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Selenium accumulation protects plants from herbivory by Orthoptera via toxicity and deterrence

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Summary

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- To investigate whether selenium (Se) accumulation in plants provides a chemical defense against generalist insect herbivores, the feeding preference and performance of a mix of orthopteran species were investigated.
- The selenium hyperaccumulator *Stanleya pinnata* and accumulator *Brassica juncea* were used in herbivory studies in the laboratory, and *S. pinnata* was also used in a manipulative field experiment.
- In laboratory studies, both crickets and grasshoppers avoided plants pretreated with selenate, while those given no choice died after eating leaves with elevated Se (447 ± 68 and $230 \pm 68 \mu\text{g Se g}^{-1}$ DW, respectively). *B. juncea* has previously been shown to accumulate selenate, while *S. pinnata* hyperaccumulates methylselenocysteine. Thus, these findings demonstrate that both inorganic and organic forms of selenium protect plants from herbivory. Grasshoppers fed *S. pinnata* contained methylselenocysteine in their midgut and absorbed this form into surrounding tissues. In a manipulative field experiment, methylselenocysteine protected *S. pinnata* from invertebrate herbivory and increased its long-term survival rate over an entire growth season.
- In native habitats of selenium hyperaccumulators, orthopterans represent a major group of insect herbivores. Protection offered by organic selenium accumulation against these herbivores may have promoted the evolution of selenium hyperaccumulation in plants.

Key words: *Brassica juncea*, deterrence, elemental defense, herbivory protection, Orthoptera, selenium (Se) hyperaccumulator, *Stanleya pinnata*, toxicity.

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Introduction

The discovery that certain plants contain extraordinarily high concentrations of trace elements dates back to the beginning of biogeochemical prospecting. In 1865 F. Risse found unusual zinc (Zn)-accumulating plants growing near the German–Belgium border, which accumulated 1.5% Zn on a dry weight (DW) leaf tissue basis (Sachs, 1865). This observation created an interest in using certain plants to indicate the presence of metalliferous soils. Seventy years later,

selenium (Se) hyperaccumulators in the genus *Astragalus*, which accumulated up to 0.6% Se in their shoot tissues, were identified in the USA (Byers, 1935). In the 1950s and 1960s, Helen Cannon, of the United States Geological Survey, plotted the distribution of hyperaccumulator *Astragalus* plants, which are indicators of Se in the western USA and normally grow associated with Se-enriched soils derived from the Morrison Formation (Emmons *et al.*, 1896; Cannon, 1960). Selenium hyperaccumulators accumulate Se preferentially over S and thus have a higher Se to S discrimination coefficient

(Se/S > 1) (Feist & Parker, 2001). Hyperaccumulator species typically have a more than 100-fold higher Se concentration than other species growing on the same site, and can accumulate Se in the range of 2000–16 000 $\mu\text{g g}^{-1}$ shoot DW (Terry *et al.*, 2000; Sors *et al.*, 2005; Galeas *et al.*, 2007). Most plant species cannot tolerate high concentrations of Se in their tissues and contain < 25 $\mu\text{g g}^{-1}$ Se DW, even on high-Se soils (White *et al.*, 2004).

While Se is essential for many organisms at low concentrations as an essential component of certain seleno-enzymes and tRNAs (Stadtman, 1990; Fu *et al.*, 2002), Se is toxic at elevated concentrations. The chemical similarity of Se to sulfur (S) leads to the nonspecific replacement of S by Se in proteins and other S compounds, causing toxicity (Stadtman, 1990). Selenium deficiency and toxicity are commonly reported problems worldwide (Terry *et al.*, 2000). Although Se has not been proven to be essential for higher plants (Fu *et al.*, 2002; Novoselov *et al.*, 2002; Sors *et al.*, 2005), plants do take up and assimilate Se. Certain specialized Se hyperaccumulator species even accumulate Se up to 1% of DW (Byers, 1935; Brown & Shrift, 1982; Feist & Parker, 2001; Sors *et al.*, 2005; Galeas *et al.*, 2007). In addition to Se, plant hyperaccumulation has now been reported for As, Cd, Co, Cu, Mn, Ni, Pb, and Zn (Reeves & Baker, 2000; Guerinet & Salt, 2001). Initial evolutionary steps leading to hyperaccumulation likely included inadvertent uptake and tolerance to elevated concentrations of a particular element. Metal tolerance in plants is known as a biological prerequisite for metal hyperaccumulation (Reeves *et al.*, 1981; Boyd & Martens, 1992). Several hypotheses have been proposed to explain why some plants evolved over time to hyperaccumulate such extraordinarily high concentrations of metals or metalloids. Hypothesized advantages conveyed by elemental accumulation are elemental allelopathy, drought resistance, elemental tolerance and protection from herbivory or infection (Reeves *et al.*, 1981; Boyd & Martens, 1992).

Consumption of Se-hyperaccumulating plants, commonly termed locoweeds, causes livestock disease and death through chronic or acute Se poisoning, called alkali disease and blind staggers (Draize & Beath, 1935; Rosenfeld & Beath, 1964; Cosgrove, 2001). This may suggest a role for Se in plant defense from vertebrate herbivory. In further support of the hypothesis that the functional significance of Se hyperaccumulation is protection from herbivory, laboratory studies have shown that supply of selenate (SeO_4^{2-} , the predominant form of bioavailable Se in soils) protects plants from herbivory by larvae of the moth *Spodoptera exigua*. The animals showed a preference to feed on artificial diet without selenate, and selenate was toxic at concentrations of *c.* 50 $\mu\text{g Se g}^{-1}$ DW in artificial media (Trumble *et al.*, 1998; Vickerman & Trumble, 1999). Alfalfa leaves that accumulated selenate caused reduced growth and survival in *S. exigua* (Vickerman & Trumble, 1999). Feeding deterrence by plant Se to caterpillars was also found for the cabbage looper (*Trichoplusia ni*), which

