

## Article

# Selenium Hyperaccumulators Facilitate Selenium-Tolerant Neighbors via Phytoenrichment and Reduced Herbivory

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## Summary

**Background:** Soil surrounding selenium (Se) hyperaccumulator plants was shown earlier to be enriched in Se, impairing the growth of Se-sensitive plant species. Because Se levels in neighbors of hyperaccumulators were higher and Se has been shown to protect plants from herbivory, we investigate here the potential facilitating effect of Se hyperaccumulators on Se-tolerant neighboring species in the field.

**Results:** We measured growth and herbivory of *Artemisia ludoviciana* and *Symphotrichum ericoides* as a function of their Se concentration and proximity to hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata*. When growing next to hyperaccumulators, *A. ludoviciana* and *S. ericoides* contained 10- to 20-fold higher Se levels (800–2,000 mg kg<sup>-1</sup> DW) than when growing next to nonaccumulators. The roots of both species were predominantly (70%–90%) directed toward hyperaccumulator neighbors, not toward other neighbors. Moreover, neighbors of hyperaccumulators were 2-fold bigger, showed 2-fold less herbivory damage, and harbored 3- to 4-fold fewer arthropods. When used in laboratory choice and nonchoice grasshopper herbivory experiments, Se-rich neighbors of hyperaccumulators experienced less herbivory and caused higher grasshopper Se accumulation (10-fold) and mortality (4-fold).

**Conclusions:** Enhanced soil Se levels around hyperaccumulators can facilitate growth of Se-tolerant plant species through reduced herbivory and enhanced growth. This study is the first to show facilitation via enrichment with a nonessential element. It is interesting that Se enrichment of hyperaccumulator neighbors may affect competition in two ways, by reducing growth of Se-sensitive neighbors while facilitating Se-tolerant neighbors. Via these competitive and facilitating effects, Se hyperaccumulators may affect plant community composition and, consequently, higher trophic levels.

## Introduction

The element selenium (Se) is a trace element for many animals as a component of selenoproteins, which are redox-active and have a variety of essential functions [1]. Although Se has not been shown to be essential for higher plants, it is a beneficial nutrient for many species [2]. Most plants take up selenate inadvertently because of its similarity to sulfate and readily metabolize it via the sulfur (S) assimilation pathway [3]. At higher levels, Se becomes toxic as a result of its chemical similarity to S. Nonspecific replacement of cysteine by selenocysteine (SeCys) in proteins disrupts protein function, leading to toxicity and death [4].

Although Se is present at low levels in most soils, it is particularly abundant in seleniferous soils such as Cretaceous shale, which typically contains 1–10 mg Se kg<sup>-1</sup> and may reach 100 mg Se kg<sup>-1</sup> [5]. Some plants native to such seleniferous soils hyperaccumulate Se to levels >1,000 mg kg<sup>-1</sup> dry weight (DW) and can even reach levels up to 15,000 mg kg<sup>-1</sup> DW (1.5%) [6]. Although most plants cannot distinguish between Se and S, hyperaccumulators preferentially take up Se over S and store Se in all plant parts. Most of the Se in hyperaccumulators is stored in the form of methyl-SeCys. This amino acid is not incorporated into protein and can therefore be safely accumulated, explaining the extreme Se tolerance of hyperaccumulators [7]. Nonhyperaccumulator plants store more toxic forms of Se such as inorganic selenate [8–10].

The functional significance of Se hyperaccumulation has been a topic of recent study. Selenium accumulation has been shown to protect plants from a wide variety of herbivores, including vertebrates and invertebrates with different feeding modes [11–16]. This protection was based on both deterrence and toxicity. Selenium-based deterrence might be due to the highly odoriferous forms of volatile Se that are emitted by Se-rich plants [3]; additionally, it is possible that Se-rich plant material has an unattractive taste. Besides Se, other hyperaccumulated elements (As, Cd, Ni, Zn) have been shown to protect plants from herbivory [17]. In addition to protecting plants from herbivores, Se accumulation has been shown to reduce infection by two pathogenic fungi [10].

