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Roles of rhizobial symbionts in selenium hyperaccumulation in *Astragalus* (Fabaceae)[1]

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- *Premise of the study:* Are there dimensions of symbiotic root interactions that are overlooked because plant mineral nutrition is the foundation and, perhaps too often, the sole explanation through which we view these relationships? In this paper we investigate how the root nodule symbiosis in selenium (Se) hyperaccumulator and nonaccumulator *Astragalus* species influences plant selenium (Se) accumulation.
- *Methods:* In greenhouse studies, Se was added to nodulated and nonnodulated hyperaccumulator and nonaccumulator *Astragalus* plants, followed by investigation of nitrogen (N)–Se relationships. Selenium speciation was also investigated, using x-ray microprobe analysis and liquid chromatography–mass spectrometry (LC-MS).
- *Key results:* Nodulation enhanced biomass production and Se to S ratio in both hyperaccumulator and nonaccumulator plants. The hyperaccumulator contained more Se when nodulated, while the nonaccumulator contained less S when nodulated. Shoot [Se] was positively correlated with shoot N in Se-hyperaccumulator species, but not in nonhyperaccumulator species. The x-ray microprobe analysis showed that hyperaccumulators contain significantly higher amounts of organic Se than nonhyperaccumulators. LC-MS of *A. bisulcatus* leaves revealed that nodulated plants contained more γ-glutamyl-methylselenocysteine (γ-Glu-MeSeCys) than nonnodulated plants, while MeSeCys levels were similar.
- *Conclusions:* Root nodule mutualism positively affects Se hyperaccumulation in *Astragalus*. The microbial N supply particularly appears to contribute glutamate for the formation of γ-Glu-MeSeCys. Our results provide insight into the significance of symbiotic interactions in plant adaptation to edaphic conditions. Specifically, our findings illustrate that the importance of these relationships are not limited to alleviating macronutrient deficiencies.

Key words: *Astragalus*; Fabaceae; γ-glutamyl-methylselenocysteine; hyperaccumulation; legume; nodulation; selenium; symbiosis; x-ray absorption spectroscopy.

The importance of mutualisms for ecosystems is well known. Mutualisms of plants with other organisms facilitate plant nutrition, pollination, and seed and spore dispersal (Boucher et al., 1982). Investigating the effects of mutualisms on stress and tolerance are increasingly important, as many ecosystems are subjected to rising stress levels. It is interesting and necessary to investigate how mutualisms persist under extreme conditions in natural ecosystems as well as to understand how environmental stress has contributed to differentiation among sympatric mutualists. In this study, we focus on a plant–endophyte symbiosis that occurs in the extreme environment of high-Se soil and Se-hyperaccumulator *Astragalus* species.

Astragalus is the largest genus in the legume family, with an estimated 3000 species of annual and perennial herbaceous plants (Osaloo et al., 2003). Livestock and wild animals commonly use members of this genus as forage, and numerous *Astragalus* species are used by humans for food, medicine, or cosmetics (Uphof, 1968). Despite the many beneficial uses of *Astragalus* species, toxins are present in some species. The three major toxic principles in the *Astragalus* genus are (1) the aliphatic nitro compounds, (2) the indolizidine alkaloids including swainsonine, and (3) selenium (Se) compounds (Rios and Waterman, 1997).

Selenium hyperaccumulation occurs in nearly 30 plant species in North America; the majority of the hyperaccumulators are species of *Astragalus* (Brooks, 1998). The criterion for Se

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hyperaccumulation is a shoot Se concentration ([Se]) above 0.1% of dry mass (≥1000 μg Se·g^{-1} DW) when growing on natural seleniferous soil. For comparison, the typical [Se] in nonaccumulator species on such soils is less than 2 μg·g^{-1}, and 100 μg·g^{-1} may be considered high (Reeves and Baker, 2000). *Astragalus* species that hyperaccumulate Se manage to amass extreme amounts of Se from seleniferous soils. Co-occurring congeners, however, do not accumulate Se to any great extent under natural conditions (Shrift, 1969; Galeas et al., 2008; Sors et al., 2009; Alford et al., 2012). Given the low concentrations of trace elements in neighboring plants and the ability of hyperaccumulators to amass trace elements at concentrations typically two orders of magnitude higher than that of the soil in which they grow, hyperaccumulation is thought to benefit the plant in some way (Boyd and Martens, 1992; Boyd, 2004). Why plants hyperaccumulate Se remains a topic of investigation, but most evidence collected to date has focused on and found support for the elemental defense hypothesis (Boyd, 2007) where Se is thought to protect plants against herbivores and pathogens (Hurd-Karrer and Poos, 1936; Hanson et al., 2003; Galeas et al., 2008; Quinn et al., 2008). In addition, Se has been implicated in elemental allelopathy where Se enrichment in soil around hyperaccumulators inhibits Se-sensitive competitors (El Mehdawi et al., 2011).

Sulfur and Se have similar physical and chemical properties; thus, they follow the same metabolic pathways in plants, including root uptake of inorganic forms, transformation into amino acids, and methylation (Barceloux, 1999; White et al., 2004; Sors et al., 2005a, 2005b). However, unlike S, Se is not required by plants (Fu et al., 2002). In bacteria and animals, Se is essential in low concentrations, but it can become toxic to organisms at higher concentrations (Barceloux, 1999). In fact, concentrations of 8.1 μg·g^{-1} in feed can cause toxicosis and death in pigs (Stowe et al., 1992), and humans have been poisoned by Se from foods produced in seleniferous regions (Yang et al., 1983; Dhillon and Dhillon, 1997; Fordyce, 2007). Since Se is essential to mammals, a lack of dietary Se also poses a threat to human and animal health (McLaughlin et al., 1999). To combat deficiency in humans and livestock, Se-fortified plants have been developed (Lyons et al., 2003; Broadley et al., 2006; Bañuelos et al., 2011). Hyperaccumulators are particularly interesting in this respect, because of their capacity to accumulate high levels of Se in an organic form that is particularly anticarcinogenic (Ellis and Salt, 2003). Most plants accumulate selenate (the most bioavailable form of Se in oxic soils), but hyperaccumulators accumulate predominantly organic methylselenocysteine (MeSeCys) (Freeman et al., 2006). To be able to use hyperaccumulators more efficiently, we need to know more about their Se accumulation mechanisms. In particular, very little is known about belowground processes that allow hyperaccumulators of Se and other elements to amass large quantities of these trace elements from soil (Alford et al., 2010; Barceló and Poschenrieder, 2011).

Interactions between soil microorganisms and root systems can have significant impacts on the ability of plant hyperaccumulators to concentrate trace elements in their shoot systems (Whiting et al., 2001; Abou-Shanab et al., 2003; Turnau and Mesjasz-Przybylowicz, 2003; Liu et al., 2005; Vogel-Mikuš et al.; 2006; Jankong et al., 2007; Li et al., 2007; Farinati et al., 2009; Alford et al., 2010; Lindblom et al., 2013a, b). Both strong reductions and strong increases in plant trace element concentration have been found in hyperaccumulators upon interaction with soil microorganisms. Li et al. (2007) found that *Burkholderia cepacia* increased cadmium concentration by 243% in *Sedum alfredii.* A reduction of 33% in arsenic concentration was found in *Pteris vittata* when it was inoculated with *Glomus mosseae* (Liu et al., 2005). However, in another study no effect of soil-derived bacteria was found on arsenic concentration in *Pityrogramma coddii* (Jankong et al., 2007).

