolved as a defense mechanism against herbivory. Se can protect plants from a wide variety of Se-sensitive invertebrate and vertebrate herbivores, due to deterrence and toxicity (for review, see Boyd, 2007, 2010; Quinn et al., 2007; El Mehdawi and Pilon-Smits, 2012). Se has been shown to protect plants from aphids (Hurd-Karrer and Poos, 1936; Hanson et al., 2004), moth and butterfly larvae (Vickerman et al., 2002; Hanson et al., 2003; Freeman et al., 2006a), crickets and grasshoppers (Freeman et al., 2007), thrips and spider mites (Quinn et al., 2010), and prairie dogs (Quinn et al., 2008; Freeman et al., 2009). The hyperaccumulators A. bisulcatus and Stanleya pinnata also harbored fewer arthropods in their native seleniferous habitat when compared with adjacent nonhyperaccumulators (Galeas et al., 2008).

Hyperaccumulator plants may host Se-tolerant herbivores, as shown in a previous study that reported a Se-tolerant moth species associated with S. pinnata (Freeman et al., 2006a). Larvae from a population of Se-tolerant Plutellidae closely resembling the diamondback moth (Plutella xylostella), a damaging agricultural pest, were collected from S. pinnata in a seleniferous area (Fort Collins, CO). These larvae were documented through laboratory tests to tolerate the hyperaccumulated Se (2,000 mg kg$^{-1}$ dry weight) in S. pinnata leaves and not to be deterred by high-Se plants. In contrast, a diamondback moth population collected and reared from a nonseleniferous area in the eastern United States quickly died after feeding on high-Se leaves and was deterred by high-Se S. pinnata plants (Freeman et al., 2006a). A potential mechanism for the difference in Se tolerance between the two moth populations was revealed by $\mu$XANES and LC-MS analyses, which demonstrated that the Se-tolerant moth accumulated MeSeCys, similar to its host plant S. pinnata, whereas the Se-sensitive population accumulated SeCys and had obvious disintegration of internal organs (Freeman et al., 2006a). SeCys is considered more toxic to organisms than MeSeCys, due to its nonspecific incorporation into proteins in the place of Cys; the resulting absence of disulfide bonds crucially affects protein structure and function (Stadtman, 1990). The same study by Freeman et al. (2006a) characterized a biochemical flux of Se into a higher trophic level, because the Se-tolerant moth larvae were actively parasitized by the microgastrine wasp Diadegma insulare (Braconidae), which also accumulated C-Se-C forms, mainly as MeSeCys.

In addition to protecting plants from generalist herbivores, accumulated Se may offer protection from Se-sensitive microbial plant pathogens. Indian mustard (Brassica juncea) plants treated with Se were less susceptible to a fungal leaf pathogen (Alternaria brassicicola) and a fungal root/stem pathogen (Fusarium sp.) compared with control plants not supplemented with Se (Hanson et al., 2003). On the other hand, it appears that a variety of Se-tolerant microbes live in association with hyperaccumulators. A litter decomposition study conducted on seleniferous soil showed that native A. bisulcatus litter naturally high in Se decomposed faster and harbored higher numbers of microbes and microarthropods than low-Se alfalfa (Medicago sativa) litter collected in the same area (Quinn et al., 2011b). Furthermore, Se-tolerant fungi were isolated from the rhizosphere of hyperaccumulators A. bisulcatus and S. pinnata (Wangelene and Reeves, 2007), and rhizosphere fungi from hyperaccumulators growing on seleniferous soil were significantly more Se tolerant than fungi from nonseleniferous areas (Wangelene et al., 2011).

The effects of hyperaccumulation have also been studied in relation to plant-plant interactions. El Mehdawi et al. (2011a) observed that soil around A. bisulcatus and S. pinnata hyperaccumulators in the field was enriched in Se, and this high-Se soil could significantly impair the germination and growth of Se-sensitive plants. Thus, Se hyperaccumulation may function as a form of elemental allelopathy against Se-sensitive neighbors. On the other hand, some apparently Se-resistant neighbors of Se hyperaccumulators were found to benefit from their enhanced Se levels around hyperaccumulators. When Symphoricarpos erioides (white heath aster) and Artemisia ludoviciana (white sage brush) were growing next to hyperaccumulators, they contained 10- to 20-fold higher Se concentrations (more than 1,000 mg Se kg$^{-1}$ dry weight), were two times larger, and had reduced herbivore loads and less herbivory damage compared with plants from the same species that were not growing next to hyperaccumulators (El Mehdawi et al., 2011b).

