SELENIUM HYPERACCUMULATION BY ASTRAGALUS (FABACEAE) DOES NOT INHIBIT ROOT NODULE SYMBIOSIS1

ÉLAN R. ALFORD2,3,6,7, ELIZABETH A. H. PILON-SMITS2,4, SIRINE C. FAKRA5, AND MARK W. PASCHE2,3

2Graduate Degree Program in Ecology, Colorado State University, Fort Collins, Colorado 80523 USA; 3Department of Forest and Rangeland Stewardship, Colorado State University, Fort Collins, Colorado 80523 USA; 4Biology Department, Colorado State University, Fort Collins, Colorado 80523 USA; 5Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, California 94720 USA

• Premise of study: A survey of the root-nodule symbiosis in Astra galus and its interaction with selenium (Se) has not been conducted before. Such studies can provide insight into how edaphic conditions modify symbiotic interactions and influence partner coevolution. In this paper plant-organ Se concentration ([Se]) was investigated to assess potential Se exposure to endophytes.

• Methods: Selenium distribution and molecular speciation of root nodules from Se-hyperaccumulators Astragalus bisulcatus, A. praelongus, and A. racemosus was determined by Se K-edge x-ray absorption spectroscopy. A series of greenhouse experiments were conducted to characterize the response of root-nodule symbiosis in Se-hyperaccumulators and non-hyperaccumulators.

• Key results: Nodules in three Se-hyperaccumulators (Astragalus crotalariae, A. praelongus, and A. preussii) are reported for the first time. Leaves, flowers, and fruits from Se-hyperaccumulators were routinely above the hyperaccumulator threshold (1,000 µg Se g⁻¹ DW), but root samples rarely contained that amount, and nodules never exceeded 110 µg Se g⁻¹ DW. Nodules from A. bisulcatus, A. praelongus, and A. racemosus had Se throughout, with a majority stored in C-Se-C form. Finally, an evaluation of nodulation in Se-hyperaccumulators and non-hyperaccumulators indicated that there was no nodulation inhibition because of plant Se tolerance. Rather, we found that in Se-hyperaccumulators higher levels of Se treatment (up to 100 µM Se) corresponded with higher nodule counts, indicating a potential role for dinitrogen fixation in Se-hyperaccumulation. The effect was not found in non-hyperaccumulators.

• Conclusions: As the evolution of Se hyperaccumulation in Astragalus developed, root-nodule symbiosis may have played an integral role.

Key words: adaptation; legume; plant-microbe; selenium

Several hyperaccumulator taxa and metallophytes are members of the legume family (Fabaceae). Many of these species are within the Papilionoideae subfamily, which has been reported to have more than 98% of its members form root nodules (Allen and Allen, 1981). Therefore, despite the challenges of being rooted in metalliferous soils, leguminous hyperaccumulators may be expected to form root-nodule symbioses. However, to date our knowledge of whether and how leguminous Se-hyperaccumulators interact with symbiotic rhizobia is limited. For metal-tolerant symbiotic legumes to evolve, tolerance needs to occur in both the plant and the bacterial partner (Antonovics et al., 1971). Symbiotic bacteria within root nodules are enclosed in the peribacterioid membrane and are exposed to consistent environmental conditions within the plant cell and may have some protection from stress, unlike free-living rhizobia that are more susceptible to stress and environmental fluctuations in the soil environment and rhizosphere (Chalk et al., 2010).

The Astragalus genus makes a good model system to evaluate how hyperaccumulation affects nodulation characteristics. The vast majority of Astragalus species do not hyperaccumulate elements, but a select number of species native to western North America do hyperaccumulate Se. The species that hyperaccumulate Se manage to amass large concentrations, while co-occurring congeners do not accumulate Se to any large extent (Shrift 1969; Galeas et al., 2007). Investigating Astragalus species may indicate if there is a coevolutionary relationship between plant hyperaccumulation and root-nodule microorganisms. Three alternative pathways could have developed: (i) plants that have evolved to hyperaccumulate Se may associate with rhizobia that have evolved to interact with high [Se] within hyperaccumulator plants and rhizospheres; (ii) the presence of Se in the system could disrupt the symbiosis entirely where Se-hyperaccumulators rarely nodulate or form ineffective partnerships; or (iii) there could be no change in interaction in response to Se.

Under the first alternative, symbiotic interactions may enhance host stress tolerance. Some plants have been shown to require

1Manuscript received 2012 March 20; revision accepted 23 October 2012.

