

Selenium accumulation in flowers and its effects on pollination

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Summary

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- Selenium (Se) hyperaccumulation has a profound effect on plant–arthropod interactions. Here, we investigated floral Se distribution and speciation in flowers and the effects of floral Se on pollen quality and plant–pollinator interactions.
- Floral Se distribution and speciation were compared in *Stanleya pinnata*, an Se hyperaccumulator, and *Brassica juncea*, a comparable nonhyperaccumulator. Pollen germination was measured from plants grown with varying concentrations of Se and floral visitation was compared between plants with high and low Se.
- *Stanleya pinnata* preferentially allocated Se to flowers, as nontoxic methyl-selenocysteine (MeSeCys). *Brassica juncea* had higher Se concentrations in leaves than flowers, and a lower fraction of MeSeCys. For *B. juncea*, high floral Se concentration impaired pollen germination; in *S. pinnata* Se had no effect on pollen germination. Floral visitors collected from Se-rich *S. pinnata* contained up to 270 $\mu\text{g g}^{-1}$, concentrations toxic to many herbivores. Indeed, floral visitors showed no visitation preference between high- and low-Se plants. Honey from seleniferous areas contained 0.4–1 $\mu\text{g Se g}^{-1}$, concentrations that could provide human health benefits.
- This study is the first to shed light on the possible evolutionary cost, through decreased pollen germination in *B. juncea*, of Se accumulation and has implications for the management of seleniferous areas.

Introduction

Selenium (Se) is both an essential nutrient and a toxin. The window between Se deficiency and toxicity is narrow in mammals, and both are problems for humans and livestock world-wide. Se supplementation has been reported to reduce the occurrence of cancer, HIV infection and heart disease (Goldhaber, 2003; Shin *et al.*, 2007; Kato *et al.*, 2010). However, Se toxicity can lead to loss of hair and nails, and, in extreme cases, death (Oliveira *et al.*, 2007).

Se serves no known essential function in higher plants. However, Se has been reported to be a beneficial element for many plant species (Pilon-Smits *et al.*, 2009). Plants take up and metabolize Se via the sulfur (S) assimilation pathway. Remarkable for a nonessential element, some so-called

Se-hyperaccumulating plants in the Brassicaceae, Asteraceae and Fabaceae families concentrate Se to concentrations > 1000 mg Se kg^{-1} dry weight (DW) and may contain up to 1% Se (Terry *et al.*, 2000; Galeas *et al.*, 2008). Non-hyperaccumulator plants suffer Se toxicity when grown on high concentrations of Se because they nonspecifically incorporate selenocysteine (SeCys) and selenomethionine (SeMet) into proteins, causing toxicity (Brown & Shrift, 1981; Anderson, 1993). By contrast, Se hyperaccumulators concentrate Se primarily as methylselenocysteine (MeSeCys), which is not incorporated into proteins and therefore does not cause toxicity.

Se is one of many elements that plants hyperaccumulate: others include arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), nickel (Ni), manganese (Mn), lead (Pb) and

zinc (Zn) (Reeves & Baker, 2000). Hyperaccumulation has been hypothesized to be a defense mechanism against herbivores and pathogens (Boyd & Martens, 1992). Elevated Se concentrations were indeed shown to protect the nonhyperaccumulator *Brassica juncea* (Indian mustard) and the Se hyperaccumulators *Stanleya pinnata* (prince's plume) and *Astragalus bisulcatus* (two-grooved milkvetch) from a variety of herbivores (Hanson *et al.*, 2003; Freeman *et al.*, 2006a; Quinn *et al.*, 2010). Moreover, a field survey revealed that Se hyperaccumulators harbored fewer arthropods than comparable non-Se hyperaccumulators in the same area (Galeas *et al.*, 2008). While Se protected plants from most herbivores, at least one, a diamondback moth variety collected in a seleniferous habitat, has disarmed this defense, probably as a result of its ability to store Se as nontoxic MeSeCys instead of the toxic SeCys that was found in Se-sensitive herbivores (Freeman *et al.*, 2006a).

Apart from the protective benefits some plants receive from Se hyperaccumulation, there may also be associated costs as relatively few species are known to hyperaccumulate Se, and Se is known to be a toxic element to many plant species. To date, no studies have been published regarding the potential costs associated with Se hyperaccumulation, such as reduced reproductive success because of decreased pollen viability and/or pollination. The effect of elevated Se in flowers has recently received media attention as a consequence of its possible toxicity to the economically important honey bee (Reilly, 2009). As Se can be toxic to many insect herbivores, insect pollinators and floral visitors may also suffer Se toxicity if they forage on high-Se flowers.

In this study, we focused on two plant species, *B. juncea* and *S. pinnata*. The crop and phytoremediation species *B. juncea*, which is a predominantly self-pollinating species and considered a secondary Se accumulator, typically accumulates 100–1000 mg Se kg⁻¹ DW when supplied with Se (Hanson *et al.*, 2003; Bañuelos *et al.*, 2007). We included *B. juncea* in this study because it is commonly used for Se phytoremediation and many *Brassica* species are grown on agricultural land rich in Se. *Stanleya pinnata*, a self-incompatible Se hyperaccumulator in the same family as *B. juncea* (Brassicaceae), can accumulate up to 5000 mg Se kg⁻¹ in the field (Galeas *et al.*, 2008), and both bumble bees and honey bees have been observed foraging on these plants in their native seleniferous habitat.

The objectives of this study were to determine: Se distribution and speciation in the flowers of *S. pinnata* and *B. juncea*; how elevated Se concentrations affect pollen viability; how floral Se affects visitation by honey bees and other floral visitors; Se distribution and speciation in floral visitors collected from hyperaccumulators in the field; and whether honey collected from Northern Colorado, a seleniferous area, has elevated concentrations of Se.

Materials and Methods

Biological material

Stanleya pinnata (Pursh) Britt. flowers and the youngest mature leaves, as well as European honey bees (*Apis mellifera*) and bumble bees (*Bombus* sp.) foraging on *S. pinnata* flowers, were collected from Pine Ridge Natural Area (40°32.70N, 105°07.87W) in Fort Collins, CO, USA during June 2008. Pine Ridge Natural Area is a seleniferous habitat with soil composed of Se-rich Cretaceous shale and harbors at least two species of Se-hyperaccumulating plants: *S. pinnata* and *A. bisulcatus* (Galeas *et al.*, 2008).

