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ORIGINAL ARTICLE

Inoculation of *Astragalus racemosus* and *Astragalus convallarius* with selenium-hyperaccumulator rhizosphere fungi affects growth and selenium accumulation

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Abstract Little is known about how fungi affect plant selenium (Se) accumulation. Here we investigate the effects of two fungi on Se accumulation, translocation, and chemical speciation in the hyperaccumulator *Astragalus racemosus* and the non-accumulator *Astragalus convallarius*. The fungi, *Alternaria astragali* (A3) and *Fusarium acuminatum* (F30), were previously isolated from *Astragalus* hyperaccumulator rhizosphere. A3-inoculation enhanced growth of *A. racemosus* yet inhibited growth of *A. convallarius*. Selenium treatment negated these effects. F30 reduced shoot-to-root Se translocation in *A. racemosus*. X-ray microprobe analysis showed no differences in Se speciation between inoculation groups. The *Astragalus* species differed in Se localization and speciation. *A. racemosus* root-Se was distributed throughout the taproot and lateral root and was 90 % organic in the lateral root. The related element sulfur (S) was present as a mixture of organic and inorganic forms in the hyperaccumulator. *Astragalus convallarius* root-Se was concentrated in the extreme periphery of the taproot. In the lateral root, Se was exclusively in the vascular core and was only 49 % organic. These findings indicate differences in Se assimilation between the two species and differences between Se and S speciation in the hyperaccumulator. The finding that fungi can affect translocation may have applications in phytoremediation and biofortification.

Keywords Plant–microbe interactions · Hyperaccumulation · *Alternaria astragali* · *Fusarium acuminatum* · μ-X-ray absorption near edge spectroscopy · μ-X-ray fluorescence mapping

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Introduction

The element selenium (Se) is chemically similar to sulfur (S). Selenium in the form of selenocysteine (SeCys) occurs in selenoproteins, which perform essential functions in many animals, bacteria, and algae (Stadtman 1990; Zhang and Gladyshev 2009). Many selenoproteins have antioxidant activity, and dietary Se supplementation can enhance antioxidant capacity which may in part explain reduced susceptibility to cancer. Studies also suggest that the chemical speciation of Se is important in determining the level of protection (Ip et al. 2000). Higher plants are thought to have lost the machinery to make selenoproteins and are therefore not known to require Se (Lobanov et al. 2009; Zhang and Gladyshev 2009). Still, many plant species have been shown to produce organic seleno-compounds and derive a physiological benefit from Se supplementation leading to increased antioxidant capacity (Hartikainen 2005; Pilon-Smits et al. 2009). Lettuce, soybean, and ryegrass biofortified with Se had greater glutathione peroxidase and superoxide dismutase activity and reduced lipid peroxidation (Hartikainen et al. 2000; Xue et al. 2001; Djanaguiraman et al. 2005).

At elevated levels, Se is toxic to most organisms. Selenium toxicity is thought to occur when SeCys is

incorporated into protein in the place of cysteine, leading to misfolding and loss of function (Stadtman 1996). In addition, inorganic forms of Se may cause oxidative stress (Grant et al. 2011). Another aspect of Se toxicity may be S starvation (Van Hoewyk et al. 2008). Because of the similarity between S and Se, they can use the same transporters and metabolic pathways. In plants, selenate is taken up via sulfate transporters and assimilated via the sulfate assimilation pathway into aminoacids and other organic compounds (Terry et al. 2000).

Some plant species stand out as having the ability to accumulate levels of Se that are 100-fold higher than surrounding vegetation, levels toxic to most organisms. These so-called hyperaccumulator plants can tolerate and actively accumulate Se up to 1.5 % of their dry weight (Beath et al. 1939; Galeas et al. 2007). Hyperaccumulation of Se has been reported for around 30 plant species, most of which are in the *Astragalus* genus (Beath 1982). One thing that sets hyperaccumulators apart is their ability to methylate SeCys via SeCys-methyltransferase (SMT) (Neuhierl and Bock 1996). Methyl-SeCys is the predominant form of Se in leaves of many hyperaccumulators (Pickering et al. 2003; Freeman et al. 2006). Roots of two hyperaccumulator species were shown recently by μ-X-ray absorption near edge spectroscopy (μ-XANES) to contain most of their Se in the form of C–Se–C compounds (Lindblom et al. 2012); most likely this was methyl-SeCys, but could also have included selenomethionine (SeMet) or selenocystathionine (SeCyst) since the spectra from these three compounds are virtually indistinguishable. The capacity of hyperaccumulators to store Se as methyl-SeCys may explain their extreme Se tolerance since this is a non-protein aminoacid and therefore does not disrupt protein function.

Several hypotheses have been proposed to explain why plants hyperaccumulate toxic elements (Boyd and Martens 1992). Most evidence supports the elemental defense hypothesis which states that hyperaccumulation protects plants from pathogens and herbivores (for a review see Boyd 2010). Indeed, Se accumulation has been shown to protect plants from a variety of herbivores (El-Mehdawi and Pilon-Smits 2012). Plant Se accumulation has also been shown to offer protection from Se-sensitive microbial plant pathogens. *Brassica juncea* (Indian mustard) plants treated with Se were less susceptible to a fungal leaf pathogen (*Alternaria brassicicola*) and a fungal root/stem pathogen (*Fusarium* sp.), compared with control plants not supplemented with Se (Hanson et al. 2003). Hyperaccumulators may also use Se as a form of elemental allelopathy, inhibiting Se-sensitive neighboring plants via Se phytoenrichment of surrounding soil (El Mehdawi et al. 2011). In addition to these ecological benefits, hyperaccumulators appear to derive a significant physiological benefit from Se: they produced two- to threefold more biomass when grown on seleniferous soil than non-seleniferous soil in a greenhouse study (El Mehdawi et al. 2012).

While hyperaccumulators negatively affect Se-sensitive ecological partners, they offer a niche for Se-resistant ecological partners, including rhizosphere and endophytic microbes, litter decomposers, herbivores, and neighboring plants (El Mehdawi and Pilon-Smits 2011). Many microbes can live in association with hyperaccumulators. For instance, there is evidence of Se-tolerant litter-decomposing microbes in seleniferous areas. In a litter decomposition study on seleniferous soil, the high-Se litter from hyperaccumulator *Astragalus bisulcatus* lost weight faster and harbored significantly more microbes than low-Se litter of *Medicago sativa* (Quinn et al. 2011). In addition, several fungi have been successfully cultured from the roots of hyperaccumulators, *A. bisulcatus* and *Stanleya pinnata* (Wangeline and Reeves 2007). When these rhizoplane fungi were grown in the presence of Se, they were significantly more tolerant to Se than fungi from non-seleniferous areas (Wangeline et al. 2011).

Relatively little is known about whether and how hyperaccumulator-associated microbes influence plant Se uptake, metabolism and tolerance. In one study, inoculation with rhizosphere bacteria collected from a high-Se area enhanced Se accumulation in the non-hyperaccumulator *Brassica juncea* (de Souza et al. 1998a). Other results suggestive of a potential influence of microbes on plant Se metabolism were obtained in a recent XANES study, where field-collected roots of Se hyperaccumulators were shown to contain a relatively high percentage of elemental Se (Se^0), compared with greenhouse-grown hyperaccumulator roots, where Se^0 was found only in areas associated with microbial activity, particularly in nodules (Lindblom et al. 2012). Since Se^0 has not been reported in plants before, but has been found to be produced by many microbes, the Se^0 found inside field roots may be due to the presence of microbial endophytes. If Se hyperaccumulator-associated microbes indeed affect Se speciation and accumulation, they may also have a profound effect on plant growth and Se tolerance.