Many legumes form mutualistic relationships with dinitrogen fixing rhizobia in root nodules. Adaption to stress in legumes, such as that imposed by a high Se environment, is expected to be complex because successful tolerance requires adaptation of both the plant and bacterial symbionts (Antonovics et al., 1971). In addition, plant survival during stress may be dependent on symbiotic interactions (Rodriguez et al., 2008). Although symbionts can have strong effects on their partners, the role of symbionts in host adaptation to stress is not clear. In *Astragalus,* nodulation has been found in nearly every species that has been investigated (Allen and Allen, 1981), but the influence of mutualistic rhizobia on Se hyperaccumulators has never been investigated.

In this study, we tested the hypothesis that symbiotic rhizobia increase plant Se accumulation in both Se hyperaccumulators and nonhyperaccumulators. The mechanism by which this increase may occur could be from increased root surface area in nodulated plants, which controls root access to soil elements. An alternative and/or complementary mechanism would be that rhizobia alter plant Se metabolism by affecting selenoamino acids levels through N input.

MATERIALS AND METHODS

Experiment 1: Relationship of N and Se—*Astragalus bisulcatus*, *A. convallarius*, *A. drummondii*, *A. praelongus*, and *A. shortianus* were obtained commercially from Western Native Seed (Coaldale, Colorado, USA), and *A. racemosus* from Prairie Moon Nursery (Winona, Minnesota, USA). The seed coats were nicked with a razor, and scarified seeds were soaked overnight in tap water. The following day, seeds were sown in a 2:1 mixture by volume of washed sand and field soil (sieved 2 mm). The field soil was collected from up to a 10 cm depth directly underneath the canopy of each species (Table 1) during the growing season. Under greenhouse conditions, 16 plants of each species received treatments of 100 μM sodium selenate (Na_2SeO_4) and a N-free fertilizer solution based on Huss-Danell (1978) (0.4 mM K_2SO_4, 4.475 μM $FeCl_3$, 4.225 μM Na_2-EDTA, 0.5 mM $MgSO_4$, 0.042 mM KH_2PO_4, 0.208 mM K_2HPO_4, 5.775 μM H_3BO_3, 1.15 μM $MnSO_4$, 0.2 μM $ZnSO_4$, 0.075 μM $CuSO_4$, 0.05 μM $NaMoO_4$, and 0.125 μM $CoSO_4$). Treatments began 4 mo post germination, where Se and fertilizer were applied twice per week. Treatments continued for the duration of the experiment (2 additional months). Shoots, roots, and nodules were harvested and then dried at 50°C for several days until dry mass remained unchanged.

Experiment 2: Nodulation effect on shoot [Se]—Scarified (razor-scored seed coat) and surface sterilized seeds (2 min with bleach and 2 min with 90% ethanol) of *A. bisulcatus* and *A. drummondii* (Western Native Seed,) were grown in 164-mL Ray Leach "Cone-tainers" (Stewe & Sons, Tangent, Oregon, USA) containing HCl-washed sand mixed at a 1:1 ratio (by volume) with fritted clay. Huss-Danell (1978) fertilizer solution at 1/4 strength (1.6 mM K_2SO_4, 0.358 mM NH_4NO_3,

TABLE 1. Soil collection locations in Colorado for the six *Astragalus* species used in experiment 1.

Species	Closest town	Latitude (N)	Longitude (W)	Elevation (m a.s.l.)
A. bisulcatus	Fort Collins	40°42′ 37.45″	105°06′ 39.28″	1597
A. convallarius	Meeker	39°34′ 35.24″	108°24′ 45.67″	2466
A. drummondii	Livermore	40°45′ 59.77″	105°21′ 06.21″	1946
A. praelongus	Uravan	38°22′ 28.93″	108°44′ 40.93"	1504
A. racemosus	Limon	39°21′ 00.59″	103°51′ 27.43"	1749
A. shortianus	Livermore	40°46′ 00.57″	105°21′ 04.29″	1940

17.9 μM $FeCl_3$, 16.9 μM Na_2-EDTA, 2 mM $MgSO_4$, 0.169 mM KH_2PO_4, 0.833 mM K_2HPO_4, 0.6 mM $CaSO_4$, 0.5 mM $CaCl_2$, 23.1 μM H_3BO_3, 4.6 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, 0.2 μM $NaMoO_4$, and 0.5 μM $CoSO_4$) was applied twice a week beginning 1 mo post germination. Plants grew for 2 mo under greenhouse conditions with fertilizer additions. Then fertilizer application was suspended for 2 wk to induce N deficiency before inoculation and selenium treatments were applied. Plants were treated with 20 μM sodium selenate (Na_2SeO_4) and were subjected to one of two inoculation treatments (live soil slurry or autoclaved soil slurry) with 10 replications. We did not use specific strains of rhizobia in our experiment because there were no cultures available from *A. bisulcatus* or *A. drummondii* hosts in the USDA-ARS National *Rhizobium* Germplasm Collection. In addition, we have been unsuccessful in isolating rhizobia from nodules of *A. bisulcatus*. Instead, two soil samples, one from directly underneath the canopy of each *Astragalus* species, were collected from plants growing in a mixed prairie community in Fort Collins, Colorado, USA (13T 488406 m E, 4490140 m N) to make soil slurries for inoculation (20 g soil in 1 L sterile, distilled water). Inoculant was added to pots directly or was autoclaved (121°C, 117 kPa) for 30 min before application. Twenty milliliters of inoculant was added to each pot by matching species with soil type. Six days after inoculation, fertilizer treatments were restarted with 1/4-strength fertilizer twice per week. After another 6 d, weekly Se treatments (50 mL of 20 μM Na_2SeO_4) were started. One month after Se treatments began, plants started 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). Shoots were harvested and dried at 50°C for 1 wk before analysis.

Experiment 3: Selenium localization and speciation—Leaves were obtained from axenic *A. convallarius*, *A. drummondii*, and *A. racemosus* growing under controlled conditions in a growth chamber (24°C) on half-strength Murashige and Skoog (1962) agar medium supplemented with 50 μM sodium selenate. *Astragalus praelongus* was grown under greenhouse conditions in field-collected soil amended with 50 μM sodium selenate. Leaves were separated from the plant, washed in water to remove external Se, and frozen in liquid N_2. Samples were kept frozen until analysis was complete to prevent changes in Se distribution and speciation. Se distribution and speciation in leaves were determined using microfocused x-ray fluorescence (μXRF) mapping and Se K-edge x-ray absorption near-edge structure (μXANES) spectroscopy with the hard x-ray microprobe Beamline 10.3.2 at the Advanced Light Source of the Lawrence Berkeley National Laboratory (Marcus et al., 2004). Samples were mounted onto a Peltier stage kept at −27°C to minimize beam radiation damage. μXRF elemental maps were recorded at 13 keV, using a 15 μm (H) × 6 μm (V) beam, 15 μm × 15 μm pixel size, and 50 ms dwell time per pixel. A seven-element germanium (Ge) solid-state detector (Canberra Co., Concord, Ontario, Canada) was used to record the maps and spectra. The chemical forms of Se in selected areas were investigated using Se K-edge XANES. Spectra were deadtime corrected, pre-edge background subtracted, and postedge normalized using standard procedures (Kelly et al., 2008). Red Se (white line position set at 12 660 eV) was used to calibrate each spectrum. Least-square linear combination (LSQ) fitting of the experimental spectra was performed in the 12630–12850 eV range using a library of well-characterized Se standard compounds to identify chemical species (Pickering et al., 1999). The best LSQ fit was obtained for minimum normalized sum of squares of residuals: $NSS = \Sigma(\mu_{exp} - \mu_{fit})^2/\Sigma(\mu_{exp})^2$, where μ represents normalized absorbance. Error on the percentages of species present was estimated to ±10%. Se standards included Na_2SeO_4, Na_2SeO_3, SeCystine and SeMet purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and MeSeCys, γ-Glu-MeSeCys, SeCysth, and $SeGSH_2$ purchased from PharmaSe (Austin, Texas, USA). SeCys was obtained by reducing SeCystine at 25°C overnight in 100 mM sodium borohydride at a 1 : 1 molar ratio. Gray and red elemental Se were provided by Amy Ryser and Dan Strawn (University of Idaho). A suite of custom LabVIEW (National Instruments) programs available for the Beamline at the Lawrence Berkeley National Laboratory were used for all data processing and analyses.

