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Influence of microbial associations on selenium localization and speciation in roots of *Astragalus* and *Stanleya* hyperaccumulators

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ABSTRACT

Selenium (Se) hyperaccumulator plants can accumulate and tolerate Se up to 1% of their dry weight. Since little is known about below-ground processes of Se uptake and metabolism in hyperaccumulators, X-ray absorption spectromicroscopy was used to characterize the chemical composition and spatial distribution of Se in roots of *Astragalus* and *Stanleya* hyperaccumulators. Selenium was present throughout the roots, with the highest levels in the cortex. The main form of Se (48–95%) in both species collected from naturally seleniferous soil was an organic C—Se—C compound, likely methyl-selenocysteine. In addition, surprisingly high fractions (up to 35%) of elemental Se (Se^0) were found, a form so far not reported in plants but commonly produced by Se-tolerant bacteria and fungi. Four fungi collected from hyperaccumulator roots were characterized with respect to their Se tolerance and ability to produce Se^0, and then used to inoculate hyperaccumulators in a controlled greenhouse study. The roots of the greenhouse-grown *Astragalus* and *Stanleya* contained mainly C—Se—C; in most plants no Se^0 was detected, with the exception of *Astragalus* nodules and roots of *Astragalus* inoculated with *Alternaria astragali*, an Se^0-producing fungus. Apparently, Se^0-producing endosymbionts including nitrogen-fixing bacteria and endophytic fungi or bacteria in the root can affect Se speciation in hyperaccumulator roots. Microbes that affect plant Se speciation may be applicable in phytoremediation and biofortification, especially if they are promiscuous and affect Se tolerance in crop species.

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1. Introduction

Selenium (Se) is a naturally occurring element in most soils, and can be found at very high levels in alkaline soils where Cretaceous shale or other seleniferous rocks are the soil parent material (Byers, 1935, 1936; Rosenfeld and Beath, 1964; Beath, 1982). Non-seleniferous soils contain less than 1 mg Se kg^{-1}, whereas seleniferous soils can range between 2 and 100 mg Se kg^{-1} (Mikkelsen et al., 1989; Beath et al., 1939). Selenate is thought to be the predominant bioavailable form of Se in seleniferous soil (Rosenfeld and Beath, 1962; Zhao et al., 2005).

Selenium is an essential nutrient for many animals including mammals, yet is also toxic at higher levels, leaving a narrow margin between deficiency and toxicity (Chen et al., 1980; Hoffmann and Berry, 2008; Li et al., 2009). Selenium toxicity is thought to occur because of the similarity of Se to sulfur (S) (Brown and Shrift, 1982; Stadtman, 1990; Smith et al., 1995; Terry et al., 2000). Selenium is metabolized into selenocysteine (SeCys), or selenomethionine (SeMet); these Se-amino acids can be non-specifically incorporated into proteins instead of cysteine or methionine, causing the proteins to lose function (Brown and Shrift, 1982; Stadtman, 1990). Plants that accumulate Se may be used to both clean up excess Se from the environment (phytoremediation) and to prevent Se deficiency in consumers (biofortification). Crop species that are particularly good Se accumulators and that may be used for biofortification are garlic, onion, and broccoli (Fairweather-Tait et al., 2011).

Selenium enters the food chain primarily through plants that inadvertently take up and assimilate selenate via S transporters and enzymes (Terry et al., 2000; Sors et al., 2005). Plant species differ in their capacity to accumulate and tolerate Se. The so-called Se hyperaccumulating plants can accumulate more than 0.1% of their

Abbreviations: XAS, X-ray absorption spectroscopy; XRF, X-ray fluorescence mapping; XANES, X-ray absorption near-edge structure; MeSeCys, methyl-selenocysteine.

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dry weight as Se, without showing any symptoms of toxicity (Beath et al., 1939; White et al., 2007). Most plant species that are Se hyperaccumulators occur within the genus *Astragalus*; examples are *Astragalus bisulcatus* and *Astragalus racemosus* (Beath et al., 1939). The genus *Stanleya* also contains at least one Se hyperaccumulating species, *Stanleya pinnata* (Feist and Parker, 2001). Hyperaccumulators detoxify Se by adding a methyl group to SeCys via the enzyme SeCys methyltransferase (SMT); the resulting methyl-SeCys (MeSeCys) can be accumulated safely since it is a non-protein amino acid (Neuhierl and Bock, 1996). Methyl-SeCys is the primary form of Se found in young and old leaves of *A. bisulcatus* as well as in young leaves and flowers of *S. pinnata* (Pickering et al., 2003; Freeman et al., 2006a; Quinn et al., in press). Within hyperaccumulator leaves the Se was found predominantly in the periphery, such as the margins, epidermis, and trichomes (Freeman et al., 2006a). Non-hyperaccumulator plant species accumulate Se mainly in the vascular tissues, in the form of selenate (de Souza et al., 1998a; Van Hoewyk et al., 2005; Freeman et al., 2006a); since this form of Se is more toxic, these species are less Se tolerant. Selenium speciation in plant roots has so far not been reported for any species, including hyperaccumulators.

In contrast to many other organisms, Se has not been shown to be essential for higher plants (Brown and Shrift, 1982; Zhang and Gladyshev, 2009). Selenium does appear to be a beneficial nutrient for many plants, especially hyperaccumulators, which can reach two-fold higher biomass in the presence of Se (Pilon-Smits et al., 2009). Thus, the functional significance of Se hyperaccumulation may be to offer better growth, perhaps due to better oxidative stress resistance (Cartes et al., 2005; Hartikainen, 2005). An additional benefit of Se hyperaccumulation is enhanced resistance to Se-sensitive herbivores and pathogens (Vickerman et al., 2002; Hanson et al., 2003, 2004; Freeman et al., 2006b, 2007, 2009; Quinn et al., 2007, 2008, 2010). Thus, Se hyperaccumulation may be considered a form of elemental defense (Boyd and Martens, 1992; Boyd, 2007, 2010). As with any plant defense, herbivores and pathogens are likely to overcome it over time. There is indeed evidence of Se-tolerant herbivores and rhizosphere microbes (Freeman et al., 2006b; Wangeline and Reeves, 2007; Wangeline et al., 2011). A Se-tolerant diamond back moth was found to feed on *S. pinnata* containing 0.2% Se dry weight without ill effects. It accumulated MeSeCys, like its host, explaining its tolerance. Se-tolerant microbes can use different Se tolerance mechanisms, including Se reduction to insoluble, non-toxic elemental Se (Se^0), volatilization, or conversion to MeSeCys (Frankenberger and Karlson, 1994; Losi and Frankenberger, 1997; de Souza et al., 2001; Hunter and Manter, 2009). Some plant-associated microbes have been shown to affect plant Se accumulation and volatilization (de Souza et al., 1998b). The capacity of microbes to affect plant Se speciation has not been investigated.