In the greenhouse study reported here, two *Astragalus* species, one hyperaccumulator and one non-hyperaccumulator, were inoculated with Se-hyperaccumulator rhizoplane fungi isolated from *Astragalus* hyperaccumulators in the field, and the effect on plant growth and the accumulation of Se and other elements was investigated. Moreover, μ-X-ray fluorescent mapping (XRF) and μ-XANES were used to investigate localization and speciation of Se and other elements in both species.

Materials and methods

Biological material

Astragalus racemosus and *Astragalus convallarius* seeds were obtained from Western Native Seed, Coaldale, CO, USA. The two fungal isolates were obtained and described previously by Wangeline and Reeves (2007), Wangeline et al. (2011) from hyperaccumulators growing on naturally seleniferous soil. They are (1) *Fusarium acuminatum* (F30), collected from *A. racemosus* in Lysite, WY and (2) *Alternaria astragali* (A3) collected from *A. bisulcatus* in Laramie, WY.

Fungal growth

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, USA) supplemented with 30 μm sodium selenate.

Plant growth

The *A. racemosus* and *A. convallarius* seeds were first scarified with sandpaper and then surface-sterilized by rinsing for 20 min in 20 % bleach, followed by five 10-min rinses in sterile water. 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.

Co-cultivation

The *A. racemosus* and *A. convallarius* 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, mycelia from F30 and mycelia and spores of A3 were macerated in sterile water in 1.5-mL tubes using sterile glass beads and a micropestle. Fragments of hyphae from F30, as well as spores and spore chains of A3 were quantified using a hemocytometer to estimate mm hyphae, spores, and spore chains mL^{-1} water. F30 and A3 were diluted to a final concentration of 2,000 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. This Se concentration was chosen so as to not induce toxicity in the non-hyperaccumulator. After inoculation, plants and fungi were co-cultivated for 10–13 weeks before harvest.

Elemental analysis

At harvest, the plant roots were washed of sand that was bound in the root mass and then dried for 48 h at 45 °C. Samples were digested in nitric acid as described by Zarcinas et al. (1987). Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used to determine Cu, Fe, Mg, Se, and S concentrations in the acid digest (Fassel 1978).

Elemental distribution and speciation

X-ray microprobe analysis was performed on intact frozen root material from *A. racemosus* and *A. convallarius* supplied with 12.5 μM Na_2SeO_4. For each species and each inoculation treatment, one taproot and one lateral root were analyzed (i.e. in total three taproots and three lateral roots for each species). Elemental tissue distribution and chemical speciation were determined using μXRF mapping and μXANES spectroscopy, respectively, both as described by Lindblom et al. (2012).

Statistical analysis

The software JMP-IN (3.2.6, SAS Institute, Cary, NC, USA) was used for statistical data analysis. Analysis of variance followed by a post hoc Tukey–Kramer test was used to compare multiple means. It was verified that the assumptions underlying these tests (normal distribution, equal variance) were met.

Results

Plant growth of Se hyperaccumulator *A. racemosus* and non-accumulator *A. convallarius* was affected by co-cultivation with hyperaccumulator-derived rhizosphere fungi. When grown in the absence of Se, *A. racemosus* shoots grew significantly larger in the presence of the A3 fungus; this positive effect was not observed in the presence of Se (Fig. 1a). Shoots and roots of F30-treated *A. racemosus* were similar in biomass to un-inoculated plants and were only different in that the Se-treated, F30-inoculated plant roots were significantly smaller than A3-treated roots

grown without added Se (Fig. 1a, b). *A. convallarius* plants were more affected by the fungal co-cultivation than by the addition of Se. Inoculation with A3 had a negative effect on shoot biomass, but when Se was added, *A. convallarius* plant shoots grew to a similar size as those of uninoculated plants (Fig. 1c). The same trend was observed for the roots of *A. convallarius*: A3 appeared to negatively affect root growth when Se was absent from the system, as judged from the observation that the A3-inoculated roots were smaller compared with the un-inoculated roots, although not quite significant at the 0.05 level ($p = 0.075$) (Fig. 1d).

Selenium and sulfur concentrations in *A. racemosus* shoots and roots, and the ratio of shoot concentration divided by root concentration differed with fungal co-cultivation treatment. Shoot Se levels in *A. racemosus* inoculated with either of the two fungi were somewhat lower than those left uninoculated, but this was not statistically significant (Fig. 2a). The average root Se was somewhat higher in the inoculated plants (Fig. 1b), but this was also not significant. Due to the combined effects of lower shoot Se and higher root Se levels, however, the ratio of shoot to root Se was significantly reduced in the F30-inoculated *A. racemosus* plants (Fig. 2c).

Sulfur levels in the shoots of *A. racemosus* were lower in the Se-treated plants compared with plants grown without Se for the uninoculated group and the F30-inoculated group, but were unaffected by Se in the A3-treated group (Fig. 2d). In the presence of Se, plants co-cultivated with F30 contained less S in the shoots compared with control plants. Sulfur in the shoots of A3 plants treated with Se was similar to that in the control plants (Fig. 2d). Sulfur concentration in the roots of plants inoculated with A3 or F30 was higher than that in the control plants within the Se-treated plant groups. In the absence of Se, A3 did not appear to affect S levels in the roots, but F30-treated plants showed significantly reduced root S levels (Fig. 2e). The fraction of S that was moved to the shoot, as determined by the concentration ratio of shoot S and root S, was significantly lower in the fungus-inoculated plants than in the uninoculated plants in the presence of Se; in the absence of Se there was no effect of fungal co-cultivation on the S root-to-shoot ratio (Fig. 2f).

There were no inoculation-related differences in Se concentration in the shoots (Fig. 3a) or roots (Fig. 3b) of *A. convallarius*, nor were there differences in the ratio of shoot:root Se concentration (Fig. 3c). There were, however, differences in shoot S levels in the absence of Se. Shoots of *A. convallarius* co-cultivated with the A3 fungus contained higher S levels than those within the F30 group (Fig. 3d). The shoots of *A. convallarius* that were given Se showed no differences in shoot S levels between the inoculated and uninoculated plants (Fig. 3d). The roots of *A. convallarius* co-cultivated with the A3 fungus and supplied with Se contained a higher S level in the roots compared with uninoculated plants (Fig. 3e). There were no other differences observed for S levels in *A. convallarius* roots due to either the presence of Se or fungal treatment (Fig. 3e). The shoot:root S concentration ratio was