preferred to feed on *Brassica juncea* (Indian mustard) plants without Se rather than on plants containing selenate at 465 $\mu\text{g g}^{-1}$ DW (Bañuelos *et al.*, 2002). Also, *B. juncea* containing selenate was better defended against herbivory by larvae of the cabbage white butterfly, *Pieris rapae* (Hanson *et al.*, 2003). *Brassica juncea* plants supplied with selenate also had enhanced resistance to two phytopathogenic fungi: the *Brassica*-specific leaf pathogen *Alternaria brassicicola* and a general root/stem pathogen *Fusarium* species (Hanson *et al.*, 2003). Accumulation of Se was also shown to protect plants against green peach aphids, *Myzus persicae* (Hurd-Karrer & Poos, 1938; Hanson *et al.*, 2004). Intriguingly, in one instance, Se accumulation made plants more preferable to herbivory, by the land snail *Mesodon ferrissi* (Hanson *et al.*, 2003). *Brassica juncea* is considered a secondary Se accumulator, that can accumulate up to *c.* 1000 $\mu\text{g Se g}^{-1}$ shoot DW; when supplied with selenate it accumulates Se primarily as selenate (Bañuelos *et al.*, 1997; de Souza *et al.*, 1998). Hyperaccumulators, on the other hand, predominantly accumulate the organic selenocompound methyl-selenocysteine (MeSeCys) (Shrift & Virupaksha, 1965; Pickering *et al.*, 2000, 2003; Freeman *et al.*, 2006a). All plants can produce volatile selenocompounds (Terry *et al.*, 2000), which have a strong pungent odor that may play a role in the observed herbivory deterrence.

The objective of this study was to investigate whether both inorganic selenate and organic methyl-selenocysteine (MeSeCys) accumulation in plants protects them against herbivory by different orthopteran species. There is some evidence that one species from this order normally feeds on Se hyperaccumulators: in 1938 *Melanoplus bivittatus* (Say) was found principally eating the Se hyperaccumulator *Astragalus bisulcatus* (Byers, 1938). The authors concluded that this grasshopper showed evidence of Se tolerance, because it accumulated up to 78 $\mu\text{g g}^{-1}$ DW Se in its tissues, which is lethal to mammals. The protective effect of hyperaccumulation against orthopteran herbivory has so far only been studied for Zn and Ni. Hyperaccumulation of both Zn and Ni were shown to protect hyperaccumulator plants from herbivory by grasshoppers (Pollard & Baker, 1997; Jhee *et al.*, 2005).

Materials and Methods

Plant material and growth

Brassica juncea (L.) Czern & Coss (Indian mustard, accession no. 173874, Brassicaceae) was obtained from the North Central Regional Plant Introduction Station (Ames, IA, USA). *B. juncea* has been previously used as a model Brassicaceae species in herbivory experiments testing the protective effect of Se because it is a good secondary accumulator of Se and highly palatable (Bañuelos *et al.*, 1997; Hanson *et al.*, 2003, 2004). The *B. juncea* seeds were germinated on vermiculite. The plants were grown for 1 month under glasshouse conditions at 25°C and a 16 h photoperiod, supplied with

half-strength Hoagland's nutrient solution (Hoagland & Arnon, 1938) One month after germination the (+) Se treatment was started. Plants of equal sizes were placed into two groups ($n = 50$); 20 μM sodium selenate was supplied in the nutrient solution to the (+) Se test group, while nutrient solution without (-) Se was supplied to the control group. After 7 d of treatment, the plants were used for herbivory experiments as described below.

Stanleya pinnata (Pursh) Britton (Prince's plume, Brassicaceae) was obtained from two different sources: Western Native Seed (WNS; Coaldale, CO, USA) and Plants of the South-west (PoSW; Santa Fe, NM, USA). The WNS accession originates from Colorado and has a twofold higher Se accumulation ability than the PoSW accession, which originates from New Mexico (Feist & Parker, 2001). Seeds from the two accessions were germinated on filter paper and then seedlings were transferred to soil. The plants were grown in Scott's Metro Mix-350 at 24 : 20°C, 10 : 14 h light : dark, 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PPF. The plants were watered every other day with half-strength Hoagland's nutrient solution. After 1 month, plants of equal sizes were placed into two groups ($n = 40$) for each accession. For the laboratory experiments, a test group of each accession received 40 μM $\text{Na}_2\text{SeO}_4^{2-}$ (high Se) twice weekly in nutrient solution, while 2 μM $\text{Na}_2\text{SeO}_4^{2-}$ was supplied to the low-Se control group. The low-Se treatments were given instead of no Se, because without any Se, the hyperaccumulator plants did not grow as well and were more susceptible to glasshouse pests. After 12 wk of treatment the plants were used for the laboratory experiments to be described. For the manipulative field experiment the plants were treated twice weekly with high Se (40 μM $\text{Na}_2\text{SeO}_4^{2-}$) or low Se (2 μM $\text{Na}_2\text{SeO}_4^{2-}$) for 8 wk before transplantation into the field.

Orthoptera material

Brown crickets (*Acheta domestica* L.) were chosen for the initial study because they are generalist orthopteran herbivores with chewing mouthparts. The crickets were obtained from a local pet store. The grasshoppers used were collected in bulk using sweepnets from the same field site (northwest of Fort Collins, CO, USA) described in the manipulative field study (see next section). The collection contained individuals from the following genera: Amphitornus, Arphia, Aulocara, Cordillacris, Dissosteira, Hesperotettix, Melanoplus, Mermiria, Spharagemon, Trachyrhachys and *Trimerotropis*. This mixture of Orthoptera species collected from a Se hyperaccumulator habitat was used to simulate Orthoptera herbivory experienced by these plants under natural conditions.

Cricket experiments

Choice feeding experiment In 250 ml aerated plastic containers, two freshly cut 2.5 × 2.5 cm *B. juncea* leaf sections were

placed at opposing ends in the containers, one from a plant pretreated with Se as described earlier, and one without Se. The +Se leaves contained $546 \pm 38 \mu\text{g Se g}^{-1} \text{ DW}$ while the -Se leaves contained nondetectable (ND) Se concentrations (detection limit $c. 1 \mu\text{g Se g}^{-1} \text{ DW}$). Wet cheesecloth was used to line the bottom of each container to keep the leaf sections moist, and to serve as a source of water for the crickets. There were no visible differences between the plants treated with Se and those that were not. In each container, one cricket was placed in the center between the leaf sections ($n = 24$). The orientation of the containers was randomized. After 1 h the initial feeding choice was scored.