In the study described here, Se speciation and distribution were characterized in roots of *Astragalus* and *Stanleya* hyperaccumulators with the goal to obtain better insight into below-ground Se metabolism and the potential of microbial symbionts to affect Se speciation and distribution in host tissues.

2. Materials and methods

2.1. Biological material

A. racemosus seeds were obtained from Western Native Seed, Coaldale, CO, USA. *S. pinnata* seeds were collected in the summer (June–July) of 2009 at Pine Ridge Natural Area, a seleniferous site in Fort Collins, CO, USA. *A. bisulcatus* and *S. pinnata* tap roots were collected from Pine Ridge Natural Area in June of 2009.

The four fungal isolates were obtained and described previously by Wangeline and Reeves (2007) and Wangeline et al. (2011) from hyperaccumulators growing on naturally seleniferous soil. They are (i) *Fusarium acuminatum* (F30), collected from *A. racemosus* in Lysite, WY, (ii) *Alternaria astragali* (A3) collected from *A. bisulcatus* in Laramie, WY, (iii) *Aspergillus leporis* (AS117), isolated from *S. pinnata* at Pine Ridge in Fort Collins, CO and (iv) *Alternaria seleniiphila* (A1), isolated from *S. pinnata* at Pine Ridge in Fort Collins, CO.

2.2. Field root preparation for XAS

Plants roots with rhizosphere soil attached were collected from Pine Ridge Natural Area in Fort Collins, CO. Live, intact roots were cut approximately 10 cm below the crown (top of the tap root). The tap root was inserted into flexible plastic tubing with a diameter of 2 cm. A two-component silicon epoxy was inserted into the tubing to fill up the space around the root, and to fix soil particles in place. After the epoxy was set, the tubing containing the root plus rhizosphere soil was cut into 0.5–1 mm thick cross-sections that were then frozen and stored at −80 °C for XAS analysis.

2.3. Fungal cultivation

All fungi were cultivated under continuous fluorescent light at 22 °C in sealed Petri dishes containing 0.5 strength malt extract agar (0.5 MEA, Difco, Detroit, MI) supplemented with different Se concentrations as specified below.

For determination of Se tolerance, the fungal isolates were supplied with 0, 30, 300 or 600 mg L^{-1} Se as Na_2SeO_4. After 5 days of growth, colony diameter measurements were taken to quantify Se tolerance; at this point none of the colonies had reached the edge of the petri dish.

Fungi for X-ray absorption spectroscopy (XAS) were grown for 5 days on 0.5 MEA with either Na_2SeO_3 or Na_2SeO_4 at 30 μM unless otherwise indicated. Mycelium was carefully collected, avoiding agar medium, rinsed with sterile distilled water, frozen in liquid nitrogen and stored at −80 °C for XAS.

Fungi to be used for plant inoculation were initially cultivated on 0.5 MEA medium with 30 μM Na_2SeO_4.

2.4. Plant preparation for fungal co-cultivation studies

S. pinnata seeds were surface-sterilized by rinsing for 20 min in 20% bleach, followed by five 10 min rinses in sterilized water. The *A. racemosus* seeds were first scarified with sandpaper and then surface-sterilized. Seeds were germinated on half-strength Murashige and Skoog medium with 10 g L^{-1} sucrose (Murashige and Skoog, 1962) under continuous light at 23 °C in a plant growth cabinet. After 14 days the seedlings were carefully transferred to 1 L cones filled with steam-sterilized gravel (in the bottom) and coarse sand (on top). The plants were watered bi-weekly with ¼ Hoagland's solution (Hoagland and Arnon, 1938) for 2 weeks and then inoculated with fungi as described below.

2.5. Co-cultivation and harvest

S. pinnata was inoculated with either fungal isolate A1 or AS117; a control treatment received no inoculum. The *A. racemosus* inoculation treatments consisted of fungal isolates A3, F30, or no inoculum. Plants were inoculated with a standard quantity of fungal hyphae and/or spores. The fungi were grown on 0.5 strength MEA plates for 5 days, and fungal materials were collected at the perimeter of the fungal colony. In preparation for plant inoculation, mycelium from F30 and mycelium and spores of A1 and A3 were macerated in sterile water in 1.5 mL tubes using sterile glass beads and a micro pestle. Spores from AS117 were placed in a

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tube with sterile water and 0.05% Tween-20 to break the water surface tension. Fragments of hyphae from F30, as well as spores and spore chains of A1 and A3 were quantified using a hemocytometer to estimate mm hyphae and spores and spore chains mL^{-1} water. Spores from an AS117 colony were quantified in the same way and diluted to 2000 spores mL^{-1} water. F30, A1, and A3 were diluted to a final concentration of 2000 mm mL^{-1} water. The inocula were delivered via peat moss to the plants in the greenhouse. Each plant received 1.5 mL of peat moss saturated with 1 mL of inoculum, or peat moss with sterile water for the control treatments. The Se treatment was delivered to the plants via watering with 12.5 μM Na_2SeO_4 twice weekly beginning at the time of inoculation. After inoculation, plants and fungi were co-cultivated for 12 weeks before harvesting. On the day of harvest, the plants were gently washed to remove adhering sand from the roots, and the roots were frozen in liquid nitrogen and stored at −20 °C for XAS. Root cross sections were made on frozen roots using a chilled razor blade, a Petri dish, and a block of dry ice to maintain the integrity of the sample.

