Selenium Hyperaccumulator Plants *Stanleya pinnata* and *Astragalus bisulcatus* Are Colonized by Se-Resistant, Se-Excluding Wasp and Beetle Seed Herbivores


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**Abstract**

Selenium (Se) hyperaccumulator plants can concentrate the toxic element Se up to 1% of shoot (DW) which is known to protect hyperaccumulator plants from generalist herbivores. There is evidence for Se-resistant insect herbivores capable of feeding upon hyperaccumulators. In this study, resistance to Se was investigated in seed chalclids and seed beetles found consuming seeds inside pods of Se-hyperaccumulator species *Astragalus bisulcatus* and *Stanleya pinnata*. Selenium accumulation, localization and speciation were determined in seeds collected from hyperaccumulators in a seleniferous habitat and in seed herbivores. *Astragalus bisulcatus* seeds were consumed by seed beetle larvae (*Acanthoscelides fraterculus* Horn, Coleoptera: Bruchidae) and seed chalclid larvae (*Bruchophagus mexicanus*, Hymenoptera: Eurytomidae). *Stanleya pinnata* seeds were consumed by an unidentified seed chalclid larva. Micro X-ray absorption near-edge structure (µXANES) and micro-X-Ray Fluorescence mapping (µXRF) demonstrated Se was mostly organic C-Se-C forms in seeds of both hyperaccumulators, and *S. pinnata* seeds contained ~24% elemental Se. Liquid chromatography–mass spectrometry of Se-compounds in *S. pinnata* seeds detected the C-Se-C compound seleno-cystathionine while previous studies of *A. bisulcatus* seeds detected only the C-Se-C compounds methyl-selenocysteine and γ-glutamyl-methyl-selenocysteine. Micro-XRF and µXANES revealed Se ingested from hyperaccumulator seeds redistributed throughout seed herbivore tissues, and portions of seed C-Se-C were biotransformed into selenocysteine, selenodiglutathione, selenate and selenite. *Astragalus bisulcatus* seeds contained on average 5,750 µg Se g⁻¹, however adult beetles and adult chalclid wasps emerging from *A. bisulcatus* seed pods contained 4–6 µg Se g⁻¹. *Stanleya pinnata* seeds contained 1,329 µg Se g⁻¹ on average; however chalclid wasp larvae and adults emerging from *S. pinnata* seed pods contained 9 and 47 µg Se g⁻¹. The results suggest Se resistant seed herbivores exclude Se, greatly reducing tissue accumulation; this explains their ability to consume high-Se seeds without suffering toxicity, allowing them to occupy the unique niche offered by Se hyperaccumulator plants.

**Citation:** Freeman JL, Marcus MA, Fakra SC, Devonshire J, McGrath SP, et al. (2012) Selenium Hyperaccumulator Plants *Stanleya pinnata* and *Astragalus bisulcatus* Are Colonized by Se-Resistant, Se-Excluding Wasp and Beetle Seed Herbivores. PLoS ONE 7(12): e50516. doi:10.1371/journal.pone.0050516

**Editor:** Martin Heil, Centro de Investigación y de Estudios Avanzados, Mexico

**Received** May 16, 2012; **Accepted** October 23, 2012; **Published** December 3, 2012

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**Funding:** Funding for these studies was provided by National Science Foundation grant #DBS-0817748 to EAHP. The Advanced Light Source is supported by the Office of Science, Basic Energy Sciences, and Division of Materials Science of the U.S. Department of Energy (DE-AC02-05CH11231). Rothamsted Research received grant-aided support from the Biotechnology and Biological Sciences Research Council of the UK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

Selenium (Se) occurs naturally in certain soils, such as Cretaceous shale, at levels between 1 and 100 mg Se kg⁻¹ [1,2]. Selenium is biologically important because it is both an essential element to animals and toxic at high concentrations. Some plant species grow almost exclusively on seleniferous soils, and are characterized by extremely high Se concentrations in their tissues, reaching levels between 0.1 and 1.5% of dry weight (1,000–15,000 mg Se kg⁻¹ DW). These plants, called Se hyperaccumulators, typically contain 100-fold higher Se levels than surrounding vegetation [1,2].

Selenium hyperaccumulation defends plants via both deterrence and toxicity from a wide variety of herbivores (for a recent review see [3]). These include prairie dogs [4], phloem-sucking aphids [5], leaf chewing caterpillars [6], crickets and grasshoppers [7], and cell disrupting thrips and spider mites [8]. When given a choice, Se-sensitive herbivores avoid feeding on Se hyperaccumulator plants, and when forced to feed on high-Se leaves they suffer visible signs of Se toxicity and often die. The Se hyperaccumulator plants *Astragalus bisulcatus* (two-grooved milkvetch, Fabaceae) and *Stanleya pinnata* (Prince’s plume, Brassicaceae) harbor few arthropods in native seleniferous habitats compared to neighboring non-hyperaccumulator plants [9]. Selenium hyperaccumulator plants also cause devastating Se toxicity to livestock (e.g., cows, sheep and horses) [10].