Stanleya pinnata and *Brassica juncea* (L.) were grown in glasshouse conditions (24°C : 20°C day : night; 16-h photoperiod; 300 μmol m⁻² s⁻¹ photosynthetic photon flux). *Stanleya pinnata* seeds were surface-sterilized and grown on pre-washed Turface MVP (Profile Products LLC, Buffalo Grove, IL, USA), a large particle size clay-based substrate. After 3 wk of germination, half of the germinated plants received twice-weekly high-Se fertilizer treatment, composed of 1 g of fertilizer (Miracle-Gro Excel, 15 : 5 : 15 Cal-Mag; The Scotts Co., Marysville, OH, USA) per liter of water blended with 80 μM Na₂SeO₄ (selenate is the dominant form of bioavailable Se in most soils). We used an 80 μM Na₂SeO₄ treatment to obtain Se concentrations similar to those found in *S. pinnata* in the field, and therefore the most ecologically relevant. The other half of the plants that germinated received the same fertilizer treatments two times a week, but without Se. From 6 to 18 months after germination, all plants received fertilizer treatments without Se. After 18 months of growth, Se treatments were resumed for the same half of the plants that were previously treated with Se. After 19 months of growth, all *S. pinnata* plants were placed in a cold room for 4 wk to induce flowering. Plants flowered after a total of 20 months of growth and were then used for pollen germination and floral visitor experiments (as described in the sections 'Pollen germination studies' and 'Floral visitor studies').

Surface-sterilized *B. juncea* seeds were germinated in Pro Mix BX (Premier Horticulture, Quakertown, PA, USA) and grown in a glasshouse. Two weeks after germination, half of the plants were watered three times a week with Se, either 80 μM Na₂SeO₄ for pollen germination experiments or 20 μM Na₂SeO₄ for floral visitor studies and pollen germination experiments. The other half of the plants were given water three times a week as a control. An 80 μM Na₂SeO₄ treatment was used for pollen germination experiments to obtain plant Se concentrations in *B. juncea* similar to those found in Se hyperaccumulators. A 20 μM Na₂SeO₄ treatment was used for *B. juncea* for floral visitor studies to obtain plant Se concentrations more similar to those found in field phytoremediation settings.

Brassica juncea plants flowered 5–6 wk after germination and were used for pollen germination and floral visitor studies as described in the sections ‘Pollen germination studies’ and ‘Floral visitor studies’. In addition, flowers from plants treated with 20 μM Na_2SeO_4 were used to investigate Se distribution and speciation. Flowers from all experiments were collected to determine S and Se concentrations. Separate groups of plants were used to investigate Se and S concentrations in *B. juncea* leaves and flowers or in flower parts.

Se concentration, speciation and distribution

Whole mature flowers, flower parts (sepals, petals, stamens, pistils and immature seeds) and the youngest mature leaves were collected from *S. pinnata* from Pine Ridge Natural Area and *B. juncea* grown under glasshouse conditions and processed for Se and S analysis. Plant samples were rinsed with distilled water and then dried at 45°C for 48 h. Five honey bees and two bumble bees foraging on Se-rich *S. pinnata* at Pine Ridge Natural Area were also collected and analyzed for Se concentration. To determine Se concentration in nectar, 1- μl nectar samples ($n = 3$ samples from three plants each) were collected from glasshouse-grown *S. pinnata* treated with 80 μM SeO_4^{2-} using one of the methods described by Morrill *et al.* (2009). In short, the nectar droplets were dissolved into 10 μl of distilled water using a micropipette, then further diluted into 2.5 ml of distilled water and analyzed for Se concentration as described earlier (Pilon-Smits *et al.*, 1999). Furthermore, honey was collected from five different locations in Northern Colorado in Larimer and Adams counties, which contain on average 0.55 ± 0.35 and 0.73 ± 0.70 mg Se kg^{-1} , respectively, in soil and stream sediment samples (USGS) and thus can be considered seleniferous areas. All samples were digested in nitric acid as described by Zarcinas *et al.* (1987). Inductively coupled plasma atomic emission spectrometry (ICP-AES) was used as described by Fassel (1978) to determine Se and S concentrations in all samples. Se, in the form of selenium dioxide (SeO_2), obtained from SPEX (Metuchen, NJ, USA), and S, in the form of sulfuric acid (H_2SO_4), obtained from CPI International (Santa Rosa, CA, USA), were used to make elemental standards that were used during ICP-AES.

Se distribution and speciation were determined using micro-focused X-ray fluorescence (μXRF) mapping and X-ray absorption near-edge structure (μXANES) spectroscopy, respectively. Flowers of *S. pinnata* and *B. juncea* were immediately flash-frozen in liquid nitrogen (LN_2) and then shipped on dry ice for microprobe analyses at the Advanced Light Source beamline 10.3.2 of the Lawrence Berkeley National Lab (Marcus *et al.*, 2004). Bumble bee and honey bee samples were frozen at -80°C without being washed first so that we could determine speciation of Se in the bee

pollen baskets. Frozen samples were transferred onto a Peltier stage kept at -33°C to reduce potential beam radiation damage. μXRF elemental maps were recorded at 13 keV, using a 15 μm (H) \times 6 μm (V) beam, 15 $\mu\text{m} \times 15 \mu\text{m}$ pixel size, and 50 ms dwell time per pixel. The chemical form of Se in particular areas of interest (such as pollen and ovules in flowers; thorax, abdomen and pollen baskets in bees) were further investigated using Se K-edge XANES. XANES provides information about the oxidation state and, when compared with well-characterized Se standard compounds, information about its chemical speciation (Pickering *et al.*, 1999). μXRF maps and μXANES spectra were recorded with a seven-element germanium (Ge) solid-state detector (Canberra, ON, Canada). Spectra were deadtime corrected, pre-edge background subtracted, and post-edge normalized using standard procedures (Kelly *et al.*, 2008). Red Se (white line position set at 13074.73 eV) was used to calibrate the spectra. Least square linear combination (LSQ) fitting of Se XANES spectra was performed in the 12 630–12 850 eV range, using a library of standards selenocompounds. The error on the percentages of species present is estimated to $\pm 10\%$. Se standards used included: Na_2SeO_4 (S8295), Na_2SeO_3 (S1382), SeCystine (S1650) and SeMet (S3132) purchased from Sigma-Aldrich, and MeSeCys, gGMeSeCys, SeCysth and SeGSH₂ 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. All data processing and analyses were performed with a suite of custom LABVIEW (National Instruments, Austin, TX, USA) programs available at the beamline.

Additional XANES speciation of *B. juncea* flowers (averaged over several flowers) was carried out on the Structural Molecular Biology beamline 9-3 at the Stanford Synchrotron Radiation Lightsource (SSRL), as described in Andrahennadi *et al.* (2007). Flowers were ground under liquid nitrogen, packed frozen into custom cuvettes, and measured at *c.* 10 K in a liquid helium cryostat. To identify the form of organic Se found in *B. juncea* flowers, strong anion exchange high-performance liquid chromatography (SAX-HPLC) coupled with real-time inductively coupled plasma mass spectrometry (ICP-MS) was performed on extracts from *B. juncea* flowers as described by Bueno *et al.* (2007). Before running SAX-HPLC-ICP-MS, a methanol chloroform water (MCW) extraction was performed on *B. juncea* flowers, with an efficiency of obtaining 65% of all selenocompounds.