The authors thank the student crew at the Restoration Ecology laboratory at Colorado State University for assistance with root washing. We also acknowledge the assistance of L. Bodistow for help with root excavations in the field. The operations of the Advanced Light Source at Lawrence Berkeley National Laboratory are supported by the Director, Office of Basic Energy Sciences, U.S. Department of Energy under contract number DE-AC02-05CH11231. National Science Foundation contract number DE-AC02-05CH11231. National Science Foundation grant IOS-0817748 provided funding to EAHPS.

2Current address: H. T. Harvey & Associates, 983 University Ave., Building D, Los Gatos, California 95032 USA

7Author for correspondence (e-mail: elan.reine@gmail.com)

doi:10.3732/ajb.1200124

symbiotic associations under certain conditions of environmental stress that are not limited to low nutrient availability. As an example, thermal tolerance and salt tolerance were only achieved in plants growing in symbiosis with mycorrhizae (Rodriguez et al., 2008). Yet, under the second alternative described above, stress may disrupt symbiotic relationships. An example of the disruptive influence of abiotic stress has been shown in the legume genus *Acacia* where more salt-tolerant plant species had less of a growth increase in response to rhizobial inoculation than did salt-sensitive host species; therefore plant salt-tolerance results in reduced dependence on the symbiotic bacteria in nodules are subjected to Se within these plants.

From previous reports it is known that the Se-hyperaccumulators *A. bisulcatus* (Hook.) A. Gray, *A. pectinatus* (Douglas ex Hook.) Douglas ex G. Don, and *A. racemosus* Pursh are nodulated (Wilson and Chin, 1947; Lindblom et al., 2012), but no one knows how the symbiotic associations respond to Se. In *A. bisulcatus*, root [Se] can reach levels that are used to define plants as Se-hyperaccumulators (>1,000 µg Se g⁻¹ DW shoot) (Galeas et al., 2007; Barillas et al., 2011). With roots having high [Se], nodules of hyperaccumulators potentially experience similar [Se]. We wanted to compare the effect of Se in nodulation of *Astragalus* species that have evolved to hyperaccumulate Se to those that have not. In this study, we address the question—does plant Se-hyperaccumulation affect root-nodule symbiosis?

We checked for the presence of nodulation and investigated the [Se] in organs of several *Astragalus* hyperaccumulators and leguminous nonhyperaccumulators. We hypothesized that Se-hyperaccumulation inhibited the symbiotic interaction, where hyperaccumulators have lost some of their ability to effectively interact with rhizobia. We expected nodulation in Se-hyperaccumulators and nonhyperaccumulators to be reduced with Se addition. To address this hypothesis we conducted field surveys and examined differences in nodulation between Se-hyperaccumulators and nonhyperaccumulators in greenhouse studies. Our studies also included Se localization and speciation analyses within root nodules from the three Se-hyperaccumulators *A. bisulcatus*, *A. paelongus*, and *A. racemosus* to determine how bacteria in nodules are subjected to Se within these plants.

### Materials and Methods

**Nodulation occurrence in Se-hyperaccumulators—**Seeds from *Astragalus crotalariae* (Benth.) A. Gray were obtained from the Desert Legume Program

### Table 1. Collection locations of the plants used to assess organ [Se] in the field including the closest Colorado town and soil Se type, plant species and hyperaccumulation type, UTM coordinates (NAD83/WGS84), and site elevation (m).

<table>
<thead>
<tr>
<th>Site and Species</th>
<th>Type</th>
<th>Location</th>
<th>UTM Zone</th>
<th>Easting</th>
<th>Northing</th>
<th>Elevation (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fort Collins</td>
<td>Seleniferous site</td>
<td>Hyperaccumulator</td>
<td>13N</td>
<td>490631</td>
<td>4506616</td>
<td>1597</td>
</tr>
<tr>
<td><em>Astragalus bisulcatus</em></td>
<td>Hyperaccumulator</td>
<td>Meeker</td>
<td>12N</td>
<td>722225</td>
<td>4383948</td>
<td>2466</td>
</tr>
<tr>
<td><em>Astragalus argophyllus</em></td>
<td>Hyperaccumulator</td>
<td>Pueblo</td>
<td>13N</td>
<td>539143</td>
<td>4242337</td>
<td>1536</td>
</tr>
<tr>
<td><em>Astragalus convallarius</em></td>
<td>Hyperaccumulator</td>
<td>Urran</td>
<td>12N</td>
<td>697009</td>
<td>4249798</td>
<td>1504</td>
</tr>
<tr>
<td><em>Melilotus officinalis</em></td>
<td>Hyperaccumulator</td>
<td>Hyperaccumulator</td>
<td>13N</td>
<td>490631</td>
<td>4506616</td>
<td>1597</td>
</tr>
<tr>
<td><em>Lotus purshianus</em></td>
<td>Hyperaccumulator</td>
<td>Hyperaccumulator</td>
<td>12N</td>
<td>722225</td>
<td>4383948</td>
<td>2466</td>
</tr>
<tr>
<td><em>Melilotus albus</em></td>
<td>Hyperaccumulator</td>
<td>Hyperaccumulator</td>
<td>12N</td>
<td>697009</td>
<td>4249798</td>
<td>1504</td>
</tr>
<tr>
<td><em>Lotus hispidus</em></td>
<td>Hyperaccumulator</td>
<td>Hyperaccumulator</td>
<td>12N</td>
<td>697009</td>
<td>4249798</td>
<td>1504</td>
</tr>
<tr>
<td><em>Oxytropis sericea</em></td>
<td>Hyperaccumulator</td>
<td>Hyperaccumulator</td>
<td>12N</td>
<td>697009</td>
<td>4249798</td>
<td>1504</td>
</tr>
</tbody>
</table>