Selenium can be toxic because plants inadvertently take up selenate (SeO₄²⁻) via sulfate transporters, and assimilate it into
seleno-amino acids via the sulfur assimilation pathway (for a review see [11]). Selenate is first reduced via selenite (SeO$_3^{2-}$), which is then incorporated into selenocysteine (SeCys) and selenomethionine (SeMet). One of the reasons Se is toxic is its similarity to sulfur (S), which can lead to the non-specific incorporation of Se into S-containing proteins and other metabolically important S compounds [12]. In addition to oxidative stress caused by the conjugation of glutathione to SeO$_3^{2-}$, the replacement of S in sulfhydryl groups or thiols (critical for disulide bond formation) with Se can lead to a lack of normal protein conformation and result in structural malformation or loss of enzymatic activity [12]. Selenium hyperaccumulators circumvent Se toxicity by methylating SeCys via the enzyme SeCys methyl-transferase (SMT) and the resulting methyl-selenocysteine (MeSeCys) accumulates in a free pool because MeSeCys is not readily incorporated into proteins [13].

Micro-focused X-ray fluorescence (µXRF) mapping and Energy Dispersive X-ray Spectroscopy (EDS) demonstrated that Se hyperaccumulator plants preferentially hyperaccumulate Se in the periphery of leaves, in leaf hairs (called trichomes), or in vacuoles of leaf epidermal cells [14,15]. Selenium X-ray absorption near-edge structure (µXANES) demonstrated that the majority (~90%) of the Se in hyperaccumulator leaves consisted of organic carbon-selenium-carbon (G-Se-C) forms. Liquid chromatography mass spectroscopy (LCMS) only detected and quantified the G-Se-C compounds MeSeCys and γ-glutamyl-MeSeCys in a 1:1 ratio in A. bisulcatus leaves, and MeSeCys and selenocystathionine (SeCyst) in a 4:1 ratio in S. pinnata leaves [14]. Roots, florets and fruit of both hyperaccumulators harvested from the field also contained mainly organic G-Se-C forms [16,17].

Although they deter many Se-sensitive herbivores, there is mounting evidence that Se hyperaccumulator plants may provide a niche occupied by Se-tolerant herbivores. For example a population of Se-tolerant Platellidae closely resembling the diamondback moth (Platella stylostella), was discovered in a seleniferous area near Fort Collins, CO, U.S.A. and was shown in laboratory tests not to avoid plants containing hyperaccumulated Se, and to readily oviposit and voraciously feed, on S. pinnata leaves that contained more than 2,000 µg Se g$^{-1}$ DW, without suffering Se-toxicity [18]. In contrast, a population of diamondback moth originally collected from a non-seleniferous area in the Eastern U.S.A. preferred to oviposit and feed on S. pinnata plants containing trace Se concentrations, and suffered Se-toxicity and quickly died when fed Se rich leaves [18]. Potentially explaining the biochemical mechanism for the observed difference in Se tolerance, the Se-tolerant moth was found to accumulate MeSeCys, similar to its host plant S. pinnata, while the Se-sensitive population accumulated the de-methylated form SeCys and showed deterioration of multiple internal organs [18]. In the same study, the Se-tolerant Stanleya moth larvae were found to be actively parasitized by a Se-tolerant microgastrine wasp, Diadegma insulare (Braconidae), which also accumulated MeSeCys. Thus, the co-evolution of Se hyperaccumulator plants, Se-tolerant herbivores, and Se-tolerant predators may represent a unique portal for Se to move up into higher trophic levels.