2.6. X-ray absorption spectroscopy

Selenium distribution and speciation were determined using micro-focused X-ray fluorescence (μXRF) mapping and X-ray absorption near edge structure (μXANES) spectroscopy, respectively. Intact biological samples were flash-frozen in liquid nitrogen and shipped on dry ice for microprobe analyses at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Lab (Marcus et al., 2004). Frozen samples (plant roots and fungal mycelia) were transferred onto a Peltier stage kept at −33 °C to reduce potential 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, 50 ms dwell time per pixel. The chemical forms of Se in particular areas of interest were further investigated using Se K-edge XANES. XANES provides information about the oxidation state and, when compared to well-characterized Se standard compounds, information about its chemical speciation (Pickering et al., 1999). μXRF maps and μXANES spectra were recorded with a 7 element Ge solid state detector (Canberra, ON, Canada). Spectra were deadtime corrected, pre-edge background subtracted, and post-edge normalized using standard procedures (Kelly et al., 2008). Red selenium (white line position set at 12,660 eV) was used to calibrate the spectra. Least square linear combination (LSQ) fitting of Se XANES spectra was performed in the 12,630–12,850 eV range, using a library of standard seleno-compounds. Se standards used include: Na_2SeO_4, Na_2SeO_3, SeCystine, SeMet purchased from Sigma–Aldrich (St Louis, MO, USA), MeSeCys, γ-GMeSeCys, SeCysth, and $SeGSH_2$ purchased from PharmaSe (Austin, TX, USA). SeCys was obtained by reducing SeCystine overnight at 25 °C in 100 mM sodium borohydride at a 1:1 molar ratio; reduction was verified enzymatically using SeCys lyase as described by Pilon-Smits et al. (2002). Gray and red elemental Se were provided by Amy Ryser and Dan Strawn. All data processing and analyses were performed with a suite of custom LabVIEW (National Instruments, Austin, TX, USA) programs available at the beamline.

3. Results

In field-collected plants from two Se hyperaccumulating species, *A. bisulcatus* and *S. pinnata*, Se was distributed throughout the roots, as judged from XRF mapping (Fig. 1). In both plant species the highest Se signal intensity was found in the peripheral (cortex) region, with relatively lower signal in the center. Soil particles attached to the roots are visible in panels A and C, tri-color coded elemental maps showing Ca (in green), Fe (in blue) and Se (in red). There is not a clear Se signal in the isolated soil particles, but at the root–soil interface both plant species show a ring of Se that overlaps with Ca- and Fe-rich soil particles. It cannot be distinguished whether this Se-rich area corresponds with the epidermis, the rhizosphere, or both.

The speciation of Se in the roots and in the Se-rich ring at the root–soil interface was determined by XANES (Table 1; the numbers in Fig. 1 correspond to the locations where spectra were collected). In all of the spots analyzed for *A. bisulcatus* and all but one of the *S. pinnata* spots the predominant form of Se (49–95%) appears to be an organic form of Se with a C—Se—C configuration (SeMet, MeSeCys, or SeCysth). The one spot in *S. pinnata* (#11) that showed a different speciation fitted best with SeCys. Interestingly, all but one of the XANES spectra from the *A. bisulcatus* root and all of the spectra from the *S. pinnata* root indicate a high amount of elemental selenium (Se^0), a form not previously reported in plants. Se^0 was the second most abundant form of Se in these field-collected roots (5–35%). The one spot that did not contain Se^0 (#1) was on the periphery of the root. Selenite was the third most abundant form of Se (4–18%), found in all XANES spots on the *A. bisulcatus* roots and all spots on *S. pinnata* except peripheral spot #9. On average, the speciation in the *A. bisulcatus* root was 9% SeO_3^{2-}, 76% C—Se—C, and 16% Se^0. The average Se composition across all the *S. pinnata* spots (not taking into account outlier #11) is 9% SeO_3^{2-}, 68% C—Se—C, and 23% Se^0. The Se signal was high enough throughout the *S. pinnata* root that a transect of XANES spots could be analyzed, which revealed interesting differences in Se speciation between the cortex (spots 8–10, 16) and the stele (spots 12–15) (Fig. 1, Table 1). The stele contained a significantly higher percentage of Se^0 than the cortex (32 + 1 vs. 14 + 4; $p = 0.0026$), and relatively more SeO_3^{2-} (14 ± 1 vs. 4 + 2, $p = 0.005$), while containing a lower percentage of C—Se—C than the cortex (53 + 2 vs. 81 + 5; $p = 0.0032$). The Se signal in the stele of *A. bisulcatus* was too low to obtain a good XANES spectrum, so speciation cannot be compared with the cortex. However, the cortex showed a similar relative distribution of organic vs. inorganic Se as the cortex of *S. pinnata*.

Since Se^0 has not been reported to be produced or found in plants before, but is well-known to be produced by many microbes, it is possible that the Se^0 in the roots of both Se hyperaccumulators is produced by microbial endophytes. To investigate this possibility, several fungi were selected and tested for their effects on root Se speciation. These fungi come from a collection isolated from the rhizoplane of *Astragalus* and *Stanleya* hyperaccumulators as described by Wangeline et al. (2011). Four isolates were selected for controlled inoculation studies with *Astragalus* and *Stanleya*. Two of these fungi were isolated from *S. pinnata*: *A. seleniiphila* (A1) a newly described small-spored species (Wangeline and Reeves, 2007), and a Se-tolerant *A. leporis* (AS117). The other two fungi were isolated from *Astragalus* species: *A. astragali* (A3) was isolated from *A. bisulcatus* and newly described by Wangeline and Reeves (2007). *F. acuminatum* (F30) was isolated from *A. racemosus*.

The four fungal isolates were tested for their Se tolerance by quantifying their growth on agar media supplied with 0–600 mg L^{-1} Na_2SeO_4. Judged from this test F30 is the most Se tolerant, growing better in the presence of Se than without Se (Fig. 2). A1 and AS117 are also highly Se tolerant, showing only a marginal growth reduction up to 600 mg Se L^{-1} (Fig. 2). Among the four fungi, A3 was the least tolerant, with ~40% growth reduction at 600 mg Se L^{-1}. This is still ten-fold more Se-tolerant than many other fungi tested in earlier studies (Hanson et al., 2003; Wangeline et al., 2011). Preliminary measurements of Se accumulation in the selenate-supplied fungi indicate that A1 is a particularly good Se accumulator, and A3 and F30 are moderate Se accumulators. AS117 is a low Se accumulator; this may be related to the observation that AS117 appears to volatilize Se at high levels, based on