In order to better characterize Se distribution and speciation in A. bisulcatus, we used $\mu$XRF elemental...
mapping and μXANES to characterize the Se distribution and speciation in all organs of mature *A. bisulcatus* as well as in seeds and seedlings. In addition, we studied the Se distribution and speciation in several belowground and aboveground ecological partners of *A. bisulcatus*. The objectives of this study were to present an in-depth analysis of the Se distribution across different organs of a hyperaccumulator and to obtain better insight into how Se hyperaccumulation affects ecological interactions and how ecological partners may affect Se hyperaccumulation.

## RESULTS

### Total Se and S Concentrations in Different *A. bisulcatus* Plant Parts

The *A. bisulcatus* plants collected in the field hyperaccumulated Se to concentrations ranging from 704 to 4,661 mg kg\(^{-1}\) dry weight in different organs (Table I). The highest Se concentrations were measured in the flowers, followed by stems, leaves, and roots, with a 1.5-fold difference between flowers and leaves and a 6.5-fold difference between flowers and roots (\(t = 3.06, P < 0.05\)). Within *A. bisulcatus* flowers, the highest Se level was measured in the sepals and petals, followed by pistils and stamens; immature seeds collected from developing seed pods had an intermediate Se level (Table I). Because Se is chemically similar to S and develop ing seed pods had an intermediate Se level by pistils and stamens; immature seeds collected from developing seed pods had an intermediate Se level (Table I). The objectives of this study were to present an in-depth analysis of the Se distribution across different organs of a hyperaccumulator and to obtain better insight into how Se hyperaccumulation affects ecological interactions and how ecological partners may affect Se hyperaccumulation.

### Distribution and Speciation of Se in Different Plant Parts and Ecological Partners

#### Roots and Nodules

The distribution of Se in the upper portion of the taproot from a field-collected *A. bisulcatus* (Fig. IA; Supplemental Figure S1) was fairly homogeneous, as shown by μXRF, with a higher Se signal in the cortex and stele than in the periderm (perimeter). μXANES analysis of Se in the cortex of the taproot demonstrated that 89% was in an organic C-Se-C form. This C-Se-C may be MeSeCys, γ-glutamyl-MeSeCys, selenocystathionine and/or seleno-Met, because the μXANES spectra for these compounds are virtually indistinguishable. The remaining 11% of the Se in the upper taproot was detected as elemental Se (Se\(^0\); Fig. 1B).

In the smaller, lateral roots, the strongest Se signal was found at the tip and along the periphery of the root, likely the epidermis (Fig. 1C). There was an equally strong Se signal in the associated nodule (Fig. 1C). Interestingly, there was a clear difference in Se speciation between the root and the nodule. In the root, Se was 100% C-Se-C (Fig. 1, C [white circles] and D), while in the nodule, 46% was C-Se-C, 31% was Se\(^0\), and 23% was selenite (Fig. 1, C [black circles] and E).

#### Stems and Leaves

In the (hollow) woody stem of *A. bisulcatus*, Se was localized throughout the cortex (Fig. 2A; Supplemental Figure S2). Within the cortex, there is not enough resolution to distinguish vascular tissue from ground tissue, but some of the more Se-rich spots appear to be arranged in a ring, which is best visible along the right side of the image, and may correspond with vascular bundles. The rim along the outside of the cross-section is also particularly elevated in Se (best visible on the lower left) and likely corresponds with the epidermis (Fig. 2A). In the cortex, Se was detected as 50% C-Se-C, 31% Se\(^0\), and 16% selenite (Fig. 2B). Leaves of *A. bisulcatus* concentrated Se to extreme levels in downy trichomes (Fig. 2C), as reported previously (Freeman et al., 2006b). An estimated 70% of total leaf Se was located in trichomes, predominantly (98%) in the form of C-Se-C, consisting of the two compounds MeSeCys and γ-glutamyl-MeSeCys, as identified by LC-MS (Fig. 2D). The remaining 30% of the total leaf Se, outside of the trichomes, consisted of 70% C-Se-C, 20% selenate, and 10% selenite. When these two fractions were combined, the entire leaf was estimated to contain 91% C-Se-C, 6% selenate, and 3% selenite (Fig. 2D; Freeman et al., 2006b). Here, it is worth mentioning that speciation fractions below 10% should be interpreted with caution because of the error margin of the least square linear combination analysis of μXANES spectra.