Seeds contain the highest Se concentrations of all the organs of Se hyperaccumulator plants [14,16,19]. Selenium is thought to be actively transported from ageing leaves to reproductive organs, and to be highly concentrated in seeds. The form(s) of Se remobilized to seeds of hyperaccumulator plants may be organic, since A. bisulcatus seeds have been reported to accumulate both MeSeCys and γ-glutamyl- MeSeCys [20]. It may enhance the reproductive success of a Se hyperaccumulator plant if Se is concentrated in the seed, where it can protect both seed and the newly germinating seedlings from herbivory or pathogen infection. For example, Acanthoscelides mixtus and Acanthoscelides pulsus seed beetle larvae, hatched from eggs oviposited by adults, were found feeding on seeds inside seedpods of the Se hyperaccumulator plant A. praelongis, but intriguingly the adults never successfully emerged. However, these same seed beetle larvae successfully completed their lifecycle and emerged from seedpods after consuming seeds of several non-accumulator Astragalus species that do not contain high concentrations of Se [21]. A third seed beetle species, Acanthoscelides aureoles, was found to consume seeds of both Se hyperaccumulator and nonaccumulator plant species with equal success. This finding led the authors to hypothesize that some seed beetles may have co-evolved with Se hyperaccumulators and evolved Se resistance. Trelease and Trelease [22] also reported the presence of a seed beetle consuming seeds of Se hyperaccumulator A. bisulcatus containing 1,475 µg Se g$^{-1}$, which they identified as Acanthoscelides fraterculus (Horn), a species very closely related to A. aureoles. Furthermore, Trelease and Trelease observed large numbers of seed chalcids, small wasp-like hymenopteran insects eating A. bisulcatus seeds, which they identified as Bruchophagus mexicanus (Ashmead) [22]. Lavigne and Littlefield [23], in their annotated list of insects associated with Astragalus species, report that the seed beetle A. fraterculus has been found in seed pods of at least three Se hyperaccumulator plant species: A. bisulcatus, A. racemosus, and A. petiotanus. Lavigne and Littlefield mention Bruchophagus mexicanus in seed pods of the closely related, often neighboring Se hyperaccumulator A. racemosus, as well as in several non-accumulating Astragalus species [23].

In order to investigate Se resistance observed in the insect herbivores of Se hyperaccumulator seeds at the molecular level we mapped the distribution of Se and analyzed the forms of Se accumulated in seeds of A. bisulcatus and S. pinnata, and three associated insect herbivores feeding on these plants. The results provide insight into the Se resistance mechanism, at a molecular level, of the seed herbivores and help assess the potential for them to bio-transfer Se to higher trophic levels in seleniferous ecosystems.

**Materials and Methods**

**Collection of Biological Material**

Astragalus bisulcatus (Hook.) A. Gray and S. pinnata (Pursh) Britton seeds were collected in the summer (June-July) at Pine Ridge Natural Area, a seleniferous site west of Fort Collins, CO, USA that has been described before [9,19]. Seeds that were used for elemental analysis were dried, ground in a mortar and pestle, acid-digested and analyzed for total Se and S via inductively coupled plasma atomic emission spectrometry as described below (n = 22...
Measurement of Total Se and S Concentrations, and Identification of Non-protein Organic Selenium Compounds

Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used to determine the concentrations of total Se and S [24]. The whole biological material was rinsed with distilled water and dried for 48 h at 45°C. Samples were then finely ground using a mortar and pestle and digested in nitric acid as described by Zarcinas et al. [25]. Liquid Chromatography Mass Spectrometry (LC-MS) was used to determine the chemical speciation of the Se-compounds in S. pinnata seeds, as described by Freeman et al. [14,18].

Table 1. Chemical forms of Se found in seeds of S. pinnata and A. bisulcatus.

<table>
<thead>
<tr>
<th></th>
<th>SS (x10⁻⁴)</th>
<th>SeO₃²⁻</th>
<th>Se(GSH)₂</th>
<th>C-Se-C</th>
<th>Se°</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pinnata</td>
<td>0, 1, 2 embryo, root</td>
<td>3.3</td>
<td>3%</td>
<td>nd</td>
<td>77%</td>
</tr>
<tr>
<td>3, 4, 5 embryo, cotyledon</td>
<td>4.1</td>
<td>3%</td>
<td>nd</td>
<td>73%</td>
<td>24%</td>
</tr>
<tr>
<td>6, 7 seed coat</td>
<td>1.8</td>
<td>5%</td>
<td>nd</td>
<td>77%</td>
<td>19%</td>
</tr>
<tr>
<td>8, 9 embryo, herbivore damage</td>
<td>3.1</td>
<td>nd</td>
<td>nd</td>
<td>100%</td>
<td>nd</td>
</tr>
<tr>
<td>A. bisulcatus</td>
<td>0 embryo, cotyledon</td>
<td>3.7</td>
<td>3%</td>
<td>nd</td>
<td>97%</td>
</tr>
<tr>
<td>1 embryo, root</td>
<td>4.0</td>
<td>3%</td>
<td>nd</td>
<td>96%</td>
<td>nd</td>
</tr>
<tr>
<td>2 seed coat</td>
<td>9.8</td>
<td>8%</td>
<td>28%</td>
<td>63%</td>
<td>nd</td>
</tr>
</tbody>
</table>