### Table 2. Collection locations for soil from beneath six *Astragalus* species used as inoculum in the experiment examining the effects of Se on nodulation including the closest Colorado town, UTM coordinates (NAD83/WGS84), and site elevation (m).

<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Location</th>
<th>UTM Zone</th>
<th>Easting</th>
<th>Northing</th>
<th>Elevation (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. bisulcatus</em></td>
<td>Hyperaccumulator</td>
<td>Fort Collins</td>
<td>13N</td>
<td>490631</td>
<td>4506616</td>
<td>1597</td>
</tr>
<tr>
<td><em>A. convallarius</em></td>
<td>Nonhyperaccumulator</td>
<td>Meeker</td>
<td>12N</td>
<td>722225</td>
<td>4383948</td>
<td>2466</td>
</tr>
<tr>
<td><em>A. drummondii</em></td>
<td>Nonhyperaccumulator</td>
<td>Livermore</td>
<td>13N</td>
<td>470315</td>
<td>4512908</td>
<td>1946</td>
</tr>
<tr>
<td><em>A. praelongus</em></td>
<td>Hyperaccumulator</td>
<td>Limon</td>
<td>13N</td>
<td>598433</td>
<td>4356258</td>
<td>1749</td>
</tr>
<tr>
<td><em>A. racemosus</em></td>
<td>Hyperaccumulator</td>
<td>Limon</td>
<td>13N</td>
<td>598433</td>
<td>4356258</td>
<td>1749</td>
</tr>
<tr>
<td><em>A. shortianus</em></td>
<td>Nonhyperaccumulator</td>
<td>Livermore</td>
<td>13N</td>
<td>470360</td>
<td>4512932</td>
<td>1940</td>
</tr>
</tbody>
</table>
sodium selenate solution (20 mL) was applied weekly for the remaining 4 mo, after which the plants were removed from their pots and examined for nodules. Root systems of *A. bisulcatus* (Fort Collins, Colorado), *A. praelongus* (Uravan, Colorado) and *A. racemosus* (Pueblo, Colorado) growing in their native habitats were also excavated to search for root nodules under natural conditions.

![Histograms of organ [Se] in four Se-hyperaccumulator species (*Astragalus bisulcatus*, *A. praelongus*, *A. racemosus*, and *A. rafaelensis*) as shown for (A) leaf, (C) flower, (E) fruit, (G) root, and (I) nodule. The organ [Se] in six nonhyperaccumulator legumes (*A. argophyllus*, *A. convallarius*, *A. missouriensis*, *Melilotus albus*, *M. officinalis*, and *Oxytropis sericea*) are also shown for (B) leaf, (D) flower, (F) fruit, (H) root, and (J) nodule.](image)

Fig. 1. Histograms of organ [Se] in four Se-hyperaccumulator species (*Astragalus bisulcatus*, *A. praelongus*, *A. racemosus*, and *A. rafaelensis*) as shown for (A) leaf, (C) flower, (E) fruit, (G) root, and (I) nodule. The organ [Se] in six nonhyperaccumulator legumes (*A. argophyllus*, *A. convallarius*, *A. missouriensis*, *Melilotus albus*, *M. officinalis*, and *Oxytropis sericea*) are also shown for (B) leaf, (D) flower, (F) fruit, (H) root, and (J) nodule.
Organ [Se] from field collections—To assess potential Se exposure for microbial endophytes we determined plant organ [Se] from excavated root systems of several nonhyperaccumulators and several Se-hyperaccumulators during the growing season (Table 1). Aboveground organs including leaves, flowers, and fruits were separated from the belowground organs in the field. Within one day of collection the samples were returned to the laboratory and the belowground organs were washed and separated into roots and nodules. All parts were dried at 40°C, weighed, and ground. For inductively coupled plasma atomic emission spectrometry (ICP-AES), 20 mg of dried plant samples were digested in 1 mL nitric acid for 2 hours at 60°C and then 130°C for 6 hours (Zarcinas et al., 1987). Organ [Se] was determined by ICP-AES on this acid digest after a 10-fold dilution with water (Fassel, 1978). An 8-element standard mix was run as a quality control every 15 samples, if the standards failed (outside of ±20% acceptance margin) we restandardized and reran the samples. We used 1,000 ppm elemental standards from SPEX CertiPrep, Inc. (Metuchen, New Jersey, USA), diluted to 1 to 2 ppm.