#### Leaf Herbivores

Larvae of two different moth species were found feeding, and apparently thriving, on leaves of *A. bisulcatus*

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**Table I. Se and S concentrations (mg kg\(^{-1}\) dry weight) in different organs and floral parts of *A. bisulcatus***

<table>
<thead>
<tr>
<th>Sample</th>
<th>Se (mg kg(^{-1}))</th>
<th>S (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td>704 ± 236 a</td>
<td>3,388 ± 1,270 b</td>
</tr>
<tr>
<td>Stems</td>
<td>4,557 ± 1,233 b</td>
<td>3,622 ± 1,735 b</td>
</tr>
<tr>
<td>Leaves</td>
<td>3,045 ± 927 b</td>
<td>16,254 ± 1,709 a</td>
</tr>
<tr>
<td>Flowers</td>
<td>4,661 ± 1,243 b</td>
<td>6,733 ± 927 b</td>
</tr>
<tr>
<td><strong>Floral parts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sepals</td>
<td>6,095 ± 3,637 a</td>
<td>9,355 ± 911 a,c</td>
</tr>
<tr>
<td>Petals</td>
<td>4,163 ± 2,504 a</td>
<td>6,223 ± 802 a,b</td>
</tr>
<tr>
<td>Stamens</td>
<td>1,817 ± 1,239 a</td>
<td>5,614 ± 1,126 a,b</td>
</tr>
<tr>
<td>Pistils</td>
<td>3,575 ± 2,290 a</td>
<td>3,950 ± 520 b</td>
</tr>
<tr>
<td>Immature seeds</td>
<td>3,153 ± 1,356 a</td>
<td>11,837 ± 1,486 c</td>
</tr>
</tbody>
</table>

Values shown for organs and floral parts were from different plants and are means ± se; \(n = 6\) except for roots and stems, where \(n = 3\). Letters indicate significant differences among plant organs for each element using the Tukey-Kramer honestly significant difference (\(P < 0.05\)).
in the field. The first species, collected during late summer, was identified using PCR as *Apamea sordens* (Noctuidae). The larva of *A. sordens* was shown by μXRF mapping to accumulate Se in the anterior head lobes (vertex), the lateral portion of the abdomen (spiracular band), and the hindgut (Fig. 3A; Supplemental Figure S3). The vertex and the spiracular band contained 96% organic C-Se-C (Fig. 3, A [white circles] and B). The hindgut contained 76% C-Se-C and 24% selenate (Fig. 3, A [white circles] and C). Larvae collected from the field were allowed to pupate, and the emerging adult (Fig. 3D) was mapped for Se accumulation by μXRF. It accumulated Se preferentially in the lower abdomen and the hindgut (Fig. 3E). μXANES analysis demonstrated that Se in the lower abdomen and hindgut consisted of 76% C-Se-C, 20% Se⁰, and 4% selenite (Fig. 3F). Some of the *A. sordens* larvae collected in the field hatched a parasitoid wasp that was identified based on morphology to be in the family Ichneumonidae, subfamily Cryptinae (Fig. 3G). μXRF mapping of this wasp showed a substantial Se signal in the head, thorax, and abdomen (Fig. 3H). μXANES analyses of the head and abdomen demonstrated that 78% of the total Se was C-Se-C, 12% was selenodiglutathione, and 10% was selenite (Fig. 3I). The thorax differed in that it contained relatively less C-Se-C (59%) and more Se⁰ (26%) and selenite (15%; Fig. 3J).

A second moth species (Fig. 4, A and D; Supplemental Figure S4) was observed feeding on *A. bisulcatus* in the late summer and early fall. This moth was collected and tentatively identified as belonging to the family Gelechiidae, based on the upcurved labial palpi (mouth parts) of the adult and the overall morphology of the adult and larva. μXRF mapping showed that Se was present throughout the larval and adult tissues, with a stronger concentration in what appears to be the digestive tract (Fig. 4, B and D). μXANES analyses showed that Se was present as organic forms with a C-Se-C configuration (98%–100%) in both larva and adult and both in the animal tissues and in the apparent digestive tract (Fig. 4, C and F).

**Flowers**

The *A. bisulcatus* flower (Fig. 5A; Supplemental Figure S5) was shown by μXRF to contain Se in the petals (Fig. 5B) as well as in the pistil and stamen (Fig. 5C). Within the petals and pistil, Se appears to be distributed fairly homogeneously, with a slight concentration in the ovules (Fig. 5, B and C). The anthers show a strong Se signal at the base as well as in apparent vascular tissue connecting the base with the anther. Se speciation was similar in the different flower parts, averaging 90% C-Se-C, 7% Se⁰, and 3% selenite (Fig. 5D).
Seeds, Seedlings, and a Seed Endosymbiont