Experiment 4: Selenoamino acids study—*Astragalus bisulcatus* was grown from seeds collected near Fort Collins. This seed source was used to have maximal ecological relevance. Seeds were scarified (razor-scored seed coat) and sterilized for 2 min in 95% ethanol followed by 2 min in 50% bleach. After several rinses with sterile water, seeds were soaked overnight before planting. Seeds were planted the next day in pots containing a 1 : 1 mixture by volume of sand and sieved (2 mm) field soil collected from the same population of *A. bisulcatus* (Table 1). Soil in the pots was from one of two treatments: autoclaved to kill soil microorganisms (treatment) or containing live soil microorganisms (control). Treatment soil underwent three consecutive rounds of 30 min of autoclaving (121°C, 117 kPa) followed immediately by freezing (−20°C) for at least 12 h. Control soil was not manipulated. Seeds were allowed to germinate over 2 mo on a mist bench. After the germination period, plants were allowed to acclimate to greenhouse conditions for 10 d. Then, plants were treated with Se (100 μM sodium selenate, Na_2SeO_4) and a low-N fertilizer to induce nitrogen stress and promote nodulation (0.08 mM NH_4NO_3) on a weekly basis for 9 wk. Shoots were harvested, and two leaflets per leaf were removed and immediately frozen in liquid N_2. Leaflets were stored at −80°C until amino acid extraction. The remaining shoot was dried at 50°C. Roots were frozen and stored at −20°C until nodule counts and root surface area measurements. Frozen roots were thawed and subsequently washed. Nodules were removed, and root surfaces were immediately scanned for area calculation using WinRHIZO (Regent Instruments, Quebec City, Quebec, Canada).

To extract amino acids from leaflets, we followed the method described by Freeman et al. (2006). Frozen leaf tissues were ground in a mortar and pestle under liquid nitrogen. Two milliliters of 50 mM HCl was added to the ground sample, and the sample was left to thaw. The sample was then centrifuged for 20 min at 13 200 rpm. Resulting supernatant was run through a C18 Sep-Pak (Waters, Milford, Massachusetts, USA) that had been previously charged with 5 mL acetonitrile and rinsed with 20 mL mass spectrometry grade water. The sample was stored at −80°C until analysis. Samples were run on a Waters quadrupole time of flight mass spectrometer coupled with ultra performance liquid chromatography (Q-TOF UPLC) at the Proteomics and Metabolomics Facility at Colorado State University. Chemical standards of MeSeCys and γ-Glu-MeSeCys were included to verify peak retention times and calibrate concentrations.

Plant elemental analysis—Twenty or 50 mg of dried and ground shoot samples from experiments 1, 2, and 4 were digested in 1 mL nitric acid for 2 h at 60°C and then 130°C for 6 h. After dilution with 9 mL of distilled water, [Se] and [S] in the digest was determined by inductively coupled plasma atomic emission spectrometry (ICP-AES) at the Soil, Water, and Plant Testing Laboratory

TABLE 2. Pearson correlation coefficients (*r*) from experiment 1 including physiological, fitness, and nodulation traits for three *Astragalus* hyperaccumulator species (*N* = 40) and three *Astragalus* nonhyperaccumulator species (*N* = 33). Significant coefficients are shown by asterisk (α = 0.05).

Variable	[Se]	[S]	Shoot mass	Root mass	Nodule mass	No. nodules
Hyperaccumulators						
[S]	0.529*					
Shoot mass	−0.199	−0.574*				
Root mass	−0.166	−0.447*	0.873*			
Nodule mass	−0.049	−0.452*	0.869*	0.857*		
No. nodules	0.045	0.145	−0.083	−0.051	0.124	
% N	0.403*	0.165	−0.169	−0.263	0.039	0.133
Nonhyperaccumulators						
[S]	0.639*					
Shoot mass	−0.423*	−0.577*				
Root mass	−0.636*	−0.363*	0.450*			
Nodule mass	−0.395*	−0.534*	0.660*	0.503*		
No. nodules	0.081	0.300	−0.268	−0.130	−0.199	
% N	0.159	−0.083	0.045	0.335	0.047	0.094

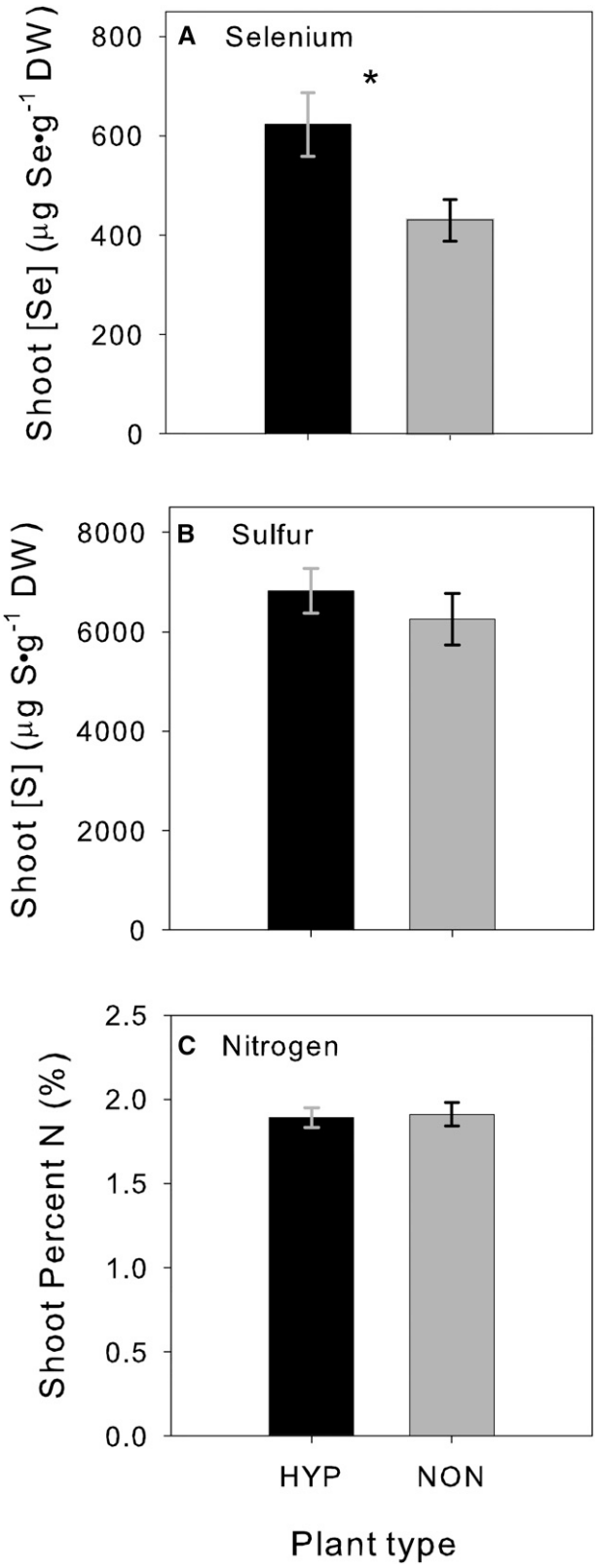

Fig. 1. A comparison of three Se-hyperaccumulator species (HYP; $N = 40$) and three nonhyperaccumulator species (NON; $N = 33$) for shoot concentrations per gram dry mass (DW) of the trace element (A) Se and the essential nutrients (B) S and (C) the percentage of N. Shown are means and standard error of the mean. Significant differences by t tests ($\alpha = 0.05$) are shown by asterisk.

at Colorado State University. For shoot [N], 50–100 mg dried and ground shoot material was combusted in a Leco Tru-Spec (St. Joseph, Michigan, USA) at the Colorado State University Natural Resource Ecology Laboratory.