Nonchoice experiment In 250 ml aerated plastic containers with wet cheesecloth lining the bottoms, crickets of equal size were placed individually into either the test or the control group ($n = 10$). The crickets were weighed at day 0 and given one intact *B. juncea* leaf from a plant pretreated with Se ($447 \pm 68 \mu\text{g Se g}^{-1} \text{ DW}$) or not treated with Se (containing no detectable Se, Fig. 1d); these leaves were replaced daily. At the end of 11 d the crickets were weighed and collected for Se analysis as described later. The plant material fed to the crickets was also analyzed for Se.

Grasshopper experiments

Choice feeding experiment Grasshoppers from the mix described previously were collected from the field ($n = 100$). Grasshopper adults in addition to third, fourth and fifth instar nymphs were hand-selected and put into a metal screened cage (23 × 76 cm) with *S. pinnata* plants (accession WNS) that had been treated with a high (40 μM) or low (2 μM) Se concentration of Na_2SeO_4 ($n = 15$ plants each). *S. pinnata* (WNS) high-Se plants (pretreated with 40 μM Na_2SeO_4) had tissue concentrations of $230 \pm 68 \mu\text{g Se g}^{-1} \text{ DW}$, while low-Se (2 μM) plants had $1 \pm 33 \mu\text{g Se g}^{-1} \text{ DW}$. After 7 d, the percentage of each individual plant eaten was carefully estimated by three independent researchers, by visually comparing the eaten plants to control plants that had not been placed in a cage.

Nonchoice experiment In total, 140 grasshoppers from the 11 genera listed earlier were collected in the field and ($n = 70$) were put into each of the two separate screened cages (23 × 76 cm) with *S. pinnata* WNS and PoSW plants treated with either high Se (40 μM) or low Se (2 μM) ($n = 13$ plants each). The *S. pinnata* plants (accession PoSW) pretreated with a high Se concentration (40 μM) had tissue concentrations of $145 \pm 38 \mu\text{g Se g}^{-1} \text{ DW}$, while low-Se plants (pretreated with 2 μM) contained $3 \pm 0.6 \mu\text{g Se g}^{-1} \text{ DW}$. After 7 d the percentage of each plant eaten was recorded as described earlier. Grasshopper molts, and the numbers of dead grasshoppers in each cage were counted. The grasshoppers were collected for Se analysis as described later.

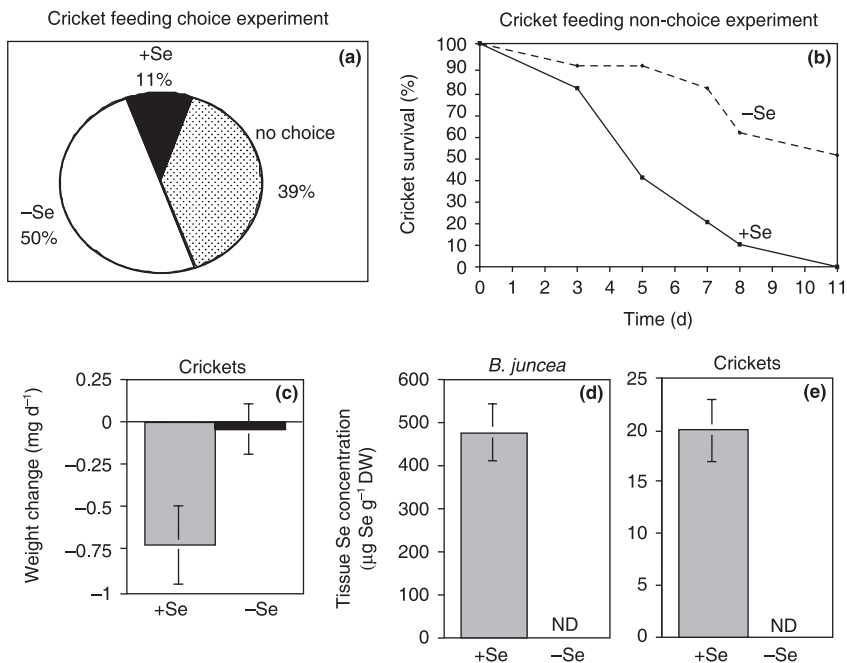


Fig. 1 Cricket (*Acheta domestica*) choice and nonchoice feeding experiments. (a) Crickets preferred to feed on leaves that did not contain selenium (Se) when given a choice between leaf sections from *Brassica juncea* plants grown with (+) Se or without (-) Se (chi-squared test, $\alpha = 0.05$). (b–e) In a nonchoice feeding experiment, crickets that were fed *B. juncea* leaves containing (+) Se showed lower survival (b) and greater weight loss (c) than those that fed on leaves that did not contain (-) Se (*t*-test, $P < 0.05$). (d, e) Plant and animal tissue Se concentration in the nonchoice experiment. Values are means \pm SE. ND, nondetectable.

μ -XRF/ μ -XAS

After having fed exclusively on high-Se *S. pinnata* plants, the grasshoppers that were still alive were lethargic and had dark-colored bands in their muscular tissues that we attributed to Se toxicity. These survivors were flash-frozen in LN₂ and shipped on dry ice to the Advanced Light Source at Lawrence Berkeley National Laboratory for micro-XAS analysis on Beamline 10.3.2 (Marcus *et al.*, 2004). The spatial distribution of Se in the animal's abdomen was imaged by scanning the grasshopper in the focused X-ray beam at 13 000 eV. An intact frozen insect was mounted on to a Peltier stage using a small amount of silicone grease, and kept at -33°C to reduce radiation damage. Microscanning X-ray fluorescence (μ -SXRF) mapping of Se was performed on one representative grasshopper abdomen. This map used a $7 \times 7 \mu\text{m}$ beam at 13 000 eV sampled in $5 \times 5 \mu\text{m}$ pixels. Micro-X-ray absorption near-edge structure (μ -XANES) at the Se K-edge was performed as described by Pickering *et al.* (2000) at two locations on a grasshopper midgut (Fig. 4a, Spot #1, $n = 6$ scans), and at two locations on the immediately surrounding tissues (Fig. 4a, Spot #2, $n = 6$ scans), to gain insight into the molecular speciation of Se at specific spots. Aqueous solutions of various selenocompounds listed in Freeman *et al.* (2006a) were again used as standard materials for generating reference spectra.