The seeds of *A. bisulcatus* (Fig. 6A; Supplemental Figure S6) concentrated Se in the embryo and not in the seed coat (Fig. 6B). The form of Se was almost exclusively organic, with a C-Se-C configuration (Fig. 6C). Surface-sterilized seeds of *A. bisulcatus* showed evidence of an endophytic fungus, which appeared in more than 50% of the germinating surface-sterilized seeds (Fig. 6D). Based on morphology and internal transcribed spacer (ITS) sequence (data not shown), it was identified as a small-spored filamentous fungus belonging to the genus *Alternaria*. μXRF analysis demonstrated Se accumulation in the fungal hyphae that emerged from the seeds (Fig. 6E). The Se in the mycelia consisted of 59% C-Se-C, 22% Se⁰, and 19% selenite (Fig. 6F). The cotyledons of the infected seed embryo contained 74% C-Se-C, 20% Se⁰, and 6% selenite (Fig. 6G). Thus, the fungus-infected seedling contained relatively less organic and more inorganic Se compared with the seed proper, and the fungus-infected seed contained relatively more inorganic Se than the uninfected seed. Uninfected seeds and cotyledons showed little or no Se⁰.

In order to investigate the movement and biochemical transformation of Se in seedlings, *A. bisulcatus* seeds were germinated on selenate-containing growth medium and the resulting seedlings were flash frozen for x-ray absorption spectroscopy (XAS) analysis at two different time points after germination. The mature seedling pictured in Figure 7A and Supplemental Figure S7 represents the last harvested seedling at day 5 after germination. μXRF mapping demonstrated that *A. bisulcatus* had a fairly uniform distribution of Se in seedling cotyledons, hypocotyls, and roots, including root hairs (Fig. 7B). Twenty-four hours after germination, the seedling contained 100% C-Se-C in cotyledons and root (Fig. 7B; note that the root tip was desiccated during the XAS analysis). Five days after germination, the cotyledon (Fig. 7D, white circle) contained 95% C-Se-C and 5% selenite, whereas the root contained relatively less C-Se-C (70%) and a substantial fraction of inorganic Se: 18% Se⁰ and 12% selenite (Fig. 7D, black circle).

**DISCUSSION**

Our survey of Se distribution and speciation in different organs of the Se hyperaccumulator *A. bisulcatus* demonstrates that Se speciation varies between organs, as summarized in Figure 8 and Supplemental Figure S8. Particularly interesting was the finding that *A. bisulcatus* roots, nodules, stems, and flowers contained a substantial fraction of Se⁰, up to 34%, a novel finding for plants. Because Se⁰ is produced by many fungi and bacteria, the Se⁰ found here may be a chemical signature from microbial detoxification inside the plant, derived from endophytic microbial partners. These results build on an earlier study by Freeman et al. (2006b) but are novel because, in the earlier study, only the leaf of *A. bisulcatus* was analyzed for Se speciation.
A. bisulcatus was analyzed and no Se$^0$ was found. Here, we analyzed all organs and found significant levels of Se$^0$ in several of them. This study also provides novel information about Se-resistant microbial and insect ecological partners of A. bisulcatus and their respective Se distribution and speciation patterns (Fig. 8). The picture that emerges from these, as well as earlier, studies is that Se hyperaccumulators live in association with a variety of Se-resistant ecological partners.

The predominant form of Se in all A. bisulcatus organs was organic Se with a C-Se-C configuration (Fig. 8). The finding that A. bisulcatus roots contain mainly C-Se-C is in agreement with a study by Pickering et al. (2000), who reported that Se was mainly present in organic form in A. bisulcatus roots. For comparison, the nonhyperaccumulators Indian mustard and wheat (Triticum aestivum) accumulated predominantly selenate in their roots when supplied with selenate (Pilon-Smits et al., 1999; Li et al., 2008). At the whole-plant level, A. bisulcatus appeared to preferentially accumulate Se in its reproductive tissues; the same pattern was observed for the Se hyperaccumulator S. pinnata but not for the nonaccumulator Indian mustard, which had higher levels in leaves than in reproductive organs (Quinn et al., 2011a). While the form of Se in flowers was 90% C-Se-C for both A. bisulcatus and S. pinnata, the Se distribution within flowers was somewhat different for the two hyperaccumulator species (Quinn et al., 2011a). In A. bisulcatus, the Se concentration was higher in the petals and sepals than in the stamen and pistil, whereas in S. pinnata, the opposite pattern was observed. Furthermore, in anthers of A. bisulcatus, Se was concentrated at the base and in apparent vascular tissue, whereas in S. pinnata anthers, Se was distributed more homogeneously throughout the pistil. Furthermore, in anthers of A. bisulcatus, Se was concentrated at the base and in apparent vascular tissue, whereas in S. pinnata anthers, Se was highly concentrated in the pollen (Quinn et al., 2011a).