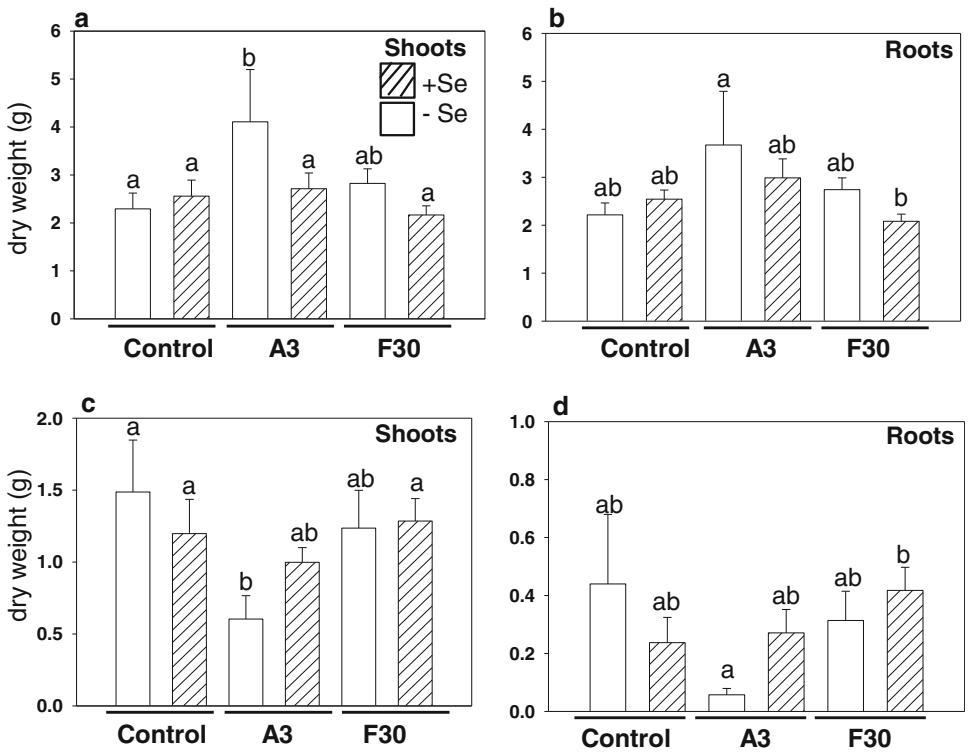

Fig. 1 Biomass of *A. racemosus* shoots (**a**) and roots (**b**) and *A. convallarius* shoots (**c**), and roots (**d**) that were either uninoculated, or inoculated with rhizosphere fungi A3 or F30. Shown values are the mean ± standard error of the mean (SEM). *Lower case letters above bars* indicate statistically significant differences (ANOVA with post hoc Tukey–Kramer analysis, $p < 0.05$)

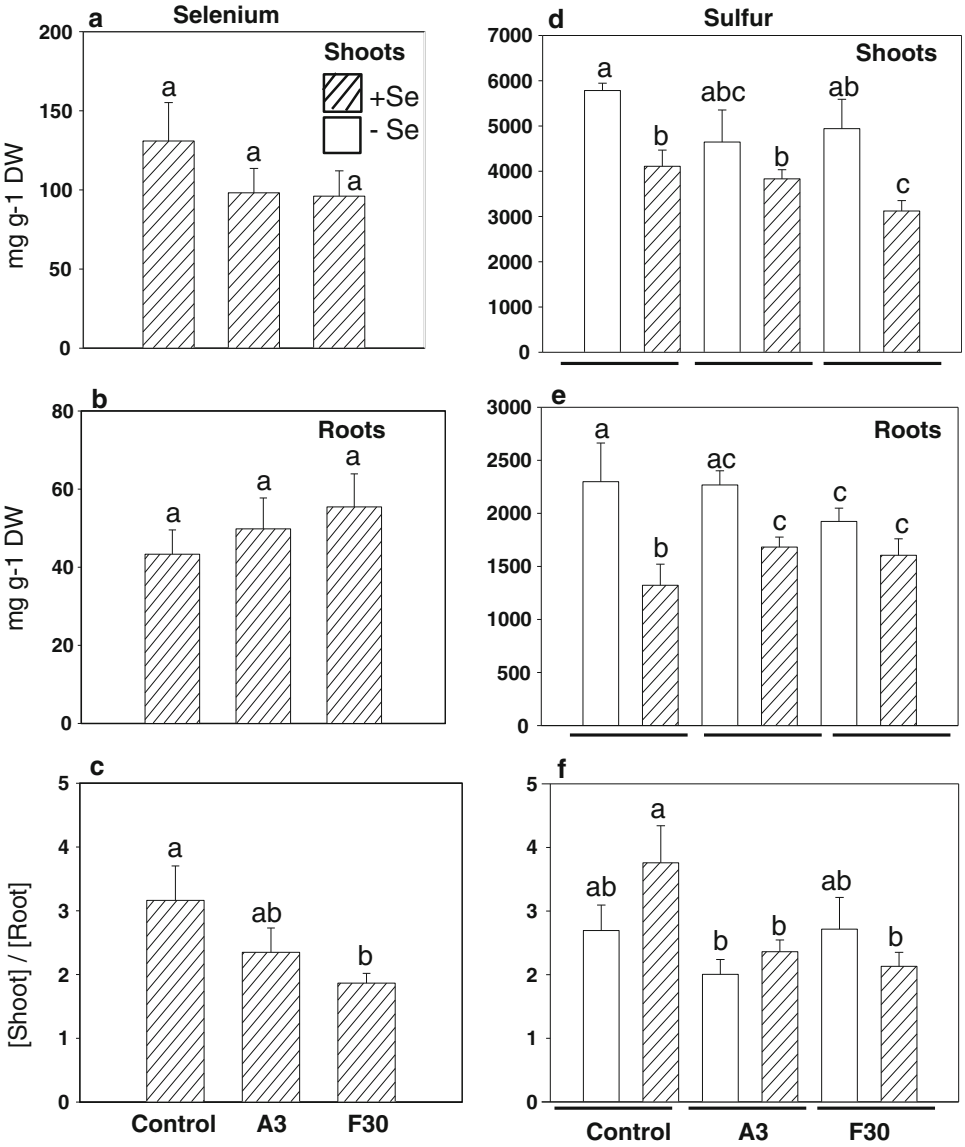

Fig. 2 Selenium and sulfur concentration in the shoots (**a**, **d**) and roots (**b**, **e**) of *A. racemosus* plants, either uninoculated or inoculated with rhizosphere fungi A3 or F30. **c**, **f** The shoot/root ratio of the Se and S concentration, respectively. Shown values are the mean ± SEM. *Lower case letters above bars* indicate statistically significant differences (ANOVA with post hoc Tukey–Kramer analysis, $p < 0.05$)

lower in the A3-inoculated plants compared with uninoculated *A. convallarius* when grown in the presence of Se (Fig. 3f). As for the F30-inoculated plants, there were no differences in shoot: root S compared with the uninoculated plants (Fig. 3f).

Both the fungal and Se treatments also affected the levels of other nutrients in *A. racemosus*. The shoot and root Cu levels were higher in the Se–treated plants than in plants grown without Se, for the uninoculated group (Fig. 4a, b). Furthermore, inoculation with A3 or with F30 was associated with a decreased shoot Cu concentration in the presence of Se (Fig. 4a). There was also a lower root Cu level in A3-inoculated plants compared with uninoculated plants in the presence of Se (Fig. 4b). Shoot Fe levels were lower in Se-treated plants compared with plants grown without Se and lower in A3- or F30-inoculated plants than in uninoculated plants (Fig. 4c). Root Fe levels were also lower in Se-treated plants compared with plants grown without Se for the uninoculated and F30-inoculated groups; no such difference was observed for the A3-inoculated group (Fig. 4d). Finally, the levels of Mg in both the shoots (Fig. 4e) and roots (Fig. 4f) were significantly lower in the Se-treated plants than in plants grown without Se; Mg levels were not affected by fungal inoculation.