Results from least-squares linear combination fitting of each samples XANES spectra in comparison to standard selenium compounds. The regions where the spectra were collected are indicated in Figure 1. SeO₃²⁻: selenite; Se(GSH)₂: seleno-diglutathione; C-Se-C: methyl-selenocysteine, seleno-methionine or seleno-cystathionine. Se°: red or gray elemental Se. SS: normal sum of squares (quality of fit; 0 = perfect fit; nd: compound not detectable. Additional standard compounds included in the fit but not detected in any location were selenate, seleno-cystine and seleno-cysteine. Note: fractions do not always add up to exactly 100% because the margin of error can be up to 10%.

doi:10.1371/journal.pone.0050516.t001

Energy-dispersive X-ray Spectroscopy

Energy Dispersive Spectrometry (EDS) and was used to investigate the localization of Se in cryo-fractured and cryo-planned samples. For cryo-fracturing the seeds were mounted on a cryo stub using OCT compound (Sakura-Netherlands) and plunged-frozen in pre-slushed liquid nitrogen (LN₂). The seeds were transferred under vacuum to the GATAN Alto 2100 cryo chamber (Gatan UK) with temperature maintained at −180°C. Here they were fractured using the cold blade mounted in the prep chamber and any contaminating ice was removed through sublimation by raising the stage temperature to −95°C for 1 minute. The heater was then turned off and the stage temperature was allowed to recover to −160°C. For cryo-planning the insect samples were embedded in OCT compound (Sakura-Netherlands), plunged into liquid nitrogen and then mounted in the cryostat LeicaCM1850 where the surface was planed using a steel blade at −30°C. This technique is easy to stop once the plane of interest has been reached and the sample is then transferred under liquid nitrogen to the prep chamber attached to the microscope for etching and coating. The specimens were then sputter coated with...
Au for 60 sec, (approximate thickness 10 nanometers) and transferred to the JSM LV6360 (Jeol UK) scanning electron microscope stage for examination. The microscope stage was maintained at −160 °C and after imaging the parameters were set for EDS analysis using the OXFORD INCA 2000 microanalysis system (Oxford Instruments, UK). An accelerating voltage of 5000V (Se L2 line detected) instead of the higher voltage needed to see the Se K line was chosen for the analysis, as it is less damaging to fully hydrated frozen biological material, reduces movement of labile elements and avoids interference from other elements such as the Au coating. Air-dried samples were also prepared and examined on the dry stage at room temperature for selenium.

Results

Micro-XRF mapping shows that in seeds of both *S. pinnata* and *A. bisulcatus* Se was mainly concentrated throughout the embryo and a much lower level was present in the seed coat (Fig. 1B, 1C). Within the embryo, Se was fairly evenly distributed, although the Se signal appears somewhat stronger in the seed embryo vascular tissue. Zinc (Zn) and iron (Fe) were also present in the embryo but excluded from the seed coat; they were strongly concentrated in the vascular tissue, particularly at the root tip (Fig. 1C, 1D). One of the *S. pinnata* seeds showed mandible scrape scars indicative of larval herbivory, part of the seed embryo was eaten (Fig. 1B) and Se rich larval frass (i.e. feces) was found in the seed pod (Fig. 1B). Micro-XANES analysis revealed that the majority of Se (~63–100%) in both the *S. pinnata* and *A. bisulcatus* seeds was in C-Se-C forms, such as SeMet, MeSeCys, γ-glutamyl-MeSeCys or seleno-cystathionine (Table 1). In addition to C-Se-C forms, the *S. pinnata* seeds contained elemental Se (19–24%) and a trace of selenite (up to 5%), and the *A. bisulcatus* seeds also contained a trace of selenite (3–8%) (Table 1). Further analyses by LC-MS detected and identified seleno-cystathionine as the only detectable C-Se-C form in *S. pinnata* seeds (Fig. S1). In *A. bisulcatus* seeds the form of Se was reported in the literature to be MeSeCys and γ-glutamyl-MeSeCys [28], which is in agreement with our C-Se-C XANES data. EDS analysis indicated the presence of Se in cells of the fractured surfaces of both seed types (Fig. 2). In Figure 2D the Se X-ray line-scan across the fractured surface of *A. bisulcatus* demonstrates how the concentration starts off low at the seed coat (testa) edge, increases across the endosperm region and then drops down again at the other seed coat edge. Fractures across the air-dried whole seeds of *A. bisulcatus* and *S. pinnata* (Fig. 2A and 2E) show acquisition points across the endosperm all positive for Se at varying levels. The cryo-fractured fully hydrated seeds at higher magnification (Fig. 2B, 2C and 2F) all gave positive readings for Se including one from the seed coat layers (spectra available in Material S1).