More recently, evidence was found that Se hyperaccumulators phytoenrich their surrounding soil with Se and that this may serve as a form of elemental allelopathy against Se-sensitive neighboring plants [18]. Soil collected around Se hyperaccumulators *Astragalus bisulcatus* and *Stanleya pinnata* was toxic to the Se-sensitive species *Arabidopsis thaliana*, and resulted in enhanced Se accumulation as compared to soil collected around nonhyperaccumulators in the same seleniferous area. The Se concentration in hyperaccumulator soil appeared to be high enough to be responsible for the observed toxicity, as judged from agar experiments with similar Se concentrations. In the field, neighboring plants of hyperaccumulators also showed enhanced Se levels [18]. Although the degree of ground cover was slightly lower around hyperaccumulator species in the field [18], there was no apparent toxicity in neighboring plants of hyperaccumulators, in contrast to the laboratory experiments using *A. thaliana*. This observation prompted us to further study two neighboring species that contained particularly elevated Se levels without ill effects: *Artemisia ludoviciana* and *Symphotrichum ericoides*. Because enhanced plant Se accumulation has been shown to provide ecological benefits, particularly protection from herbivores, we hypothesized that the enhanced Se levels in Se-tolerant neighbors of hyperaccumulators may be facilitative for these species.

In facilitation, benefactors (also called nurse plants) can benefit neighboring plants (beneficiaries) in several ways. Direct facilitative effects may involve giving protection from sun, wind, extreme temperatures, or herbivores, better access

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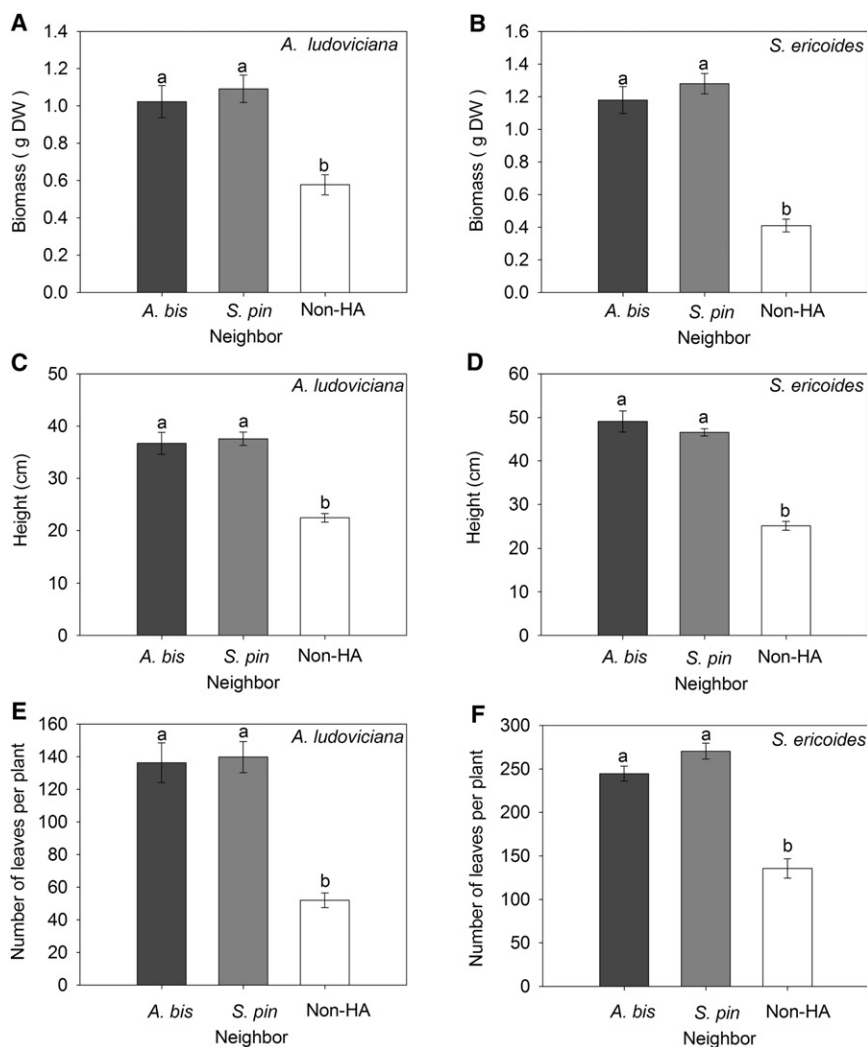