There were no significant differences in *A. convallarius* shoot (Fig. 5a) or root (Fig. 5b) Cu levels between any of the treatment groups. Shoot Fe levels were twofold higher in the A3-inoculated plants compared with the uninoculated plants, but only when grown in the absence of Se (Fig. 5c). Root Fe levels were lower in plants treated with Se than in plants grown without Se, independent of inoculation treatment (Fig. 5d). Similarly, shoot Mg concentration was lower in the presence of Se than in its absence, independent of inoculation treatment (Fig. 5e) There was

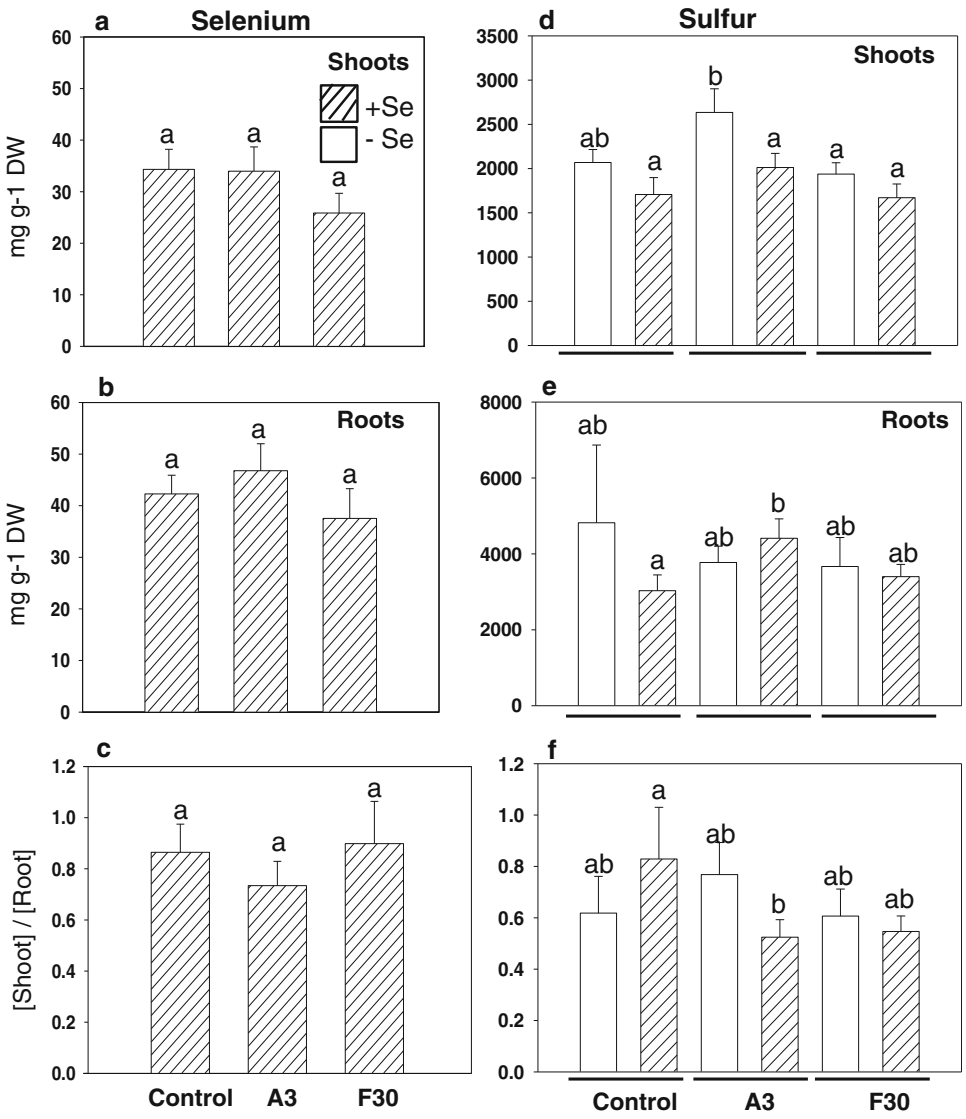

Fig. 3 Selenium and sulfur concentration in the shoots (**a**, **d**) and roots (**b**, **e**) of *A. convallarius* plants, either uninoculated or inoculated with rhizosphere fungi A3 or F30. **c**, **f** The shoot/root ratio of the Se and S concentration, respectively. Shown values are the mean ± SEM. *Lower case letters above bars* indicate statistically significant differences (ANOVA with post hoc Tukey–Kramer analysis, $p < 0.05$)

no effect of Se treatment or fungal inoculation on Mg levels in the roots of *A. convallarius* (Fig. 5f).

Comparing the nutrient composition between the two *Astragalus* species (Figs. 2, 3, 4, 5) there is a striking difference in S distribution. There appeared to be less S translocated to the shoots of *A. convallarius* than in *A. racemosus*, judged from the twofold higher root S levels and twofold lower shoot S levels in *A. convallarius* (Figs. 2, 3). The levels of Cu, Fe and Mg were similar in the two species (Figs. 4, 5).

The Se distribution in *A. racemosus* roots was not affected by the fungal treatments. Representative roots are shown in Fig. 6. In the cross-sectioned tap root (a), Se is shown to be distributed throughout the root, with a somewhat higher concentration in the peripheral tissues (cortex and periderm) relative to the central stele. Calcium showed a similar distribution as Se. Sulfur, on the other hand, appeared to be present at equal levels in stele, cortex and periderm. Iron was concentrated greatly in the cortex and periderm, with negligible levels in the central stele. In the lateral root (b), both Se and S were distributed throughout the root. Calcium was also found throughout the lateral root as well, while Fe was present in specks along the outside of the root.

In *A. convallarius*, the different fungal treatments also did not appear to affect the root Se distribution pattern (Fig. 7). As can be seen in the representative cross-section of a tap root (a), Se was localized to a great extent in the extreme periphery, in what appears to be the periderm. Also, the Se appeared to be present at somewhat higher level in the cortex than the stele. Calcium and S followed a similar distribution pattern. Iron was also concentrated in the extreme periphery, and within the root appears to be present in two concentric rings, one in the cortex and one in the stele. In the lateral root (b), Se was less concentrated in the periphery of the root, but rather was concentrated in