Seeds of *S. pinnata* and *A. bisulcatus* that had been collected from a seleniferous field site were dissected and found to contain three different herbivorous insect species: the *S. pinnata* seeds contained an unknown seed chalcid wasp, and the *A. bisulcatus* seeds harbored a larger seed chalcid wasp as well as a seed beetle (Figs. 3, 4 and 5). Based on morphology we tentatively identified the *A. bisulcatus* seed beetle as *Acanthoscelides fraterculus* Horn, Coleoptera: Bruchidae (Figs. 3, 5) and the seed chalcid wasp resembled *Bruchophagus mexicanus*, Hymenoptera: Eurytomidae (Figs. 3, 4). Energy-dispersive X-ray spectroscopy (EDS) detected very little Se in the herbivore tissues, except for a very weak signal in the mid-abdomen and gut of the bruchid beetle and the posterior abdomen region of the cryo-fractured samples (Figs. 2, 4). The seed beetle mouth region indicated by the red rectangle in Figure 2G was surprisingly negative for Se even though Se was detected inside the beetle’s body. External surfaces of the seed chalcid wasp, seed chalcid wasp larva and the seed beetle were also targeted for Se analysis using EDS. Very low levels of Se were detected on upper leg segment spines of the chalcid wasp and around the spiracle and some bristles/spines on the seed chalcid larva.

Micro-XRF mapping of the larvae and adult seed chalcid wasp that emerged from the *S. pinnata* seed pods demonstrated that Se was present throughout all tissues in both stages (Fig. 4E–H). In the seed chalcid wasp larva Se was uniformly distributed (Fig. 4F) and in the seed chalcid wasp adult the Se concentration was elevated in the thorax and abdomen, and lower in the wings and exoskeleton (Fig. 4G, 4H). Iron was concentrated in discrete locations along the adult’s exoskeleton and on wings; some of these Fe “hot spots” also contained Ca (Fig. 4G, 4H). Zinc was concentrated in the intestine of the larva and in mouth parts (mandibles) of the adults (Fig. 4E–H). XANES analysis (Table 2) showed that the high-Se frass deposited by the seed chalcid wasp larvae in the *S. pinnata* seed contained almost exclusively (96%) C-Se-C forms, the same forms in the seed embryo (Table 2, *S. pinnata* spectra 0 and 1). On the other hand, the chalcid wasp larva that emerged from *S. pinnata* seeds contained only 46% C-Se-C forms in tissues and no C-Se-C forms were detected in the midgut (Table 2). A large fraction (43–57%) of the Se in the larva was Se-diglutathione (Se(GSH)2) and 10–16% was selenite (Table 2). The Se XANES spectrum obtained from the midgut also indicated the presence of SeCystine (29%), however this compound did not have a very good fit likely due to the relatively low Se concentrations. In the adult chalcid wasp the Se speciation varied somewhat between thorax and abdomen (Table 2). The Se XANES spectrum collected at the thorax contained 70% C-Se-C, while the abdominal spectrum had only 28% C-Se-C; furthermore, the abdomen contained SeCys (25%) and trace levels of SeCystine (8%) while these compounds were not detected in the thorax. Both thorax and abdomen also contained fairly large fractions of Se(GSH)2 (21–29%) and trace levels of selenite (8–9%).

Micro-XRF mapping of the seed chalcid wasp and seed beetle adults after emerging from the *A. bisulcatus* seed pods demonstrated that Se was present throughout the insects (Fig. 5). In the seed chalcid wasp, the Se did not appear to be concentrated in any particular area, but in the beetle Se was apparently accumulated in the hind gut (Fig. 5D). Zinc was concentrated in the mandibles of both animals, and in the intestine (Fig. 5B, 5D). Unfortunately, the Se signal from the seed chalcid wasp adult was too low to obtain XANES spectra. The Se in the seed beetle adult, however, was concentrated enough for spectra to be obtained in three locations, which all gave similar results and demonstrated that the forms of...
Figure 4. Localization of selected elements in seeds of Se hyperaccumulator *S. pinnata* and a seed herbivorous chalcid wasp. Photographs of (a) *S. pinnata* seed, (b) seed chalcid wasp larvae and adults associated with (c) *S. pinnata* seeds. (d) Tricolor-coded μXRF map of the *S. pinnata* seed showing Se (in red), Zn (in green) and Ca (in blue). The Se-rich areas are frass from the seed chalcid wasp larvae (e) Tricolor-coded μXRF
Se were SeCystine (53%), Se(GSH)_2 (34%) and selenite (14%) (Table 2).