Pollen germination studies

Brassica juncea plants grown with 0, 20 or 80 μM Se and *S. pinnata* grown with either 0 or 80 μM Se (as described) were used in pollen germination studies to determine if elevated floral Se affected pollen viability. Anthers of high-Se

and low-Se plants were collected and pollen was placed on semi-solid agar medium containing 18% sucrose, 0.01% boric acid, 1 mM CaCl_2 , 1 mM $\text{Ca}(\text{NO}_3)_2$ and 1 mM MgSO_4 as described by Carlson *et al.* (2009). After 24 h of growth, the total number of pollen grains and the number of pollen grains that had germinated were counted and the percentage of pollen germination calculated.

Floral visitor studies

Floral visitor experiments were conducted with colonies of the European honey bee (*Apis mellifera*) at a nonseleniferous field site in central Fort Collins. We provided the honey bees with a choice between high-Se and low-Se *B. juncea* or *S. pinnata* plants treated as described in the section entitled 'Biological material'. For experiments using *B. juncea*, a group of 18 flowering high-Se and a group of 18 flowering low-Se plants (treated with 20 or 0 μM Se, respectively) in individual pots were placed 10 m from each other and 10 m from the hive, a design modified from Naug & Arathi (2007). High-Se and low-Se plants were of similar size and had a similar number of flowers. To investigate floral visitors' preferences between high-Se and low-Se treated plants, the number of plants visited by honey bees and other flying insects and the number of individual honey bees that visited each group of plants were recorded simultaneously (by two observers) for high- and low-Se plants in 45-min observation periods. In addition, honey bee visits > 5 s were recorded to compare bee foraging for respective treatments. Bees are thought to quickly pass over unrewarding flowers (Pyke, 1978). We assume that the more time a bee spends at a flower the more likely pollination will occur and we define 5 s, the minimum amount of time most honeybee visits last when bees are collecting honey or nectar, as a quality visit (Ellis & Delaplane, 2009). This experiment was repeated 13 times.

For experiments using *S. pinnata*, one high-Se plant and one low-Se plant (treated with 80 and 0 μM Se, respectively) growing in pots were placed 10 m from a group of four hives and 10 m from each other (Naug & Arathi, 2007). One high-Se and one low-Se *S. pinnata* plant was used, compared with 18 high-Se and 18 low-Se *B. juncea* plants, because the plume of flowers on one *S. pinnata* plant was comparable in size, based on observation, to the collective flowers produced by 18 individual *B. juncea* plants. In addition, *S. pinnata* plants are often found growing solitary in the field and *B. juncea* plants are more often found growing in groups. High-Se and low-Se *S. pinnata* plants were of similar size and had a similar number of flowers. Floral visitor preference was determined by calculating the percentage of floral visitors and the percentage of total honey bees that visited either high- or low-Se plants. In addition, the total number of bees that visited high- and low-Se treated plants was recorded. This experiment was repeated 22 times.

All of the *B. juncea* floral visitor experiments were conducted between 09:30 and 12:30 h between 25 June and 30 July 2008. The *S. pinnata* experiments were performed between 09:00 and 12:00 h from 5 June to 30 August 2010. For each observation period, one person collected data from high-Se treated plants and another individual from low-Se treated plants. To reduce possibilities of bees habituating to the location of the plants, the groups of plants were removed from the site after the 45-min observation period and returned to the field site 15 min later but with the high- and low-Se positions switched. Individuals observing remained in the same location after plants were switched. Four researchers were used to prevent potential bias during data collection. The observations were then repeated as described in this paragraph.

Data analysis

The software JMP-IN (version 3.2.6; SAS Institute, Cary, NC, USA) was used for statistical data analysis. A Student's *t*-test was used when two means were compared. A Brown–Forsythe test for unequal variances and a normal quantile plot were performed to ensure that the data conformed to the assumptions of our statistical analyses. When more than two means were compared, a one-way ANOVA was conducted followed by a Tukey–Kramer test.

Results

Se concentration, speciation and distribution in flowers of *S. pinnata* and *B. juncea*

The hyperaccumulator *S. pinnata* contained more Se in flowers than in leaves (Fig. 1a; $P < 0.001$). As Se is thought to be taken up by plants via the S assimilation pathway, we also measured S concentration. Flowers and leaves showed similar S concentrations (Fig. 1b; $P = 0.77$). Within the *S. pinnata* flower, Se was preferentially allocated to the sex stamens and pistil and to the immature seeds (Fig. 1c; $P < 0.05$). Nectar from *S. pinnata* flowers with 2323 ± 275 mg Se kg^{-1} DW (*c.* 550 mg Se kg^{-1} FW) contained 244 ± 33 μl Se ml^{-1} FW. In contrast to Se, S was more evenly distributed across *S. pinnata* flower parts (Fig. 1d; $P < 0.05$).

Leaves of the secondary Se accumulator *B. juncea* contained 1.5-fold more Se than flowers (Fig. 1e, $P = 0.02$) and S concentrations followed a similar pattern (Fig. 1f; $P < 0.001$). The Se distribution in different flower parts was further investigated using another group of *B. juncea* plants; sepals, the most vegetative-like part of the flower, had 2-fold more Se than petals, stamens, pistils and immature seeds (Fig. 1g; $P < 0.05$). Sepals also had the highest concentrations of S, but the differences were less pronounced