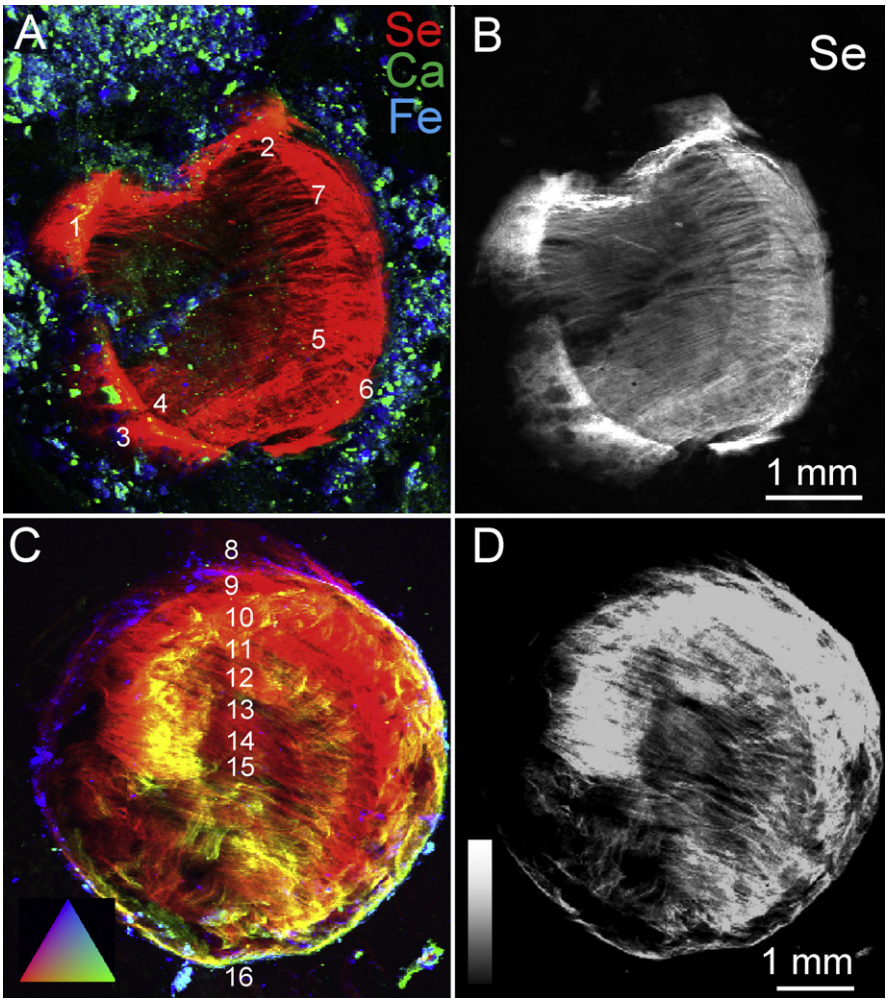

Fig. 1. X-ray fluorescence elemental mapping of *Astragalus bisulcatus* and *Stanleya pinnata* roots with soil particles attached, collected from the field. (A and B) Crown root of an *A. bisulcatus* plant. (B and D) Root of *S. pinnata*. (A and C) Tricolor-coded maps, with Se in red, Ca in green, and Fe in blue. (B and D) Black and white Se distribution map (high Se = white). Numbers indicate locations of XANES analyses.

Table 1
Results from least squares linear combination fitting of experimental XANES spectra with standard seleno-compounds. The points where the spectra were collected are indicated in Fig. 1. C—Se—C: methyl-selenocysteine, Se-methionine or Se-cystathionine. Se^0: red or gray elemental Se. SS: normal sum of squares (quality of fit; 0 = perfect fit); nd: compound not detectable.

	SS ($\times 10^{-4}$)	SeO_4^{2-}	SeO_3^{2-}	$SeGSH_2$	SeCysteine	SeCystine	C—Se—C	Se^0
A. bisulcatus								
1	9.30	nd	5%	nd	nd	nd	95%	nd
2	4.30	nd	16%	nd	nd	nd	50%	35%
3	1.76	nd	11%	nd	nd	nd	64%	26%
4	1.77	nd	5%	nd	nd	nd	88%	8%
5	3.43	nd	6%	nd	nd	nd	89%	6%
6	2.11	nd	11%	nd	nd	nd	66%	24%
7	1.86	nd	7%	nd	nd	nd	80%	14%
S. pinnata								
8	4.78	nd	4%	nd	nd	nd	84%	13%
9	2.96	nd	nd	nd	nd	nd	93%	7%
10	2.67	nd	9%	nd	nd	nd	67%	24%
11	3.88	nd	4%	nd	70%	nd	nd	22%
12	3.57	nd	15%	nd	nd	nd	50%	34%
13	2.30	nd	13%	nd	nd	nd	56%	31%
14	2.41	nd	11%	nd	nd	nd	59%	31%
15	5.05	nd	18%	nd	nd	nd	48%	34%
16	2.00	nd	5%	nd	nd	nd	82%	13%

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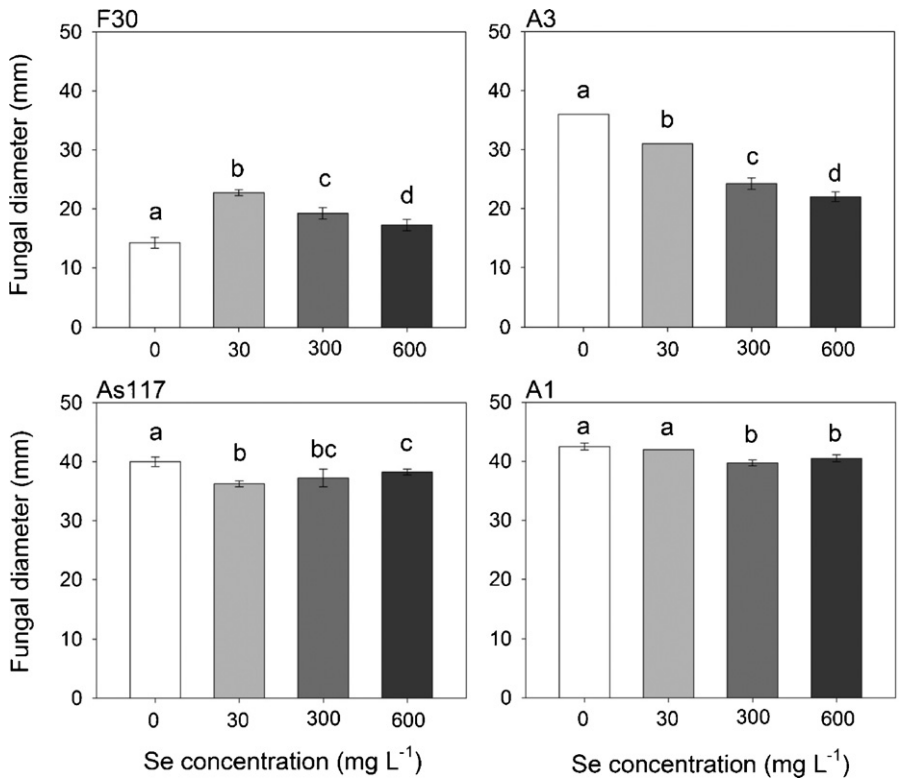

Fig. 2. Selenium tolerance of the four fungi used in this study, F30 (*Fusarium acuminatum*), A3 (*Alternaria seleniiphila*), AS117 (*Aspergillus leporis*), A1 (*Alternaria astragali*), quantified as colony diameter after 5 days of growth on 0.5-strength malt extract agar supplied with either 0, 30, 300 or 600 mg L^{-1} sodium selenate. Values shown represent the means, and the error bars are the standard error of the mean. Lower case letters above bars indicate statistically different means (ANOVA, $p < 0.05$).

qualitative assessment of the strength of the distinct volatile Se smell (Wangeline, unpublished results).