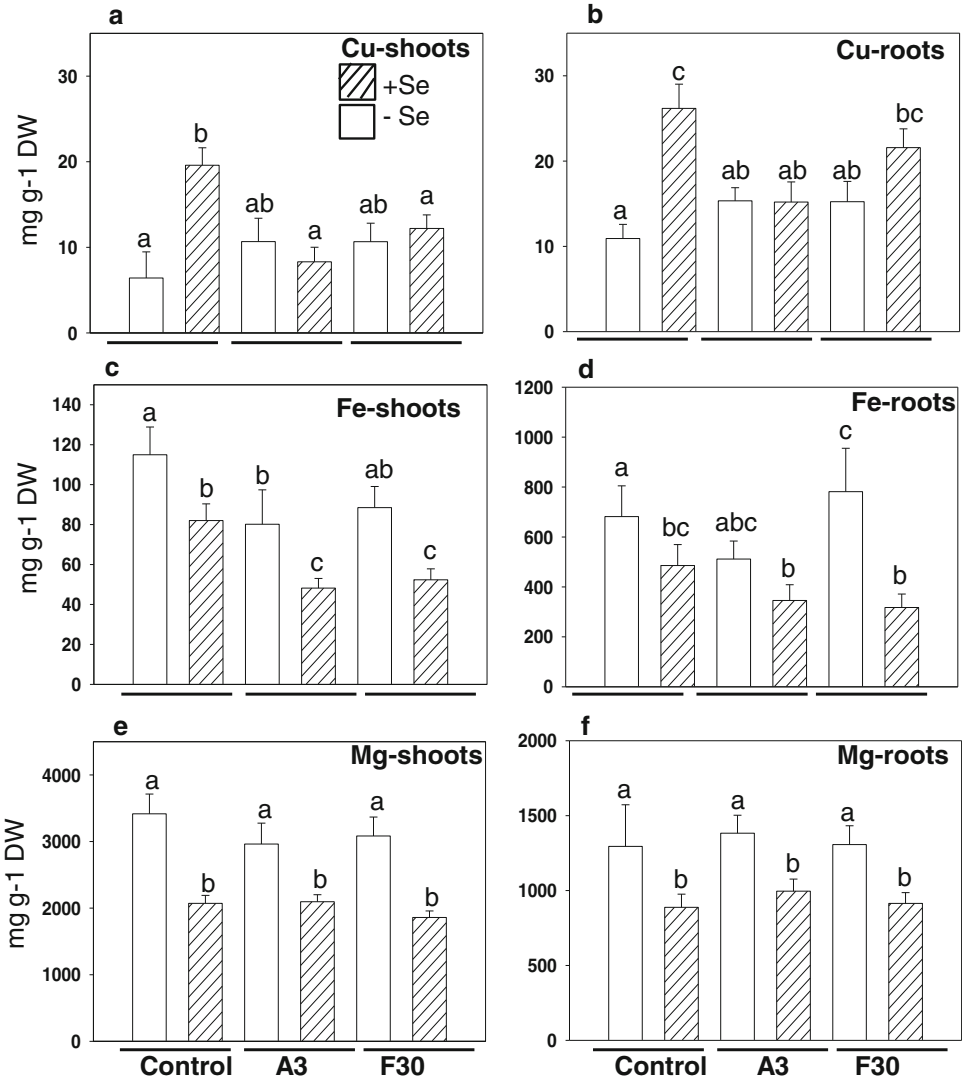

Fig. 4 Copper (**a**, **b**), iron (**c**, **d**), and magnesium (**e**, **f**) concentration in the shoots (**a**, **c**, **e**) and roots (**b**, **d**, **f**) of *A. racemosus* either uninoculated or inoculated with rhizosphere fungi A3 or F30. Shown values are the mean ± SEM. *Lower case letters above bars* indicate statistically significant differences (ANOVA with post hoc Tukey–Kramer analysis, $p < 0.05$)

what appears to be vascular tissue. Calcium and Fe, on the other hand, appear to be present to a large extent in the periphery of the lateral root. Sulfur does not show a particular localization pattern.

Like Se distribution, the Se speciation in roots of *A. racemosus* and *A. convallarius* did not appear to be affected by the fungal treatments. Typical K-edge Se XANES spectra are shown in Fig. 8a. The spectrum from the *A. racemosus* root showed a high degree of similarity to the SeMet standard. The spectrum of the *A. convallarius* root, on the other hand, showed similarity to a combination of the spectra from selenate, selenite and SeMet. Least square linear combination (LSQ) analysis showed that lateral roots of *A. racemosus* contained predominantly organic Se (91 % of total Se), mainly in the C–Se–C configuration (Fig. 8b). *A. convallarius* contained 49 % organic Se (mainly C–Se–C); its large inorganic Se fraction consisted roughly equally of selenite and selenate (Fig. 8c). Sulfur speciation was also analyzed using S XANES on the *A. racemosus* tap root shown in Fig. 6. In the stele and the cortex, S was mostly present as a compound with an absorbance peak around 2,482 eV (Fig. 9). The XANES spectra collected at the periphery of the root (periderm) showed two peaks, one at 2,482 eV and one at 2,474 eV (Fig. 9).

Discussion

The inoculation of Se hyperaccumulator, *A. racemosus* and non-accumulator, *A. convallarius* with rhizoplane fungi isolated from hyperaccumulators significantly affected plant growth and accumulation of Se and S. The effects of fungal inoculation on plant growth were generally positive for *A. racemosus* and negative for *A. convallarius* and were Se-dependent. The fungi reduced shoot/root Se ratio in *A. racemosus* and shoot/root S ratio in both species. The observed effects of fungal inoculation on Se accumulation