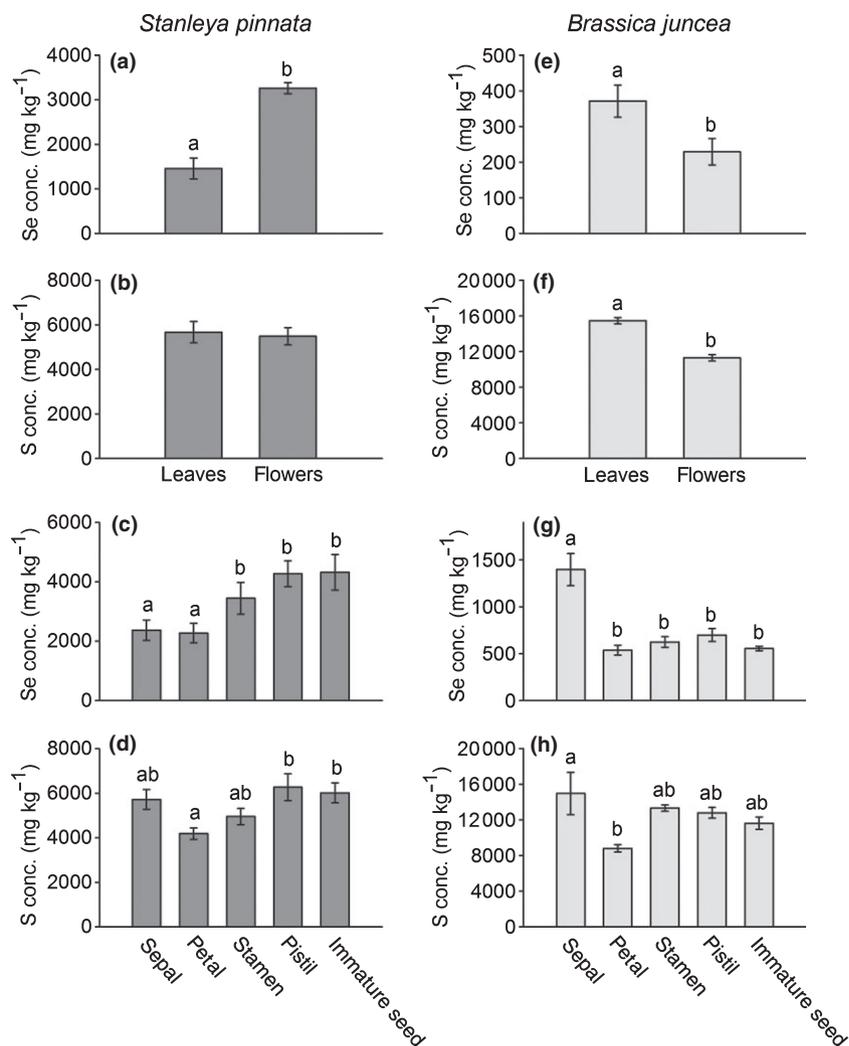


Fig. 1 Comparison of selenium (Se) and sulfur (S) concentrations and distributions in *Stanleya pinnata* and *Brassica juncea*. (a–d) Se and S concentrations in *S. pinnata* leaves and flowers (number of repetitions (n) = 10) and flower parts (n = 9). (e–h) *Brassica juncea* Se and S concentrations in leaves and flowers (n = 20) and flower parts (n = 20). Values are mean \pm SE; a different letter above bars represents a significant difference (α = 0.05).

(Fig. 1h; $P < 0.05$). Thus, while floral S distribution patterns were similar for *S. pinnata* and *B. juncea*, Se distribution showed opposite patterns in this hyperaccumulator and secondary accumulator.

Se distribution maps of *S. pinnata* flowers (Fig. 2a) revealed that Se was primarily in the ovules in the pistil (Fig. 2b) and in pollen grains on the tips of the anthers (Fig. 2c). Se in petals and sepals of *S. pinnata* was distributed in a more diffuse pattern (Fig. 2d). *Stanleya pinnata* primarily accumulated Se in the form of an organic R-Se-R compound, consistent with the MeSeCys standard (Fig. 2e; similar Se speciation results were found for all flower parts). MeSeCys is known to also be the predominant form of Se in leaves of *S. pinnata* (Freeman *et al.*, 2006a).

Brassica juncea flowers (Fig. 2f) showed a diffuse distribution of Se in all parts, including the pistil (Fig. 2g), stamen (Fig. 2h), petal (Fig. 2i) and sepal (Fig. 2j). Bulk XANES analyses revealed that *B. juncea* flowers contained a variety of Se species (Fig 2k; Supporting Information Fig. S1).

The major form was an R-Se-R species, consistent with either selenomethionine (SeMet) or MeSeCys. Results of LSQ fitting of Se XANES spectra also included selenocystine (R-Se-Se-R) and some selenate and selenite, which can be toxic to plants at elevated concentrations (Hopper & Parker, 1999). To distinguish between SeMet and MeSeCys, SAX-HPLC coupled with ICP-MS was performed on extracts of the *B. juncea* flowers, which showed that the R-Se-R species in *B. juncea* flowers was MeSeCys (Fig. S2a). The SAX-HPLC-ICP-MS analysis of the *B. juncea* flower extract, which comprised 65% of total floral Se, found selenate to be the primary form of Se (46%), followed by MeSeCys (32%) and selenocystine (14%). In addition, small amounts of selenite (1.5%) and selenomethionine (0.5%) were found by SAX-HPLC-ICP-MS (Fig. S2b). Thus, the hyperaccumulator sequestered Se primarily in its reproductive organs as nontoxic MeSeCys while the secondary accumulator showed uniform Se concentrations throughout the flowers and contained a mixture of selenocompounds.

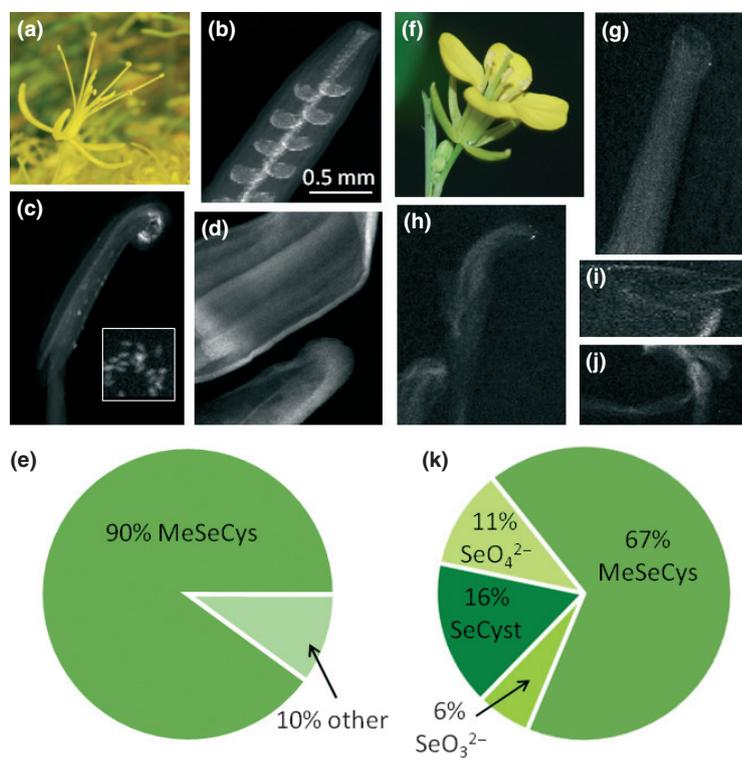


Fig. 2 Distribution and speciation of selenium (Se) in *Stanleya pinnata* and *Brassica juncea* flowers. (a) *Stanleya pinnata* flower, and (b, c, d) micro-focused X-ray fluorescence (μ XRF) distribution maps of Se in *S. pinnata*, showing that Se is (b) concentrated in the ovules of the pistil, (c) concentrated in pollen grains on the anther of the stamen and (d) diffusely distributed in petals and sepals. (e) The primary form of Se found in *S. pinnata* flowers was methylselenocysteine. (f) *Brassica juncea* flower. (g–j) μ XRF distribution maps of Se in *B. juncea* flowers showing that Se was diffusely distributed in all flower parts, (k) with a variety of selenocompounds present.

Pollen germination studies

To investigate whether elevated Se affects pollen quality, pollen germination was compared between high- and low-Se plants of *S. pinnata* and among high-, medium- and low-Se plants of *B. juncea*. Pollen from high-Se *S. pinnata* germinated at the same rate as pollen from low-Se *S. pinnata* (Fig. 3a; $P = 0.20$; Fig. 3b; $P < 0.001$). Pollen collected from high-Se *B. juncea* plants ($2200 \text{ mg Se kg}^{-1}$) germinated significantly less often than pollen from low-Se ($300 \text{ mg Se kg}^{-1}$) or no-Se plants (Fig. 3c; $P < 0.05$; Fig. 3d; $P < 0.05$).