Figure 1. Size Comparison of *A. ludoviciana* and *S. ericoides* Growing around Hyperaccumulator Species *A. bisulcatum* and *S. pinnata* or Less Than Four Meters from Hyperaccumulator Vegetation in Seleniferous Habitat in Fort Collins, Colorado

(A) and (B) show shoot biomass, (C) and (D) show stem length, and (E) and (F) show number of leaves. Values shown represent means  $\pm$  standard error of the mean (SEM) ( $n = 16$ ); different lowercase letters above bars indicate significantly different means ( $p < 0.05$ ).

hyperaccumulator *Sedum alfredii*, which improved the growth of *Zea mays* [23]. In this study, we investigated the facilitative effects of two Se hyperaccumulators, *A. bisulcatum* and *S. pinnata*, on the neighboring species *A. ludoviciana* and *S. ericoides*, all growing in their natural seleniferous habitat. We measured the neighbors' Se concentration and size, as well as their susceptibility to herbivory, as a function of their proximity to hyperaccumulators.

## Results

### Selenium Hyperaccumulators *A. bisulcatum* and *S. pinnata* Positively Affect Growth and Se Accumulation in Neighbors

In their natural seleniferous habitat, the species *A. ludoviciana* and *S. ericoides* were 2- to 3-fold taller and had more leaves when growing next to the hyperaccumulators *A. bisulcatum* and

to nutrients, or protection from toxins. Facilitated nutrient access may be achieved via nutrient pumping,  $N_2$  fixation, or excretion of metal chelators. Indirect facilitative effects may involve a negative effect of the benefactor on competitors or herbivores of the beneficiary [19, 20]. Facilitation is especially important during the most sensitive seedling stage of the beneficiary. It is most prevalent in areas where the beneficiary is at the edge of its geographical range, and more generally in harsh environments with respect to water supply, temperature, exposure, soil quality, and herbivory pressure. Under such adverse conditions, competition is thought to become a less important and facilitation a more important plant-plant interaction [21]. The benefactor plant may experience the relationship with its beneficiary as neutral (commensalism) or may experience competition, particularly when the beneficiary increases in size.

So far there is little information on the possible effects of plant elemental accumulation on plant-plant interactions, particularly with respect to facilitation. In cocropping experiments on metal-polluted soil, in the context of phytoremediation, metal accumulating species were found to facilitate the growth of less metal-tolerant neighbors by removing the toxic metal. This was found for *Salix caprea*, which improved the growth of *Carex flava* [22], and for the Zn/Cd

*S. pinnata* than when growing next to nonhyperaccumulators (Figure 1). There was also a pronounced difference in leaf Se concentration in *A. ludoviciana* and *S. ericoides* plants depending on their proximity to hyperaccumulators: leaf Se levels were 10- to 20-fold higher when they were growing next to hyperaccumulators as compared to when they were growing away from them (Figures 2A and 2B). As a result, overall Se accumulation per plant (concentration  $\times$  biomass) was 20- to 40-fold higher for *A. ludoviciana* and *S. ericoides* growing next to hyperaccumulators. Because Se hyperaccumulators are known to contain not only higher Se levels but also higher S levels than other vegetation on seleniferous soils [6], we also compared the S levels of the *A. ludoviciana* and *S. ericoides* plants under study. Leaf S levels in *A. ludoviciana* were significantly higher (by 40%–50%) when growing next to hyperaccumulators (Figure 2C); in *S. ericoides* the S level was also somewhat elevated (25%) when growing next to *A. bisulcatum* but not next to *S. pinnata* (Figure 2D). The soil Se levels around *A. bisulcatum* and *S. pinnata* were 7- to 13-fold higher compared to those in soil collected around nonhyperaccumulators (Figure 2E). The soil S levels were 3- to 5-fold higher around the hyperaccumulators than around nonaccumulators (Figure 2F), but this difference was only significant for *A. bisulcatum*.

