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# Getting to the Root of Selenium Hyperaccumulation—Localization and Speciation of Root Selenium and Its Effects on Nematodes

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# Getting to the Root of Selenium Hyperaccumulation—Localization and Speciation of Root Selenium and Its Effects on Nematodes

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**Abstract:** Elemental hyperaccumulation protects plants from many aboveground herbivores. Little is known about effects of hyperaccumulation on belowground herbivores or their ecological interactions. To examine effects of plant selenium (Se) hyperaccumulation on nematode root herbivory, we investigated spatial distribution and speciation of Se in hyperaccumulator roots using X-ray microprobe analysis, and effects of root Se concentration on root-associated nematode communities. Perennial hyperaccumulators *Stanleya pinnata* and *Astragalus bisulcatus*, collected from a natural seleniferous grassland contained 100–1500 mg Se $kg^{-1}$ root dry weight (DW). Selenium was concentrated in the cortex and epidermis of hyperaccumulator roots, with lower levels in the stele. The accumulated Se consisted of organic (C-Se-C) compounds, indistinguishable from methyl-selenocysteine. The field-collected roots yielded 5–400 nematodes $g^{-1}$ DW in Baermann funnel extraction, with no correlation between root Se concentration and nematode densities. Even roots containing > 1000 mg Se $kg^{-1}$ DW yielded herbivorous nematodes. However, greenhouse-grown *S. pinnata* plants treated with Se had fewer total nematodes than those without Se. Thus, while root Se hyperaccumulation may protect plants from non-specialist herbivorous nematodes, Se-resistant nematode taxa appear to associate with hyperaccumulators in seleniferous habitats, and may utilize high-Se hyperaccumulator roots as food source. These findings give new insight into the ecological implications of plant Se (hyper)accumulation.

**Keywords:** *Astragalus bisulcatus*; elemental defense; herbivory; *Stanleya pinnata*

## 1. Introduction

Selenium (Se) is a trace element that naturally occurs in soils, at concentrations that vary with geology and climate. Across the Western United States, Se levels are typically high, sometimes in excess of 10 mg Se $kg^{-1}$ soil [1]. Soil Se concentration determines the associated plant Se concentration, which in turn is very important for the health of associated human and animal populations [2]. Selenium is an essential element for these populations, but can be toxic at elevated concentration, with a narrow window between deficiency and toxicity [2,3]. For plants, Se is a beneficial element, but toxic to most plant species at tissue concentrations above 100 mg Se $kg^{-1}$ dry weight (DW); for crop plants the toxicity threshold can be as low as 10 mg Se $kg^{-1}$ DW [2,3]. However, some plants, the so-called hyperaccumulator species, can accumulate Se to concentrations as high as 10,000 mg Se $kg^{-1}$ DW, or 1% of the dry weight of the plant without experiencing toxicity [4].

Selenium is chemically similar to sulfur (S), and all plants can take up selenate from soil via sulfate transporters, and assimilate it via the sulfate assimilation pathway [3,5]. Hyperaccumulators are different from other plants in that they take up Se preferentially over sulfur (S) and translocate relatively more Se to their shoot [3]. In non-hyperaccumulators Se is toxic because it becomes incorporated into selenomethionine (SeMet) and selenocysteine (SeCys), which may be non-specifically incorporated into proteins (replacing Met and Cys), causing improper protein folding and, hence, impaired function [6]. The predominant form of Se in hyperaccumulators is methyl-selenocysteine (MeSeCys). This seleno-aminoacid does not get non-specifically incorporated into proteins and therefore, does not cause toxicity [5]. Freeman and colleagues investigated the distribution and speciation of Se in leaves of hyperaccumulators and found Se focused in high concentrations in the leaf periphery: along the margins, in the epidermis, or leaf hairs. In non-hyperaccumulators, selenate was the predominant form of Se accumulated, and the Se was concentrated in the vascular tissues [7,8]. Thus, conversion of Se to MeSeCys and specific sequestration in the leaf periphery may both contribute to Se hyperaccumulation and hypertolerance.

Selenium hyperaccumulator species are perennials and the Se distribution in the plant fluctuates with the season. Galeas and colleagues [4] found that Se is translocated from the roots to the young leaves in the spring and early summer, from old leaves to young leaves and reproductive structures in the late summer, and to the roots and seeds in the fall. The seasonal fluctuation patterns in leaf Se concentration differed between hyperaccumulators and non-accumulator species growing in the same habitat. Hyperaccumulator Se levels peaked in spring while non-accumulators peaked in summer; leaf S levels peaked in summer for both species [4].

One proposed hypothesis for the functional significance of hyperaccumulation is basic elemental defense [9]. Selenium in plants is toxic to most animals at concentrations well below those found in all of the organs of hyperaccumulators. Plants with elevated shoot Se concentrations were toxic to, and deterred, a wide variety of aboveground herbivores including prairie dogs, grasshoppers, caterpillars, spider mites, thrips and aphids [10–15]. The specialized high-Se localizations found by Freeman [8] along the hyperaccumulator leaf periphery are most likely the first tissues encountered by these aboveground herbivores, and may function to protect the plant from this biotic stress. Herbivores such as a Colorado population of the diamondback moth, however, have evolved tolerance to the high levels of Se in hyperaccumulators and are no longer deterred by Se [16].

In contrast to the considerable study of the aboveground ecological interactions of herbivores and hyperaccumulator plants, the numerous belowground interactions of hyperaccumulators and herbivores remain relatively unexplored. There have been several studies on the effects of Se on below-ground plant-plant and plant-microbe interactions that have shown that hyperaccumulator-associated Se can have toxic effects on Se-sensitive plant- and microbial partners, while facilitating Se-resistant partners [17–24]. Effects of hyperaccumulation on root herbivores are still lacking, particularly the effect on root-nematode interactions [9,25–27].