Floral visitor studies

To investigate the effect of elevated floral Se concentrations on visitation by honey bees and other floral visitors, insect visits were compared between high- and low-Se treated *B. juncea* or *S. pinnata* plants. The same percentage of high-Se and low-Se *B. juncea* plants were visited by total floral visitors as well as by honey bees (Fig. 4a; $P < 0.001$; Fig. 4b; $P = 0.86$; Fig. 4c; $P = 0.54$). Moreover, the same number of honey bees visited high- and low-Se plants per 45-min observation period (Fig. 4d; $P = 0.95$), and the number of quality foraging visits ($> 5 \text{ s}$) was the same for high- and low-Se treated plants, averaging 21 and 22 per 45 min, respectively ($P = 0.82$). Similar results were found for hyperaccumulator *S. pinnata*. High- and low-Se plants received similar fractions of total floral visitors or honey bee visits, and an equal num-

ber of bee visits per plant (Fig. 5a; $P < 0.001$; Fig. 5b; $P = 0.98$; Fig. 5c; $P = 0.62$; Fig. 5d; $P = 0.77$). Thus, we found no evidence that floral visitors avoided high-Se flowers.

Se accumulation and speciation in bees

Five individual honey bees foraging on high-Se *S. pinnata* flowers (containing $3261 \text{ mg Se kg}^{-1}$) at Pine Ridge Natural Area were analyzed for Se concentration. Two of the five bees contained detectable Se concentrations, with 13.9 and $15.7 \text{ mg Se kg}^{-1} \text{ DW}$, respectively. Some of this Se may have been from exterior pollen. However, X-ray absorption spectroscopy revealed that Se was distributed throughout the body of the honey bee (Fig. 6a,b). Determining the chemical form of Se in the honey bee was challenging because of its low Se concentration, but the primary form (58%) of Se appeared to be an R-Se-R compound resembling MeSeCys, the same form found in *S. pinnata* flowers; the remainder was best fitted as SeGSH₂ (30%) and SeO_3^{2-} (10%). Two bumble bees foraging on the same *S. pinnata* plants contained 228 and $274 \text{ mg Se kg}^{-1} \text{ dry weight (DW)}$, respectively. Se was distributed throughout the body of the bumble bee with a relatively high concentration on the rear legs, probably present in pollen baskets (Fig. 6c,d). The bumble bees accumulated 96% of their Se as R-Se-R, probably MeSeCys, both on their legs and in their body. Honey from five locations in Northern Colorado (from counties known to contain seleniferous soils) was analyzed for Se concentration,

Fig. 3 Pollen germination of *Stanleya pinnata* and *Brassica juncea* pollen grains from plants with either high, medium or low concentrations of selenium (Se). (a) Pollen germination rates of high- and low-Se *S. pinnata* ($n = 10$ flowers from high-Se plants and $n = 11$ flowers from low-Se plants) and (b) Se concentration in flowers of the corresponding plants ($n = 2$ for high Se; $n = 4$ for low Se). (c) Pollen germination rates of high-, medium- and low-Se *B. juncea* plants ($n = 21$ for high Se; $n = 30$ for low Se) and (d) Se concentration in flowers of the corresponding plants ($n = 11$ for high and low Se). Values are mean \pm SE; different letters above bars indicate significant differences ($\alpha = 0.05$).

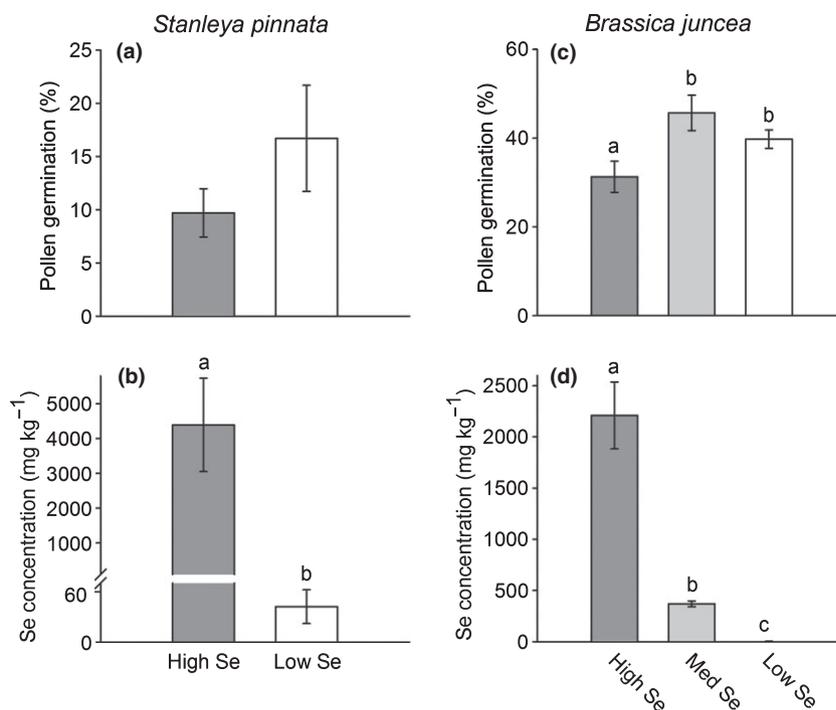
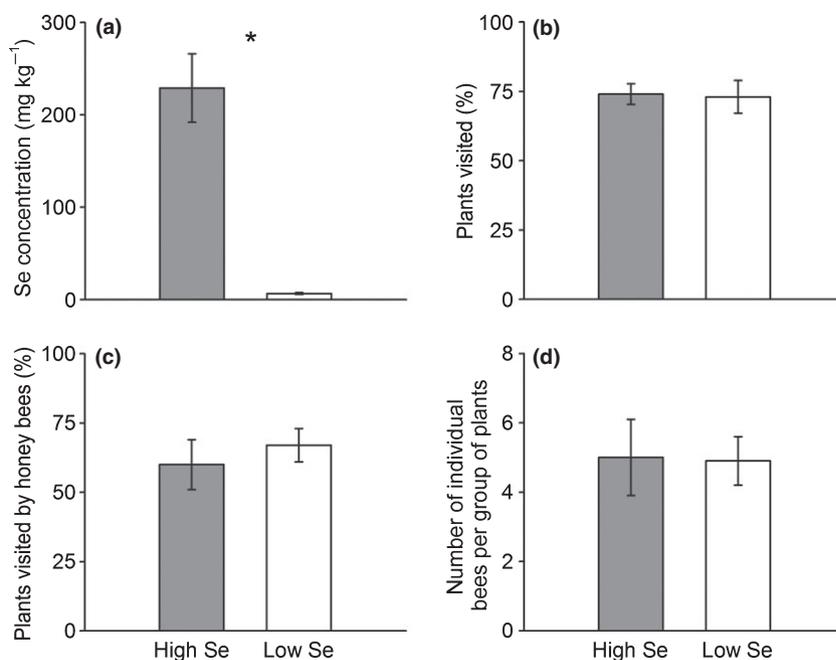


Fig. 4 Floral visitation results for *Brassica juncea* with high or low selenium (Se). (a) Se concentration in flowers of high- and low-Se *B. juncea* used for floral visitation studies. (b) Percentage of plants (of a group of 18) visited by any floral visitor, and (c) by honey bees only. (d) Number of individual honey bee visits per plant over 45-min time periods. Values are mean \pm SE; an asterisk between bars represents a significant difference ($\alpha = 0.05$).



and found to contain between 0.4 and 1.0 mg Se kg⁻¹ FW. Thus, bees forage on high-Se flowers and accumulate detectable concentrations of Se in their tissues and honey.