To test whether these four fungi can produce Se^0 when supplied with selenate or selenite, the fungi were grown on media containing one of these compounds, and their speciation determined by XANES. Indeed, with the exception of F30, all fungi accumulated Se^0, which comprised 28–98% of their total Se; F30 accumulated organic forms of Se (Table 2). Based on these characteristics with respect to Se tolerance and metabolism, it was decided to use all four fungal species for hyperaccumulator inoculation studies.

To test whether production of Se^0 in hyperaccumulator roots is associated with the presence of microbial symbionts, hyperaccumulators *A. racemosus* and *S. pinnata* were grown from surface-sterilized seeds in sterilized growth medium, with or without the fungi described above. Each plant species was inoculated with two different fungi that were isolated previously from plants in the same genus. The reason why *A. racemosus* was chosen over *A. bisulcatus* was that 50–80% of the *A. bisulcatus* seeds contained an endophytic fungus, which might confound the results; *A. racemosus* seeds did not show any evidence of endophytes. In the *A. racemosus* roots from all three treatments (no inoculum, A3 inoculum and F30 inoculum) Se was found throughout the roots, with a higher signal coming from the cortex compared to the stele (Fig. 3), similar to what was observed in field-collected *A. bisulcatus* roots. The majority of *A. racemosus* plants from all greenhouse treatments had root nodules at the time of harvest. Nodules can be seen on the lateral root of *A. racemosus* inoculated with F30 (Fig. 3D), and in the cross-section of *A. racemosus* inoculated with A3 (Fig. 3F). The Se signal is very high in the nodules of these roots compared to the rest of the root. There are no obvious differences in Se distribution between the three treatments.

Selenium speciation in the roots and root nodules was determined using Se K-edge XANES. The numbers in Fig. 3 indicate where XANES spectra were collected; Se speciation results can be found in Table 3. The most abundant form of Se in the *A. racemosus* roots from the greenhouse had a C—Se—C configuration. A small fraction of Se was SeO_3^{2-} in most of the XANES spots. Spots 2–8 and 12–20 all gave similar Se spectra with on average 95% C—Se—C and 5% SeO_3^{2-}. Interestingly, in the majority of root spectra collected from the greenhouse plants there is a noted absence of Se^0 (which was a significant Se species in the field-collected *A. bisulcatus* roots). The only spots from the greenhouse roots that contained Se^0 (Table 3) were located in the root nodule of *A. racemosus* inoculated with F30 (Fig. 3D, spots 9 and 10, 28–45% Se^0), on the lateral root mass of *A. racemosus* inoculated with A3 (Fig. 3E, spot 11, 22% Se^0), and on the root-nodule interface of *A. racemosus* inoculated with A3 (Fig. 3F, spots 15–17, <5% Se^0). The XANES fit from spot 1 (Fig. 3A) was notably different from the others, and is likely unreliable based on the poor quality of the fit (sum of squares value of 91.1×10^{-4}).

Fig. 4 shows the μ-XRF elemental distribution maps of the root sections of the greenhouse-grown *S. pinnata*. Selenium was predominantly localized in the cortex, and lower or absent in the center of *S. pinnata* plants inoculated with A3 or F30 (Fig. 4B and C). The same may be true for the uninoculated root (Fig. 4A), but this cannot be determined from this sample due to the angle of the cross-section. XANES spectra from the un-inoculated root (Fig. 4A) reveal that all of the Se is present in a C—Se—C configuration (Table 4). The XANES spectra obtained from the root inoculated with AS117 indicated that the Se was also almost exclusively present as C—Se—C, with a small fraction of selenite (Fig. 4B, Table 4). The spot on the cortex showed relatively more inorganic Se compare to the spot on the cortex–stele interface, but the fit of the cortex spot was less reliable. The Se present in the cortex of the root inoculated with A1 was also almost completely C—Se—C, with small fractions (up to 4%) of Se^0 and selenite (Fig. 4C, Table 4). Thus, similar to *Astragalus*,

Table 2
Results from least squares linear combination fitting of experimental XANES spectra with standard seleno-compounds. XANES fits were obtained from fungi originally isolated from hyperaccumulator roots in the field, grown on plates with Se. C—Se—C: methyl-selenocysteine, Se-methionine or Se-cystathionine. Se^0: red or gray elemental Se. SS: normal sum of squares (quality of fit); nd: compound not detectable.

	SS ($\times 10^{-4}$)	SeO_4^{2-}	SeO_3^{2-}	$SeGSH_2$	SeCysteine	SeCystine	C—Se—C	Se^0
A. bisulcatus fungal isolates								
F30 on 30 μM SeO_3^{2-}								
1	12.1	nd	10%	38%	nd	nd	56%	nd
2	16.2	nd	6%	35%	nd	nd	63%	nd
3	5.47	nd	6%	40%	nd	nd	55%	nd
A3 on 30 μM SeO_3^{2-}								
1	3.12	1%	nd	16%	43%	nd	nd	38%
2	2.56	nd	4%	11%	nd	nd	58%	29%
3	3.73	nd	5%	12%	nd	23%	21%	40%
S. pinnata fungal isolates								
AS117 on 30 μM SeO_4^{2-}								
1	6.57	nd	nd	nd	nd	37%	nd	61%
2	12.9	nd	nd	nd	nd	37%	nd	60%
AS117 on 360 μM SeO_3^{2-}								
1	8.89	nd	nd	nd	nd	nd	nd	98%
A1 on 30 μM SeO_3^{2-}								
1	2.53	nd	nd	13%	nd	nd	nd	87%
2	2.01	nd	4%	17%	nd	22%	nd	57%
3	1.25	nd	nd	11%	nd	nd	nd	90%

Table 3
Results from least squares linear combination fitting of experimental XANES spectra with standard seleno-compounds. XANES fits were obtained at the points indicated in Fig. 3 on the roots of greenhouse-grown *A. racemosus* plants. C—Se—C: methyl-selenocysteine, Se-methionine or Se-cystathionine. Se^0: red or gray elemental Se. SS: normal sum of squares (quality of fit); nd: compound not detectable.