Se localization and speciation in nodules—Root nodules of hyperaccumulators were obtained from Astragalus bisulcatus, growing in the field, along with A. praelongus Sheldon and A. racemosus, growing under greenhouse conditions exposed to 50 µM sodium selenate (as described above under Nodulation occurrence in Se-hyperaccumulators). Nodules were separated from the majority of the roots, washed in water to remove external Se, frozen in liquid N₂, and then sliced in half. The samples were kept frozen until analysis. Nodule Se localization and speciation were determined using microfocused x-ray fluorescence (µXRF) mapping and x-ray absorption near-edge structure (µXANES) spectroscopy at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Laboratory (Marcus et al., 2004). Frozen nodule samples were placed onto a Peltier stage kept at −27°C and then 130°C for 6 hours (Zarcinas et al., 1987). Organ [Se] was determined by ICP-AES on this acid digest after a 10-fold dilution with water (Fassel, 1978). An 8-element standard mix was run as a quality control every 15 samples, if the standards failed (outside of ±20% acceptance margin) we restandardized and reran the samples. We used 1,000 ppm elemental standards from SPEX CertiPrep, Inc. (Metuchen, New Jersey, USA), diluted to 1 to 2 ppm.

Se effect on nodulation—We grew Astragalus bisulcatus, A. convallarius Greene, A. drummondii Douglas ex Hook., A. praelongus, and A. shortianus Nutt. obtained from Western Native Seed (Coaldale, Colorado, USA), and A. racemosus from Prairie Moon Nursery (Winona, Minnesota, USA) in a mixture of 2:1 (v:v) washed sand and field soil (sieved through a 2 mm mesh, Table 2). Soil collected from Se-hyperaccumulators and nonhyperaccumulators were similar in total (1.21 – 2.02 µg g⁻¹) and extractable (0.71 to 0.90 µg g⁻¹) Se (Sparks et al., 1996). The plants received 0, 50, or 100 µM sodium selenate (Na₂SeO₃) and an N-free fertilizer solution (0.4 mM K₂SO₄, 4.475 µM Na₂MoO₄, and 0.125 µM Na₂-EDTA) twice per week. After another 6 d, Se treatments (20 µM Na₂SeO₃) were started. Sodium selenite solutions were mixed in distilled water and supplied as 50-ml applications once every week. One month after Se treatments began the plants began to brown so fertilizer was reduced to once a week and Se treatments were reduced to once every 2 wk for the duration of the experiment (10 more weeks). Plants were harvested and separated into roots, shoots, and nodules. Each sample was dried at 50°C for one week before analysis.

Root and nodule [Se] was determined in samples from the greenhouse experiment as well as samples from Astragalus bisulcatus, A. praelongus, and A. racemosus collected from the field (as described under Organ [Se] from Field Collections). As previously described, ICP-AES was used to determine organ [Se] on digested samples. For statistical analysis we compared root and nodule [Se] in each species by t-test in Systat v.12 (Systat Software, Chicago, Illinois, USA).

Se localization and speciation in nodules—Root nodules of hyperaccumulators were obtained from Astragalus bisulcatus, growing in the field, along with A. praelongus Sheldon and A. racemosus, growing under greenhouse conditions exposed to 50 µM sodium selenate (as described above under Nodulation occurrence in Se-hyperaccumulators). Nodules were separated from the majority of the roots, washed in water to remove external Se, frozen in liquid N₂, and then sliced in half. The samples were kept frozen until analysis. Nodule Se localization and speciation were determined using microfocused x-ray fluorescence (µXRF) mapping and x-ray absorption near-edge structure (µXANES) spectroscopy at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Laboratory (Marcus et al., 2004). Frozen nodule samples were placed onto a Peltier stage kept at −27°C and then 130°C for 6 hours (Zarcinas et al., 1987). Organ [Se] was determined by ICP-AES on this acid digest after a 10-fold dilution with water (Fassel, 1978). An 8-element standard mix was run as a quality control every 15 samples, if the standards failed (outside of ±20% acceptance margin) we restandardized and reran the samples. We used 1,000 ppm elemental standards from SPEX CertiPrep, Inc. (Metuchen, New Jersey, USA), diluted to 1 to 2 ppm.