Discussion

This study is the first to investigate the ecology of floral elemental hyperaccumulation. Our results reveal differences in

Se distribution and speciation between flowers of the Se hyperaccumulator *S. pinnata* and the secondary accumulator *B. juncea*. *Stanleya pinnata* contained a less toxic form of Se and preferentially allocated it to its reproductive parts, including pollen and ovules. High Se concentrations significantly reduced pollen viability for *B. juncea* but not for *S. pinnata*. Floral visitors showed no preference between high- and low-Se plants of either species, and both bees and

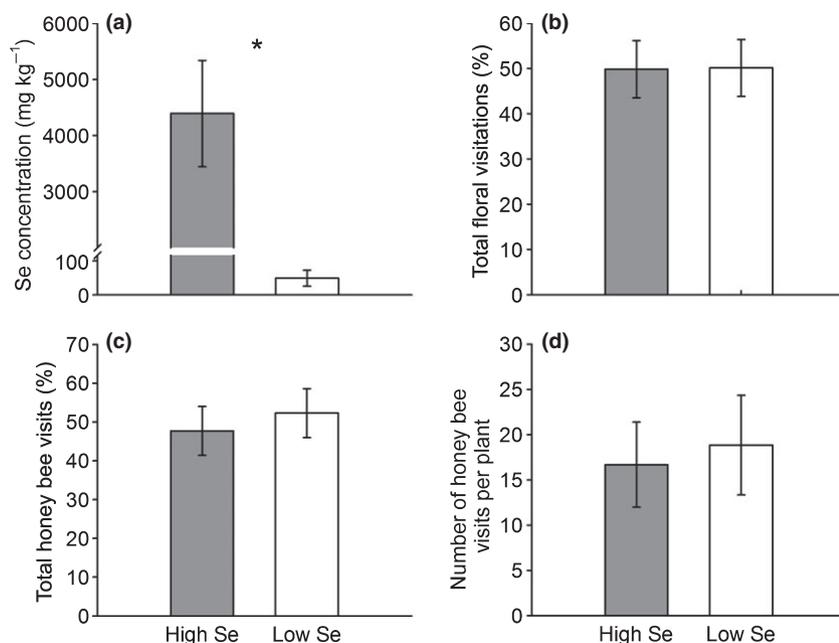


Fig. 5 Floral visitation results for *Stanleya pinnata* with high or low selenium (Se). (a) Se concentration in flowers of high- and low-Se *S. pinnata* used for floral visitation studies. (b) Percentage of floral visitations to high- or low-Se plants. (c) Percentage of honey bees visiting high- or low-Se plants. (d) Number of individual honey bee visits per plant during 45-min observational periods. Values are mean \pm SE; an asterisk between bars represents a significant difference ($\alpha = 0.05$).

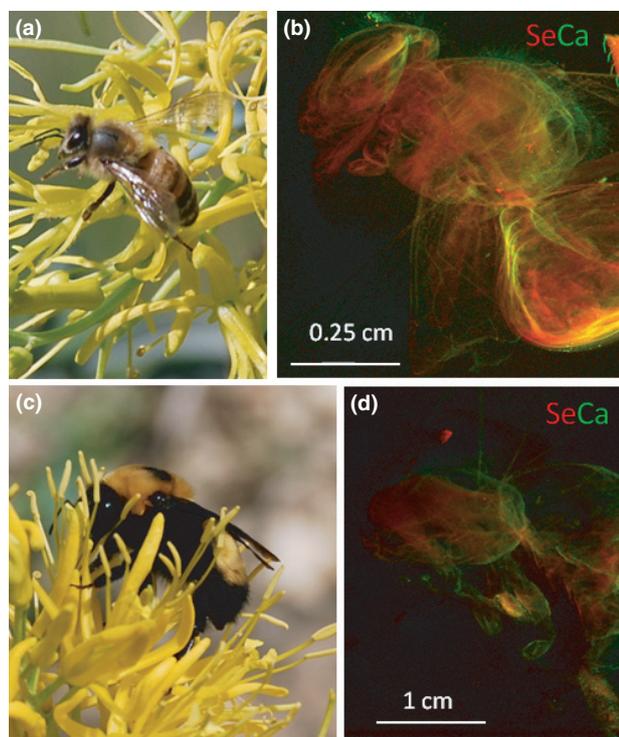


Fig. 6 Bees, and distribution of selenium (Se) and calcium (Ca) in bees, foraging on Se-rich *Stanleya pinnata*. (a, b) Honey bee foraging on *Stanleya pinnata* and bicolor-coded μ XRF distribution maps of Se (in red) and Ca (in green) in a honey bee collected from *S. pinnata*. (c, d) Bumble bee foraging on *S. pinnata* and Se (in red) and Ca (in green) in a bumble bee collected from *S. pinnata*. Bees from photographic images are not the same bees used for XAS Se maps.

honey from seleniferous habitats contained elevated Se concentrations.

Se accumulation in flowers was substantial, particularly in the hyperaccumulator *S. pinnata*, with field concentrations over 3200 mg Se kg⁻¹. *Stanleya pinnata* preferentially allocated Se to reproductive tissues, perhaps as a protection against herbivore or pathogen attacks. It is also interesting that *S. pinnata* nectar contained elevated concentrations of Se. The 244 μ l Se ml⁻¹ FW found in nectar in this study is similar to a previous report that showed nectar from *S. pinnata* contained 141 μ l Se ml⁻¹ FW (Hladun & Trumble, 2009). These concentrations may have implications for pollinator health and for Se entering the food chain and honey, as discussed on the following page. *Brassica juncea* flowers could also accumulate high concentrations of Se, but did not preferentially store Se in reproductive organs. The finding that Se and S distribution patterns were very similar for *B. juncea* but not *S. pinnata* may indicate that the Se hyperaccumulator can distinguish between Se and S. *Stanleya pinnata* flowers accumulated Se in the form of MeSeCys, which is relatively nontoxic compared with the inorganic forms of Se commonly found in nonhyperaccumulators (de Souza *et al.*, 1998; van Hoewyk *et al.*, 2005; Freeman *et al.*, 2006b). *Brassica juncea* flowers contained primarily MeSeCys, but also contained the potentially toxic forms selenate, selenite and selenocystine. In leaves of *B. juncea* the majority of Se was found previously to be selenate (de Souza *et al.*, 1998; Pilon-Smits *et al.*, 1999) and, therefore, it is interesting that *B. juncea* flowers contained such a high fraction of MeSeCys (58%).