Manipulative field experiment

Ten field test plots were planted with *S. pinnata* plants grown in a glasshouse and pretreated with high or low Se concentrations.

Each test plot contained four high-Se plants (two from accession WNS and two from accession PoSW) pretreated for 8 wk with high Se concentration ($40 \mu\text{M Na}_2\text{SeO}_4$), and four low-Se plants (two from each accession) pretreated for 8 wk with $2 \mu\text{M Na}_2\text{SeO}_4$. The 10 groups of eight plants were planted in $1 \times 1 \text{ m}$ plots each, alternating high- and low-Se pairs within each group. There was at least 10 m between each group of eight plants, and the total area harboring the 10 plots was $100 \text{ m} \times 200 \text{ m}$. The planting site was a grassy area in northwest Fort Collins, CO, USA that contained the Se hyperaccumulator species *A. bisulcatus*; *S. pinnata* was located close by, but off site. The site also sustained a variety of native grasses and several other herbaceous dicotyledon species. *S. pinnata* plants averaging 12 large leaves were transplanted from the glasshouse at the end of May 2005. All *S. pinnata* plants survived the transplant. The planting site was observed to support a large population of grasshoppers. Little mammalian herbivory or presence was observed in this area. As a control, a wooden-framed mammal exclusion cage with 1.8 cm^2 wire mesh covered one test plot in the middle of the area. Herbivory rates were the same inside and outside of this cage (DNS). The grasshoppers appeared to be eating *S. pinnata* plants at the same rate inside and outside the mammal exclusion cage. Based on the observed grasshopper abundance on the site, together with the observed herbivory patterns on the leaves, typical for chewing mandibles and matching those observed in the laboratory grasshopper experiments, we concluded that grasshoppers were the major herbivores of *S. pinnata* on these plots during this study. *S. pinnata* shoot herbivory was measured on all plots through the majority of the growing season (at 0, 1, 3, 5, 7, 9 and 16 wk), as was total

grasshopper abundance. At the end of the season, leaf samples were taken to analyze plant Se concentrations. Total plant survival percentages were calculated from the number of live remaining plants at the end of the season.

Selenium quantification in plant and insect tissues

Plant and insect tissue samples were rinsed with distilled water and dried at 50°C for 48 h. Individual animals or 100 mg leaf samples were acid-digested according to Zarcinas *et al.*, 1987) and analyzed for Se by inductively coupled plasma atomic emission spectrometry (ICP-AES) according to Fassel (1978).

Data analyses

Statistical analyses, chi-square test, analysis of variation and Student's *t*-test were performed using the software package JMP-IN (version 3.2.6) from the SAS institute (Cary, NC, USA). X-ray absorption spectroscopy data analysis was performed using a suite of LabView programs (National Instruments) available at beamline 10.3.2 and freely available at <http://xraysweb.lbl.gov/uxas/Beamline/Software/Software.htm>

Results

Selenate accumulation in *B. juncea* and its effects on cricket herbivory

A choice feeding experiment was conducted in which individual crickets were placed between two recently cut leaf sections, one from a plant grown with selenate and one grown without. Nearly five times as many crickets chose to feed on the leaf that contained no Se compared with those that fed on the leaf containing Se (Fig. 1a). In order to test the toxicity of plant Se to crickets, a nonchoice feeding experiment was performed in which half of the crickets were fed leaf material containing elevated (+) Se and the other half were fed leaf material that did not contain (-) Se (Fig. 1d). The crickets on the high (+) Se leaves showed a higher mortality rate throughout the experiment, and all 24 died by the end of the experiment (Fig. 1b). The crickets on the high (+) Se leaves lost significant weight during the experiment (*c.* 10% or 0.74 ± 0.24 mg d⁻¹), while those that were not fed Se did not (Fig. 1c). The crickets that fed on the (+) Se-rich plant material accumulated 20 ± 3 μg Se g⁻¹ DW, which likely led to the observed toxicity (Fig. 1e).

MeSeCys accumulation in *S. pinnata* and its effects on grasshopper herbivory

For further investigations, the Se hyperaccumulator plant *S. pinnata* was used, which in the wild can accumulate up to 3000 μg Se g⁻¹ DW in its shoot. To test the hypothesis that the organic MeSeCys in *S. pinnata* protects it from grasshopper

herbivory via deterrence and toxicity, plants treated in the laboratory with high and low Se were used in choice and nonchoice grasshopper herbivory experiments.

The leaf Se concentration in the *S. pinnata* plants used for these (and subsequent) laboratory experiments is reported in the Materials and Methods section and shown in Fig. 2(a). For 1 wk grasshoppers were given a choice to feed on *S. pinnata* plants with high or low Se, and consumed significantly less from the high-Se plants, for both *S. pinnata* accessions (Fig. 2b). After 9 d, all the leaves from the low-Se plants were completely eaten by the grasshoppers, as shown in Fig. 2(c), while the high-Se plants were virtually untouched. To test whether plant accumulation of MeSeCys has a protective effect against herbivory as a result of toxicity, a nonchoice herbivory experiment was conducted. Grasshoppers provided with plants containing high Se concentrations (229 ± 70 μg g⁻¹ DW) consumed less than 10% of the total plant leaf area, while those given low-Se plants ate *c.* 70% of the total leaf area in 7 d (*t*-test, *n* = 13, *P* < 0.05, Fig. 3a). Grasshoppers that fed on leaves with high Se accumulated significantly more Se in their tissues than animals that fed on low-Se plants (Fig. 3b). The majority of grasshoppers that ate high-Se leaves died during the experiment, indicating that the 12 ± 2 μg Se g⁻¹ DW they accumulated was lethal. Indeed, grasshoppers fed *S. pinnata* plants with high Se concentrations for a week had a survival rate of only 10%, while 70% of grasshoppers fed *S. pinnata* plants with a low Se concentration survived. Moreover, no molts were produced in the cage with the high-Se plants, while 47 molts were produced by the 70 animals in the low-Se plant cage, suggesting that Se affected the animals' growth and development.