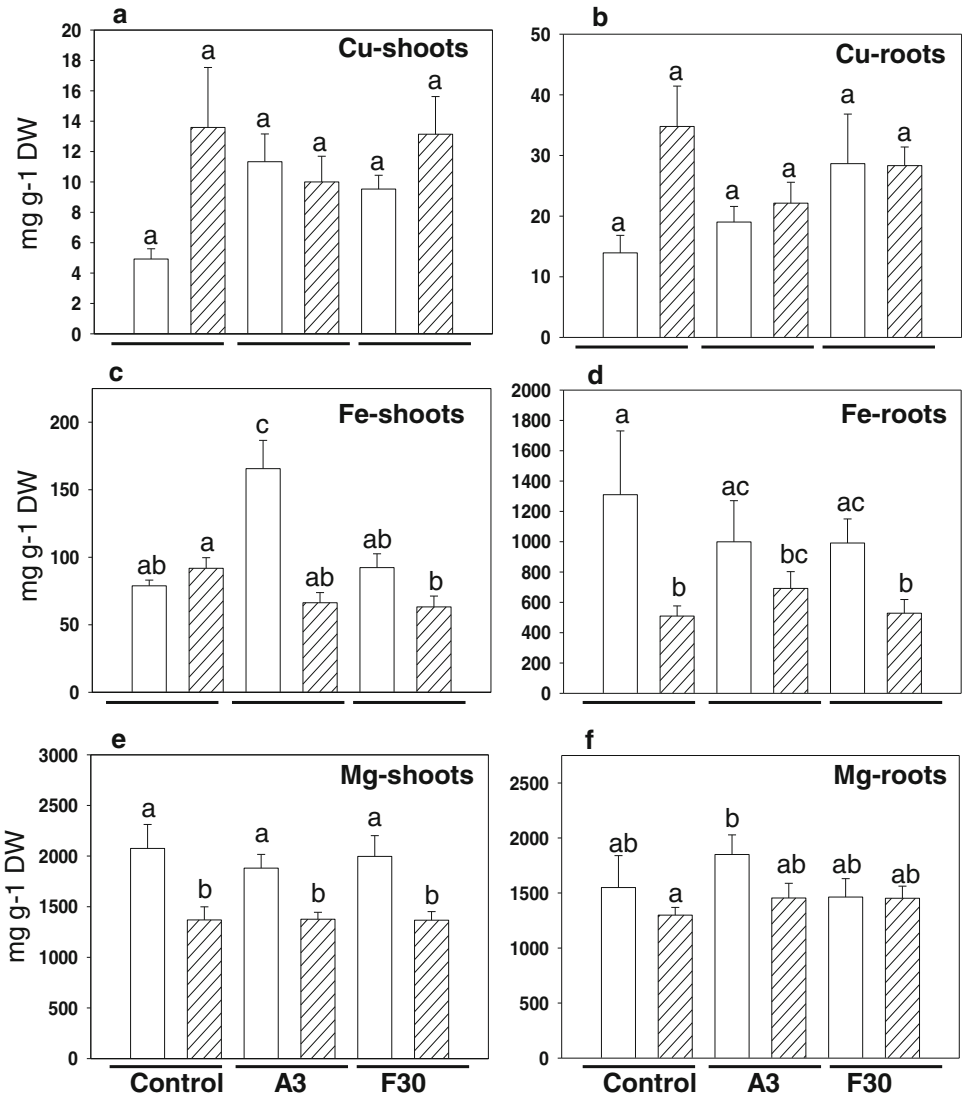

Fig. 5 Copper (**a**, **b**), iron (**c**, **d**) and magnesium (**e**, **f**) concentration in the shoots (**a**, **c**, **e**) and roots (**b**, **d**, **f**) of *A. convallarius* either uninoculated or inoculated with rhizosphere fungi A3 or F30. Shown values are the mean ± SEM. *Lower case letters above bars* indicate statistically significant differences (ANOVA with post hoc Tukey–Kramer analysis, $p < 0.05$)

could not be explained by differences in Se speciation and localization, as judged from μ-XANES and μXRF analysis. There were, however, differences in Se localization and speciation between the two *Astragalus* species; furthermore, Se and S showed different localization patterns in both species.

The effect of A3, *Alternaria astragali*, on plant growth was Se-dependent as well as species-dependent. When no Se was added, A3 had a positive effect on *A. racemosus* but a negative effect on *A. convallarius*. Thus, A3, originally isolated from hyperaccumulator *A. bisulcatus*, promoted growth of a related hyperaccumulator, but appeared to be pathogenic to the related nonaccumulator. In the presence of Se, these growth effects were not observed. The positive and negative growth effects did not correlate with root or shoot Cu, Fe, Mg or S levels; the underlying mechanisms are not clear but may involve fungal production of growth-affecting hormones.

The observation that there was a negative growth effect of A3 on *A. convallarius* in the absence but not in the presence of Se, suggests that Se accumulation may protect *A. convallarius* from the pathogenicity of this fungus. Selenium accumulation was shown earlier to be able to protect *B. juncea* from two Se-sensitive pathogenic fungi, one *Alternaria* and one *Fusarium* species (Hanson et al. 2003). A3 was shown earlier (Lindblom et al. 2012) to be inhibited by Se at the lowest concentration tested, 30 mg/L. For comparison, the tissue Se concentration in *A. convallarius* root and shoot was 30–40 mg Se/kg DW, or ~3–4 mg Se/kg FW. In *A. racemosus*, the tissue Se levels were 50–100 mg Se/kg DW. The positive effect of A3 on the growth of this species was also only observed in the absence of Se, again indicating that this fungus may be inhibited by Se. Of course, the comparison is not so straightforward because the form of Se was not the same in the two plant species and the medium. In *A. racemosus* Se

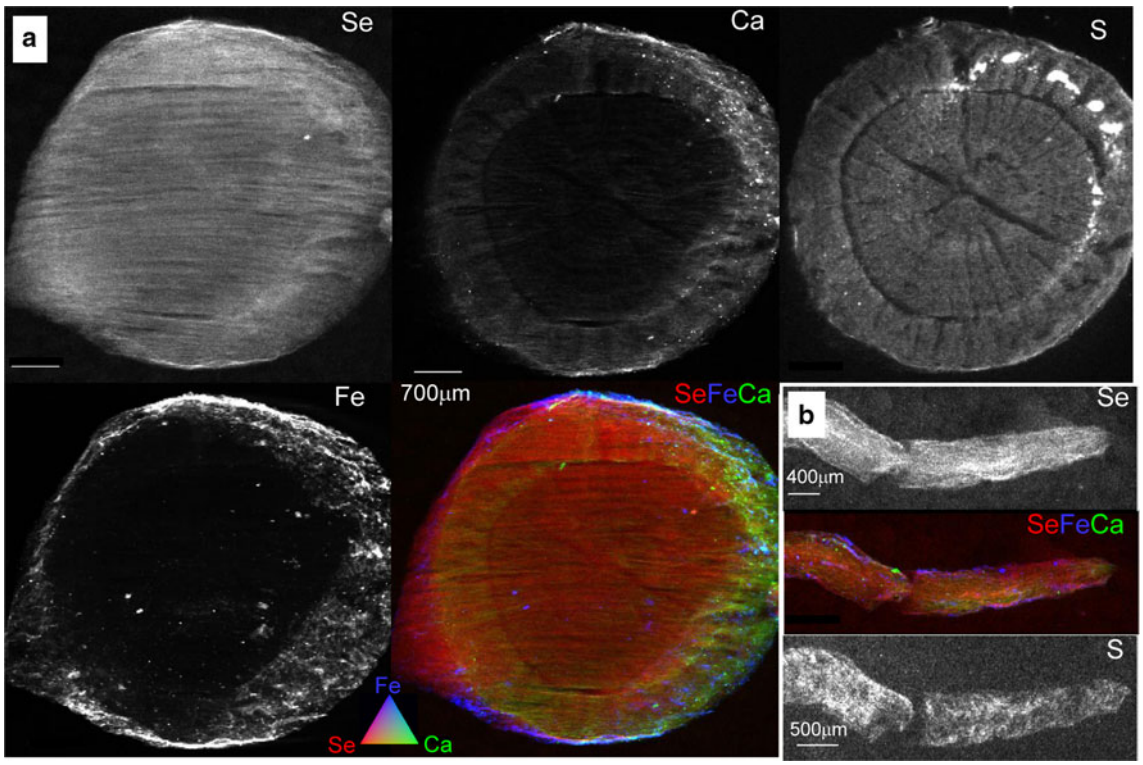

Fig. 6 XRF maps showing distribution of Se, Ca, S, and Fe in root cross-sections (**a**) and lateral roots (**b**) of uninoculated A. *racemosus* plants