The finding of these extremely high Se concentrations in flowers raises the question of whether this Se may have

physiological and ecological consequences. As Se hyperaccumulation is rare, and Se is toxic, it is possible there is a reproductive cost associated with floral Se accumulation. As far as physiological consequences are concerned, we found no reduction in pollen germination in *B. juncea* plants containing 370 mg Se kg⁻¹, a concentration relevant for the field as it represents the highest field concentrations observed (Bañuelos *et al.*, 2005; Dhillon & Dhillon, 2009). Pollen germination was affected in plants containing 2200 mg Se kg⁻¹, a concentration that is not found in the field but mimics hyperaccumulator concentrations (Dhillon & Dhillon, 2009). The variance of pollen germination rate was much larger for *S. pinnata* than for *B. juncea* (perhaps because *S. pinnata* has more genetic variation as an obligatory outcrosser and a wild species) so that, although it may appear that Se reduced pollen germination to a similar extent in the two species, the means were not statistically different for *S. pinnata*. As the *S. pinnata* used contained 4400 mg Se kg⁻¹ (a very high concentration even for this hyperaccumulator) our results suggest that the floral Se concentrations in *S. pinnata* in the field do not impair pollen germination. Thus, if there is a reproductive cost associated with Se hyperaccumulation, it does not appear to be via reduced pollen germination. It is possible that Se hyperaccumulation affects other reproductive processes. In future studies it would be interesting to reciprocally cross high- and low-Se plants to further investigate the effects of Se on male and female reproductive functions *in vivo*.

We found no evidence of an ecological cost of floral Se accumulation in terms of floral visitations, suggesting that non-Se-hyperaccumulating plants grown in seleniferous areas will probably not suffer reduced fitness as a consequence of decreased floral visitations. Plants with high and low Se were visited equally by floral visitors. In agreement with these results, preliminary data on a limited number of sampled honey bees (five) and bumble bees (two) foraging on Se-rich *S. pinnata* indicate elevated concentrations of Se. Bumble bees contained up to 274 mg Se kg⁻¹ DW, an order of magnitude higher than concentrations that were toxic to other insects (Hanson *et al.*, 2003; Freeman *et al.*, 2006a). The bumble bees accumulated Se as nontoxic MeSeCys, the same form found in an Se-tolerant herbivore (Freeman *et al.*, 2006a). The nonnative honey bee contained up to 15.7 mg Se kg⁻¹, an order of magnitude lower than the bumble bees, and a lower fraction was MeSeCys.

Se is thought to be an essential element for many insects (Zhang & Gladyshev, 2010), and thus Se ingestion may have a beneficial effect on bees at low concentrations while being toxic at higher concentrations. As honey bee benefits to agriculture are estimated to be over \$200 billion annually world-wide (Gallai *et al.*, 2009) it will be important to investigate whether Se accumulated by honey bees affects their health, either positively or negatively. The Se accumulated by honey bees may also have implications for human

health. The concentrations of Se found in honey (up to 1 µg g⁻¹) are high compared with Se concentrations previously reported for honey collected from Turkey (0.038–0.113 µg g⁻¹; Tuzen *et al.*, 2007). The recommended intake of Se for a healthy human adult is 50–70 µg d⁻¹ (Thomson, 2004), and 200–300 µg d⁻¹ was reported to help prevent some types of cancers (Rayman, 2005). One serving (*c.* 21 g) of honey with 1 µg Se g⁻¹ provides a healthy adult with 30–42% of the recommended daily intake of Se. Future studies investigating the chemical speciation of Se in honey would be helpful to further determine the nutritional benefits of Se in honey.

This study gives us insights into differences between Se hyperaccumulator and nonhyperaccumulator floral ecology and potential reproductive costs, in the form of reduced pollen germination for *B. juncea* with elevated Se concentrations, that may have prevented Se hyperaccumulation from evolving in more species. It is possible that Se-hyperaccumulating plant species have physiological adaptations that allow them to avoid some of the costs associated with elevated Se concentration, such as decreased pollen germination. These studies provide a foundation for future experiments on the ecological and evolutionary aspects of floral hyperaccumulation. In addition, these results have important implications for the management of seleniferous habitats and for the cultivation of Se-rich plants for phytoremediation or as Se-fortified food. Our finding that Se-rich plants do not deter floral visitors may have both positive and negative implications for agriculture. On the one hand, productivity of Se-rich crops, which usually contain between 2 and 10 mg Se kg⁻¹, and up to 200 mg Se kg⁻¹ (Dhillon & Dhillon, 2009), is not expected to suffer from reduced floral visitation. On the other hand, if floral visitors, particularly honey bees, should suffer Se toxicity after foraging on Se-rich crops, this may affect pollination and thus plant productivity in the entire region. Therefore, future studies investigating honey bee Se sensitivity would be beneficial.