Data analysis—Statistical data analysis was performed using Systat v.12 (Systat Software, Chicago, Illinois, USA). In some instances, transformations were required to correct for normality and equality of variances in the data. In experiment 1, the relationship of N and Se, correlation analyses were conducted separately for the three Se hyperaccumulators and three nonhyperaccumulators using physiological attributes (log shoot [Se] and log shoot [S]), fitness attributes (log shoot mass and log root mass), and nodulation attributes (log nodule mass, square root nodule number, and log shoot [N]). Comparisons of log-transformed shoot [Se], shoot [S], and shoot [N] were conducted between Se hyperaccumulators and nonhyperaccumulators. In experiment 2, nodulation effect on shoot [Se], nodulation effects on *A. bisulcatus* and *A. drummondii* were compared by t-test for log-transformed shoot dry mass, the log ratio of [Se] to [S], log shoot [Se], and shoot [S]. For experiment 3, the Se localization and speciation experiment, difference between Se compositions in each *Astragalus* species was compared by ANOVA. Differences in the molecular Se composition between hyperaccumulators (*A. praelongus* and *A. racemosus*) and nonhyperaccumulators (*A. convallarius* and *A. drummondii*) were compared by t tests. Finally for the selenoamino acids study, *A. bisulcatus* plants in different soil treatments (autoclaved or live soil) were compared by t test for log shoot dry mass, root surface area, log shoot [Se], log shoot [MeSeCys], and log shoot [γ-Glu-MeSeCys]. The amount of total shoot Se derived from selenoamino acids was determined by converting fresh mass amino acid concentration to dry mass concentrations for each treatment.

RESULTS

Experiment 1: Relationship of N and Se—Shoot [N] was positively correlated with shoot [Se] in the three hyperaccumulator *Astragalus* species ($r_{HYP} = 0.403$), but there was no such correlation in the three nonhyperaccumulator species (Table 2). In both Se-hyperaccumulator species and nonhyperaccumulator species, shoot sulfur concentration [S] was positively correlated with shoot [Se] ($r_{HYP} = 0.529$; $r_{NON} = 0.639$). In both hyperaccumulators and nonhyperaccumulators, significant negative correlations were detected between shoot [S] and shoot mass ($r_{HYP} = -0.574$; $r_{NON} = -0.577$), root mass ($r_{HYP} = -0.447$; $r_{NON} = -0.363$), and total nodule mass ($r_{HYP} = -0.452$; $r_{NON} = -0.534$). In the nonhyperaccumulators shoot mass ($r_{NON} = -0.423$), root mass ($r_{NON} = -0.636$), and nodule mass ($r_{NON} = -0.395$) were also negatively correlated with shoot [Se], while no significant correlations were detected for these attributes in the hyperaccumulators. In both plant types, there were significant positive correlations between shoot and root mass ($r_{HYP} = 0.873$; $r_{NON} = 0.450$), shoot and nodule weight ($r_{HYP} = 0.869$; $r_{NON} = 0.660$), and root and nodule mass ($r_{HYP} = 0.857$; $r_{NON} = 0.503$). Hyperaccumulators had a significantly higher ($t = 2.619$, df = 94, $P = 0.010$) shoot [Se] than nonhyperaccumulators (Fig. 1A). No differences in shoot [S] (Fig. 1B; $t = 0.831$, df = 94, $P = 0.408$) or shoot [N] (Fig. 1C; $t = -0.272$, df = 72, $P = 0.786$) were detected between hyperaccumulators and nonhyperaccumulators.

Experiment 2: Nodulation effect on shoot [Se]—The hyperaccumulator *A. bisulcatus* and the nonhyperaccumulator *A. drummondii* were used to determine whether nodulation status affected shoot [Se]. As shown in Fig. 2, both species produced nodules, and the nodulation was associated with increased shoot biomass (*A. bisulcatus,* $t = -3.819$, df = 16, $P = 0.002$; *A. drummondii,* $t = -5.899$, df = 14, $P < 0.001$).

Both *A. bisulcatus* ($t = -3.240$, df = 12, $P = 0.007$) and *A. drummondii* ($t = -2.392$, df = 8, $P = 0.044$) had a significantly higher shoot Se to S ratio when nodulated (Fig. 3A, 3B). Although the effect of nodulation was similar overall, changes in

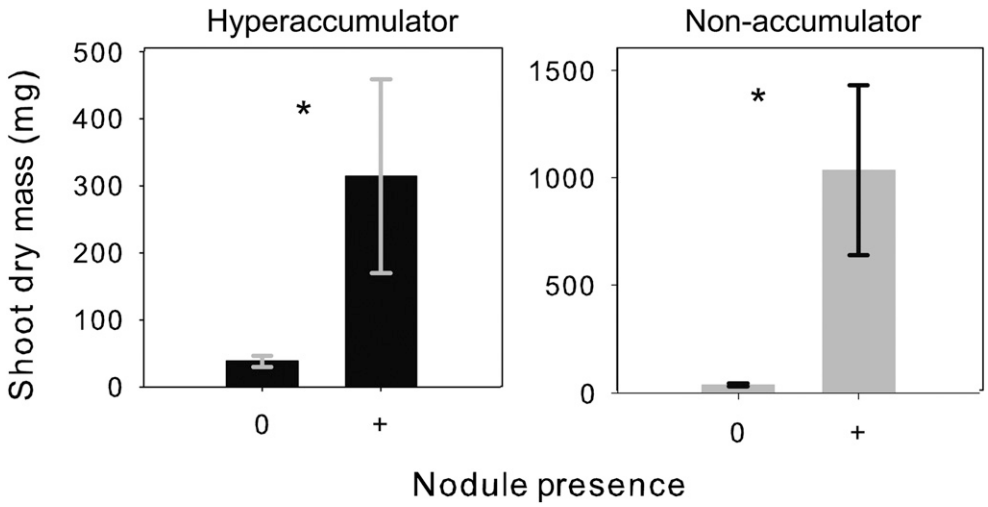

Fig. 2. The effect of nodulation on shoot dry mass in the hyperaccumulator *Astragalus bisulcatus* and the nonhyperaccumulator *A. drummondii*. Shown are means and standard error of the mean. Nodule absence (0) and nodule presence (+) were compared by *t* test ($\alpha = 0.05$); significant differences are indicated by asterisk. (*A. bisulcatus* $n_0 = 12$, and $n_+ = 6$; *A. drummondii* $n_0 = 10$, and $n_+ = 6$).

elemental concentrations differed between the two species. While in *A. bisulcatus* shoot [Se] was higher in nodulated *A. bisulcatus* plants than nonnodulated plants (+74.3%; $t = -2.382$, df = 12, $P = 0.033$; Fig. 3C), there was no such difference in *A. drummondii* ($t = 0.266$, df = 12, $P = 0.795$; Fig. 3D). Shoot [S] did not differ with nodulation in *A. bisulcatus* (Fig. 3E; $t = 0.429$, df = 12, $P = 0.675$), but was significantly lower in nodulated plants of *A. drummondii* than in nonnodulated plants (−39.4%; $t = 2.198$, df = 12, $P = 0.048$; Fig. 3F).