Nematodes are one of the most abundant animals on earth, are species-rich in soils and have a diversity of feeding habits; many are root-feeding plant parasites or herbivores [28,29]. Plant-parasitic nematodes have been well-studied in agriculture worldwide, and can affect plant productivity through root damage, altered root architecture and root rot [30]. In hyperaccumulator roots, Se concentrations are commonly between 100 and 1500 mg Se $kg^{-1}$ DW [4] which may be toxic to many soil dwelling invertebrate herbivores including soil nematodes [10,11,16,31–34]. Most pertinently, Se nanoparticles were reported to protect tomato plants from root-knot nematodes by inducing systemic resistance [32]. In further analogy with aboveground herbivores that can evolve resistance to hyperaccumulator Se levels [16], it is also possible that some soil nematode species have evolved tolerance to Se and can successfully forage and complete their life cycle on hyperaccumulator roots.

The main objective of this study was to determine how variation in root Se concentration in hyperaccumulators affects nematode colonization. Two approaches were used: a field survey combined with a manipulative greenhouse experiment. To explore the relationship of soil nematode and Se in

two hyperaccumulator plant species, we examined speciation and distribution of Se in the roots of two hyperaccumulator species, conducted a seasonal field survey of the same plant species for root Se levels, and determined the densities of root-associated nematodes. In addition, in a follow-up greenhouse study we examined root nematode abundance in relation to plant Se concentration. The results from these collective studies provide new insight into root Se physiology and ecology, specifically the role of Se hyperaccumulation in protecting plants from root herbivory by nematodes.

## 2. Materials and Methods

### 2.1. Field Nematode Collections

Pine Ridge Natural Area in Fort Collins, Colorado, USA (40.550° N–105.142° W) is a naturally seleniferous grassland that is home to Se hyperaccumulator plant species *Astragalus bisulcatus* (Fabaceae) and *Stanleya pinnata* (Brassicaceae). Entire plants including taproots were excavated from this field location, 5 individuals per species in the fall and 5 individuals per species in the spring. The plants that were harvested were small, mature plants more than one year of age with large, intact taproots. Because of the limited number of plants from each species growing on the field site and the slow reestablishment rate of both species, 10 individuals (approximately 20% of the population) were considered the maximum number of plants that could be extracted from the field without impairing the health of the population. Plants were harvested from varying locations within the study site and were not collected closer than 10 m from each other, to try to avoid interactive effects between plants on each other's nematode composition. After sampling in separate bags, the plants were transported at room temperature to the laboratory (15 min from field) to be processed as described below.

For Se analysis, root and leaf samples were washed, dried at 50 °C, acid-digested and analyzed via inductively-coupled plasma optical emission spectrometry (ICP-OES), all as described [35–37]. For each plant part, multiple acid digestions were done, to cover variation in Se concentration across the different leaf and root parts. For each sample, 100 mg dry weight was digested in concentrated nitric acid, diluted 10-fold and then analyzed by ICP-OES.

### 2.2. Nematode Extraction and Quantification

All nematode extractions were carried out using the taproot and large lateral roots, using the Baermann Funnel extraction procedure, immediately after collection from the field [38]. Roots were washed gently prior to placing on the Baermann Funnel. In this method live nematodes move from the root into water where they are collected in three consecutive 15 mL fractions at 24 h increments, for a total extract of 45 mL. The nematodes were allowed to settle in the vial at room temperature for another 24 h, after which the volume was reduced by removing the top 40 mL, to a final volume of 5 mL. To this water-nematode mix, 5 mL of 90% formaldehyde at 90 °C was added to preserve the nematodes, with the exception of nematodes that were later used for inoculation to plants [39]. The total number of nematodes was counted. The nematodes were further observed microscopically and identified by feeding types, with particular interest in plant parasitic nematodes, including both migratory and sedentary endo- and ectoparasites. The taproots and large lateral roots that had been used for nematode extractions as described below, were dried and the dry weight obtained to calculate the number of nematodes per g DW root.

### 2.3. Nematode Greenhouse Studies

From the nematodes collected from hyperaccumulators harvested from the field, 1/3 fraction was used for a plant inoculation experiment, immediately after their extraction and characterization. Nematodes were used to inoculate 3-year-old *S. pinnata* plants that had been growing in a greenhouse (24/20 °C day/night, 16-h photoperiod, 300 μmol $m^{-2}$ $s^{-1}$ photosynthetic photon flux) on a non-sterile 1:1 (v/v) mixture of Pro mix BX (Premier Horticulture, Quakertown, PA, USA) and Turface® (MVP, Premier Products LLC, Buffalo Grove, IL, USA). The plants were watered three times a week; once a

week fertilizer was supplied at 1 g $L^{-1}$ Miracle-Gro Excel (15:5:15 Cal-Mag, The Scotts Co., Marysville, OH, USA). For the Se test, half the plants received 80 µM $Na_2SeO_4$ twice a week, while the other half received an equal amount of tap water. Selenate was chosen because it is the main form of bioavailable Se in soils. Each Se treatment was further divided into nematode treatments: no nematodes, nematodes extracted from *S. pinnata* roots from the field, and nematodes extracted from *A. bisulcatus* roots from the field. Inoculation involved placement of twenty nematodes in 0.5 mL of $H_2O$ on the soil surface next to the plant root. There were 3 plants per inoculation in each Se treatment, for a total of 18 plants. Twenty-four weeks after nematodes were inoculated, root and leaf material from each plant was harvested. Nematodes were extracted from fresh roots as described below. For Se analysis, root and shoot material was dried, acid-digested and elemental concentrations determined via ICP-OES.