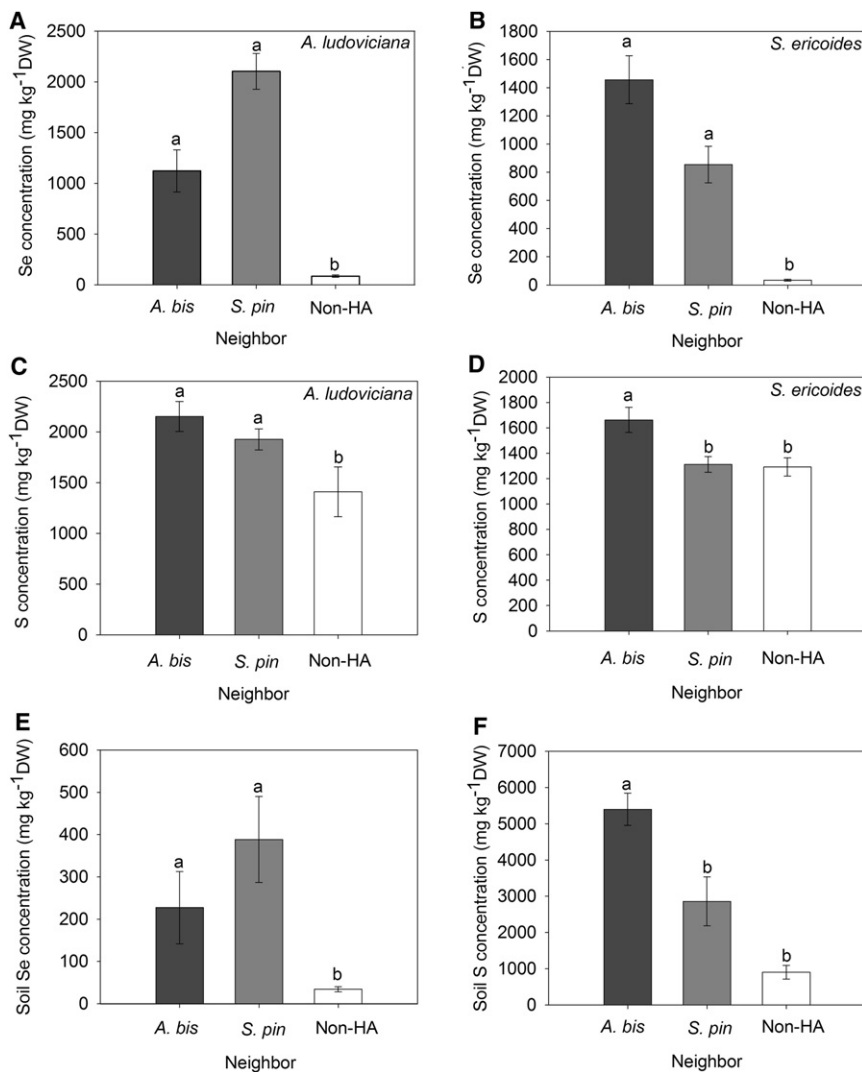


Figure 2. Selenium and Sulfur Concentration in Leaves and Soil near Hyperaccumulators and Nonhyperaccumulators

(A–D) Selenium (Se) and sulfur (S) concentration in leaves of *A. ludoviciana* and *S. ericoides* collected from around hyperaccumulators (*A. bisulcatus* and *S. pinnata*) or from around non-hyperaccumulator vegetation in the same seleniferous habitat.

(E and F) Soil Se and S concentration adjacent to the hyperaccumulators and nonhyperaccumulators. Values shown represent means  $\pm$  SEM (n = 16); different lowercase letters above bars indicate significantly different means (p < 0.05).

degree of herbivore damage were surveyed on the high- and low-Se *A. ludoviciana* and *S. ericoides* plants growing next to or far away from hyperaccumulators. The number of arthropods per plant was 3- to 4-fold lower on plants growing next to hyperaccumulators than on plants growing away from hyperaccumulators (Figures 4A and 4B). Similarly, the number of damaged leaves per plant was ~2-fold lower for plants growing next to hyperaccumulators (Figures 4C and 4D).