***Experiment 3: Selenium localization and speciation*—**Selenium was present throughout leaves of each *Astragalus* species (Fig. 4). In the nonhyperaccumulator *A. convallarius*, most Se was present in vascular bundles (Fig. 4A, 4B). In the hyperaccumulator *A. praelongus*, the highest Se signal was observed in trichomes (Fig. 4E, 4F). The nonhyperaccumulators *A. drummondii* and *A. convallarius* had trichomes (rich in Ca) as evidenced in the bicolor-coded maps in Fig. 4A and 4C, but did not store Se in them. Selenium speciation data from the XANES analysis was grouped by organic and inorganic forms for both nonhyperaccumulators (Fig. 4I) and Se hyperaccumulators (Fig. 4J). Nonhyperaccumulators contained significantly higher ($t = -3.900$, df = 10, $P = 0.003$) amounts of inorganic Se forms and significantly less ($t = 2.3096$, df = 10, $P = 0.044$) organic Se than the Se hyperaccumulators. Table 3 shows the mean and standard error for each Se species identified in plants by XANES. Nonhyperaccumulators contained significantly more selenate ($t = -5.067$, df = 10, $P < 0.001$) and selenite ($t = -4.774$, df = 10, $P = 0.001$) than hyperaccumulators. Significant differences in selenate levels were observed between species ($F_{3, 8} = 11.214$, $P = 0.003$). *Astragalus convallarius* had the highest amount of selenate, while *A. racemosus* did not contain any detectable amount. Significant differences in selenite concentrations were observed between individual species ($F_{3, 8} = 24.158$, $P < 0.001$). *Astragalus drummondii* had the most selenite of the four species, and none was detected in *A. praelongus*. The Se hyperaccumulators contained significantly more organic Se in the form of C-Se-C ($t = 2.574$, df = 10, $P = 0.028$). The form of C-Se-C may be either selenomethionine or MeSeCys since they are indistinguishable by Se K-edge XANES. Differences between C-Se-C concentrations were not distinguishable by species ($F_{3, 8} = 2.390$, $P = 0.100$). No differences in elemental Se concentrations were detected between hyperaccumulators and nonhyperaccumulators as a whole ($t = 1.195$, df = 10, $P = 0.260$) or individually by species ($F_{3, 8} = 1.386$, $P = 0.315$). Data from individual XANES spots analysis are shown in Appendix S1 (see Supplemental Data with the online version of this article).

***Experiment 4: Selenoamino acids study*—**To obtain better insight into the contribution of symbiotically fixed N_2 to Se hyperaccumulation, we grew *A. bisulcatus* in the presence or absence of rhizosphere inoculant obtained from the same species growing in its natural habitat; a control treatment received autoclaved (dead) inoculum. As shown in Table 4, live inoculum soil resulted in substantial nodulation, while autoclaved inoculum did not result in any nodules. Nodulated Se hyperaccumulators did not differ in shoot [Se] from plants without nodules ($t = 1.863$, df = 12, $P = 0.087$) but did reach a significantly higher shoot dry mass ($t = -12.912$, df = 40, $P < 0.001$) and root surface area ($t = -4.393$, df = 20, $P < 0.001$) (Table 4). Interestingly, shoot concentration of the seleno-diamino acid γ-Glu-MeSeCys was 10-fold higher in nodulated plants ($t = -3.792$, df = 6, $P = 0.009$) than in plants without nodules (Table 4). By contrast, shoot concentration of the selenoamino acid MeSeCys was not significantly different between nodulated and nonnodulated plants ($t = 1.592$, df = 8, $P = 0.150$).

To estimate what fractions of total shoot Se were γ-Glu-MeSeCys and MeSeCys, we used the percentage dry mass of samples and converted γ-Glu-MeSeCys and MeSeCys concentrations from a fresh mass basis to a dry mass basis. We then calculated what fraction of total, acid-digestible Se was γ-Glu-MeSeCys or MeSeCys (Fig. 5). A similar fraction of shoot Se was MeSeCys in nodulated plants grown in live soil (50.3%) compared with nonnodulated plants grown in autoclaved soil (56.9%). However, the shoot fraction of Se present as γ-Glu-MeSeCys was approximately 13 times higher in

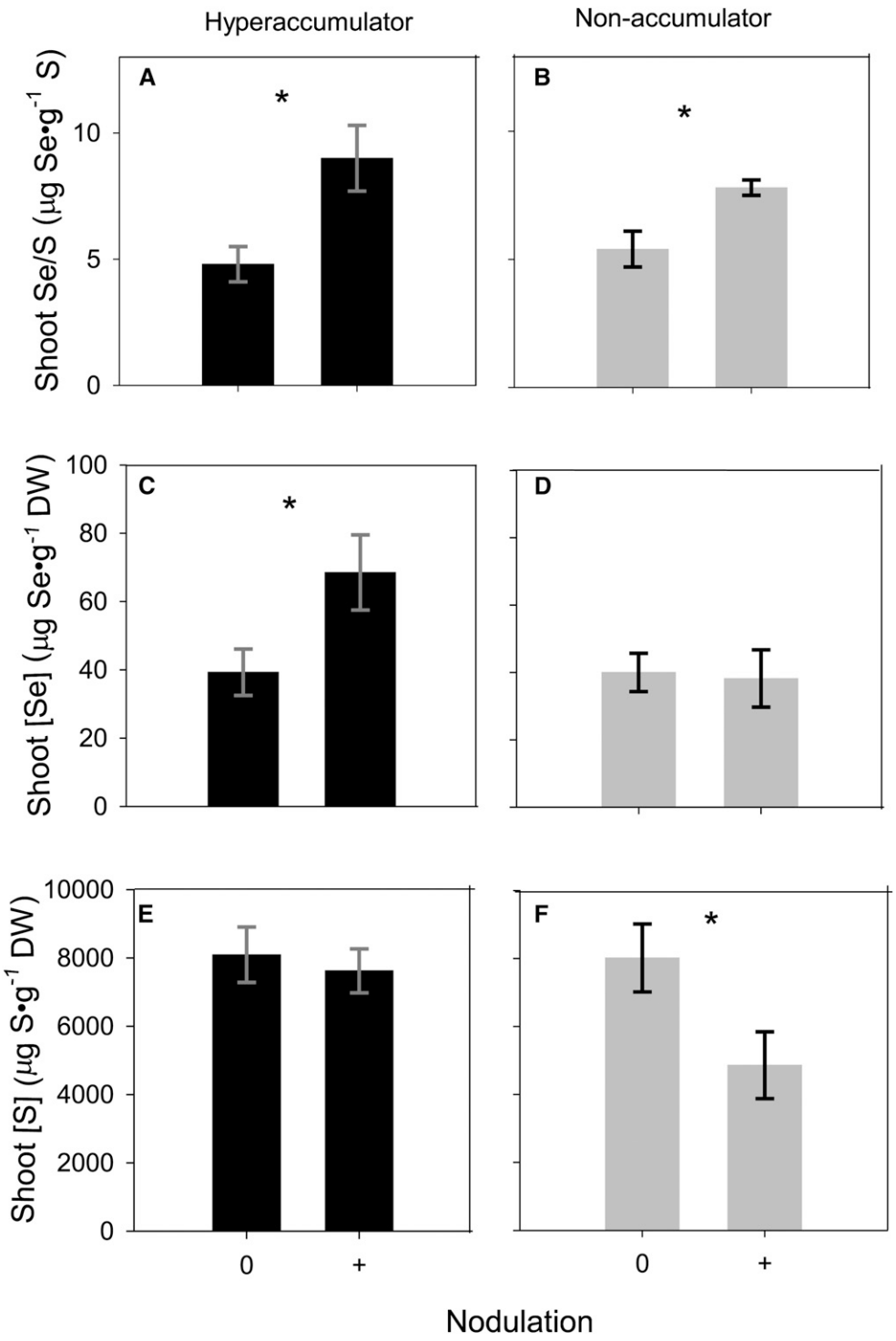

Fig. 3. Effect of nodule absence (0; $N = 8$) or presence (+; $N = 6$) on shoot Se and S concentration in the Se-hyperaccumulator *Astragalus bisulcatus* and the nonhyperaccumulator *A. drummondii*. Shown are means and standard error of the mean per gram dry mass (DW).