### 2.4. Selenium Distribution and Speciation

Selenium distribution in hyperaccumulator roots was determined by micro-focused X-ray fluorescence (µXRF) mapping and the forms of Se accumulated were investigated using Se K-edge X-ray absorption near-edge structure (XANES) spectroscopy [40]. Specifically, field-collected taproots of *A. bisulcatus* and *S. pinnata* were sliced into approximately 0.5 mm cross-sections with a frozen razor blade over dry ice, flash-frozen in liquid nitrogen ($LN_2$) and shipped on dry ice for microprobe analyses at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Laboratory, Berkeley, CA [41]. The frozen root sections were analyzed at −33 °C using a Peltier stage to reduce beam damage. µXRF elemental maps and µXAS spectra were recorded with a 7 element Ge solid state detector (Canberra). Maps were recorded at 13 keV incident energy, with a 15 × 6 $\mu m^2$ beam spot size and 15 × 15 $\mu m^2$ pixel size. For each biological replicate, XANES spectra were collected in QXAS mode, at the spots indicated on the XRF maps. In QXAS mode, the monochromator is scanned on the fly, with thirty lines averaged. To determine the identity and relative abundance of selenocompounds in the tissues, least-square linear combination (LSQ) fitting of the XANES spectra was performed using an XAS library of standard selenocompounds. Se standards included $Na_2SeO_4$, $Na_2SeO_3$, SeCystine and SeMet purchased from Sigma-Aldrich (St. Louis, MO, USA) and MeSeCys, γ-Glu-MeSeCys, Se-Cysth, and $SeGSH_2$ purchased from PharmaSe (Austin, TX, 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). All spectra were processed using standard procedures [42] and calibrated using red amorphous elemental selenium (white line position set at 12,660 eV). All data processing was performed using a suite of custom LabVIEW programs available at the beamline. The error for LSQ fitting is ±10%. As a measure for goodness of fit, minimum normalized sum-squares residuals is provided; $NSS = 100 \times \{\Sigma(\mu_{exp} - \mu_{fit})^2/\Sigma(\mu_{exp})^2\}$ where µ represents the normalized absorbance.

### 2.5. Statistical Analyses

All statistical analyses were performed using JMP-IN (version 3.2.6, SAS Institute, Cary, NC, USA) or SAS software (9.2, SAS Institute, Cary, NC, USA). Tests for normal distribution and equal variance of data sets were conducted. Multiple linear regression ANCOVA models were used to analyze the effect of multiple variables and their interactions. Correlational analyses were conducted to investigate if any sort of relationship existed between parameters. One-way ANOVA was used to compare several means and Student's t-tests were used to compare two means. All ANOVAs and ANCOVAs were post-hoc evaluated using Tukey-Kramer tests for significance at $\alpha = 0.05$.

## 3. Results

### 3.1. Selenium Concentration, Speciation and Distribution in Field-Collected Hyperaccumulator Roots

There was substantial variation in root Se concentration in hyperaccumulators *S. pinnata* and *A. bisulcatus* growing in this naturally seleniferous grassland; this heterogeneity is known from the

literature [4,43]. Some of the plants did not reach the Se concentration threshold commonly used to classify a species as Se hyperaccumulator, i.e., >1000 mg $kg^{-1}$ DW. Root Se concentration further varied seasonally. The range in Se levels in the collected plants demonstrates the variability in root Se concentration that nematodes encounter. The observed variation in root Se concentration allowed a correlative analysis of nematode abundance in relation to Se concentration.

In both species and independent of season, Se concentrations were higher in the cortex (peripheral part of the root) than in the central stele tissue ($p < 0.0001$, Figure 1a–d), as determined via ICP-OES. The magnitude of this difference in Se concentration increased in the spring. On average, *S. pinnata* root cortex had a 3.7 times higher Se concentration than the corresponding stele in the spring, compared to 2.2 times higher Se level than stele in the fall. Similarly, *A. bisulcatus* root cortex had a 7.6 times higher Se level than the stele in the spring compared to 1.5 times higher Se concentration than the stele in the fall. For A. bisulcatus, the Se concentration in the root was higher compared to that in the leaves in the fall ($p = 0.0377$, Figure 1a); the reverse was seen in the spring when the Se concentration was higher in the leaves compared to the root ($p = 0.0004$, Figure 1c,d). The Se concentration in *S. pinnata* leaves was higher than the root Se concentration in the spring ($p < 0.0001$ Figure 1d). In the fall, there was not sufficient *S. pinnata* leaf material to collect for ICP; therefore, there is not enough information to compare Se partitioning in spring and fall for *S. pinnata* (Figure 1b).