To further gain a molecular understanding into how these seed insect herbivores can feed on seeds containing such extremely high levels of Se, ICP-AES analysis was done on seeds and herbivores in order to quantify total Se and S levels and then compare and contrast them to one another. Sulfur was included in the analysis because of its biochemical similarity to Se. Nineteen S. pinnata seed samples were analyzed that were collected from at least one plant each. The seeds contained on average 1,329 mg Se kg\(^{-1}\), with a range between 261 and 3,293 mg Se kg\(^{-1}\) (Table 3). This was in stark contrast to the Se levels in the seed chalcid wasp that had fed on S. pinnata seeds. For example, when compared to the S. pinnata hyperaccumulator plant seeds, the seed chalcid wasp larva and adult contained on average 148- and 28-fold lower Se concentrations at 9 and 47 mg Se kg\(^{-1}\) respectively (Table 3).

Similarly, for A. bisulcatus seeds and its seed insect herbivores a vast difference in Se concentration was observed. Intriguingly, the two herbivore species contained Se concentrations that were three orders of magnitude lower than the Se concentrations in the seeds they had just fed on (Table 3). This study is the first to investigate the form of Se in S. pinnata seeds and the presence of only one form, seleno-cystathionine, is remarkable based on previous research showing multiple forms of Se in other S. pinnata tissues and organs. Leaves of S. pinnata were found earlier to contain a substantial fraction of Se as seleno-cystathionine, but the majority of Se in leaves was MeSeCys [14]. Thus, assuming the speciation data from independent studies using plant material collected at different time points can be compared; Se speciation

**Discussion**

In this study the accumulation, distribution and chemical forms of Se were analyzed in seeds of two Se hyperaccumulators, A. bisulcatus and S. pinnata, as well as in three associated seed herbivore species. Both plant species hyperaccumulated Se to extraordinarily high concentrations throughout the seed embryo and endosperm, however, little Se was detected in the seed coat, as judged from mXRF and EDS analyses. XANES demonstrated that the forms of Se in seeds were C-Se-C, identified as Se-cystathionine in S. pinnata seeds, and previously reported by Nigam and McConnell [20] to be MeSeCys and \(\text{c-Glu-MeSeCys}\) in A. bisulcatus seeds. This study

Figure S. Localization of Se, Zn and Ca in two seed herbivores of Se hyperaccumulator A. bisulcatus, a seed herbivorous chalcid wasp and a seed beetle. (a) Photograph of a chalcid wasp that emerged from A. bisulcatus seed. (b) Tricolor-coded \(\mu\)XRF map of the chalcid wasp showing Se (in red), Zn (in green) and Ca (in blue). (c) Photograph of a seed beetle that emerged from seed of A. bisulcatus. (d) Tricolor-coded \(\mu\)XRF map of the seed beetle showing Se (in red), Zn (in green) and Ca (in blue). The locations where XANES spectra were collected are indicated with numbered circles and results from XANES analyses are displayed in Table 2.
hyperaccumulators can make elemental Se(0) themselves or in the greenhouse [17]. At this point it is not clear whether these the native ecosystem on seleniferous soil, but this was not observed discovered in roots of S. pinnata [31]. 

Distinctively, seeds both contain MeSeCys and-cystathionine. SS: normal sum of squares (quality of fit; 0 = perfect); nd: compound not detectable. No elemental Se⁶ was found in these samples. Note: fractions do not always add up to exactly 100% because the margin of error can be up to 10%.

doi:10.1371/journal.pone.0050516.t002

Table 2. Chemical forms of Se found in herbivore insects, and frass obtained from least squares linear combination fitting of each samples XANES spectra in comparison to standard selenium compounds.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>SS (x10⁻⁴)</th>
<th>SeO₄²⁻</th>
<th>SeO₃²⁻</th>
<th>Se(GSH)₂</th>
<th>SeCysteine</th>
<th>SeCystine</th>
<th>C-Se-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astragalus bisulcatus Seed Insects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4D (0, 1) Seed wasp larva frass inside seed</td>
<td>11</td>
<td>2%</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>96%</td>
</tr>
<tr>
<td>4F (0, 1) Seed wasp larva, gut</td>
<td>19</td>
<td>nd</td>
<td>16%</td>
<td>57%</td>
<td>nd</td>
<td>29%</td>
<td>nd</td>
</tr>
<tr>
<td>4F (2, 3) Seed wasp larva, tissue</td>
<td>5.2</td>
<td>nd</td>
<td>10%</td>
<td>43%</td>
<td>nd</td>
<td>nd</td>
<td>46%</td>
</tr>
<tr>
<td>4GH (0, 1) Seed wasp adult, thorax</td>
<td>4.8</td>
<td>nd</td>
<td>9%</td>
<td>21%</td>
<td>nd</td>
<td>nd</td>
<td>70%</td>
</tr>
<tr>
<td>4GH (2, 3) Seed wasp adult, abdomen</td>
<td>6.3</td>
<td>nd</td>
<td>8%</td>
<td>29%</td>
<td>25%</td>
<td>8%</td>
<td>28%</td>
</tr>
<tr>
<td>Seed Herbivores – Selenium Hyperaccumulator Plants</td>
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</tbody>
</table>