In addition to organic C-Se-C, A. bisulcatus roots, nodules, stems, and flowers contained up to 34% Se$^0$. Se$^0$ is not typically found in plants, although plants have been shown to contain SeCys lyase activity, which

Figure 5. A, Photograph of an A. bisulcatus flower. B, μXRF map showing the distribution of Se (in red) and calcium (Ca; in green) in the petals of the flower. C, μXRF map showing the distribution of Se (in red) and calcium (in green) in the pistil and stamen of the flower as well as in the seed pod (inset). D, Composition of Se in all floral parts, as determined by XANES. Circles in B and C show where XANES scans were performed to determine Se composition.

Figure 6. A, Photograph of an A. bisulcatus seed. B, μXRF map showing the distribution of Se (in red), zinc (Zn; in green), and calcium (Ca; in blue) in the seed. C, Se composition in the embryo within the seed (white circles in B), as determined by XANES. D, Photograph of a fungal endophyte emerging from a germinating A. bisulcatus seed. E, μXRF map showing the distribution of Se (in red) and calcium (in green) in an endophyte-containing A. bisulcatus seed as well as in the mycelium of the emerging endophytic fungus (inset). F, Se composition in fungal mycelium growing out from the seed (white circles in E), as determined by XANES. G, Se composition in the endophyte-containing seed (black circle in E), as determined by XANES.
can produce Se\(^0\) (Pilon-Smits et al., 2002). In contrast to plants, Se\(^0\) is produced widely by fungi and bacteria (Gharieb et al., 1995; Losi and Frankenberger, 1997). Because Se\(^0\) is insoluble and less toxic than many other forms of Se, many microbes produce Se\(^0\) as a means of Se tolerance, either via reduction of selenate or selenite or by SeCys lyase activity (Sarles et al., 1935; Losi and Frankenberger, 1997; Hunter and Kuykendall, 2007). Thus, the Se\(^0\) found in these field-collected A. bisulcatus plants may result from the activity of microbial endosymbionts. A recent study by Lindblom et al. (2012) suggests an environmental influence on Se speciation in roots of Se hyperaccumulators: A. bisulcatus and S. pinnata roots collected in the field contained up to 35% Se\(^0\), but greenhouse-grown plants from the same two species contained almost exclusively C-Se-C. Moreover, several root-associated fungi isolated from Se hyperaccumulators were shown to accumulate high levels of Se\(^0\) (Lindblom et al., 2012). This study provides some indication that microbial symbionts of A. bisulcatus may be responsible for the observed Se\(^0\) in the plant tissues. The root nodule, site of a nitrogen-fixing bacterial endosymbiont, was one of the structures that had a very high Se\(^0\) fraction (31%), which was around three times higher than in the root itself. Furthermore, a fungal seed endophyte (Alternaria sp.) was shown to accumulate a large fraction of the Se in its mycelia in the form of Se\(^0\) while growing on an A. bisulcatus seed; within the infected seed, there was also a substantial (although somewhat lower) Se\(^0\) fraction, but an uninfected seed and a 24-h-old seedling contained exclusively C-Se-C. An Alternaria endophyte could be isolated from surface-sterilized stem and root explants cultivated on fungal growth medium. Considering the life cycle and host colonization of vertically transmitted fungal endophytes, it is possible that the same endophyte occurs throughout the plant. Systemic endophytic colonization in the apoplas of leaves, stems, and reproductive structures has been documented for several Astragalus species (Ralphs et al., 2008). If this is the case here, it may explain the occurrence of Se\(^0\) in the seedling root, the taproot, and particularly the stem.

Figure 7. A, Photograph of an A. bisulcatus seedling, with an empty seed coat to its left. B, \(\mu\)XRF map showing the distribution of Se (in red) and calcium (Ca; in green) in a 24-h-old A. bisulcatus seedling, C, Se composition in an endophyte-containing seedling 24 h after germination, as determined by XANES at the points indicated by circles in B. D, \(\mu\)XRF map showing Se (in red) and calcium (in green) distribution in a 5-d-old A. bisulcatus seedling, E, Se composition in the cotyledons of a 5-d-old endophyte-containing seedling (white circle in D), F, Se composition in the root of a 5-d-old endophyte-containing seedling (black circle in D), as determined by XANES.