Selenium mapping and speciation in grasshoppers using μ-XRF and μ-XAS

While the storage location and form of Se accumulated in *S. pinnata* are known (Freeman *et al.*, 2006a), the form of Se that is accumulated and causes toxicity in grasshoppers fed *S. pinnata* was unknown. The spatial Se distribution in the abdomen of the grasshoppers as well as the chemical forms of Se accumulated by the animals inside and immediately around the midgut were investigated using μ-SXRF and μ-XANES, respectively. μ-SXRF of Se in grasshoppers fed high-Se *S. pinnata* revealed that Se was present in the upper abdomen at a high concentration in what appears to be the midgut, and at a lower concentration in surrounding tissues (Fig. 4a). To determine which form of Se was being digested in the grasshopper midgut and accumulated in its tissues, we performed Se K-edge XANES. When compared with standard Se compounds, this technique provides information about the chemical form of Se. μ-XANES spectra were collected at four different locations on the grasshopper as indicated by the black circles in Fig. 4(a). These spectra (Fig. 4b) indicate that in the midgut and the immediately surrounding tissues,

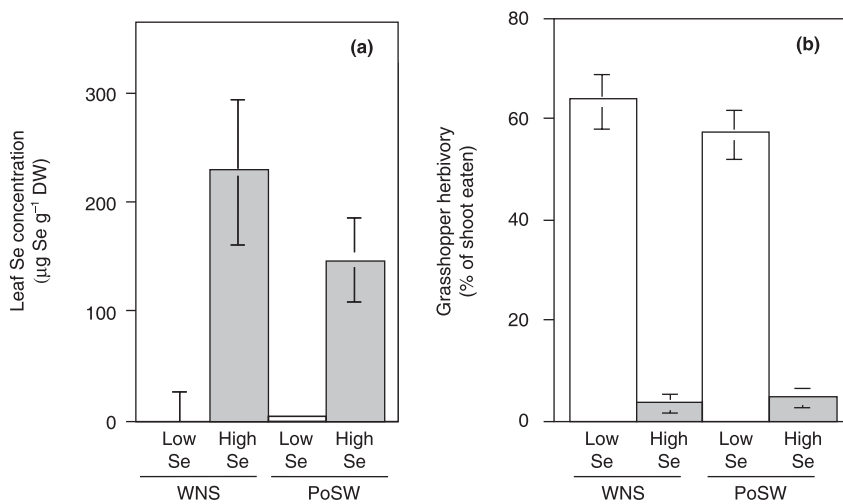


Fig. 2 Grasshopper herbivory choice experiment using *Stanleya pinnata* plants accessions Western Native Seed (WNS) and Plants of the South-west (PoSW) grown with high ($40 \mu\text{M}$) or low ($2 \mu\text{M}$) concentration of selenium (Se). (a) *S. pinnata* shoot tissue Se concentration. (b) Herbivory quantified as percentage of *S. pinnata* shoot eaten over 7 d for *S. pinnata* accessions WNS and PoSW pretreated with high or low Se concentration. (c) Ultimate grasshopper herbivory results pictured after 9 d for WNS *S. pinnata* with low or high Se concentration. Values are means \pm SE.

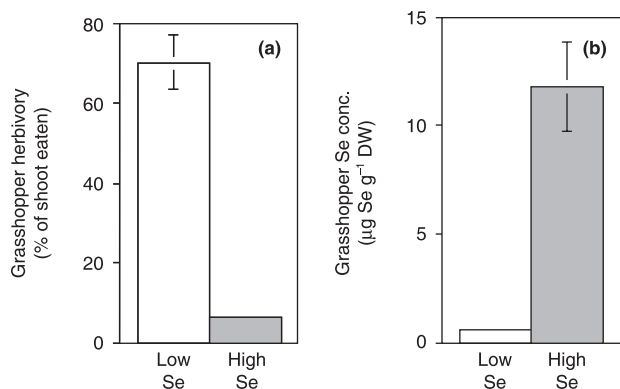
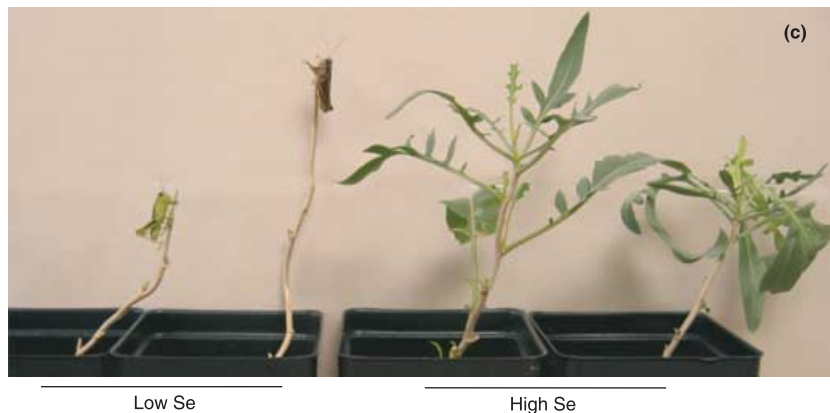


Fig. 3 Grasshopper nonchoice herbivory experiment using *Stanleya pinnata* plants accession Western Native Seed (WNS) pretreated with high ($40 \mu\text{M}$) or low ($2 \mu\text{M}$) selenium (Se) concentration. (a) Herbivory quantified as percentage of *S. pinnata* shoot eaten after 7 d. (b) Grasshopper tissue Se concentration after feeding on low- or high-Se plants. Values are means \pm SE.

MeSeCys constituted 99 and 97% of the total Se, respectively. The remaining Se was inorganic selenate.