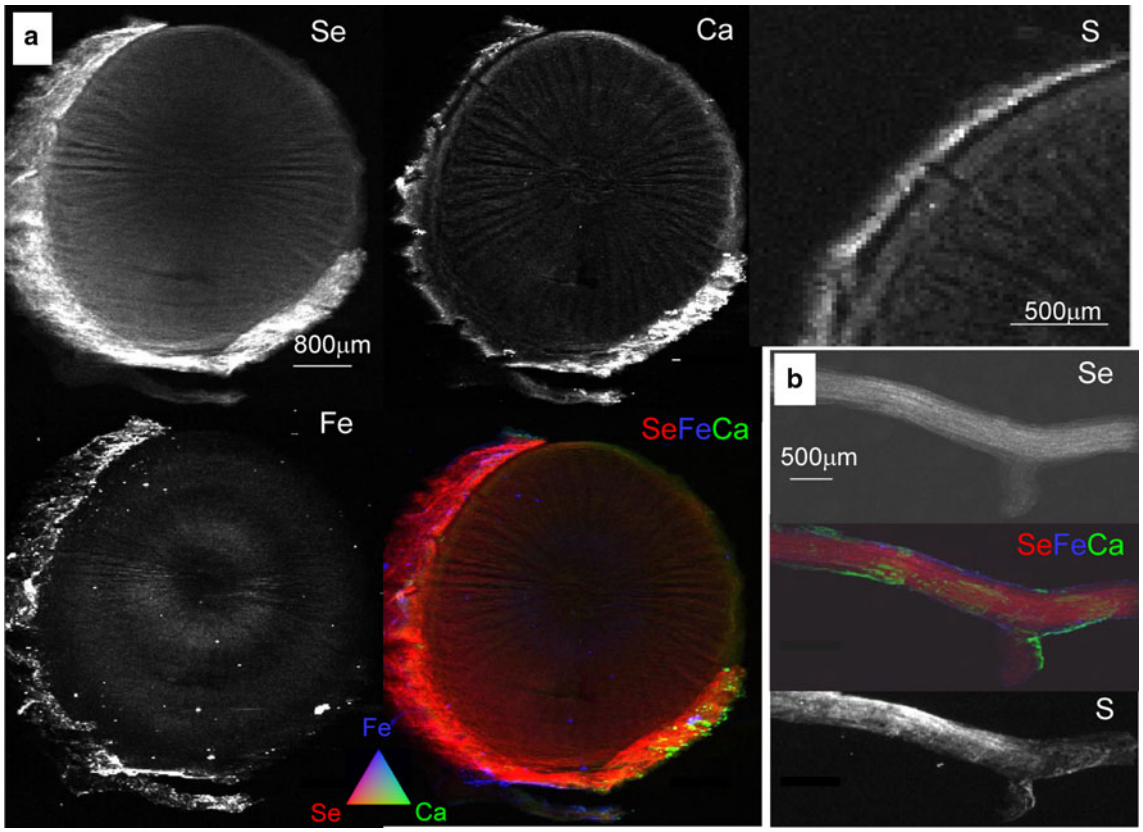

Fig. 7 Micro-XRF maps showing distribution of Se, Ca, S, and Fe in root cross-sections (**a**) and lateral roots (**b**) of uninoculated A. *convallarius* plants

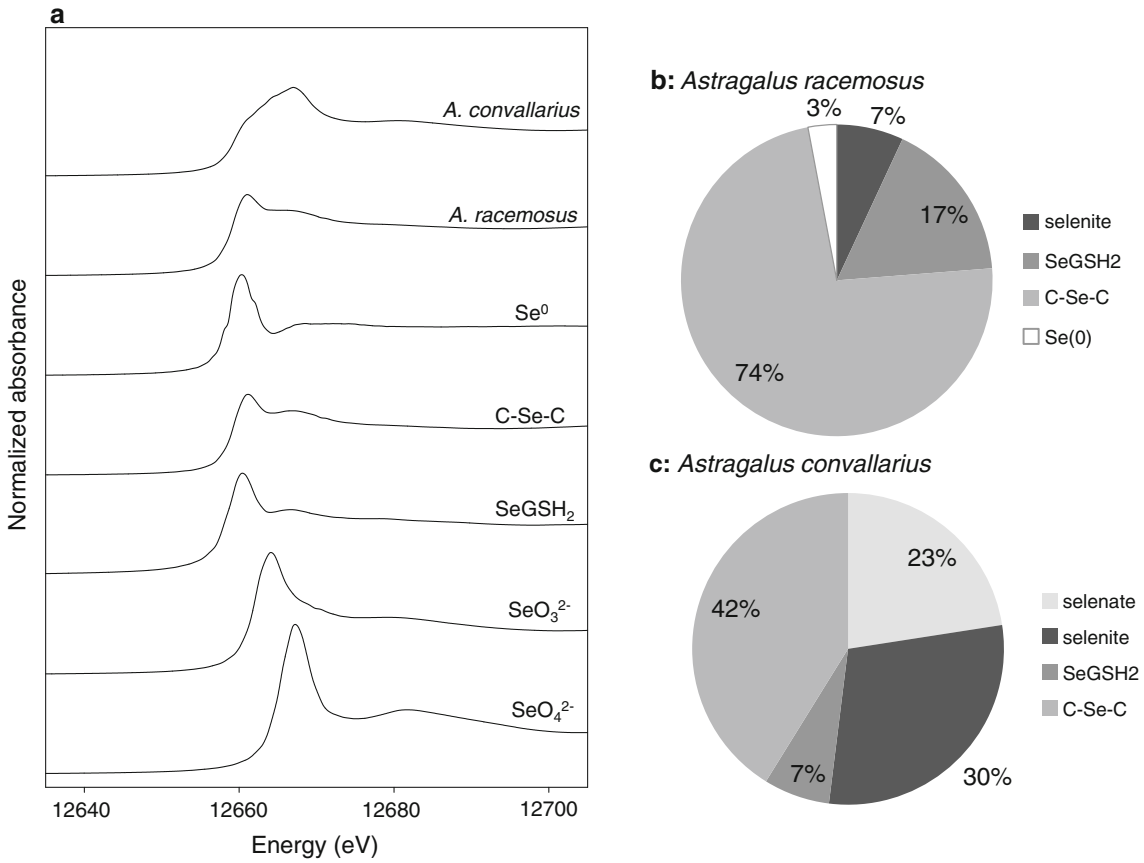

Fig. 8 XANES K-edge Se spectra from representative lateral roots of *A. racemosus* and *A. convallarius* as well as from the isolated F30 and A3 fungi grown on selenite, in comparison with spectra from five standard seleno-compounds: *gray* elemental Se, selenate, selenite, seleno-glutathione, and seleno-methionine

was present mainly in C–Se–C forms, whereas in *A. convallarius* there was a large proportion of inorganic Se and in the cocultivation experiments selenate was provided.