*S. ericoides* plants growing next to hyperaccumulators had two distinct leaf types, prompting us to further investigate herbivory and Se concentration in the small versus big leaves. Even though the plants had many more small leaves than big leaves (~17-fold, Figure S1A), herbivory damage was 3-fold more prevalent on big than small leaves (Figure S1B). Overall, ~75% of the big leaves showed herbivory, versus only 2.5% of the small leaves (Figure S1C).

Interestingly, the Se concentration was 10- to 25-fold higher in the small leaves than in the big leaves (Figure S1D).

To compare herbivory on *A. ludoviciana* and *S. ericoides* as a function of their proximity to hyperaccumulators under more controlled conditions, we collected plants from both species next to *A. bisulcatus* or away from hyperaccumulators, transferred to pots, and taken to the laboratory. Grasshoppers were also collected from the same field site. Before being offered to grasshoppers in choice and nonchoice experiments, the plants were characterized in terms of height, number of leaves, and Se concentration. Similar to our earlier survey, plants of both species were taller and had more leaves and a higher Se concentration (16- to 22-fold) when growing next to the hyperaccumulator than when growing next to nonhyperaccumulators (Figure S2).

In the choice experiment, the grasshoppers preferentially targeted the low-Se plants collected next to nonhyperaccumulators rather than high-Se plants of the same species collected next to hyperaccumulators (Figures 5A–5C). *Symphytotrichum ericoides* showed significantly less stem height loss and less leaf loss for the high-Se plants. For *A. ludoviciana*, this preference was only significant for leaf loss but not for stem height loss, but we noticed that its stems were in several cases clipped by the grasshoppers, but the clippings were left

### *A. ludoviciana* and *S. ericoides* Roots Grow toward Hyperaccumulator Neighbors

The finding that *A. ludoviciana* and *S. ericoides* appear to benefit from their elevated Se levels when growing next to hyperaccumulators in terms of above-ground biomass led us to investigate below-ground root-root interactions. The taproots of both *A. ludoviciana* and *S. ericoides* were directed predominantly (70%–90%) toward their hyperaccumulator neighbor when growing next to *A. bisulcatus* (Figures 3A and 3B) or *S. pinnata* (Figures 3C and 3D; see also Table S1 available online). In contrast, roots of both species did not grow in any particular horizontal direction when the plants were situated next to the nonaccumulator legume *Medicago sativa* (Figures 3E and 3F; Table S1). Although the angle of root growth was horizontal in almost all cases when the plants were growing next to a hyperaccumulator, it was vertical in about a third of the plants growing next to *M. sativa*.

### High-Se Neighbors of Hyperaccumulators Are Protected from Herbivory

Because Se accumulation has been found to protect other plant species from herbivory, the number of herbivores and