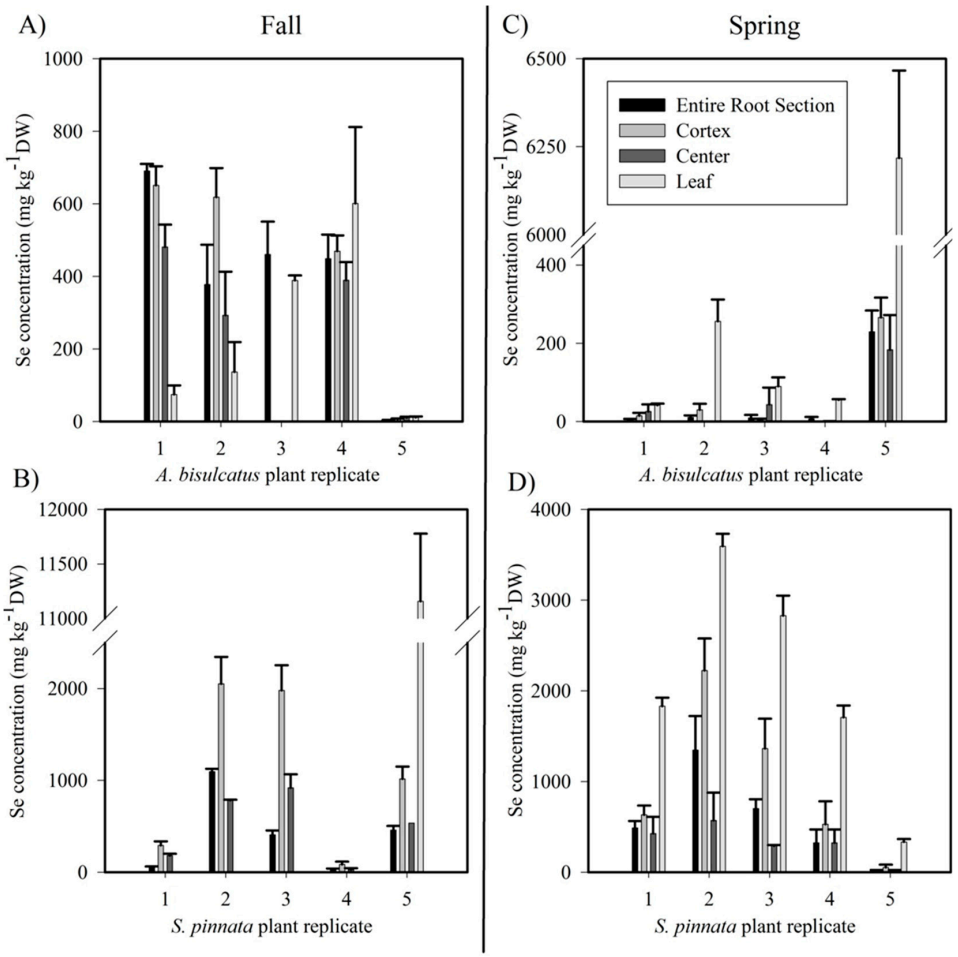


**Figure 1.** Selenium (Se) concentration (mg Se $kg^{-1}$ dry weight (DW)) in roots and leaves of individual hyperaccumulator plants growing in their natural seleniferous habitat, collected in the fall or the spring. (**A**) *Astragalus bisulcatus* collected in the fall. (**B**) *Stanleya pinnata* collected in the fall. (**C**) *A. bisulcatus* collected in the spring. (**D**) *S. pinnata* collected in the spring. Five plants per species were collected at each time point, and their Se levels are shown individually. Roots were analyzed as intact sections, as well as separate cortex and stele (center) sections. Values are means +/− standard error of the mean (SEM).

In the *S. pinnata* taproot, μXRF mapping showed that Se was more concentrated in the cortex compared to the central stele (Figure 2), in agreement with the ICP-OES results. μXANES analysis showed that Se in both cortex and stele was almost exclusively organic Se (C-Se-C, indistinguishable from the MeSeCys standard, Table 1). On the interface of soil and epidermis (XANES spots indicated in Figure 2) there was also predominantly organic Se, but also 17% selenate (Table 1). In the *A. bisulcatus* root cross-section, the Se signal was also higher in the cortex than in the stele (Figure 2). The form of Se in cortex and stele was 95%–97% C-Se-C, and the remainder was selenate (Table 1). On the soil-epidermis interface, three spots showed a similar Se composition as the interior of the root (94%–100% C-Se-C), while one spot showed large fractions of selenite and elemental Se. This spot was relatively more peripheral, and thus reflect more the rhizosphere Se speciation.

**Table 1.** Selenium speciation in root material, determined from Least-Square Linear Combination (LSQ) fitting of XANES spectra. The values shown below are percentages of total Se ± standard deviation (SD). The best LSQ fit was obtained for minimum normalized sum-squares residuals (NSS) = $100 \times \{\Sigma(\mu_{exp} - \mu_{fit})^2/\Sigma(\mu_{exp})^2\}$ where μ represents the normalized absorbance. ND: not detectable. C-Se-C: MeSeCys, γ-Glu-MeSeCys, SeMet, or SeCysth (indistinguishable). Forms of Se that were not detected in any of the samples, so not tabulated: SeCys, Se-Cystine, $Se(GSH)_2$. Elemental Se, also included as a standard, was found in one spot (14%), indicated by [1].

| XANES Spot | NSS ($\times 10^{-4}$) | C-Se-C (%) | $SeO_4^{2-}$ (%) | $SeO_3^{2-}$ (%) |
|---|---|---|---|---|
| *Stanleya pinnata* (Figure 2) | | | | |
| Cortex: | | | | |
| 4 | 5.5 | 99 | ND | ND |
| 5 | 4.1 | 100 | ND | ND |
| 6 | 4.9 | 99 | ND | ND |
| **Average ± SD** | **4.8 ± 0.7** | **99 ± 0.6** | **ND** | **ND** |
| Stele: | | | | |
| 1 | 3.1 | 99 | ND | ND |
| 2 | 3.3 | 99 | ND | ND |
| 3 | 3.7 | 100 | ND | 0.1 |
| **Average ± SD** | **3.4 ± 0.3** | **100 ± 0.4** | **ND** | **ND** |
| Soil/epidermis: | | | | |
| 7 | 3.6 | 100 | ND | ND |
| 8 | 5.1 | 100 | ND | ND |
| 9 | 11.8 | 48 | 49 | ND |
| **Average ± SD** | **6.8 ± 4.4** | **83 ± 30** | **17 ± 29** | **ND** |
| *Astragalus bisulcatus* (Figure 2) | | | | |
| Cortex: | | | | |
| 3 | 5.5 | 91 | 9 | ND |
| 6 | 4.2 | 96 | 3 | ND |
| 8 | 3.7 | 97 | 2 | ND |
| **Average ± SD** | **4.5 ± 0.9** | **95 ± 3** | **5 ± 4** | **ND** |
| Stele: | | | | |
| 4 | 5.2 | 96 | 2 | 1 |
| 5 | 4.3 | 98 | 1 | ND |
| **Average ± SD** | **4.8 ± 0.6** | **97 ± 2** | **2 ± 0.4** | **ND** |
| Soil/Epidermis: | | | | |
| 1 | 3.4 | 100 | 1 | ND |
| 2 | 3.7 | 97 | 3 | ND |
| 7 | 3.8 | 94 | 5 | ND |
| 9 [1] | 9.0 | 38 | ND | 48 |
| **Average ± SD** | **5.0 ± 2.7** | **82 ± 30** | **2 ± 2** | **12 ± 24** |