Manipulative field experiment gives evidence of grasshopper herbivory protection by Se in the native hyperaccumulator *S. pinnata*

In the laboratory experiments, *S. pinnata* was protected by a relatively low concentration of Se compared with the $3000 \mu\text{g Se g}^{-1} \text{ DW}$ often accumulated by these plants in the field. Based on our laboratory studies it can be hypothesized that protection from insect herbivory is a major ecological advantage of Se hyperaccumulation in the field. To test this hypothesis, *S. pinnata* plants containing high or low Se concentrations were planted on a field site, in a Se hyperaccumulator habitat, in which grasshoppers were likely to be a dominant herbivore (see Materials and Methods section for experimental details). During the course of the growing season, the plants pretreated with Se continued to accumulate Se (Fig. 5a, high Se). The source of the accumulated Se was likely the glasshouse soil attached to the roots during planting, because the -Se plants

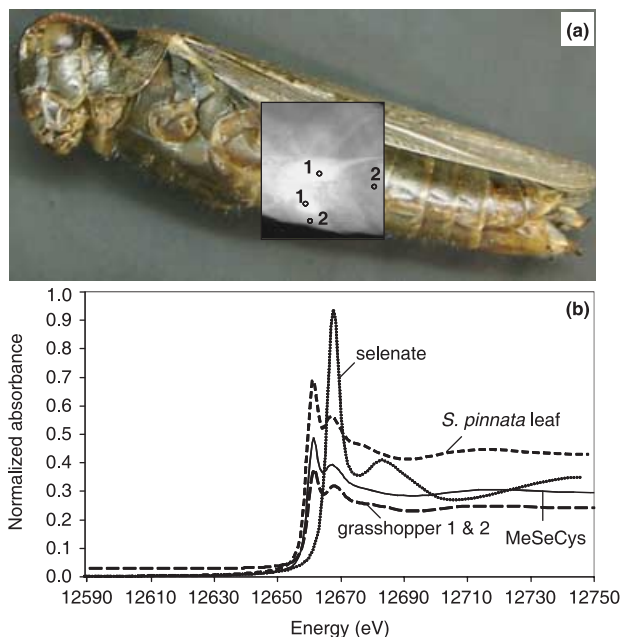


Fig. 4 Scanning X-ray fluorescence mapping (μ -SXRF) and speciation of selenium (Se) in a grasshopper abdomen. (a) Shown is spatial distribution of total Se, imaged in white for the inset. Black circles show the locations where microfocused X-ray absorption near-edge structure (μ -XANES) spectra were collected at Se K-edge, to investigate the chemical forms of Se being digested in the midgut (1) and accumulated in the surrounding tissues (2). (b) The XANES spectra collected in fluorescence mode on *S. pinnata* leaves and both grasshopper locations along with selenate and methyl-selenocysteine (MeSeCys) standards.

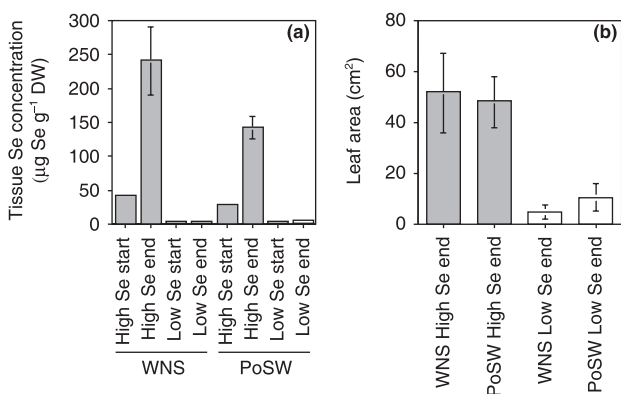


Fig. 5 Manipulative field experiment using *Stanleya pinnata* Western Native Seed (WNS) and Plants of the South-west (PoSW) pretreated with high ($40 \mu\text{M}$) or low ($2 \mu\text{M}$) selenium (Se) concentration, before transplanting to the field for long-term herbivory tests over an entire growing season. (a) *S. pinnata* leaf Se concentration at the start and end of the 16 wk season. (b) The leaf area at the end of the 16 wk growing season, reflecting herbivory. Values are means \pm SE.

did not accumulate more Se over this period of time (Fig. 5a, low Se). The leaf area per plant at the end of the growing season was measured as a parameter of herbivory. For both of the *S. pinnata* accessions used, the final leaf area was fivefold

higher for the high-Se plants than for the low-Se plants ($P < 0.05$, ANOVA, Fig. 5b), indicating that the Se did indeed protect the plants from herbivory in the field.

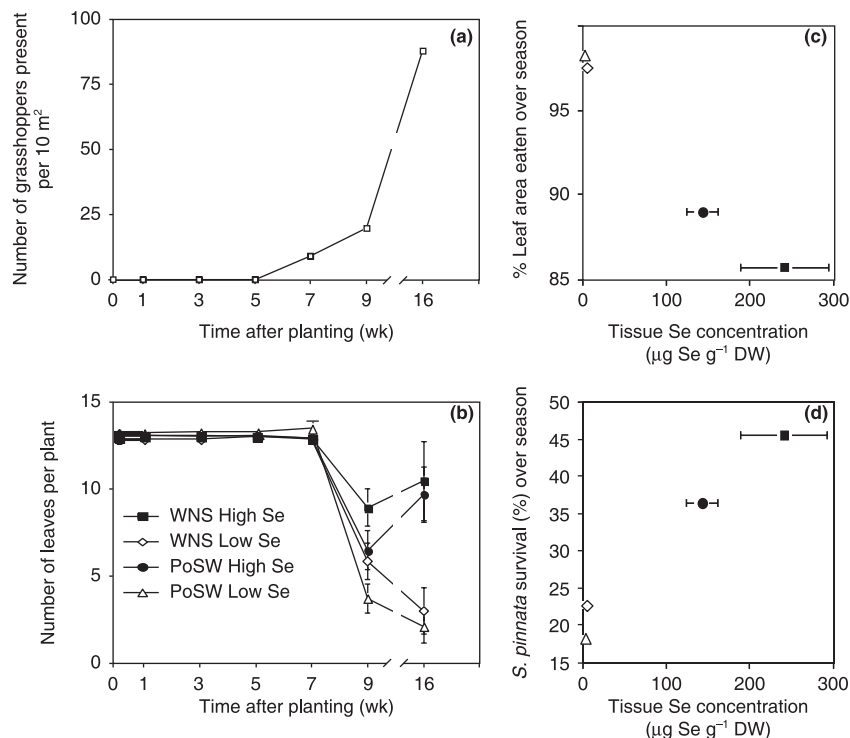
The number of grasshoppers increased dramatically from week 5 through week 16 (Fig. 6a). This increase in grasshopper abundance coincided with a pronounced drop in *S. pinnata* leaf number, which had remained relatively steady earlier that season as a result of the slow growth of hyperaccumulators, as well as the high summer temperature and arid conditions (Fig. 6b). Both accessions of *S. pinnata* pretreated with a high Se concentration ended the season with a significantly higher leaf number and a lower percentage of leaf area eaten than their low-Se-treated counterparts (Fig. 6b,c). High-Se plants also showed a higher rate of survival (Fig. 6d). Interestingly, the *S. pinnata* ecotype WNS, which accumulated *c.* 70% more Se than ecotype PoSW when pretreated with a high Se concentration, showed less herbivory (Fig. 6b,c) and a higher survival rate than PoSW (Fig. 6d).