	SS ($\times 10^{-4}$)	SeO_4^{2-}	SeO_3^{2-}	$SeGSH_2$	SeCysteine	SeCystine	C—Se—C	Se^0
No inoculum								
1	91.1	nd	nd	63%	nd	nd	8%	nd
2	10.3	nd	3%	nd	nd	nd	97%	nd
3	4.25	nd	3%	nd	nd	nd	98%	nd
4	4.47	nd	4%	nd	nd	nd	95%	nd
F30 inoculum								
5	6.71	nd	5%	nd	nd	nd	95%	nd
6	9.57	nd	6%	nd	nd	nd	96%	nd
7	6.98	nd	8%	nd	nd	nd	93%	nd
8	10.9	nd	4%	nd	nd	nd	100%	nd
9	2.92	1%	nd	15%	nd	38%	nd	45%
10	5.06	nd	8%	23%	nd	nd	41%	28%
A3 inoculum								
11	9.42	nd	18%	18%	nd	nd	46%	22%
12	5.76	nd	9%	nd	nd	nd	91%	nd
13	3.61	nd	9%	nd	nd	nd	85%	nd
14	3.68	nd	6%	nd	nd	nd	95%	nd
15	6.43	nd	7%	nd	nd	nd	91%	2%
16	5.02	nd	7%	nd	nd	nd	90%	3%
17	5.77	nd	7%	nd	nd	nd	89%	4%
18	6.21	nd	nd	nd	nd	nd	103%	nd
19	3.94	nd	3%	nd	nd	nd	99%	1%
20	4.89	nd	nd	nd	nd	nd	103%	nd

S. pinnata roots contained a substantial Se^0 fraction in the field, but not in the greenhouse.

4. Discussion

This study describes Se distribution and speciation in roots of Se hyperaccumulators, both from the field and from a greenhouse experiment. The main form of root Se was a C—Se—C compound in both *Astragalus* and *Stanleya* hyperaccumulator species, either growing in the field or the greenhouse. There was an interesting difference in Se speciation between plants collected from naturally seleniferous soil and plants germinated from surface-sterilized seeds and grown on sterilized medium in the greenhouse. The plants in the field contained a substantial fraction (up to 35%) of elemental Se (Se^0), a form usually associated with microbial activity. This form was in most cases not observed in greenhouse plants. When observed in greenhouse-grown plants, the Se^0 was found particularly in root nodules. In the field it was found throughout the roots: in the cortex, stele, and at the root–soil interface. There were no nodules on these particular field-collected roots, but other *A. bisulcatus* nodules were shown to contain around 30% Se^0 (Valdez-Barillas, Lindblom, Fakra, Marcus and Pilon-Smits, unpublished results).

Since the nodules of *A. racemosus* contained relatively high levels of Se^0 compared to the rest of the root, an as yet unidentified nitrogen fixing bacterium in the nodules appears to significantly affect Se speciation. It is interesting that the roots of the greenhouse-grown plants were in most cases nodulated, even though no attempt was made to inoculate with *Rhizobium*, and the seeds and growth medium were sterilized at the beginning of the experiment. Apparently, there were bacterial strains in the vicinity that could colonize this hyperaccumulator species and were not affected

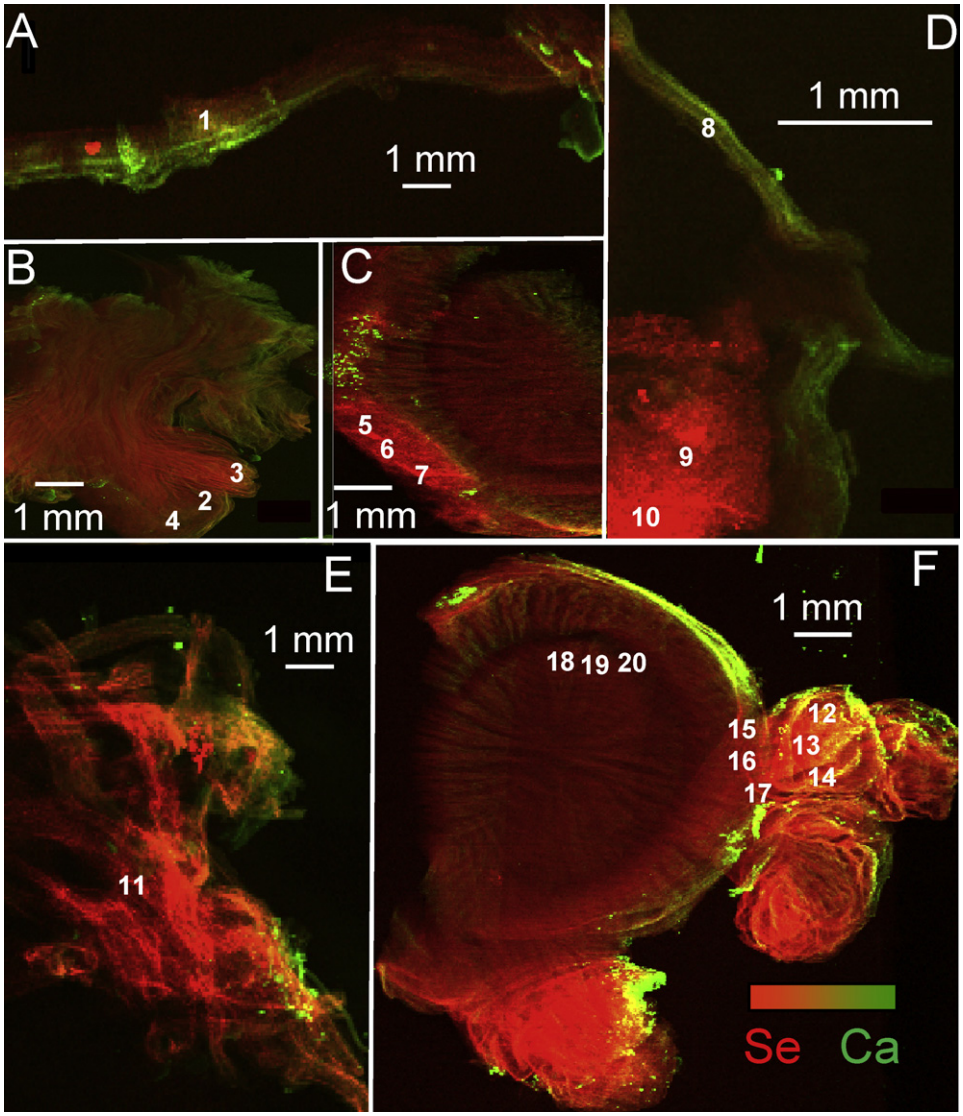

Fig. 3. X-ray fluorescence elemental mapping of *Astragalus racemosus* roots grown in greenhouse conditions. (A and B) Lateral root and root thin section respectively from an un-inoculated plant. (C and D) Thin section and lateral root with nodule, respectively, from plants inoculated with *Fusarium acuminatum* (F30). (E and F) From plants inoculated with *Alternaria astragali* (A3). All panels show bicolor-coded maps with Se in red and Ca in green. Numbers indicate locations of XANES analyses.