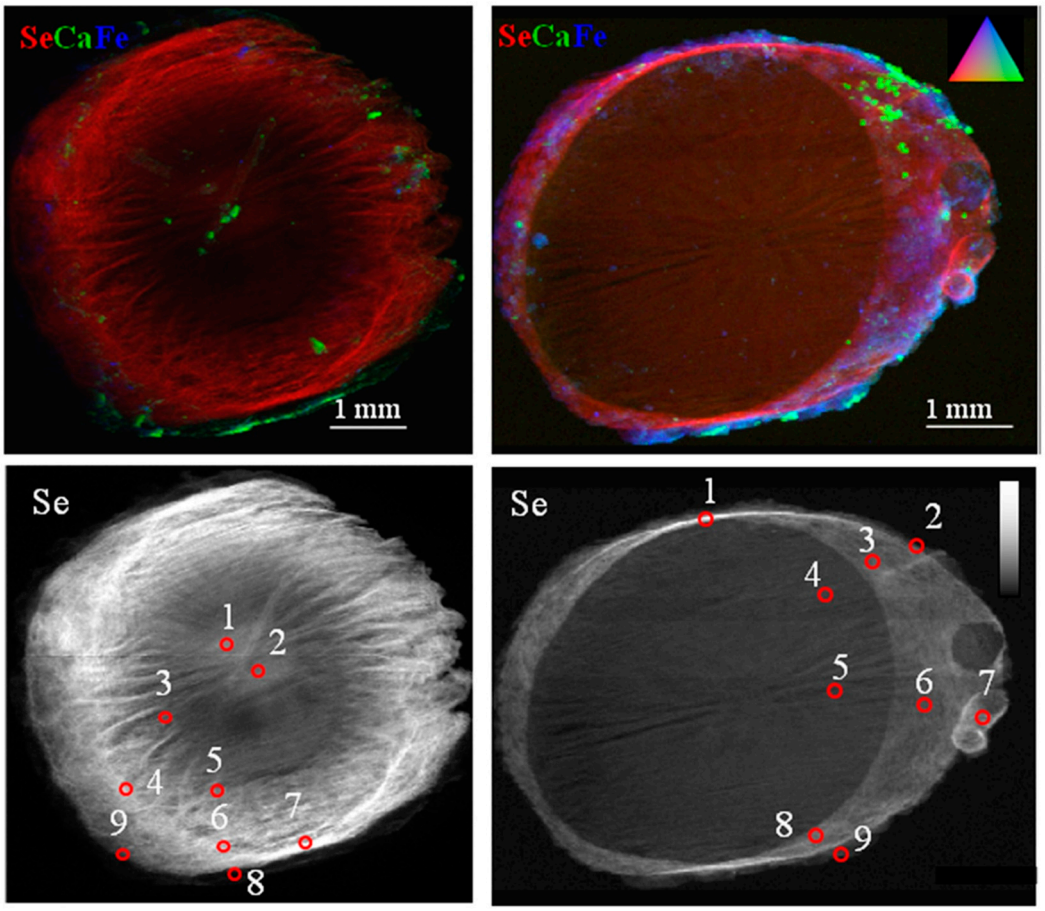


**Figure 2.** X-ray microprobe analysis of selenium (Se) distribution and speciation in cross-sections of roots of *Stanleya pinnata* (**Left side**) and *Astragalus bisulcatus* (**Right side**). Top: Micro X-ray fluorescence (μXRF) map of Se in red, calcium (Ca) in green and iron (Fe) in blue; bottom: Se μXRF map of the same root (Se in white), with numbered red circles indicating locations where Se K-edge X-ray absorption near-edge structure (XANES) spectra were collected.

### *3.2. Field Nematode Counts*

All plant hyperaccumulator taproots from the field yielded nematodes, even those with the highest Se concentrations (up to 1300 mg Se $kg^{-1}$ DW, Figure 3a). While nematodes could be collected both in fall and spring, root-associated nematode abundance was overall higher in spring than in fall (not shown). The number of plants available for this study (20 plants) does not allow for rigorous statistical correlation analysis, and based on the data available there is no significant correlation between root Se concentration and nematode presence in roots in either the fall or the spring, nor in fall and spring data combined (Figure 3a). There appears to be a downward pattern in the number of nematodes with increasing root Se concentration, but more sampling would be needed to confirm that such a trend exists. It is an interesting finding that there were still a significant number (>50) of nematodes found in close association with high-Se roots (Figure 3a). Both *A. bisulcatus* and *S. pinnata* roots yielded nematodes with a variety of feeding modes (Figure 3b). In the fall collection, the fraction of total nematodes that consisted of plant parasites (migratory and sedentary endo- and ectoparasites) was on average 16% for *A. bisulcatus* and 7% for *S. pinnata*, respectively (Figure 3b). Among the nematodes collected was *Pratylenchus* spp. (Figure 3c), a migratory endoparasite.