Figure 8. Schematic overview of Se uptake, translocation, and speciation in different A. bisulcatus organs and the associated ecological interactions.
The strength of the μXRF Se signal from the fungal mycelia on the infected seed was similar to that obtained from the root and other plant organs. This suggests that this fungal endophyte is quite Se tolerant. For comparison, two fungal pathogens (A. brassicicola and Fusarium sp.) were shown earlier to be 50% inhibited in growth on medium with 50 mg Se kg\(^{-1}\) dry weight, and Se accumulation effectively protected plants against these pathogens (Hanson et al., 2003). In a recent study by Wangeline et al. (2011), the growth of an Aspergillus leporis strain isolated from the rhizosphere of a nonhyperaccumulator plant growing in a nonseleniferous habitat was reduced by 50% when exposed to 30 mg Se L\(^{-1}\), whereas the growth of another strain of A. leporis isolated from the rhizosphere of a hyperaccumulator plant growing in seleniferous habitat was tolerant and actually showed improvement by Se in medium at levels up to 1,000 mg Se L\(^{-1}\). Thus, fungi associated with Se hyperaccumulators, like this seed endosymbiont, may have relatively high Se tolerance.

Astragalus hyperaccumulators have been reported earlier to host bacteria that can nodulate other, nonhyperaccumulator Astragalus species (Wilson and Chin, 1947). The finding that A. bisulcatus harbored root nodules shows that A. bisulcatus has a nitrogen-fixing bacterial endosymbiont, and the fact that the Se signal intensity in the nodule was similar to that in the root suggests that this bacterium is resistant to substantial Se levels. Other Rhizobiaceae have been shown before to differ in Se sensitivity. For example, nodulation and nitrogen fixation in the nonhyperaccumulator Melilotus indica were reduced by Se at 1 mg L\(^{-1}\) selenate, which may suggest that the plant and/or bacterial symbiont are Se sensitive (Wu et al., 1994). On the other hand, some Rhizobiaceae could grow on medium with 100 mM selenate or selenite (8,000 mg Se L\(^{-1}\)), on which they formed red colonies, indicative of the presence of Se\(^0\) (Kinkle et al., 1994; Hunter and Kuykendall, 2007). These species (Rhizobium selenireducens, Sinorhizobium fredii, and Sinorhizobium meliloti) are not known to nodulate Astragalus species, but it is feasible that the nitrogen-fixing symbiont in A. bisulcatus nodules can produce Se\(^0\). It may be interesting in future investigations to compare the Se tolerance of this hyperaccumulator endosymbiont (if culturable) with that of other rhizobia. If the bacterial endosymbiont of A. bisulcatus has unusually high Se tolerance, it may have evolved under the influence of Se in the roots of its hyperaccumulator host. Another interesting aspect of this nitrogen-fixing symbiosis is that it may play a role in plant Se hyperaccumulation. The Se-containing amino acid MeSeCys and the diamino acid γ-glutamyl-MeSeCys are vital components of Se tolerance in A. bisulcatus (Sors et al., 2005) as the main forms of Se stored in leaves (Freeman et al., 2006b). The microbial symbiont may provide A. bisulcatus with a portion of the nitrogen required for the production of these organic selenocompounds.

Increased metal tolerance has been reported for rhizospheric partners of several hyperaccumulators, some of which could potentially affect plant metal hyperaccumulation (for review, see Alford et al., 2010). Similarly, it appears that fungal and bacterial endosymbionts of Se hyperaccumulators have evolved enhanced Se tolerance via their capacity to convert other, more toxic forms of Se to the relatively inert Se\(^0\) form. These endophytes may not only affect plant Se speciation but also overall plant Se hyperaccumulation and tolerance. This will be an interesting area for further investigations.

Other Se-resistant ecological partners of A. bisulcatus discovered in this study include two herbivorous moth species and a parasitic wasp. One moth was identified as a member of the Gelechiidae and the other as A. sordens. To our knowledge, this is the first record of an A. pumila species feeding on A. bisulcatus. A. sordens is a medium-sized moth in the family Noctuidae. It is widely distributed across Eurasia from western Europe to Japan and also occurs in North America, from British Columbia east to Labrador and south to northeastern Oregon, Colorado, and South Carolina (Powell and Opler, 2009). Larvae of this species have been reported to feed on Poaceae (Robinson et al., 2010) and are considered to be a pest of wheat, rye (Secale cereale), barley (Hordeum vulgare), and oat (Avena sativa) in parts of Eurasia (Gričanović and Ovsyannikova, 2009). Lepidoptera species that have been reported to feed on A. bisulcatus tend to be general Fabaceae feeders and include Walshia amorphella (Cosmopterigidae), Ereynnis persius (Hesperiidae), Strymon melinus (Lycaenidae), Euxoa costata (Noctuidae), Colias alexandra (Pieridae), and Colias eurytheme (Pieridae; Robinson et al., 2010). So far, no herbivores have been reported to specialize on A. bisulcatus. More studies are needed to determine whether the two moths described here may be specialized feeders on Se hyperaccumulator plants.