Discussion

The results described here demonstrate that Se accumulation in *B. juncea* and in the hyperaccumulator *S. pinnata* deterred feeding and was toxic to members of the Orthoptera (crickets and grasshoppers, respectively, for *B. juncea* and *S. pinnata*). *B. juncea* is known to accumulate mainly selenate when supplied with selenate, while *S. pinnata* accumulates mainly MeSeCys. Therefore, this study suggests that both these inorganic and organic forms of Se are efficient at protecting plants from herbivory by these Orthoptera. After feeding on *S. pinnata*, grasshoppers accumulated a form of Se indistinguishable by XANES from MeSeCys in both the midgut and the immediately surrounding tissues. This suggests that they had not biotransformed the MeSeCys, which was the form digested and absorbed. The bioconversion and accumulation of Se in other tissues was not investigated because of the large animal size and limited beam time. However, our data suggest that a small amount of MeSeCys might be transformed into selenate in the tissues immediately surrounding the midgut.

In a manipulative field experiment, Se in *S. pinnata* appeared to protect the plants from herbivory in the field, which was observed to be mainly by Orthoptera: plants that had accumulated more Se showed increased long-term survival and fewer leaves were eaten. The observation that *S. pinnata* ecotype WNS, which accumulated *c.* 1.7-fold more Se than ecotype PoSW, showed less herbivory and a higher survival rate suggests that an intermediate Se concentration may provide an intermediate degree of herbivore protection. Based on the herbivory marks observed and the concomitance of herbivory and grasshopper abundance, it was clear that the observed reduction in plant leaf number and leaf surface area was the result of grasshopper herbivory rather than other herbivores, or leaf senescence. The fact that the low-Se-

Fig. 6 (a) Occurrence and abundance of grasshoppers throughout the field season. (b) Number of leaves present per plant throughout the season on plants of the two *Stanleya pinnata* accessions Western Native Seed (WNS) and Plants of the South-west (PoSW), pretreated with high (40 μM) or low (2 μM) selenium (Se) concentration (*t*-test, $P < 0.05$). Herbivory damage in relation to *S. pinnata* leaf Se concentration across both accessions and treatments, all measured at the end of the season. (c) Percentage of leaf area eaten in relation to shoot tissue Se concentration. (d) Percentage survival of *S. pinnata* in relation to shoot tissue Se concentration. Values shown are means \pm SE.



pretreated plants did not accumulate more Se from the native soil after planting was probably because they continued to take up their nutrients from the transplanted soil rather than the surrounding seleniferous soil; this was fortunate because the relative difference in Se concentrations could have decreased over the course of the experiment.

These results provide strong evidence in support of the elemental defense hypothesis, which states that hyperaccumulation has evolved as a protection against herbivory or infection. Even at tissue concentrations an order of magnitude lower than those commonly found in the field, Se protected both a secondary Se accumulator and a Se hyperaccumulator species from herbivory by various Orthoptera species. These results are of significance for several reasons. First, they shed light on the evolution of the intriguing phenomenon of Se hyperaccumulation. The novelty of our results compared with earlier studies is that this work is the first to include a long-term manipulative field study which provides strong evidence in support of this hypothesis. Previous studies in the field by Martens & Boyd (2002) did reveal a protective effect of Ni against a variety of insect herbivores, but unfortunately these plants were killed by larger mammalian herbivores and thus the end results were difficult to interpret. The observed interactions between these hyperaccumulator plants and these grasshoppers are ecologically relevant, as they coexist on our field site. On the prairies and grasslands of the western United States, grasshoppers represent a large group of voracious insect herbivores (Hewitt *et al.*, 1976; Burlison & Hewitt, 1982), and their herbivory may well have been a selection pressure

that has driven the evolution of Se-hyperaccumulating plants. Second, Orthoptera, especially grasshoppers, are extremely harmful agricultural pests (Burlison & Hewitt, 1982; Capinera & Roltsch, 1980), and the finding that supplying Se to both nonhyperaccumulators and hyperaccumulators protects them from herbivory by different Orthoptera may have applications in both agriculture and phytoremediation. Supply of Se to crop plants may have the beneficial side-effect that they offer added value to the crops. Selenium-fortified crops have been gaining attention lately for their anticarcinogenic properties, and as a source of Se for livestock in Se-deficient areas (Ip & Ganther, 1992; Gupta & Gupta, 2000; Whanger, 2002; Ellis & Salt, 2003).

The cricket *A. domesticus* avoided feeding on *B. juncea* leaves from plants that had been treated with (+) selenate. This deterrence effect may in part be the result of the volatile compound dimethylselenide, which is known to be emitted from selenate-treated *B. juncea* plants (de Souza *et al.*, 1998). Crickets that had no choice but to consume *B. juncea* leaves containing high concentrations of selenate did not grow, but lost *c.* 10% of their body weight over 11 d. Crickets that were fed leaves without Se also lived significantly longer than those that were fed leaves containing elevated Se. In fact, these results were even more pronounced since half of the crickets fed leaves without Se lived much longer than the 11 d time point that was the end of the experiment. Crickets that consumed leaves containing Se had at least 20 times more Se in their tissues than control animals, which contained no detectable Se. It therefore appears that selenate accumulation

in *B. juncea* leaves can protect plants from herbivory by directly causing toxicity in crickets. These results have implications for growing crops in seleniferous soils or amended with selenate, because they suggest that crops enriched in selenate will suffer less herbivory damage by orthopteran species. The total Se concentration in the *B. juncea* leaves used in these experiments ($c. 500 \mu\text{g Se g}^{-1}$ DW) was much lower than that normally found in Se hyperaccumulator species ($2000\text{--}16\,000 \mu\text{g g}^{-1}$ DW) living in nature. Therefore, it would be expected that the four to 32-fold higher Se concentration found in Se hyperaccumulators would result in even better protection.