Inoculation with either of the two fungi was associated with a reduced shoot/root Se ratio in *A. racemosus* as well as a lower shoot/root S ratio in both *Astragalus* species. The effect of the fungi on S translocation was only observed in the presence of Se. The lower shoot/root Se/S ratios were mainly the result of elevated root Se or S levels, although the shoot Se or S levels were also somewhat lower. Thus, the fungi appeared to promote Se and S uptake into the root and perhaps also root-to-shoot translocation. Enhanced root Se and S uptake under influence of the fungi may have been caused by increased root surface area or upregulation of sulfate/selenate transporters. Such effects were reported for rhizosphere bacteria (de Souza et al. 1998a). It is also feasible that the fungi affected root Se and S levels by altering Se and S speciation in the rhizosphere or root, either into a form more readily taken up by the root or a form less readily translocated to the shoot. XANES did not show a significant difference in speciation between the inoculated and uninoculated roots, but it cannot be excluded that the XANES locations examined were not colonized by fungal hyphae. Since the A3 fungus has been shown to produce elemental Se (Lindblom et al. 2012), it is feasible that some of the Se in or around the root was converted by the fungus to insoluble, elemental Se and therefore not available for translocation. The F30 fungus was not shown to be able to produce elemental Se, but did convert inorganic Se to organic Se forms, which may also affect Se mobility in the plant (Lindblom et al. 2012). Another reason the fungi affected root Se and S levels may have been that the fungal hyphae were particularly rich in Se and S and were so tightly bound to the roots that they were collected together with the root for elemental analysis. This is perhaps less likely, in view of the finding that Se accumulation was only affected by the fungi in *A. racemosus* and not in *A. convallarius*. Some other differences between the two plant species worth mentioning are that the hyperaccumulator generally showed a much higher Se and S translocation ratio compared with the non-accumulator and that S uptake and translocation were negatively affected by Se in the hyperaccumulator, but not the nonaccumulator. Hyperaccumulators of Se have been reported before to have a higher Se translocation factor (El Mehdawi et al. 2012);

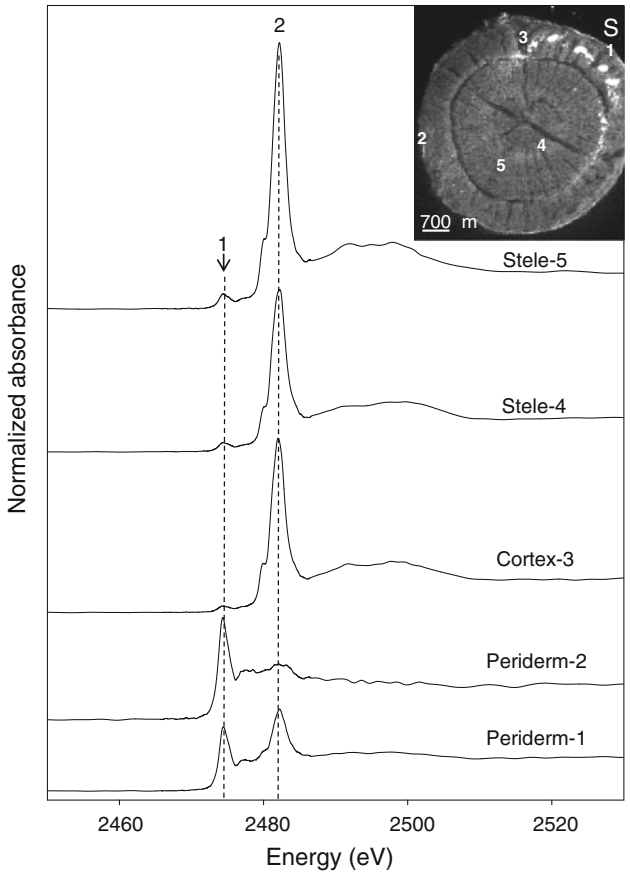

Fig. 9 XANES K-edge S spectra from the tap root of *A. racemosus*. Spectra were obtained inside the central vascular cylinder (stele); in the surrounding cortex, and in the extreme periphery (periderm)

this may be caused by different expression levels of selenate transporters or to differential speciation of Se in roots of hyperaccumulators and non-hyperaccumulators. The finding that Se impeded S uptake and translocation more in the hyperaccumulator may suggest that the hyperaccumulator sulfate/selenate transporters that mediate uptake and translocation have a higher affinity for selenate than sulfate.

The two plant species differed markedly with respect to root Se localization and speciation. Hyperaccumulator *A. racemosus* contained Se throughout its taproot and lateral root, with a slightly higher level in the cortex. Non-accumulator *A. convallarius*, on the other hand, showed a strong Se localization in the extreme periphery of the taproot (periderm and/or rhizosphere), and in the lateral root Se was strongly concentrated in the vascular core. Concentration of Se in the root cortex has been observed earlier in another *A. racemosus* plant as well as in *Astragalus bisulcatus* (Lindblom et al. 2012). The Se in *A. racemosus* lateral roots was 90 % organic, while *A. convallarius* contained only 49 % organic Se; the other half of the Se in *A. convallarius* was selenite and selenate. The organic Se in both species consisted mainly of C–Se–C compounds. This may have been SeMet, methyl-SeCys or selenocystathionine, as their XANES spectra are indistinguishable. The other non-accumulator species, *Astragalus drummondii* was shown recently to contain predominantly C–Se–C in its leaves (El Mehdawi et al. 2012). It is interesting that these non-accumulator *Astragalus* species apparently are capable of producing substantial levels of organic Se from selenate, since non-accumulators from other genera were found to predominantly accumulate selenate when supplied with selenate (de Souza et al. 1998b; Van Hoewyk et al. 2005). The speciation in this lateral root of *A. racemosus* was similar to that found earlier in the taproot of another plant of the same species growing under the same conditions, but the lateral root contained a somewhat smaller fraction of C–Se–C (90 %) compared with the taproot, which contained exclusively C–Se–C (Lindblom et al. 2012).

The root distribution of S was somewhat different from that of Se for hyperaccumulator *A. racemosus*: S was evenly dispersed throughout the taproot, while Se was most concentrated in the cortex. No such difference was observed in the taproot of *A. convallarius* where S, like Se, was strongly concentrated in the extreme periphery. The different tissue distribution patterns for Se and S in the hyperaccumulator can perhaps be explained by differences in Se and S speciation. Indeed, while the two elements were supplied in analogous forms, as selenate and sulfate, respectively, they were apparently metabolized differently in the hyperaccumulator. Most of the Se was accumulated in organic form throughout the root. Sulfur, however, appears to have been present to a large extent as inorganic sulfate in the stele and cortex: XANES spectra revealed one absorbance peak at 2,482 eV, which is what has been reported for sulfate (Vairavamurthy 1998; Jalilehvand 2006). The root periderm showed an additional peak at 2,474 eV, which may correspond to thiol compounds (Vairavamurthy 1998; Jalilehvand 2006). This suggests that the hyperaccumulator *A. racemosus* has the ability to discriminate between Se and S analogs and that it assimilates much of its Se in its root while S assimilation happens more in the shoot. Indeed, S assimilation is thought to happen mostly in mesophyll chloroplasts (Pilon-Smits and Pilon 2006).

Our finding that fungal inoculation may affect Se and S uptake and translocation may have ecological implications and potential applications. It appears that the capacity of a plant to (hyper) accumulate Se is determined not only by the attributes of the plant, but also by its microbial partners. If fungi can affect Se uptake and translocation, this could influence the above- and below-ground interactions with different ecological partners, including herbivores, pollinators, and other microbes. The finding that one fungus had

an opposite effect on growth in the hyperaccumulator and the related non-accumulator, and that this effect was Se-specific, may suggest that fungi can affect competition between plant species in the field and that this effect will be different on different soil types. In a phytoremediation setting, fungus-associated enhanced uptake and root accumulation may enhance phytostabilization efficiency. Moreover, reduced translocation of Se to the shoot may reduce movement of Se into the food chain and associated toxicity to wildlife and livestock. In future investigations it will be interesting to further explore the impact of microbial symbionts on plant Se (hyper)accumulation for better insight into the ecology of seleniferous areas and potential application in phytoremediation or biofortification.

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