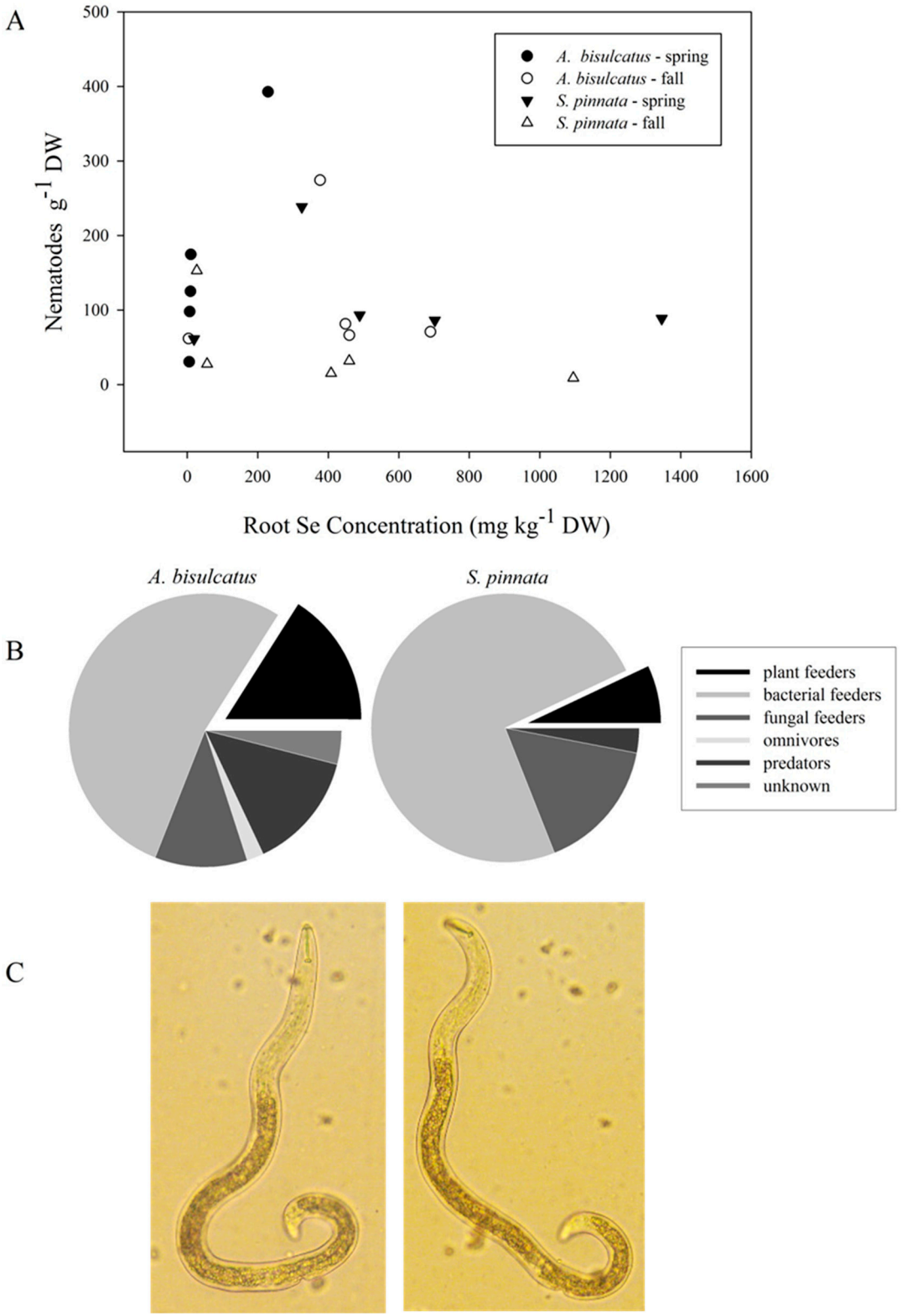


**Figure 3.** (**A**) Nematode colonization (animals per gram DW) in roots of hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* as a function of root selenium (Se) concentration (no significant correlation, $p > 0.05$). Ten plants from each species were collected while growing in their natural habitat, five in the fall and five in the spring (see Figure 1 for Se levels in each individual plant). Each of the 20 nematode counts is represented by a dot. (**B**) Feeding mode distribution among extracted nematodes from *A. bisulcatus* (left) and *S. pinnata* (right), determined microscopically. (**C**) Representative herbivorous nematodes (see feeding stylet) collected from high-Se roots of hyperaccumulators *A. bisulcatus* and *S. pinnata*.

### 3.3. Greenhouse Nematode Inoculations

A greenhouse nematode inoculation was carried out to complement the survey-type field study. This manipulative greenhouse experiment similarly explored the effect of Se on root nematode abundance. Nematodes extracted in the fall from field roots of *S. pinnata* or *A. bisulcatus* were used to inoculate greenhouse-grown *S. pinnata* plants that had been pretreated with or without (control) 80 µM $Na_2SeO_4$. The plants were harvested the following spring, 24 weeks after inoculation. The 80 µM $Na_2SeO_4$ group had on average an approximately 8 fold higher Se concentration in their leaves than the plants not treated with Se, and around 10 fold higher Se concentration in their roots ($p = 0.0392$ for leaves, $p < 0.0001$ for roots, Figure 4a,b). The root Se concentration of the Se-treated plants was around 600 mg $kg^{-1}$ DW, similar to root Se levels observed in plants growing naturally in the field (Figures 1 and 4b). Within the 80 µM $Na_2SeO_4$ treatment, both groups that had received nematode inoculations contained significantly lower leaf Se levels than the control group that had not received nematodes (Figure 4a). The different nematode inoculation treatments also showed statistically different root Se levels ($p = 0.0196$, Figure 4b). Within the Se treatment, the plants inoculated with nematodes extracted from *S. pinnata* had significantly lower root Se concentration compared to plants inoculated with nematodes from A. bisulcatus. The plant group that had not been inoculated with nematodes was not significantly different from either inoculation treatment ($p = 0.069$ and $p = 0.433$, respectively, Figure 4b).