Similar to selenate, the organic form of Se present in hyperaccumulator plants, MeSeCys, resulted in toxicity to Orthoptera. A large variety of grasshopper species collected from a grassland area in the hyperaccumulator habitat avoided eating leaves from the hyperaccumulator *S. pinnata*, which was shown earlier to contain predominantly MeSeCys and to emit volatile dimethyldiselenide. The striking result from the herbivory choice experiment (shown in Fig. 2c) clearly demonstrates the protection experienced by plants that accumulate Se. The concentrations of Se in *S. pinnata* leaves ($229 \pm 70 \mu\text{g g}^{-1}$ DW) is lower than those used in *B. juncea* for crickets, and $c. 10$ -fold lower than those observed in wild Se hyperaccumulator plants. Our results demonstrate that, even at these low concentrations, the organic Se had a protective effect against grasshopper herbivory. This protection was not only as a result of deterrence but also as a result of toxicity. Grasshoppers given no choice but to feed on *S. pinnata* plants containing high Se concentrations consumed less than 10% of the total plant leaf area and showed only a 10% survival rate, while grasshoppers supplied with low-Se plants consumed $c. 70\%$ of the total leaf area and 70% survived. Grasshoppers that fed on leaves with high Se accumulated significantly more Se in their tissues than animals fed on low-Se plants. The average grasshopper Se concentration of $12 \pm 2 \mu\text{g g}^{-1}$ DW was 20 times lower than that in the leaf material consumed, so there was no evidence of bioaccumulation in the herbivore. Similarly the crickets in this study were killed at tissue concentrations of $20 \pm 3 \mu\text{g Se g}^{-1}$ DW. The observation that this low tissue Se concentration was lethal to these orthopterans indicates that they are fairly Se-sensitive. The total absence of grasshopper molting when fed high-Se plants is further indication of the toxic effect of MeSeCys on growth and development of a variety of grasshopper species. The low lethal tissue Se concentrations observed in these orthopterans are similar to those found for the lepidopteron *Plutella xylostella* (accession G88), which was killed by MeSeCys at $10 \mu\text{g total Se g}^{-1}$ larval DW (Freeman *et al.*, 2006b). Similarly, larvae of the cabbage looper that were fed *B. juncea* containing mainly selenate at $465 \mu\text{g total Se g}^{-1}$ leaf DW were negatively affected at tissue concentrations as low as $2.9 \mu\text{g total Se g}^{-1}$ larval DW (Bañuelos *et al.*, 2002). However, these concentrations are

several-fold lower than those observed in the much larger Se-sensitive lepidopteron *Pieris rapae* (cabbage white butterfly) ($80 \mu\text{g Se g}^{-1}$ larval DW) and the apparently Se-preferring snail *M. ferrissi* ($50 \mu\text{g Se g}^{-1}$ DW in soft tissues, Hanson *et al.*, 2003).

Together these findings show that concentrations of MeSeCys in the Se hyperaccumulator *S. pinnata*, an order of magnitude lower than those typically observed in the field, are already lethal to a mixed collection of native herbivores: grasshoppers collected from areas directly around *S. pinnata* growing in the field. Also, these concentrations of Se efficiently deterred these grasshoppers when other, low-Se plant material was available. These results therefore support the elemental defense hypothesis, specifically the hypothesis that Se hyperaccumulation evolved as a protection from herbivory.

Since fairly low concentrations of Se, around $200 \mu\text{g Se g}^{-1}$ leaf DW, already offered plants protection from grasshopper herbivory, this may explain how natural selection may have favored plants with moderately elevated Se accumulation at the earlier evolutionary stages of hyperaccumulation. This finding may also have implications for Se phytoremediation. In recent phytoremediation field studies, *B. juncea* accumulated $50\text{--}100 \mu\text{g Se g}^{-1}$ DW (Bañuelos *et al.*, 2005, 2007). These concentrations may already offer protection from grasshopper herbivory, similar to results found for aphids (Hurd-Karrer & Poos, 1938; Hanson *et al.*, 2004), the diamondback moth (Freeman *et al.*, 2006b) and the cabbage looper (Bañuelos *et al.*, 2002). As mentioned earlier, fortification of crops with Se for the production of anticarcinogenic selenocompounds is another setting for which these results are relevant, as they suggest that the Se in these crops would protect them from herbivory by a variety of Orthoptera. Incidentally, the observation that these herbivores died before they had a chance to accumulate significant Se suggests that there is not a serious threat of Se moving into the food chain when plants are used as Se-fortified crops or for phytoremediation. Movement of Se into the food chain may only be of significance in natural seleniferous areas where specialist Se-tolerant herbivores and predators have had time to (co)evolve with hyperaccumulators. Such specialists potentially accumulate substantial concentrations of Se, as was recently shown for a population of Se-tolerant diamondback moths and their parasitic, microgastrine-wasp. These Se-tolerant specialists naturally accumulated toxic concentrations of Se ($160\text{--}200 \mu\text{g total Se g}^{-1}$ larva and adult DW) from highly poisonous *S. pinnata* plants growing in the wild ($1246 \mu\text{g total Se g}^{-1}$ leaf DW) without any toxicity. (Freeman *et al.*, 2006b).

In conclusion, these laboratory and field studies support the hypothesis that protection from insect herbivory is a major ecological advantage of Se hyperaccumulation. The protective effect of Se against herbivory was evident even at relatively low concentrations of Se accumulation. Also, accumulation in leaves of two very different forms of Se, inorganic selenate in a nonhyperaccumulator and organic

MeSeCys in a hyperaccumulator, had similar protective effects against herbivory by Orthoptera. Additionally, an intermediate Se concentration caused an intermediate degree of herbivore protection. These results shed light on the possible selection pressures that have driven the evolution of Se hyperaccumulation. They also provide insight into some of the possible implications of the use of Se-enriched plants as agricultural crops or for phytoremediation.

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