Table 4
Results from least squares linear combination fitting of experimental XANES spectra with standard seleno-compounds. XANES fits were obtained at the points indicated in Fig. 4 on the roots of greenhouse-grown *S. pinnata* plants. C—Se—C: methyl-selenocysteine, Se-methionine or Se-cystathionine. Se^0: red or gray elemental Se. SS: normal sum of squares (quality of fit); nd: compound not detectable.

	SS ($\times 10^{-4}$)	SeO_4^{2-}	SeO_3^{2-}	$SeGSH_2$	SeCysteine	SeCystine	C—Se—C	Se^0
No inoculum								
1	6.47	nd	nd	nd	nd	nd	100%	nd
2	7.58	nd	nd	nd	nd	nd	100%	nd
3	7.07	nd	nd	nd	nd	nd	100%	nd
4	6.46	nd	nd	nd	nd	nd	100%	nd
5	3.91	nd	nd	nd	nd	nd	100%	nd
6	6.08	nd	nd	nd	nd	nd	100%	nd
AS117 inoculum								
7	9.02	nd	6%	nd	nd	nd	101%	nd
8	38.9	nd	16%	nd	nd	nd	86%	nd
A1 inoculum								
9	4.16	nd	2%	nd	nd	nd	93%	7%
10	4.18	nd	nd	nd	nd	nd	96%	4%
11	3.51	nd	nd	nd	nd	nd	98%	nd
12	3.64	nd	1%	nd	nd	nd	100%	nd
13	3.26	nd	nd	nd	nd	nd	99%	2%
14	11.5	1%	6%	nd	nd	nd	91%	nd

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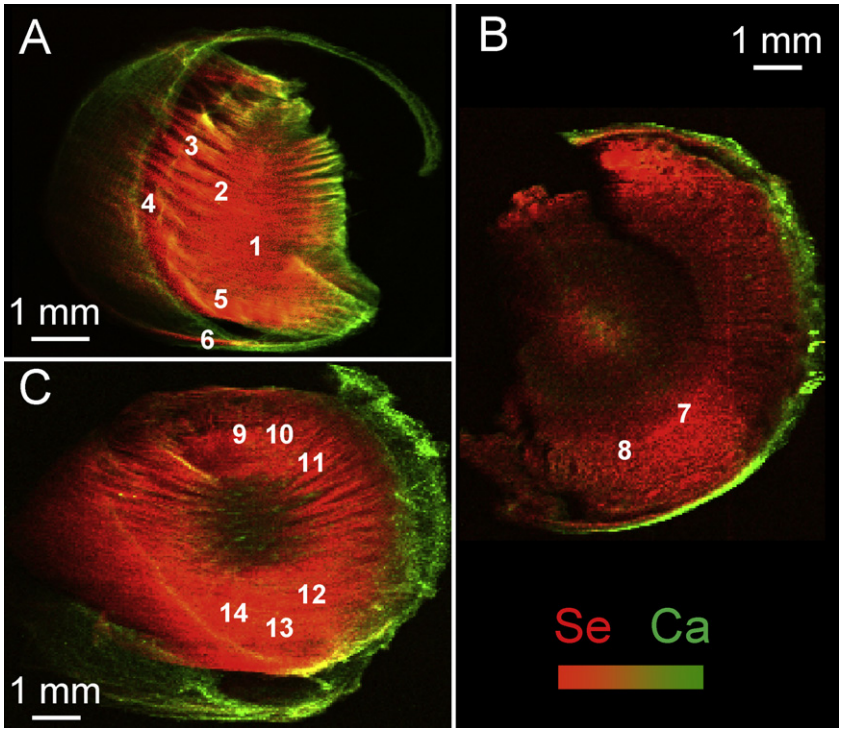

Fig. 4. X-ray fluorescence elemental mapping of *Stanleya pinnata* roots grown in greenhouse conditions. (A) Un-inoculated control, (B) plant inoculated with *Aspergillus leporis* (AS117), (C) plant inoculated with *Alternaria seleniiphila* (A1). All panels show bicolor-coded maps with Se in red and Ca in green. Numbers indicate locations of XANES analyses.

by its elevated Se levels. Selenium-hyperaccumulating *Astragalus* species have been reported before to have root nodules (Defaria et al., 1989) and to host bacteria that could nodulate other, non-hyperaccumulator *Astragalus* species (Wilson and Chin, 1947). So far these hyperaccumulator-associated Rhizobiaceae have not been characterized, and nothing is known about their relative Se tolerance. In earlier studies Rhizobiaceae associated with other plant species have been shown to differ in Se sensitivity. While 1 mg L^{-1} selenate impaired nodulation and N_2 fixation in *Melilotus indica*, a non-hyperaccumulator (Wu et al., 1994), the symbionts *Rhizobium selenireducens*, *Sinorhizobium fredii*, and *Sinorhizobium meliloti* were Se-tolerant (Kinkle et al., 1994; Hunter et al., 2007). Several Se-tolerant fungi were tested for the ability to reduce Se to Se^0; among isolates shown to have this capacity were an *Aspergillus* and a *Fusarium* species (Gharieb et al., 1995).