Table 3. Maximum [Se] (µg Se g⁻¹ DW) in different legume organs collected from the field for a survey of organ [Se] in Se-hyperaccumulators and nonhyperaccumulators. Some organs were not collected and elemental concentrations were not determined (−).

<table>
<thead>
<tr>
<th>Plant type</th>
<th>Species</th>
<th>Leaf</th>
<th>Flower</th>
<th>Fruit</th>
<th>Root</th>
<th>Nodule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonhyperaccumulator</td>
<td>Astragalus argophyllus</td>
<td>47</td>
<td>58</td>
<td>–</td>
<td>66</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Astragalus convallarius</td>
<td>77</td>
<td>–</td>
<td>–</td>
<td>37</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Astragalus missouriensis</td>
<td>83</td>
<td>52</td>
<td>48</td>
<td>48</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Melilotus albus</td>
<td>39</td>
<td>40</td>
<td>28</td>
<td>17</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>Melilotus officinalis</td>
<td>61</td>
<td>–</td>
<td>–</td>
<td>56</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Oxytropis sericea</td>
<td>12</td>
<td>20</td>
<td>5</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>Hyperaccumulator</td>
<td>Astragalus bisulcatus</td>
<td>436</td>
<td>606</td>
<td>291</td>
<td>291</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>Astragalus praelongus</td>
<td>2,925</td>
<td>2,999</td>
<td>5,405</td>
<td>1,281</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Astragalus racemosus</td>
<td>422</td>
<td>81</td>
<td>83</td>
<td>87</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Astragalus rafaelensis</td>
<td>689</td>
<td>2,151</td>
<td>1,263</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Fig. 2. Mean (±SE) [Se] in four Se-hyperaccumulator species (*Astragalus bisulcatus*, *A. praelongus*, *A. racemosus*, and *A. rafaelensis*) as shown for (A) leaf, (C) flower, (E) fruit, (G) root, and (I) nodule, and (B) leaf, (D) flower, (F) fruit, (H) root, and (J) nodule of six nonhyperaccumulator legume species (*A. argophyllus*, *A. convallarius*, *A. missouriensis*, *Melilotus albus*, *M. officinalis*, and *Oxytropis sericea*).
Fig. 3. Microfocused x-ray fluorescence (μXRF) maps derived from elemental distributions within (A) Astragalus bisulcatus, (B) A. praelongus, and (C) A. racemosus root nodules. The tricolor-coded maps show Se (in red), Ca (in green), and Fe (in blue) along with individual elemental maps (each shown in white). Microfocused x-ray absorption near-edge structure (μXANES) spectra from nodules from each of the Astragalus species along with the spectra of the standard organic Se, MeSeCys, containing carbon-Se-carbon bonds (D).
expected to have a lower value than nonhyperaccumulators. Statistical analyses were conducted with Systat v.12 (Systat Software, Chicago, Illinois, USA).

RESULTS

Nodulation occurrence in Se-hyperaccumulators—Nodulation of Astragalus bisulcatus and A. racemosus was confirmed in plants growing in the field. We observed nodulation in A. praelongus growing in its native habitat. Nodules were also observed in A. crotalariae and A. preussii inoculated with A. bisulcatus field soil under greenhouse conditions.

Organ [Se] from field collections—Ten legume species were collected from Colorado field sites (see Table 1) and analyzed for [Se] in leaves, flowers, roots, and nodules. Histograms show the number of plant samples within a range of [Se] for both Se-hyperaccumulators and nonhyperaccumulators (Fig. 1). Concentrations in hyperaccumulators and nonhyperaccumulators were variable, with nonhyperaccumulators having lower [Se] than hyperaccumulators. Except for hyperaccumulator flowers, both groups of plants have samples from each organ within the lowest ranges of [Se].

Astragalus missouriensis Nutt. had the lowest [Se] of the nonhyperaccumulators for leaf (0 µg Se g⁻¹ DW), flower (0.1 µg Se g⁻¹ DW), and fruit (0 µg Se g⁻¹ DW). A. argophyllus Nutt. had the lowest root and nodule [Se] at 0 µg Se g⁻¹ DW for both organs. Nonhyperaccumulators did not surpass a [Se] of 100 µg Se g⁻¹ DW in any organ, while every organ measured in hyperaccumulators surpassed that concentration. The highest leaf (83 µg Se g⁻¹ DW) and fruit (48 µg Se g⁻¹ DW) [Se] in nonhyperaccumulators were recorded for A. missouriensis on the seleniferous site in Fort Collins, Colorado (Table 3). A. argophyllus growing on a nonseleniferous site near Meeker, Colorado had the highest flower (58 µg Se g⁻¹ DW) and root (66 µg Se g⁻¹ DW) [Se] of the nonhyperaccumulators (Table 3). The maximum nodule [Se] found among all the nonhyperaccumulators was 29 µg Se g⁻¹ DW in Oxytropis sericea growing at a seleniferous site near Fort Collins, Colorado (Table 3).