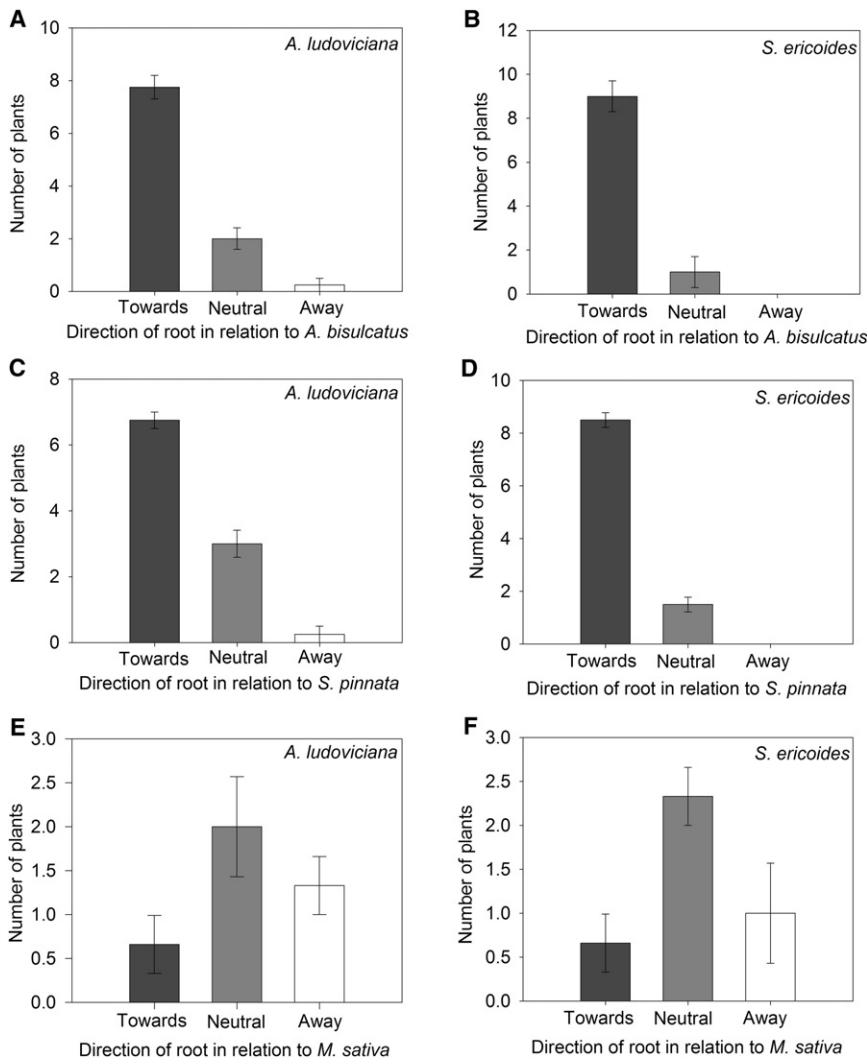


Figure 3. Root Direction of Neighboring Plants Relative to Hyperaccumulators and Nonaccumulators

*A. ludoviciana* in relation to *A. bisulcatus* (A), *S. ericoides* in relation to *A. bisulcatus* (B), *A. ludoviciana* in relation to *S. pinnata* (C), *S. ericoides* in relation to *S. pinnata* (D), *A. ludoviciana* in relation to *M. sativa* (E), and *S. ericoides* in relation to *M. sativa* (F). Values shown represent means  $\pm$  SEM ( $n = 4$  for A–D and  $n = 3$  for E and F).

on plants from the same species collected from around nonhyperaccumulators; these levels were not significantly higher, though (Figure 6F).

### Discussion

In this study, we present evidence that Se hyperaccumulators can act as benefactor plants (also known as nurse plants), facilitating the growth of Se-tolerant neighboring plants. When growing next to hyperaccumulators *A. bisulcatus* and *S. pinnata*, as compared to nonhyperaccumulator neighbors, *A. ludoviciana* and *S. ericoides* were bigger and showed reduced herbivory damage and arthropod load. These neighbors of hyperaccumulators were also better protected from grasshopper herbivory in laboratory experiments, owing to both deterrence and toxicity. The herbivory protection was likely due to Se enrichment: *A. ludoviciana* and *S. ericoides* contained 10- to 20-fold elevated Se levels (800–2,000 mg kg<sup>-1</sup> DW) when growing

uneaten. Despite the apparent avoidance of high-Se plants by the grasshoppers, grasshopper mortality over the course of the experiment was substantial: only 5%–20% survived (Figures 5D and 5E). Grasshopper Se accumulation in the animals that had fed on *A. ludoviciana* and *S. ericoides* for 6 days contained 10- and 20-fold higher Se levels, respectively, than grasshoppers from the field (Figure 5F). The grasshoppers that fed on *S. ericoides* reached higher tissue Se levels, showed lower survival, and died more rapidly than those that fed on *A. ludoviciana* (Figures 5D–5F).