In the laboratory, the capacity of field-collected larvae from both moth species to complete their life cycle on high-Se (more than 1,000 mg Se kg\(^{-1}\) dry weight) A. bisulcatus plants was confirmed, and based on μXRF Se signals, the larvae and adults contained substantial Se levels. Thus, both Se-resistant moth herbivores may actually be Se tolerant, but further studies are needed to investigate this. Both moths accumulated mainly C-Se-C, with smaller fractions as inorganic selenate or Se\(^0\). The locations where Se\(^0\) was observed often appeared to correspond with the intestinal tract. It is possible that microbial activity in the intestine of these insects is responsible for the production of the observed Se\(^0\) in the larvae. The main form of Se in the two moth herbivores described here, C-Se-C, was also shown to accumulate in a Se-tolerant diamondback moth herbivore of the hyperaccumulator S. pinnata (as MeSeCys; Freeman et al., 2006a). In contrast, a Se-sensitive population of diamondback moth accumulated the apparently demethylated product SeCys when feeding on S. pinnata. Thus, the Se-tolerant diamondback moth was hypothesized to derive its tolerance from the inability to metabolize the ingested MeSeCys from its hyperaccumulator host. A Se-resistant
parasitic wasp of the diamondback moth also accumulated MeSeCys (Freeman et al., 2006a). The same mechanism may be responsible for the Se resistance of the two moths and the parasitoid wasp described in this study, because they also accumulated C-Se-C.

These parallel findings of Se-resistant moths that feed on different Se hyperaccumulator species, each associated with parasitic wasps, suggest that Se hyperaccumulators facilitate the evolution of Se-resistant herbivores that can utilize hyperaccumulators as a food source. Such Se-accumulating herbivores may in turn facilitate the evolution of Se-tolerant parasites or predators in higher trophic levels. The accumulation of Se in the larvae and the retention of this Se in the adult may play a role in defending the herbivore against generalist predators and also may be a source of Se flux into higher trophic levels. The results from this study complement earlier studies on Se hyperaccumulators (for review, see El Mehdaoui and Pilon-Smits, 2012) and provide further evidence that Se plays a significant role in shaping the ecological interactions of hyperaccumulator plants and in seleniferous ecosystems as a whole. Through their extreme Se concentrations, hyperaccumulator plants may select against Se-sensitive organisms while driving the evolution of Se-tolerant symbiotic partners. Affected organisms may include endophytes, rhizospheric microorganisms, litter detritivores, leaf herbivores, pollinators, parasites, predators, pathogens, and neighboring plants. Through positive or negative effects on different ecological partners, Se hyperaccumulators likely have a profound effect on the overall species composition at different trophic levels and may significantly affect the entry and cycling of Se in seleniferous ecosystems.

**MATERIALS AND METHODS**

**Plant Material**

*Astragalus bisulcatus* material was collected in the summer season (June to July) at Pine Ridge Natural Area, a seleniferous site in Fort Collins, Colorado. The leaf material used for x-ray analysis was already described earlier (Freeman et al., 2006b). After collection, some of the fresh tissues were used for fungal endophyte culturing as described below. Samples for elemental analysis were dried and analyzed for total Se and S, as described below. For μXRF mapping and μXANES, the different plant parts from the field were flash frozen in liquid nitrogen, and the root and stem were cross-sectioned to a thickness of 1 mm using a new razor blade. Seeds were surface sterilized in a 100% bleach solution for 5 min, before rinsing in sterile distilled water for 5 min. Fungal endophytes were then isolated on Malt Extract Agar medium.

**Fungal Material**

One-half of the surface-sterilized seeds germinated on Murashige and Skoog medium showed evidence of colonization by an endophytic fungus. This fungus was isolated and cultured on one-half-strength Malt Extract Agar (Difco) medium. Based on its morphology and ITS 1 and ITS 2 sequences (White et al., 1990), the fungus was identified as a small-spored *Alternaria* species similar to other selenophilic *Alternaria* species (Wangelin and Reeves, 2007). Endophytes inside leaf and stem cuttings were recovered by first surface sterilizing the tissue in 90% ethanol for 5 min, followed by treatment with a 100% bleach solution for 5 min, before rinsing in sterile distilled water for 5 min. Fungal endophytes were then isolated on Malt Extract Agar medium.

**Insect Material**

Two different species of moth larvae were collected from *A. bisulcatus* while feeding on leaves at the Pine Ridge field site. Some larvae were flash frozen in liquid nitrogen for microprobe analyses, and some were kept alive in the laboratory feeding on their host, *A. bisulcatus*, collected from the field, until they pupated and enclosed. These adults were then flash frozen in liquid nitrogen for x-ray analyses. The parasitoid wasp species that emerged from one of the moth species pupae was also flash frozen for x-ray analyses.