In contrast, Astragalus praelongus growing near Urvan, Colorado had the highest leaf (2.925 µg Se g⁻¹ DW), flower (2.999 µg Se g⁻¹ DW), fruit (5.405 µg Se g⁻¹ DW), and root (1.281 µg Se g⁻¹ DW) [Se] of all the Se-hyperaccumulators (Table 3). Maximum nodule [Se] in hyperaccumulators was in A. racemosus growing near Pueblo, Colorado, USA at 109 µg Se g⁻¹ DW (Table 3). The lowest [Se] found in hyperaccumulators were 0 µg Se g⁻¹ DW in some A. racemosus leaf, fruit, and root samples. Samples of A. bisulcatus had the lowest fruit (55 µg Se g⁻¹ DW) and nodule (6 µg Se g⁻¹ DW) [Se] of the hyperaccumulators.

Of the Se-hyperaccumulators, Astragalus praelongus had the highest average [Se] for leaves (Fig. 2A), fruits (Fig. 2E), and roots (Fig. 2G). A. rafaelensis M. E. Jones had the highest average [Se] in flowers (Fig. 2C) and A. racemosus had the highest [Se] in nodules (Fig. 2I) of the hyperaccumulators. In the nonhyperaccumulators A. missouriensis had the highest average leaf [Se] (Fig. 2B); Melilotus albus Medik. had the highest average [Se] in flowers (Fig. 2D), fruit (Fig. 2F), and nodules (Fig. 2J); and M. officinalis (L.) Lam. had the highest average root [Se] (Fig. 2H).

Se localization and speciation in nodules—Selenium was distributed throughout root nodules in the three Astragalus hyperaccumulators, A. bisulcatus (Fig. 3A), A. praelongus (Fig. 3B), and A. racemosus (Fig. 3C). Clear differences in Se compartmentalization in the developmentally different distal and proximal regions of the nodule were not apparent. The distribution of calcium (Ca) and iron (Fe) was more localized than Se, which was distributed more evenly in each nodule, but proximal or distal differences in Ca and Fe were not observed either. Molecular speciation by XANES showed that the majority of Se in the nodules of each plant species was C-Se-C (Fig. 3D; Table 4). Each root nodule also contained some selenite (SeO₃²⁻). Both A. bisulcatus and A. praelongus contained some form of elemental Se (Se⁰), but A. racemosus did not (Table 4). Nodules from A. praelongus contained selenocysteine, while nodules from A. bisulcatus and A. racemosus contained selenomethionine.

Root and nodule [Se] under greenhouse and field conditions—Organ [Se] was significantly different between roots and nodules (t = −3.09; df = 8; P = 0.013) in the Se-hyperaccumulator Astragalus bisulcatus growing under greenhouse conditions (Fig. 4A). Nodules had a double the [Se] as roots in A. bisulcatus under greenhouse conditions. A similar trend (nonsignificant) where nodules had a higher [Se] than roots was observed in A. bisulcatus (Fig. 4B) and A. racemosus (Fig. 4D) growing under field conditions. In A. praelongus root [Se] was significantly higher (t = 2.213; df = 23; P = 0.037; +432%) than nodule [Se] (Fig. 4C).

Se effect on nodulation—Compared to nonhyperaccumulators, hyperaccumulators did not exhibit inhibition of nodulation characteristics (Fig. 5). Among the individual species, the nonhyperaccumulators Astragalus shortianus had the highest nodulation index, A. convallarius had the lowest, and all other species fell between these two (Fig. 5A). Additionally there was no difference in the nodulation index between the Se-hyperaccumulators and the nonhyperaccumulators as a group (Fig. 5B). The average weight of nodules was also determined. The Se-hyperaccumulators A. praelongus had the largest nodules and A. bisulcatus had the smallest nodules of all species with all other species falling between these two (Fig. 5C). No difference in nodule weight was found between the Se-hyperaccumulators and nonhyperaccumulators as a group (Fig. 5D).