In the nonchoice experiment, the high-Se *A. ludoviciana* and *S. ericoides* plants originating from around hyperaccumulators lost less stem height and fewer leaves than their low-Se counterparts collected next to nonhyperaccumulators (Figures 6A–6C; Figure 7). The animals that had fed on high-Se plants showed 20% survival after 6 days, whereas the animals that had fed on low-Se plants showed 50%–80% survival over the same time period (Figures 6D and 6E). Furthermore, the tissue Se concentrations in grasshoppers that had fed on plants collected next to hyperaccumulators were 15-fold (*A. ludoviciana*) and 40-fold (*S. ericoides*) higher than in animals collected in the field (Figure 6F). Animals that fed on plants collected from around hyperaccumulators also contained on average 2- to 10-fold higher Se levels than animals that fed

next to a hyperaccumulator neighbor. These are similar to hyperaccumulator levels and high enough to protect plants from a wide variety of herbivores [11, 12, 14–16, 24]. Indeed, for *S. ericoides*, the herbivory experienced by the high-Se neighbors of hyperaccumulators was almost exclusively on those leaves that had the lowest Se levels.

The Se enrichment of *A. ludoviciana* and *S. ericoides* may in part be explained by the finding that soil Se levels were 7- to 13-fold higher around hyperaccumulators. The beneficiary plants showed preferential root growth toward their hyperaccumulator neighbors, which was not observed toward nonhyperaccumulator *M. sativa*. Thus, it appears that when growing next to a Se hyperaccumulator, *A. ludoviciana* and *S. ericoides* actively tap into this source of Se, reaching hyperaccumulator Se levels themselves that they can tolerate and from which they derive ecological benefit. For further studies, it will be interesting to investigate the Se tolerance mechanisms of *A. ludoviciana* and *S. ericoides*, e.g., whether they store Se mainly as methyl-SeCys like their hyperaccumulator neighbors. It will also be interesting to study the mechanisms responsible for their apparent preferential root growth toward high-Se areas.

In addition to the demonstrated ecological benefit, it is feasible that *A. ludoviciana* and *S. ericoides* enjoyed



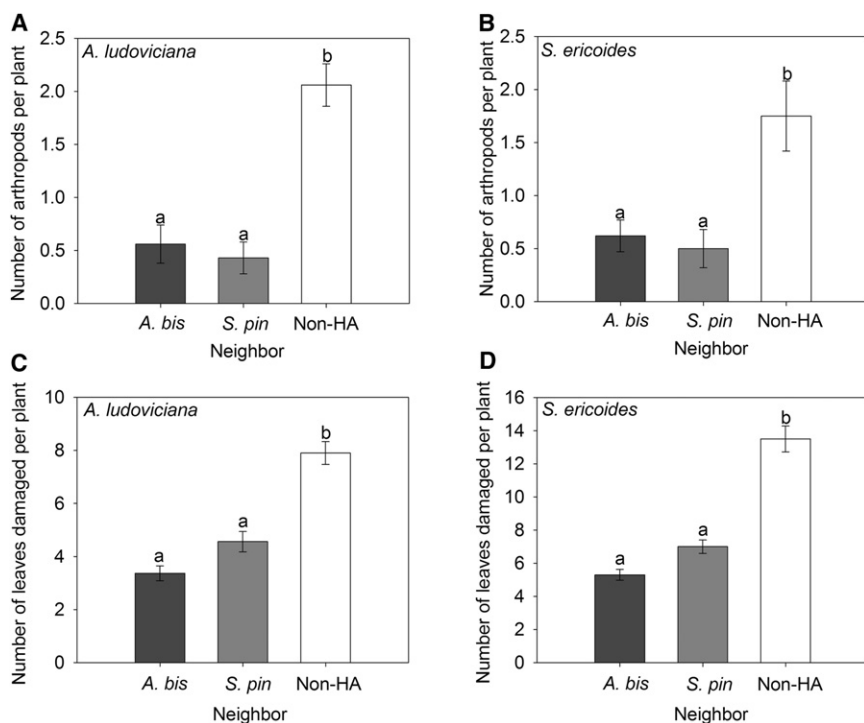


Figure 4. Relation of Arthropods and Damaged Leaves per Plant to Growing Distance from Hyperaccumulators and Nonhyperaccumulators (A) and (B) show the number of arthropods per plant on *A. ludoviciana* and *S. ericoides*, respectively; (C) and (D) show the number of leaves damaged per plant of *A. ludoviciana* and *S. ericoides*, respectively, when growing close to hyperaccumulator species (*A. bisulcatus* and *S. pinnata*) or away from hyperaccumulators (nonHA). Values shown represent means  $\pm$  SEM ( $n = 16$ ); different lowercase letters above bars indicate significantly different means ( $p < 0.05$ ).