The high levels of Se^0 in the root proper (cortex and stele) that was found only in the plants growing in the field may be due to endophytic fungi or bacteria, since these are known to colonize these tissues (Weyens et al., 2009a,b). Inoculation of greenhouse-grown plants with four rhizoplane fungi isolated from hyperaccumulators in seleniferous areas did not significantly affect root Se speciation, although one fungus gave rise to some (2–22%) Se^0 accumulation. When this fungus (A3) was grown on plates and supplied with selenite, it accumulated predominantly Se^0, so it is possible that it affected root Se speciation. It is interesting to note that the XANES spectrum indicating the highest Se^0 fraction in the greenhouse roots was on a lateral root where the X-ray beam was hitting the outside of the root; the area of initial contact with the fungus. In the field the Se^0 fractions in the roots were much higher, however, than those observed in the greenhouse. Perhaps additional microbes are present in the field that further influence root Se speciation, or perhaps root colonization by the fungus requires more time. The plants in the greenhouse experiment were only co-cultivated with the fungi for 3 months, while the field plants were several years old. The finding that most of the greenhouse-grown *A. bisulcatus* and *S. pinnata* plants did not accumulate Se^0 indicates that this form of Se is not commonly made in these hyperaccumulators, and makes it more likely that it is the result of microbial activity. Since Se^0 is insoluble and therefore not mobile, the compound is likely produced in the root itself, perhaps by an endophyte as a detoxification mechanism. Microbial conversion of Se to Se^0 could also protect the plant from Se toxicity. Furthermore, trapping of insoluble Se in the roots by microbes may reduce shoot Se concentration. Higher plant tolerance and lower shoot Se levels may benefit the practice of phytostabilization – where plants are used to prevent the movement of pollutants and prevent toxic levels from entering the food chain. In future studies it will be interesting to study the microbiome of hyperaccumulators, and to characterize the properties of the endophytes and their effects on plant metabolism.

The predominant form of Se in roots of all of the hyperaccumulators tested was C—Se—C. This may either be SeCysth, SeMet, MeSeCys, or γ-glutamyl-MeSeCys. The XANES spectra for these Se species are practically indistinguishable (Pickering et al., 1999). In young leaves the predominant form of Se (~90%) was also shown to be a C—Se—C compound (Pickering et al., 2003; Freeman et al., 2006a). Liquid chromatography mass spectrometry showed this compound to be 50% MeSeCys and 50% γ-glutamyl-MeSeCys (Freeman et al., 2006a). Pickering et al. (2000) reported that in roots of *A. bisulcatus* Se was mainly present in organic form, which is in agreement to our study. This appears to be different from non-accumulators: the non-hyperaccumulator *Triticum aestivum* (wheat) accumulated predominantly selenate in its roots when supplied with selenate, and mainly selenite and SeMet when applied with selenite (Li et al., 2008).

The roots of greenhouse- and field-grown hyperaccumulators contained a small fraction of selenite. In earlier studies, leaves of the same hyperaccumulator species showed minor fractions of selenate (Freeman et al., 2006a,b). The finding that the minor fraction of inorganic Se in roots (apart from Se^0) is selenite rather than

selenate may reflect the more reducing (anoxic) conditions in the root tissues as compared to the leaves with their large intracellular spaces. It is thought that the main bioavailable form of Se in soil is selenate, and that selenate assimilation takes place predominantly in the leaf chloroplasts (Pilon-Smits et al., 1999). It is interesting that organic Se is the main form of Se in the root, because this either suggests that selenate is quickly translocated to the shoot, transformed to organic Se and remobilized back to the root, or that the roots are capable of assimilating selenate into organic Se directly. Yet another explanation is that the form of Se in seleniferous soil in the vicinity of hyperaccumulators is already to a large extent organic, due to litter deposition and root turnover and/or exudation. It will be interesting in future studies to investigate Se speciation in soil around hyperaccumulators. In this context it is interesting to note that in an earlier XRF study rice roots were found to influence soil Fe, Mn and As distribution and speciation in their rhizosphere (Frommer et al., 2011).

In both plants from the field and plants from the greenhouse the highest Se signal intensity was found in the peripheral (cortex) region, with relatively lower signal in the center (stele). This may reflect a difference in physiology between the two tissue types, with more metabolic activity and better storage capacity in the cortex (ground tissue) than in the stele (vascular tissue). Since Se may have a defensive function, the plant may also accumulate more Se in its periphery where it can better defend the plant from attacks initiated from outside. It will be interesting in future studies to compare Se and S distribution to see if the two chemically similar elements co-localize, particularly because in earlier studies Se and S distribution and seasonal fluctuations were often shown to be correlated for non-hyperaccumulators, but not correlated in hyperaccumulators (Galeas et al., 2007; El Mehdawi et al., 2011).

In the plants from the field there was a high-Se ring at the root–soil interface of both hyperaccumulator species, which may either correspond with the epidermis or rhizosphere. Earlier, leaf Se levels were found to be especially high in the epidermis (Freeman et al., 2006a), and it is possible that the root, too, sequesters Se preferentially in the epidermis. Concentration in the periphery of organs, away from vital metabolic processes may contribute to Se tolerance, and may also offer the plant better protection from pathogens and herbivores. It is also possible that Se levels are elevated in the rhizosphere. Recently it has been reported that Se levels in rhizosphere soil of hyperaccumulators *A. bisulcatus* and *S. pinnata* were significantly higher (7–13 fold) compared to bulk soil (El Mehdawi et al., 2011). The form of Se in the Se-enriched area at the root-soil interface was mainly C—Se—C in all cases. Interestingly, in *S. pinnata* the fraction of C—Se—C was relatively higher on the root–soil interface than inside the root, while in *A. bisulcatus* the fraction of C—Se—C was relatively lower there (Fig. 1, Table 2). The *A. bisulcatus* root periphery contained a large fraction of Se^0, indicative of microbial activity. Perhaps the high-Se peripheral ring of *A. bisulcatus* reflects the rhizosphere and is highly colonized by microbes that can reduce Se into insoluble Se^0. In the *S. pinnata* field root, on the other hand, the stele had a higher Se^0 fraction than the cortex, possibly indicating colonization by endophytic Se reducing microbes in the xylem apoplast. In either case, the degree of microbial colonization may affect plant Se accumulation; this will be interesting to further investigate in future studies.

5. Conclusions

This study shows for the first time that Se is concentrated mainly in the cortex of hyperaccumulator roots, and that the form of Se is C—Se—C, like in leaves, but with selenite rather than selenate as a minor additional compound. In addition, these studies indicate that the Se speciation in the hyperaccumulators may be affected by endosymbionts that produce Se^0. This form of Se was fairly abundant in the field but not found in controlled greenhouse studies, except when the plants were inoculated with a fungus (A3) known to produce Se^0, and in nodules colonized by nitrogen-fixing bacteria. The finding that endophytes may influence plant Se metabolism (and with that, perhaps Se tolerance, uptake and translocation, and total accumulation) opens an entirely new area of study. If indeed endophytes can be isolated that affect these plant properties, they may be applicable in phytoremediation and biofortification, especially if they are promiscuous (as endophytes often are) and can also affect Se metabolism in popular crop species.

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