Exposing Astragalus species to Se under greenhouse conditions did not alter the number of nodules produced in the Se-hyperaccumulator A. bisulcatus (Fig. 6A) or the nonhyperaccumulators A. convallarius (Fig. 6D) and A. shortianus (Fig. 6F). The addition of Se significantly increased nodule production in the hyperaccumulators A. praelongus (F₂₄ = 5.629; P = 0.007; Fig. 6B) and A. racemosus (F₂₄ = 3.195; P = 0.050; Fig. 6C). Only in the nonhyperaccumulator A. drummondii did Se addition significantly reduce plant nodule production (F₂₄ = 4.775; P = 0.038; Fig. 6E). Across all three Se-hyperaccumulator species, adding Se in our greenhouse experiment significantly increased (+143%;  F₂₄ = 3.409; P = 0.036) the number of nodules formed by those plants (Fig. 6G). There was no significant effect of Se addition on nodule production when the three nonhyperaccumulators were analyzed as a group (Fig. 6H).

DISCUSSION

The results reported here do not support our hypothesis that Se hyperaccumulation inhibits nodulation in Astragalus. To our knowledge, we provide the first report of nodulation in three
Table 4. The percent of Se in each molecular species from nodules of three *Astragalus* hyperaccumulators as determined by Se K-edge x-ray absorption near edge spectroscopy (XANES).

<table>
<thead>
<tr>
<th></th>
<th>SeO(_3)</th>
<th>C-Se-C</th>
<th>SeCys</th>
<th>SeGS(_2)</th>
<th>Se(^0)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. bisulcatus</em></td>
<td>4</td>
<td>89</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td><em>A. praelongus</em></td>
<td>2</td>
<td>70</td>
<td>22</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><em>A. racemosus</em></td>
<td>19</td>
<td>45</td>
<td>0</td>
<td>32</td>
<td>0</td>
</tr>
</tbody>
</table>

*Astragalus* Se-hyperaccumulators, *A. crotalariae*, *A. praelongus*, and *A. preussii*. All form nodules under greenhouse or field conditions. We also observed nodules on the hyperaccumulators *A. bisulcatus* and *A. racemosus* under greenhouse and field conditions, consistent with reports from Wilson and Chin (1947) and Lindblom et al. (2012). In the field we had the most success finding root nodules when the roots grew around rocks or between horizontal rocky layers, in locations where moisture was retained. A consistent pattern of nodulation in all hyperaccumulators examined indicates that symbiotic nodulation interactions are not diminished by a plant’s ability to hyperaccumulate Se.

When we compared Se-hyperaccumulators to nonhyperaccumulators grown under greenhouse conditions we found no evidence that Se treatment inhibited the formation of root nodule symbiosis in hyperaccumulators. These findings are similar to the report of a successful symbiotic interaction in the metallophyte *Lotus purshianus* growing on an abandoned copper mine (Wu and Lin, 1990), except here we find that tolerance is widespread in numerous species of *Astragalus*. Our findings are in contrast to reduced symbiotic dependence found in some metal hyperaccumulators, where strong Ni-hyperaccumulators native to New Caledonia had reduced mycorrhizal colonization compared to species that are moderate or weak accumulators of Ni (Amir et al., 2007). When we added Se to hyperaccumulators under greenhouse conditions the number of nodules per plant increased with increasing Se doses. This was not the case in the nonhyperaccumulators where there was no significant effect of adding Se. These effects for nonhyperaccumulator legumes agree with the earlier report that Se had no effect on nodule number in *Melilotus indicus* (L.) All., although Se additions did reduce mean nodule dry weight in that study (Wu et al., 1994). However, in our study the Se-hyperaccumulators made 143% more nodules when they were given up to 100 µM Se, indicating that the belowground symbiosis in hyperaccumulators is positively linked in some way to Se.

We hypothesize that nodulation could be linked to Se hyperaccumulation where increased nodule production under Se treatment increases plant N content, which could promote the storage of selenoamino acids in Se-hyperaccumulators. This is feasible to imagine because *Astragalus bisulcatus* stores up to 99% of Se in young leaves as the selenoamino acid methylselenocysteine (Sors et al., 2005). Investigations of the tolerance of
for field-collected nodules of *A. bisulcatus*, where a root nodule collected from the field contained a majority of Se in the C-Se-C form (Valdez Barillas et al., 2012). Neither our report of three Se-hyperaccumulator species, nor the Valdez Barillas et al. (2012) report on *A. bisulcatus* indicates that Se is localized differently between the young, distal end of nodules and the older, proximal region.