appears to be different in seeds than in leaves of S. pinnata. It is possible that selenocystathionine is selectively translated from leaves and directed into seeds. In A. bisulcatus, however, leaves and seeds both contain MeSeCys and γ-glutamyl-MeSeCys [14,20]. Distinctively, S. pinnata seeds also contained up to 24% elemental Se⁰, which has not been reported in seeds but is often associated with microbial activity [29,30]. Recently, elemental Se⁰ was discovered in roots of S. pinnata and A. bisulcatus when growing in the native ecosystem on seleniferous soil, but this was not observed in the greenhouse [17]. At this point it is not clear whether these hyperaccumulators make elemental Se⁰ themselves or whether it results from the action of endophytic or closely associated microbes. Sun et al. [31] reported for rice grains that SeMet was the major Se species (83% of total Se), and that the grains also contained relatively small fractions of MeSeCys and SeCys. Similar to rice grains, wheat kernels accumulated mainly (72–85%) SeMet [32]. The Se hyperaccumulated in these two species seeds appeared to be mainly organic as well. The finding that the forms of organic Se in seeds of hyperaccumulators can be quite different between these plant species from two different families is of significance, since high concentrations of different selenocompounds have varying levels of Se toxicity, but also a range of antioxidant and anticarcinogenic efficacies when consumed by animals or humans at much lower, nutritionally ideal concentrations [33]. Another difference between rice and S. pinnata or A. bisulcatus was that the Se concentration in rice bran (the seed coat) was 2-fold higher than that in the corresponding polished rice [31], while in the hyperaccumulator plants Se was present mostly in the seed embryos as opposed to the much lower concentrations found in seed coats.

While not the focus of this study, it was an interesting observation that each of the three herbivores showed substantial accumulation of Zn in their mouth parts. This was also observed in the diamondback moth in earlier μ-XRF studies [18]. As mentioned by Schofield et al. [34], Zn and other heavy metals are often found in the mouth parts of arthropods. In the mandibles of leaf-cutter ants Zn accumulated with age, up to 16% of DW. The metals likely function to provide strength to the mouth parts, as mandible hardness was found to be correlated with Zn content [34].

The exact Se levels in the seeds these particular seed herbivores emerged from is not known, but based on the range in Se concentration in seeds collected from this area it appears contained Se levels several orders of magnitude lower than those in the seeds that they fed on (Table 3). This strongly suggests that all three herbivores are Se excluders. Further evidence for this hypothesis is that the frass of the seed chalcid wasp larvae in the S. pinnata seed was much more highly concentrated in Se when compared to the Se in the rest of the seed (Fig. 1). The mechanism of Se exclusion may be fairly Se-specific, although due to the chemical similarity between Se and S, biochemical analogues are thought to be metabolized and transported via similar mechanisms. However, the exclusion of S was 1–2 orders of magnitude less pronounced than that of Se, suggesting Se was specifically

Table 3. Total Se and S concentrations, and Se/S concentration ratios in seeds and seed-herbivores of S. pinnata and A. bisulcatus, as determined by ICP-AES.

<table>
<thead>
<tr>
<th>Sample Description</th>
<th>Se (mg kg⁻¹ DW)</th>
<th>S (mg kg⁻¹ DW)</th>
<th>Se/S</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pinnata seeds (n=22)</td>
<td>1,329±244</td>
<td>12,051±420</td>
<td>0.110</td>
</tr>
<tr>
<td>S. pinnata seed wasp larva (n=6)</td>
<td>9±2</td>
<td>1,574±73</td>
<td>0.006</td>
</tr>
<tr>
<td>S. pinnata seed wasp adult (n=4)</td>
<td>47±6</td>
<td>5,048±2150</td>
<td>0.009</td>
</tr>
<tr>
<td>A. bisulcatus seeds (n=6)</td>
<td>5,750±754</td>
<td>11,837±1486</td>
<td>0.486</td>
</tr>
<tr>
<td>A. bisulcatus seed wasp adult (n=3)</td>
<td>6±1</td>
<td>2,815±347</td>
<td>0.002</td>
</tr>
<tr>
<td>A. bisulcatus seed beetle adult (n=3)</td>
<td>4±1</td>
<td>2,241±101</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data shown is the mean and standard error. Replicate samples were collected from one or more individuals.