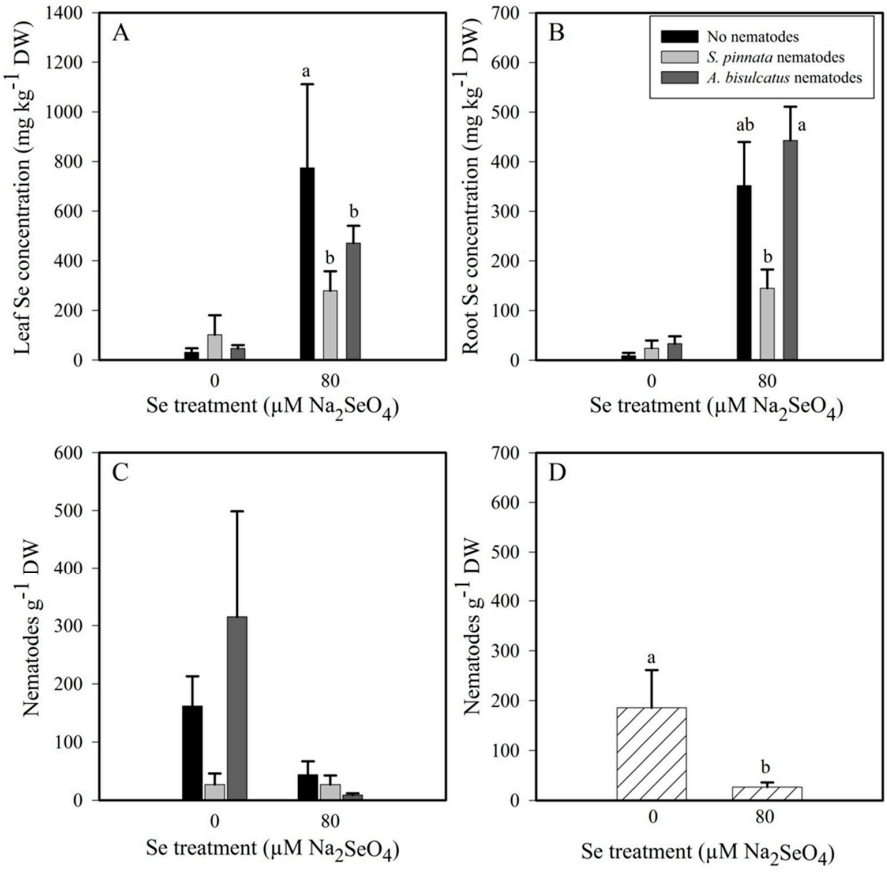


**Figure 4.** Selenium concentration (mg Se $kg^{-1}$ dry weight (DW)) and root nematode colonization (nematodes $g^{-1}$ DW) in greenhouse-grown *Stanleya pinnata* plants treated with 0 or 80 µM $Na_2SeO_4$. The plants were inoculated with nematodes extracted from *S. pinnata*, nematodes extracted from *A. bisulcatus*, or with water without nematodes ($n = 3$). (**A**) Se concentration in leaves, (**B**) Se concentration in the root, (**C**) Root nematode abundance for individual treatments, (**D**) Root nematode abundance pooled for the 0 and 80 µM $Na_2SeO_4$ treatments. Values are means +/− SE; different letters above bars represent significant differences ($\alpha = 0.05$).

When the root-associated nematodes were extracted and counted 24 weeks after inoculation, there were significantly (7-fold) more nematodes extracted from *A. bisulcatus* control plants (not treated with Se) compared to *A. bisulcatus* plants treated with Se ($p = 0.0425$, Figure 4c,d). There were no significant differences in nematode abundance between the inoculation treatments. The range of nematode abundance in the greenhouse roots was similar to that observed for field roots (Figures 3 and 4c,d).

## 4. Discussion

The main focus of the study was the effect of root Se accumulation on the abundance of root-associated nematodes. In this context, it is relevant to know the Se concentration, forms of Se and the tissue Se distribution in roots of Se hyperaccumulator species. In Se hyperaccumulators *A. bisulcatus* and *S. pinnata* growing naturally on seleniferous soil at Pine Ridge Natural Area, Fort Collins, CO, the Se was found to be concentrated in the root cortex, with lower levels in the central stele. The predominant form of Se throughout the root of both species was organic C-Se-C indistinguishable from MeSeCys, which is also the Se speciation in leaves [8].