nodulated plants (32.3%) than in nonnodulated plants (2.3%). Selenocompounds that made up the remaining shoot Se were not identified, but accounted for 17.4% in nodulated plants and 40.8% in nonnodulated plants. Figure 6 depicts our current hypothetical model describing how root nodules may influence shoot [Se].

DISCUSSION

Plant symbioses with rhizobia affect metabolism in both partners (Colebatch et al., 2004). During symbiosis, plants and bacteria engage in a cycle of amino acid exchange within nodules. Experimental evidence suggests that pea plants may supply

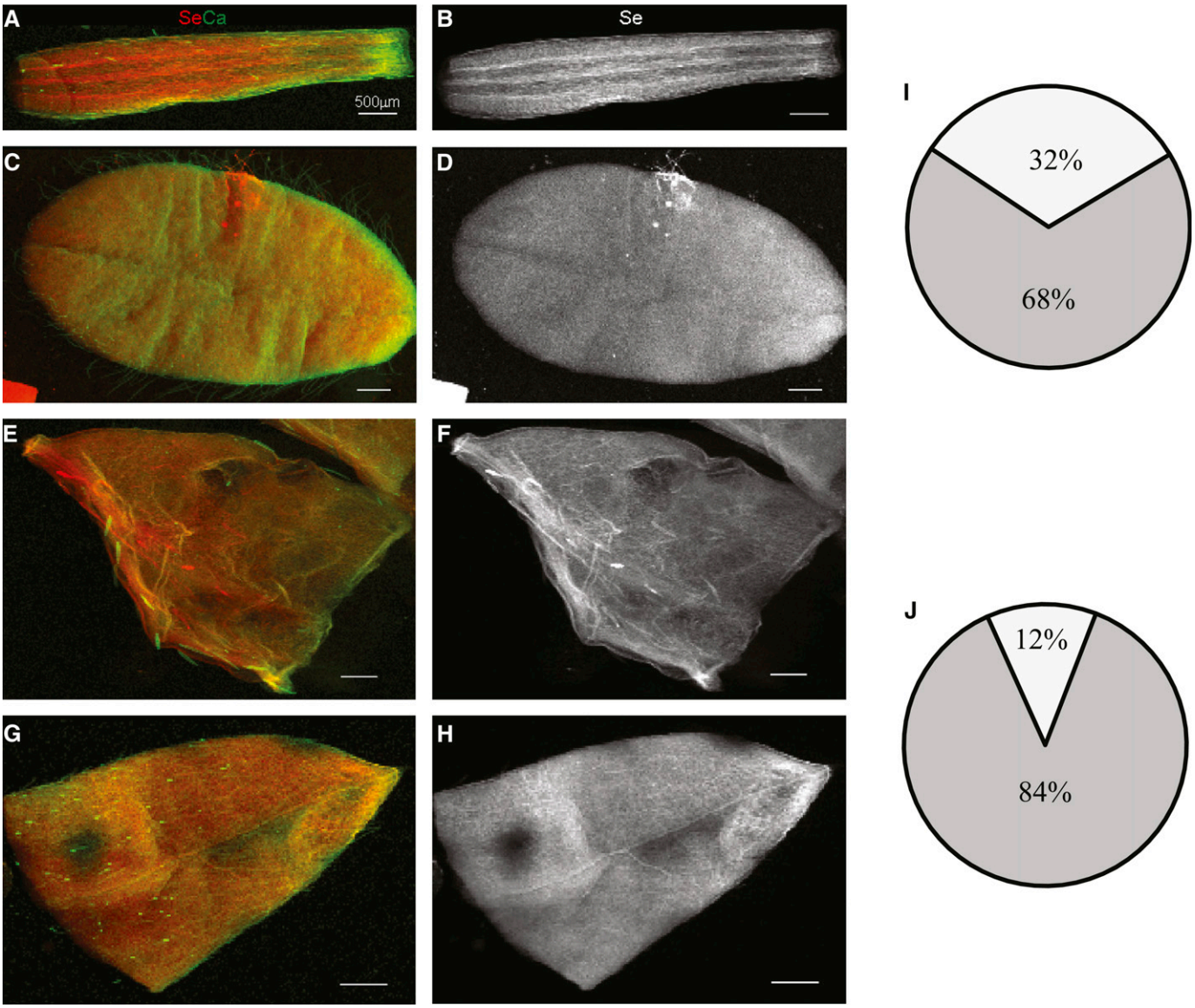

Fig. 4. Selenium (red) and Ca (green) distribution in leaves of the nonhyperaccumulators (A) *A. convallarius* and (C) *A. drummondii* and the hyperaccumulators (E) *A. praelongus* and (G) *A. racemosus*. Selenium (white) distribution for the same leaves in (B) *A. convallarius*, (D) *A. drummondii*, (F) *A. praelongus*, and (H) *A. racemosus*. *Note:* The appearance of Se in the upper portion of the *A. drummondii* leaf was due to damage from forceps during sample mounting; Se was not found in any other trichomes. Panels I and J show the Se speciation results for both nonhyperaccumulators and both Se hyperaccumulators, respectively. Inorganic Se is shown in light gray, organic Se in darker gray.

glutamate to bacteroids and the bacteroids then cycle amino acids such as aspartate back to the plants (Lodwig et al., 2003). Because *Astragalus* species hyperaccumulate Se and predominantly store Se in amino acids, we hypothesized that rhizobia contribute to Se hyperaccumulation. Using three species of Se hyperaccumulators and three nonhyperaccumulator species, we found that shoot [N] was correlated with shoot [Se] in hyperaccumulators, but not in nonhyperaccumulators. Shoot [Se] in Se-hyperaccumulator *A. bisulcatus* was 74.3% higher when nodulated. In contrast, there was no effect of nodulation on shoot [Se] in the nonhyperaccumulator *A. drummondii*. Thus, the hypothesized positive effect of nodulation on [Se] was observed but was specific to Se hyperaccumulators and did not occur in nonhyperaccumulators.

It is possible that symbiotic rhizobia increase N-based defenses in the Se-hyperaccumulator *A. bisulcatus* because Se is stored in the leaf in several forms, including selenoamino acids (Shrift, 1969; Pickering et al., 2003; Freeman et al., 2006), which are considered defensive compounds. In the *A. bisulcatus* leaf, Se is mainly stored as MeSeCys and γ-Glu-MeSeCys, with minor fractions of SeO_4^{2-} and SeO_3^{2-} (Freeman et al., 2006). In experiment 4, we did not find differences between nodulated and nonnodulated plants for total shoot [Se] or [MeSeCys], but differences were observed in shoot [γ-Glu-MeSeCys], a product of the enzymatic coupling of MeSeCys to glutamate (Ellis and Salt, 2003; Freeman et al., 2006). In contrast to SeCys or SeMet, both MeSeCys and γ-Glu-MeSeCys can safely be accumulated since they do not get nonspecifically incorporated into proteins (Neuhierl

TABLE 3. The percentage of Se atoms in various chemical forms in leaves estimated from XANES LSQ fitting (reported in Appendix S1) with standard error from each *Astragalus* species and hyperaccumulation group in experiment 3. SeCys: selenocysteine; C-Se-C: organic Se with C-Se-C configuration (methyl-SeCys, selenocystathionine or selenomethionine); Se^0: elemental Se.