doi:10.1371/journal.pone.0050516.t003
excluded by these herbivores. The mechanism of this Se-specific exclusion may be that Se and S occur in different chemical forms, and only the Se form is expelled. Alternatively, Se and S may occur as analogs of the same form, and the Se-containing molecules are somehow differentiated by the transporter from the other analogs by molecular weight or atomic size differences.

Investigation of the chemical forms of Se found that the three herbivores converted between 30–100% of the ingested C-Se-C forms into other organic and inorganic forms of Se, including SeCyS, SeCysteine, SeGSH2, and selenite. These forms are thought to be more toxic to insects than the ingested C-Se-C forms [18]. Apparently the overall levels of these individual Se-compounds in the insects were sufficiently low so as not to cause visible Se toxicity. In order to better elucidate this, further studies may focus on directly comparing different forms of Se and their relative toxicities to insects or their cell lines in growth media.

These results are of interest because they provide new evidence that Se hyperaccumulating plants live in symbiosis with a range of Se-resistant ecological partners, including these novel Se-excluding seed insect herbivores. In an earlier study by Freeman et al. [18] the mechanism of Se-resistance in a diamondback moth herbivore of S. pinnata was hypothesized to be the ability to keep ingested MeSeCyS in its parent form, or rather the inability to demethylate it into the more toxic, potentially protein-accumulated form MeSeCyS. This may enable the Colorado population of diamondback moth to tolerate Se levels in its tissues up to 250 μg Se g−1 DW [18]. In all three seed herbivore insect species investigated in the current study, the apparent Se resistance mechanism appears to be the ability to actively exclude Se from bio-accumulating in their tissues and to excrete Se as a waste in frass. While the herbivores did metabolize the ingested MeSeCyS, γ-glutMeSeCyS or selenocystathionine from the host plant seeds to relatively more toxic forms, they excluded these Se forms from their tissues, and in at least one case concentrated Se to much higher levels in frass.

Our findings demonstrate the presence of a previously unreported Se-resistance mechanism for the ecological partners of these Se hyperaccumulator plants. Furthermore, the finding that these three seed insect herbivore species do not accumulate substantial Se levels has important implications for their potential to form a Se portal which could move Se up into higher trophic levels. Based on these data their relatively low Se concentrations are not expected to lead to any biomagnification of Se in their predators.

### Supporting Information

**Figure S1** Liquid Chromatography Mass Spectrometry (LC-MS) chromatograms from 50 mM HCL extracts of two replicate batches of S. pinnata seeds collected at Pine Ridge Natural Area, identifying the only detectable Se-compound as selenocystathionine (Mw 270 [M+H]).

**Material S1** EDS spectra obtained from A. bisulcatus and S. pinnata seeds, as well as from seed chalcids and bruchid beetles that emerged from such seeds. (PDF)

### Acknowledgments

We thank Todd Gilligan and Boris Kondratieff for help with identification of the herbivores.

### Author Contributions

Conceived and designed the experiments: JLF. Performed the experiments: JLF SM CQ JD EAHP. Analyzed the data: JLF SF MM JD CQ SM. Contributed reagents/materials/analysis tools: JLF SM SQ JD EAHP. Wrote the paper: JLF EAHP.

### References


13. Neuhierl B, Bock A (1996) On the mechanism of selenium tolerance in selenium-resistant ecological partners, including these novel Se-excluding seed insect herbivores. In an earlier study by Freeman et al. [18] the mechanism of Se-resistance in a diamondback moth herbivore of S. pinnata was hypothesized to be the ability to keep ingested MeSeCyS in its parent form, or rather the inability to demethylate it into the more toxic, potentially protein-accumulated form MeSeCyS. This may enable the Colorado population of diamondback moth to tolerate Se levels in its tissues up to 250 μg Se g−1 DW [18]. In all three seed herbivore insect species investigated in the current study, the apparent Se resistance mechanism appears to be the ability to actively exclude Se from bio-accumulating in their tissues and to excrete Se as a waste in frass. While the herbivores did metabolize the ingested MeSeCyS, γ-glutMeSeCyS or selenocystathionine from the host plant seeds to relatively more toxic forms, they excluded these Se forms from their tissues, and in at least one case concentrated Se to much higher levels in frass.

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