In a greenhouse experiment, selenate-supplied *S. pinnata* plants containing root Se levels similar to those found in field settings, had significantly lower numbers of nematodes associated with their roots compared to plants of the same species grown without Se. This lower nematode abundance may reflect a protective effect of root-accumulated Se, and may also be due to the higher soil Se levels. In either case, the abundance of live nematodes associated with the plant roots appeared to be negatively affected by the applied Se. While the greenhouse experiment included different nematode inoculation treatments using field nematodes collected from hyperaccumulator roots, there were no differences in final nematode numbers between the inoculated and non-inoculated treatments. It is possible that there already were established nematode populations in the potting soil at the time of inoculation that colonized the plants earlier. Baseline counts from previous experiments showed no plant parasite nematodes in fresh potting soil (Wall, Tomasel, personal communication). However, these plants had been growing on the potting soil until maturity in the greenhouse before being inoculated with the field nematodes, and may have picked up greenhouse nematodes during this time. If so, the inoculation with 20 additional individuals may not have had a significant effect on overall nematode abundance, and the nematodes isolated from the roots of the greenhouse *S. pinnata* plants at the end of the experiment may largely have been greenhouse populations already present before inoculation. The finding that Se treatment reduced nematode abundance may be because greenhouse nematode populations are not adapted to high-Se conditions. They are likely to be more Se-sensitive than nematode populations native to seleniferous soil.

At Pine Ridge Natural Area, a seleniferous field site, even hyperaccumulator roots with very high Se levels, up to 1300 mg $kg^{-1}$ DW, had nematodes. These live nematodes were extracted from roots using Baermann funnels, and clearly lived in close association with the high-Se roots. A substantial fraction of these nematodes had plant-feeding stylets and thus could have been feeding on the high-Se roots; in most soils, nematodes with stylets are predominantly plant parasites, but some are fungivores. Thus, it appears that these high-Se hyperaccumulator plants in this seleniferous habitat are utilized by nematode communities.

Earlier studies focusing on aboveground ecological interactions have shown that Se (hyper)accumulation can protect plants from a variety of generalist herbivores, but that hyperaccumulators can also offer a niche for Se-resistant herbivores [16,27]. The below-ground interactions of hyperaccumulators with nematodes may follow a similar pattern. Herbivorous nematodes native to high-Se roots in seleniferous soils may over time have evolved Se resistance, enabling them to feed on Se hyperaccumulator plants. Similar effects on nematodes were found for other toxic elements in soil. Broeks reported nematodes from polluted soil to have elevated tolerance to cadmium and arsenic [44]. Likewise, Millward and Grant reported that high soil copper (Cu) levels selected for higher Cu tolerance in native populations of nematodes compared to nematodes on non-polluted sites [45]. Ellis and colleagues reported lower nematode abundance on a site heavily

contaminated with metals compared to a site with low levels of metals, but did not compare metal tolerance between the two sites [46].

The Se levels in root tissues were higher in the fall than the spring, and the Se levels were higher in leaves than in the root in the spring. These results are in agreement with the report by Galeas and coworkers [4], who suggested there is a seasonal flow of Se from the hyperaccumulator root to the shoot in the spring, and from the leaves to the root and the seeds in the fall. It may be advantageous for the plant to transfer the ecologically valuable Se from shoot, leaf, and floral tissues as they senesce at the end of the growing season to the root and the seeds, which persist throughout the winter. The reallocation of Se to root tissues in the fall may have a protective effect, defending the root against Se-sensitive herbivores, including non-specialist nematodes.

According to ICP-OES and XAS data, the Se in the root is concentrated in the cortex in *A. bisulcatus* and *S. pinnata*. The speciation of Se in the cortex was similar to that in the stele. Both contained primarily (95%–100%) C-Se-C, indistinguishable from MeSeCys. Only one spot at the root-rhizosphere interface of *A. bisulcatus* also contained significant levels of elemental Se and selenite. Lindblom and coworkers also found C-Se-C to be the predominant form of Se in *A. bisulcatus* and *S. pinnata* roots; furthermore, up to 30% of the Se in field-collected plants was present in the form of elemental Se [47]. This was attributed to microbial symbionts, since greenhouse-grown plants from the same species contained almost exclusively C-Se-C. Microbial associations in the field likely vary between individuals of a species, which may explain differences in Se speciation, and may also contribute to the observed intraspecific variation in Se accumulation in the field [43]. Similar to what was found here for Se speciation in roots, the Se in leaves of *A. bisulcatus* and *S. pinnata* consisted primarily of C-Se-C compounds (MeSeCys, γ-Glutamyl-MeSeCys and/or selenocystathionine) [8]. Selenium is taken up from the soil as selenate and then assimilated into organic selenocompounds [3]. The finding that the roots of *A. bisulcatus* and *S. pinnata* accumulate mainly MeSeCys suggests that in these hyperaccumulators, the root is capable of selenate assimilation, or that the plant metabolizes Se in the shoot, and then remobilizes MeSeCys back to the roots.

The observed Se distribution in hyperaccumulator roots has implications for root-associated nematodes and other root herbivores. Hyperaccumulators preferentially allocate Se to the outer section of the root, which is where herbivores first interact with root tissues. This increased concentration of Se could deter the herbivore from the root tissue and/or have a toxic effect on the herbivore, and via these mechanisms, protect the vascular tissue inside the stele without needing to accumulate much Se in stele tissues themselves. In leaves of hyperaccumulators, Freeman and colleagues also observed preferential allocation of Se to the margins and specialized epidermal structures [8]. They hypothesized this distribution would protect the plant from leaf herbivores because the margin and epidermis are the first parts of the leaf many herbivores encounter.

If root Se accumulation can protect plants from herbivorous nematodes, as suggested by the results from the greenhouse experiment described here, this may have applications in agriculture. Nematodes are a serious pest faced by farmers, and particularly in subsistence and sustainable agriculture systems, pesticides are not an option [34]. Selenium biofortification may not only enhance crop nutritional value, but also protect the plant from herbivorous nematodes. The results presented in this study also provide a framework for further ecological investigations into the rhizosphere interactions of Se hyperaccumulators as well as of Se-accumulating plants in phytoremediation or biofortification settings.

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