Plant or statistic	SeO_4^{2-}	SeO_3^{2-}	SeCys	C-Se-C	Se^0
A. convallarius	11 ± 3 a	14 ± 1 a	nd	70 ± 3 a	4 ± 3 a
A. drummondii	9 ± 2 ab	25 ± 1 b	6	60 ± 3 a	1 ± 1 a
A. praelongus	3 ± 2 b	nd	nd	78 ± 12 a	15 ± 8 a
A. racemosus	nd	5 ± 3 a	nd	88 ± 4 a	5 ± 2 a
$F_{3,8}$	11.214	24.158		2.930	1.386
P value	0.003	<0.001		0.100	0.315
Nonhyperaccumulators	10 ± 2 A	18 ± 3 A	3	66 ± 3 A	3 ± 2 A
Hyperaccumulators	2 ± 1 B	5 ± 3 B	nd	84 ± 5 B	11 ± 7 A
t value	–5.067	–4.774		2.574	1.195
df	10	10		10	10
P value	<0.001	0.001		0.028	0.260

Notes: n.d. = Se species were not detected. Letters after values in the column indicate statistically significant differences ($\alpha = 0.05$) for species (lower case) and plant group (upper case).

and Böck, 1996) and, unlike inorganic forms of Se, they do not cause oxidative stress (Grant et al., 2011). Accumulation of MeSeCys and γ-Glu-MeSeCys explains the extreme Se tolerance of hyperaccumulators. The different effect of nodulation on [Se] in hyperaccumulators and nonhyperaccumulators is likely attributable to differences in the form of Se stored in the two plant groups. An observation that may be linked to the differences in Se storage in hyperaccumulators compared with nonhyperaccumulators is shown in experiment 1 in which a significant negative correlation with shoot, root, and nodule mass and [S] was found in both hyperaccumulators and nonaccumulators. If Se follows the same metabolic pathway as S in plants, a significant negative correlation in organ mass and [Se] would be expected. This correlation was observed in nonhyperaccumulators but not in hyperaccumulators.

In the Se-hyperaccumulator *A. bisulcatus*, Se is stored in leaves as 91% organic Se in the form of the C-Se-C compounds, MeSeCys and γ-Glu-MeSeCys, 6% SeO_4^{2-}, and 3% SeO_3^{2-} (Freeman et al., 2006). In the present study, we found that both Se hyperaccumulators, *A. praelongus* and *A. racemosus,* also contained high fractions of organic C-Se-C Se forms and low amounts of inorganic forms. Both *A. praelongus* and *A. racemosus* stored Se throughout the leaf like *A. bisulcatus* (Freeman et al., 2006). In *A. praelongus*, Se was stored in trichomes as was also reported for *A. bisulcatus* (Freeman et al., 2006); for *A. racemosus*, this was not observed. The distribution and speciation of Se in leaves of nonhyperaccumulator *Astragalus* species have not been previously reported. We found that Se was stored throughout the nonhyperaccumulator leaves, but not in trichomes. The nonhyperaccumulators contained more of the reactive, oxidized inorganic Se forms than hyperaccumulators and less of the organic Se forms. This difference is of significance since inorganic forms of Se do not require N as a constituent as is the case with reduced organic Se forms. Therefore, Se accumulation in nonhyperaccumulators may be less limited by N availability and hence, nodulation, than is Se accumulation in hyperaccumulators. Higher inorganic Se levels in nonhyperaccumulaors may also explain the higher Se sensitivity of *A. drummondii* (El Mehdawi et al., 2012).

In Se hyperaccumulators, rhizobia-derived N could contribute to plant Se levels through incorporation into selenocysteine (SeCys), the precursor of MeSeCys, glutamate (the other substrate for γ-Glu-MeSeCys), or through the enzyme γ-glutamylcysteine synthetase (ECS) that likely catalyzes the formation of γ-Glu-MeSeCys (Fig. 6). The mechanism for how this exchange may operate is not known at this time and was not a focus of this study. Even though *A. bisulcatus* plants did not differ in shoot [Se] in experiment 4, where selenoamino acid levels were determined, the presence or absence of nodulation did affect their metabolic profile of Se. Nodulated plants were estimated to derive 32% of their shoot Se from γ-Glu-MeSeCys, while in plants without nodules this fraction was only 2%. This finding that nodulation affected [γ-Glu-MeSeCys] but not [MeSeCys] suggests that the contribution of the rhizobial N to overall Se accumulation in *A. bisulcatus* is primarily through providing N in the form of glutamate.

Our results suggest that the degree of nodulation with symbiotic rhizobia affects Se accumulation in *Astragalus* hyperaccumulator species, but not in nonhyperaccumulators. The finding that symbiosis with rhizobia increased [Se] in Se hyperaccumulators may indicate that rhizobia have played a role in the evolution of Se hyperaccumulation in *Astragalus*. The nodulation effect on shoot [Se] may be critical during early periods of growth in a young N_2-fixing hyperaccumulator because increased

TABLE 4. Mean values and standard error for various attributes of *Astragalus bisulcatus* plants growing in Experiment 4.

Attribute	*N*	Autoclaved soil	Live soil	*t* value	df	*P* value
Shoot mass (mg)	21	79.8 ± 4.8 a	231 ± 12 b	–12.912	40	<0.001
Root surface area (cm^2)	10	70.1 ± 8.1 a	124 ± 9.5 b	–4.393	20	<0.001
Number of nodules	21	0	21.3 ± 2.7			
[Se] ($\mu g \cdot g^{-1}$ DW)	21	3179 ± 1646 a	2402 ± 566 a	1.863	12	0.087
[MeSeCys] ($\mu g \cdot g^{-1}$ FW)	7	590 ± 102 a	413 ± 43 a	1.592	8	0.150
[γGMSC] ($\mu g \cdot g^{-1}$ FW)	7	15.3 ± 9.9 a	153 ± 21b	–5.959	8	<0.001

Notes: Different letters across the rows indicate a significant difference between the two soil treatments in a *t* test ($\alpha = 0.05$).

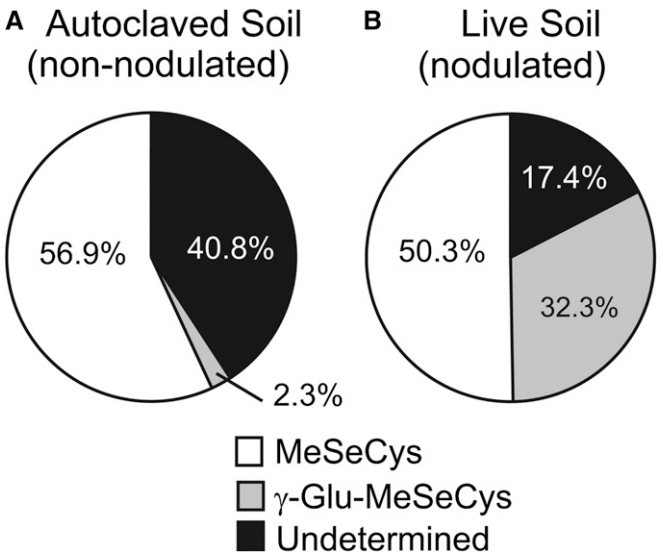

Fig. 5. Percentage of *Astragalus bisulcatus* shoot [Se] on a dry mass basis for different Se forms. Plants ($N = 7$) were grown in (A) autoclaved or (B) live (nonautoclaved) field-collected soil, resulting in nodulated and nonnodulated plants. The Se fractions in both plant groups were calculated by setting the total acid-digestible Se to 100%; LC-MS was used to measure MeSeCys and γGMSC, and the fraction of each compound was calculated relative to the total Se. The percentage of total Se unaccounted for by either amino acid is denoted as undetermined.