The leguminous nonhyperaccumulators that we investigated never breached the 100 µg Se g⁻¹ DW level in any organ, even when co-occurring with hyperaccumulators on seleniferous soil. Our finding is similar to previous reports of nonhyperaccumulator species growing on seleniferous sites (Shrift 1969, Galeas et al., 2007). Examining the nodule [Se] of nonhyperaccumulators is new, but like the other organs, [Se] was low. The maximum nodule [Se] found in nonhyperaccumulators was 29 µg Se g⁻¹ DW. In fact, an attempt to analyze the nodule of the nonhyperaccumulator *Astragalus drummondii* by µXAS was unsuccessful because of the low Se signal in the sample analyzed.

While mostly investigated aboveground, the protective effect of trace-element hyperaccumulation may also occur belowground. To our knowledge the elemental defense hypothesis (Boyd, 2007) has never been demonstrated in belowground organs, although in some species belowground organs do satisfy...
investigated root nodule herbivory in *Astragalus*. Our study was not designed to test the elemental defense hypothesis, but these greenhouse results suggest new aspects to investigate. In contrast with our greenhouse findings, when we looked at root and nodule [Se] in other *Astragalus* Se-hyperaccumulators growing under field conditions, the opposite result was also detected where root [Se] was higher than nodule [Se]. A more intensive study of belowground Se translocation must be conducted because of the known variation of Se allocation during the growing season (Galeas et al., 2007).
Selenium cycles through the plant and is proposed to move from the roots to the new leaves in the spring, from the mature leaves into the flowers and fruits, and back to the roots during dormancy (Galeas et al., 2007). This cycle does not account for root nodules. The data we collected here were from actively growing plants when Se is translocated from roots to above-ground organs. We expect there is better potential to detect differences in belowground organs when Se moves back belowground. Perennial legumes are expected to have perennial nodules (Gurusamy et al., 2000), so Se cycling within plants could manifest in nodules as well. If nodules are inactive during the overwintering process, Se may not affect nodule physiology, but may rather affect their susceptibility to herbivory. Even if nodule [Se] is elevated in hyperaccumulators, bacteria inside nodules are separated from the plant cell by the peribacterioid membrane (Brewin, 1991). Therefore, nodule [Se] may be isolated from the bacteria. However, free-living rhizobia must cross the Se-enriched rhizosphere soil from Se-hyperaccumulators to infect the host root. Even though Astragalus Se-hyperaccumulators do not show evidence of reduced reliance on symbiotic root nodule interactions, the coevolutionary effects of Se-hyperaccumulation on bacterial symbionts remain to be determined.

Astragalus nodule [Se] may be related to the promiscuity of rhizobia that associate with Astragalus hyperaccumulators and nonhyperaccumulators. We did not address this, but different nodule [Se] between Se-hyperaccumulators and nonhyperaccumulators could act as a selective force for rhizobial symbionts. Rhizosphere fungal communities from seleniferous soils have been shown to have increased Se tolerance and some may be considered habitat specialists that associate with Se-hyperaccumulators (Wangelin et al., 2011). Tolerance to inorganic Se forms at levels that will exceed the nodule [Se] in all our specimens have been observed in some rhizobia isolates in vitro (Kinkl et al., 1994). The maximum nodule [Se] found under field conditions in this study was 109 µg g⁻¹ DW, which is much lower than the 200 mM Se (IV) or 400 mM Se (VI) minimum inhibitory concentrations (MIC) determined by Kinkl et al. (1994) for Rhizobium leguminosarum bv. viciae. Since some rhizobia have a high Se tolerance, the same bacteria that infect nonhyperaccumulators have the potential to infect Se-hyperaccumulators as well. If so, co-occurring Astragalus species would not have segregated symbionts, but rather each host species promotes the growth of the same bacterial symbiont, thereby enhancing the rhizobial population within sites where the two Astragalus groups co-occur. Whether or not Se serves as a selective force, microsymbiont identities of many perennial milkvetch species in North America remains to be investigated by molecular methods.

Just like their nonhyperaccumulator congeners, Astragalus Se-hyperaccumulators form root nodule symbioses. The hyperaccumulators differed from their nonhyperaccumulator congeners in the fact that their symbiotic relationship is related to Se treatment and accumulation, while these effects did not occur in nonhyperaccumulators. We hypothesize that the increased number of nodules in Se-hyperaccumulators treated with increased Se levels may result in the symbiotic interaction helping plants acquire more N, which in turn helps the plant to store more Se as selenoamino acids in their shoots. These interactions present opportunities for further studies of evolutionary relationships. We found that the symbiotic interaction is not inhibited by the plant’s ability to hyperaccumulate Se. Because of the Se translocation cycle within perennial Astragalus Se-hyperaccumulator species, nodule [Se], symbiont exposure, and the consequences of those levels for segregation of symbionts between co-occurring plant congeners remain to be determined in a more conclusive way.

LITERATURE CITED