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References

- Anderson JW. 1993. Selenium interactions in sulfur metabolism. In: De Kok JJ, ed. *Sulfur nutrition and assimilation in higher plants: regulatory, agricultural and environmental aspects*. The Hague, the Netherlands: SPB Academic Publishing, 49–60.
- Andrahennadi R, Wayland M, Pickering IJ. 2007. Speciation of selenium in stream insects using X-ray absorption spectroscopy. *Environmental Science and Technology* 41: 7683–7687.
- Bañuelos GS, Leduc DL, Pilon-Smits EAH, Terry N. 2007. Transgenic Indian mustard overexpressing selenocysteine lyase or selenocysteine methyltransferase exhibit enhanced potential for selenium phytoremediation under field conditions. *Environmental Science and Technology* 41: 599–605.
- Bañuelos GS, Terry N, Leduc DL, Pilon-Smits EAH, Mackey B. 2005. Field trial of transgenic Indian mustard plants shows enhanced phytoremediation of selenium contaminated sediment. *Environmental Science and Technology* 39: 1771–1777.
- Boyd RS, Martens SN. 1992. The raison d'être for metal for metal hyperaccumulation by plants. In: Baker AJM, Proctor J, Reeves RD, eds. *The vegetation of ultramafic (Serpentine) soils*. Andover, UK: Intercept, 279–289.
- Brown TA, Shrift A. 1981. Exclusion of selenium from proteins in selenium-tolerant *Astragalus* species. *Plant Physiology* 67: 1951–1953.
- Bueno M, Pannier F, Potin-Gautier M. 2007. Determination of organic and inorganic selenium species using HPLC- ICP-MS. *Agilent Technologies International* 2007: 1–5.
- Carlson AL, Telligman M, Swanson RJ. 2009. Incidence and post-pollination mechanisms of nonrandom mating in *Arabidopsis thaliana*. *Sex Plant Reproduction* 22: 257–262.
- Dhillon SK, Dhillon KS. 2009. Phytoremediation of selenium-contaminated soils: the efficiency of different cropping systems. *Soil Use and Management* 25: 441–453.
- Ellis A, Delaplaine KS. 2009. Individual forager profits in *Apis mellifera* unaffected by a range of colony *Varroa destructor* densities. *Insect Sociaux. Journal of Apicultural Research and Bee World* 48: 15–18.
- Fassel VA. 1978. Quantitative elemental analyses by plasma emission spectroscopy. *Science* 202: 183–191.
- Freeman JL, Quinn CF, Marcus MA, Fakra S, Pilon-Smits EAH. 2006a. Selenium tolerant diamondback moth disarms hyperaccumulator plant defense. *Current Biology* 16: 2181–2192.
- Freeman JL, Zhang LH, Marcus MA, Fakra S, Pilon-Smits EAH. 2006b. Spatial imaging, speciation and quantification of selenium in the hyperaccumulator plants *Astragalus bisulcatus* and *Stanleya pinnata*. *Plant Physiology* 142: 124–134.
- Galeas ML, Klumper EM, Bennett LE, Freeman JL, Kondratieff BC, Pilon-Smits EAH. 2008. Selenium hyperaccumulation affects plant arthropod load in the field. *New Phytologist* 177: 715–724.
- Gallai N, Salles JM, Settele J, Vassière BE. 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics* 68: 810–821.
- Goldhaber SB. 2003. Trace element risk assessment: essentiality vs. toxicity. *Regulatory Toxicology and Pharmacology* 38: 232–242.
- Hanson B, Garifullina GF, Lindblom SD, Wangeline A, Ackley A, Kramer K, Norton AP, Lawrence CB, Pilon-Smits EAH. 2003. Selenium accumulation protects *Brassica juncea* from invertebrate herbivory and fungal infection. *New Phytologist* 159: 461–469.
- Hladun KR, Trumble JT. 2009. Selenium accumulation in the floral tissues of two Brassicaceae plant species: implications for plant–pollinator interactions. 2009 *ESA Annual Meeting Abstract*. COS 31-2.
- van Hoewyk D, Garifullina GF, Ackley AR, Abdel-Ghany SE, Marcus MA, Fakra S, Ishiyama K, Inoue E, Pilon M, Takahashi H *et al.* 2005. Overexpression of AtCpNifS enhances selenium tolerance and accumulation in Arabidopsis. *Plant Physiology* 139: 1518–1528.
- Hopper JL, Parker DR. 1999. Plant availability of selenite and selenate as influenced by the competing ions phosphate and sulfate. *Plant and Soil* 210: 199–207.
- Kato MA, Finley DJ, Lubitz CC, Zhu BX, Moo TA, Loeven MR, Ricci JA, Zarnegar R, Katdare M, Fahey TJ. 2010. Selenium Decreases thyroid cancer cell growth by increasing expression of GADD153 and GADD34. *Nutrition and Cancer-An International Journal* 62: 66–73.
- Kelly SD, Hesterberg D, Ravel B. 2008. Analysis of soils and minerals using X-ray absorption spectroscopy. *Methods of Soil Analysis, Part 5. Mineralogical Methods* 367.
- Marcus MA, MacDowell AA, Celestre R, Manceau A, Miller T. 2004. Beamline 10.3.2 at ALS: a hard X-ray microprobe for environmental and materials sciences. *Journal of Synchrotron Radiation* 11: 239–247.
- Morrant DS, Schumann R, Petit S. 2009. Field sampling and storing nectar from flowers with low nectar volumes. *Annals of Botany* 103: 533–542.
- Naug D, Arathi HS. 2007. Sampling and decision rules used by honey bees in a foraging arena. *Animal Cognition* 10: 117–124.
- Oliveira KD, Franca TN, Nogueira VA, Peixoto PV. 2007. Diseases associated with selenium poisoning in animals. *Pesquisa Veterinaria Brasileira* 27: 125–136.
- Pickering IJ, George GN, Van Fleet Stalder V, Chasteen TG, Prince RC. 1999. X ray absorption spectroscopy of selenium-containing amino acids. *Journal of Biological Inorganic Chemistry* 6: 791–794.
- Pilon-Smits EAH, Hwang SB, Lytle CM, Zhu YL, Tai JC, Bravo RC, Leustek T, Terry N. 1999. Overexpression of ATP sulfurylase in *Brassica juncea* leads to increased selenate uptake, reduction and tolerance. *Plant Physiology* 119: 123–132.
- Pilon-Smits EAH, Quinn CF, Tapken W, Malagoli M, Schiavon M. 2009. Physiological functions of beneficial elements. *Current Opinions in Plant Biology* 12: 267–274.
- Pye GH. 1978. Optimal foraging in bumblebees and coevolution with their plants. *Oecologia* 36: 281–293.
- Quinn CF, Freeman JL, Reynolds RJB, Lindblom SD, Cappa JJ, Marcus MA, Fakra SF, Pilon-Smits EAH. 2010. Selenium hyperaccumulation offers protection from cell disruptor herbivores. *BMC Ecology* 10: 19.
- Rayman MP. 2005. Selenium in cancer prevention: a review of the evidence and mechanism of action. *Proceedings of the Nutrition Society* 64: 527–542.
- Reeves RD, Baker AJM. 2000. Metal accumulation in plants. In: Raskin I, Ensley BD, eds. *Phytoremediation of toxic metals: using plants to clean up the environment*. New York, NY, USA: Wiley, 193–222.
- Reilly M. 2009. *Toxic pollen, nectar could sting bees*. *Discovery News*, [WWW document]. URL <http://dsc.discovery.com/news/2009/07/29/ bees-selenium.html> [accessed on ?? 20??]
- Shin SH, Yoon MJ, Kim M, Kim JI, Lee SJ, Lee YS, Bae S. 2007. Enhanced lung cancer cell killing by the combination of selenium and ionizing radiation. *Oncology Reports* 17: 209–216.
- de Souza MP, Pilon-Smits EAH, Lytle CM, Hwang S, Tai JC, Honma TSU, Yeh L, Terry N. 1998. Rate limiting steps in Se assimilation and volatilization by *Brassica juncea*. *Plant Physiology* 117: 1487–1494.
- Terry N, Zayed AM, de Souza MP, Tarun AS. 2000. Selenium in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 51: 401–432.

- Tuzen M, Silici S, Mendil D, Soylak M. 2010. Trace element levels in honeys from different regions of Turkey. *Food Chemistry* 103: 325–330.
- Thomson CD. 2004. Assessment of requirements for selenium and adequacy of selenium: a review. *European Journal of Clinical Nutrition* 58: 391–402.
- Zarcinas BA, Cartwright B, Spouncer LR. 1987. Nitric acid digestion and multi element analysis of plant material by inductively coupled plasmaspectrometry. *Communications in Soil Science and Plant Analysis* 18: 131–146.
- Zhang Y, Gladyshev VN. 2010. General trends in trace element utilization revealed by comparative genomic analyses of Co, Cu, Mo, Ni, and Se. *Journal of Biological Chemistry* 285: 3393–3405.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Normalized Se K-edge XANES spectra of *B. juncea* flowers.

Fig. S2 (a) Strong anion exchange high-performance liquid chromatography coupled with inductively coupled plasma mass spectroscopy spectra of *B. juncea* flowers; (b) identification of Se species and relative abundance, based on retention times of standard compounds.

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