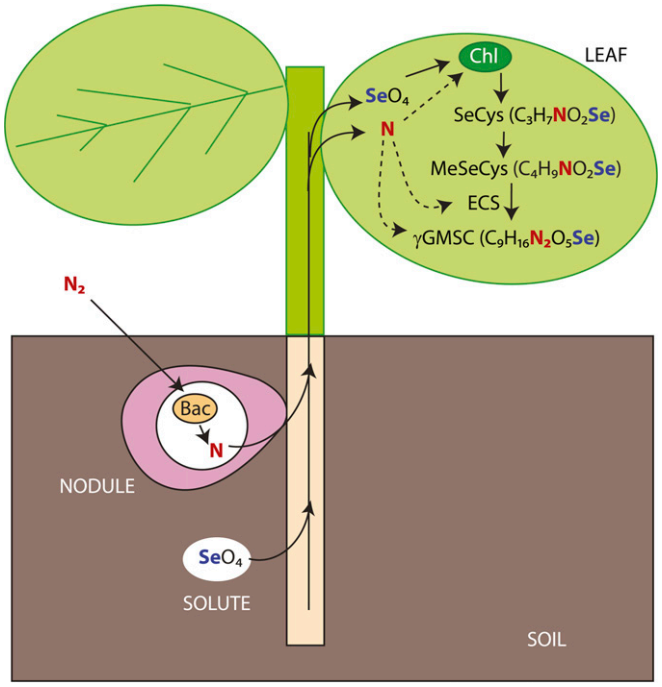

Fig. 6. Model of how rhizobia in *Astragalus* hyperaccumulators may contribute to Se hyperaccumulation. Bacteriods (Bac) within the nodule symbiotically fix N_2 and inorganic Se enters the plant through the root system. Although N can enter through the root system, the pathway is not represented because it is not mediated by symbiotic rhizobia. The elements are transferred through the xylem and enter the leaf where they can be used as building blocks for selenoamino acid synthesis in the mesophyll chloroplast (Chl) and cytosol. Both Se and N can be incorporated into selenocysteine (SeCys) in the chloroplast. This selenoamino acid can subsequently be methylated to form methylselenocysteine (MeSeCys). Another N atom could be added when glutamate is added to MeSeCys to form γ-glutamyl-methylselenocysteine (γGMSC). The likely enzyme mediating this process is γ-glutamyl-cysteine synthetase (ECS), which occurs in both the cytosol and the chloroplast. N may also be used to produce ECS itself.

shoot [Se] helps defend plants from herbivores and pathogens (Hurd-Karrer and Poos, 1936; Hanson et al., 2004; Galeas et al., 2008; Quinn et al., 2008). Previous work has shown that the leguminous symbiosis enhanced plant defenses in other species (Johnson et al., 1987; Briggs, 1990; Valdez Barillas et al., 2007; Dean et al., 2009; Kempel et al., 2009), especially N-containing defensive compounds. Nitrogen-fixing symbioses are known to contribute to plant secondary metabolites, such as alkaloid production in *Oxytropis sericea* (Valdez Barillas et al., 2007). Linkage of Se hyperaccumulation to N nutrition, and thus symbiotic N_2 fixation, was further supported by our finding that shoot [Se] was positively correlated with shoot [N]. This correlation was not significant in the nonhyperaccumulator *Astragalus* species. Thus, N_2 fixation facilitates Se sequestration in hyperaccumulators but not in nonhyperaccumulators. Nodule number and total nodule mass were not significantly correlated with shoot [Se]; therefore, shoot [N] may be a better predictor of shoot [Se] in *A. bisulcatus* than the degree to which the plant is colonized by rhizobia.

To determine whether the positive correlation found between shoot [Se] and shoot [N] in hyperaccumulators was a result of differences in nutrient or trace element levels between Se hyperaccumulators and nonhyperaccumulators, we compared these attributes. The two groups of *Astragalus* species did not differ in S or N concentrations, indicating that plant nutrition levels were similar between the two groups. Rather, they only differed in shoot [Se], where, as expected, the Se hyperaccumulators had a higher concentration than the nonhyperaccumulators. In the nodulation study, the ratio of Se to S increased with nodulation in both *A. bisulcatus* and *A. drummondii,* but the change in the ratios was driven by different elements. An increase in shoot [Se] was found to cause the enhanced Se to S ratio in the hyperaccumulator *A. bisulcatus,* while a decrease in shoot [S] caused the enhanced Se to S ratio in the nonhyperaccumulator *A. drummondii*.

Astragalus bisulcatus and *A. drummondii* occurred on the same study site, and soil beneath these plants was similar in total and water-extractable Se (results not shown). We used soil slurry to inoculate plants in the greenhouse experiment; therefore, soil properties other than microorganisms and water-soluble compounds should not have had much influence on these results. The use of fertilizer in the experiment was intended to minimize effects from other nutritional root symbioses, such as mycorrhiza. However, we cannot rule out that the specific symbiotic interactions reported here showing differences in patterns of nodulation and [Se] in the two species resulted from different microorganism identities in the two soil inocula. It has been shown that different strains of rhizobia produced different effects in nodulated legumes; for example, swainsonine production differed in *Oxytropis sericea* when it was nodulated with different strains (Valdez Barillas et al., 2007). Rather, we demonstrated that in both *Astragalus* species, nodulation created the expected benefit of increasing plant biomass, but only in *A. bisulcatus* did nodulation influence accumulation of the nonessential element Se. In the experiment comparing six *Astragalus* species with respect to the relationship between N and Se, all

plants were grown in the same soil, which was a composite derived from each species. Se hyperaccumulators were found to differ from nonhyperaccumulators in that they showed a positive correlation between shoot [Se] and shoot [N].

Based on our results from this short-term study, rhizobial symbionts appear to play an important supporting role in Se hyperaccumulation in *Astragalus* species by providing N for γ-Glu-MeSeCys synthesis. Future studies on other hyperaccumulators should also consider the influence of belowground root–microbe symbioses on uptake and accumulation of trace elements by plants. Such studies may reveal rhizobia that have the ability to enhance phytoremediation or biofortification potential. These unique hyperaccumulator *Astragalus* species may provide further biotechnological benefits by yielding new Se-resistant rhizobial isolates. Some rhizobia isolates have been shown to have high Se tolerance (Kinkle et al., 1994), but rhizobial isolates from Se hyperaccumulators have never been tested in this regard.

Mutualisms play a large role in the ecology of ecosystems because they provide vital services such as nutrient exchange, pollination, and seed and spore dispersal (Boucher et al., 1982). Exploring mutualisms within the context of stress and tolerance is increasingly important because ecosystems are subjected to rising levels of stress as human-induced pressures build. Therefore, it would be beneficial to learn how mutualisms persist under extreme conditions in natural ecosystems as well as to understand how environmental stress has contributed to differentiation among sympatric mutualists.

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