To identify one of the Lepidoptera species, DNA was extracted using a Qiagen DNeasy Blood and Tissue kit. One-half of an early-instar larva was ground, incubated at 56°C overnight in 100 μL of Adam-Evans buffer, and DNA was then eluted following the manufacturer’s protocol. The DNA barcode region of the *cytochrome c oxidase* I (COI) gene (Hebert et al., 2003a, 2003b) was PCR amplified using an Eppendorf gradient Mastercycler 5331. PCR was performed using ExTaq HS polymerase (Takara Bio) in a 50-μL final volume including the ExTaq buffer and deoxyribonucleotide triphosphate mixture. The primers LCO-1490 (5'-GGTCAACAATATCATAAGATATTGG-3') and HCO-2198 (5'-TAAACTCAGGGTGACCAAAAAATCA-3'; Folmer et al., 1994) amplified a 688-bp DNA segment. PCR on the Mastercycler involved a 3-min denaturation step at 94°C, followed by 32 cycles of 20 s at 94°C, 30 s at 50°C, 30 s at 72°C, and a 5-min extension step at 72°C. Amplicons were purified using a QiAquick PCR Purification kit (Qiagen) and sequenced at the University of Chicago Cancer Research Center. Contigs were assembled and trimmed using Geneious Pro 5.3.4 (Biomatters). The COI DNA sequence was BLAST searched against the BOLD Systems animal identification (COI) species barcode database (Ratnasingham and Hebert, 2007).

**Measurement of Total Se and S Concentrations**

Before elemental analysis, the biological material was rinsed with distilled water and dried for 48 h at 45°C. Samples were digested in nitric acid as described by Zarcinas et al. (1987). Inductively coupled plasma atomic emission spectrometry was used to determine the concentrations of Se and S in the acid digest (Fassel, 1978).

**X-Ray Spectroscopy Studies**

Se distribution and speciation were investigated using μXRF and μXANES, respectively, as described by Manceau et al. (2002) and Quinn et al. (2011a). Fresh, intact biological samples were flash frozen in liquid nitrogen and shipped on dry ice to the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Laboratory for microprobe analyses (Marcus et al., 2004). Due to the time-intensive nature of μXRF and μXANES studies and the limited beamtime available to individual research groups, one biological replicate was analyzed unless stated otherwise. Frozen samples were placed on a −33°C Peltier stage to reduce radiation damage caused by the x-ray beam. μXRF elemental maps were recorded at 13 keV using a 15-μm (height) × 6-μm (width) beam spot size, 15-μm × 15-μm pixel size, and 50-ms dwell time per pixel. The chemical forms of Se in particular areas of interest were analyzed using μXANES. μXRF maps and μXANES spectra were recorded with a seven-element Ge solid-state detector. Spectra were deadtime corrected, preedge background subtracted, and postedge normalized using standard procedures (Kelly et al., 2008). Red Se (white light energy set at 12,600 eV) was used to calibrate each spectrum. Least square linear combination analysis of μXANES spectra was performed in the 12,630- to 12,830-eV range through fitting to a library of standard Se compounds. The error on the fit percentages of Se species was estimated at ±10%. Standards used were as follows: Na2SeO3, Na2SeO4, selenocysteine, and seleno-Met purchased from Sigma-Aldrich and MeSeCys, γ-glutamyl-MeSeCys, selenocystathionine, and selenodiglutathione purchased from PharmaSe. SeCys was obtained by reducing selenocysteine overnight at 25°C in 100 μm sodium borohydride at a 1:1 ratio. Gray and red Se3+ standards were provided by Amy Ryser and Dan Strawn. Data processing and analyses were performed using custom LabVIEW (National Instruments) software programs available at the beamline.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1. XAS analyses of an A. bisulcatus taproot cross section, lateral root, and root nodule.**

**Supplemental Figure S2. XAS analyses of the cross-section of an A. bisulcatus stem and a leaflet.**
Supplemental Figure S3. XAS analyses of moth larva, adult moth, and the parasitic wasp.

Supplemental Figure S4. XAS analyses of a gelechiid moth, collected from A. bisulcatus.

Supplemental Figure S5. XAS analyses of an A. bisulcatus flower and seed pod.

Supplemental Figure S6. XAS analyses of an A. bisulcatus seed and endophytic fungus.

Supplemental Figure S7. XAS analyses of an A. bisulcatus seedling, endophyte-containing seedling, and 1- and 3-d-old seedlings.

Supplemental Figure S8. Schematic overview of Se uptake, translocation, and speciation in different A. bisulcatus organs and associated ecological interactions.

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LITERATURE CITED


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