a physiological benefit from their enhanced Se levels, because they were so much taller (2-fold) next to hyperaccumulators. Selenium is a beneficial nutrient for many plants, particularly for hyperaccumulators, perhaps via protection from oxidative stresses [2, 25]. The hyperaccumulators may also provide other benefits like shelter from wind or extreme temperatures or better access to other elements besides Se. In this context, it is interesting to note that S levels were 3- to 5-fold elevated in hyperaccumulator soil and up to 1.5-fold elevated in the beneficiaries; S levels were shown earlier to be higher in Se hyperaccumulators than in nonhyperaccumulators on the same site [18]. There may also be an indirect facilitating effect on *A. ludoviciana* and *S. ericoides* if hyperaccumulators use Se as a form of elemental allelopathy to reduce competition from Se-sensitive neighbors, as indicated by results from our earlier work [18]. In addition, intraspecific competition within *A. ludoviciana* and *S. ericoides* may play a role: we observed that the density of *A. ludoviciana* and *S. ericoides* was lower around hyperaccumulators than away from hyperaccumulators (data not shown). Perhaps there is genetic variation with respect to Se tolerance within *A. ludoviciana* and *S. ericoides*. If so, the more sensitive individuals may be selected against around hyperaccumulators, leaving the tolerant individuals with less competition, resulting in better growth.

The *A. bisulcatus* and *S. pinnata* nurse plants did not show any obvious positive or negative effects when growing next to their beneficiaries. *A. bisulcatus* and *S. pinnata* are substantially bigger than *A. ludoviciana* and *S. ericoides*, so the relationship of the hyperaccumulators to their beneficiaries is likely neutral, unless the beneficiaries tap so much Se from the hyperaccumulators that it would compromise the physiological and ecological benefits the hyperaccumulators derive from the Se.

The Se levels in the beneficiary plants were increased 10- to 20-fold, whereas the levels in the soil were only 7- to

13-fold elevated. As mentioned, roots of *A. ludoviciana* and *S. ericoides* grew preferentially toward their hyperaccumulator neighbors, which may have enabled them to maximize their access to Se. The preferential root growth of *A. ludoviciana* and *S. ericoides* toward Se hyperaccumulators may indicate that they have positive chemitropism toward Se. Plant roots are well known to respond positively or negatively to soil pockets with elevated levels of nutrients or toxins, as well as to the presence

of roots from neighboring plants of the same or different species [17, 26, 27]. In several earlier reports, hyperaccumulator roots were shown to preferentially proliferate in soil containing the hyperaccumulated element; this was found for the Zn hyperaccumulator *Thlaspi caerulescens* [28, 29], the Cd/Zn hyperaccumulator *Sedum alfredii* [30], and the Se hyperaccumulator *S. pinnata* [31]. Because the soil Se levels around hyperaccumulators were found to be elevated compared to soil around nonaccumulators in the same area, it is possible that the neighboring *A. ludoviciana* and *S. ericoides* responded positively to this soil Se gradient. However, it is also possible that the hyperaccumulator plants provide some other positive stimulus that affects their neighbors' root growth, e.g., higher levels of the nutrient S. The stimulus does not appear to be nitrogen, because the related and similarly sized nonaccumulator legume *M. sativa* did not influence the direction of root growth in *A. ludoviciana* and *S. ericoides*.

An additional explanation for the finding that the Se levels in the companion plants were increased by 10- to 20-fold and those in the soil only 7- to 13-fold may be that the soil Se around hyperaccumulators is particularly bioavailable. It is interesting to note in this respect that the Se/S ratio in *S. ericoides* and *A. ludoviciana* was elevated when they were growing next to hyperaccumulators (0.5 next to *A. bisulcatus* and 1.2 next to *S. pinnata*, as compared to 0.07 when growing next to a nonhyperaccumulator). The soil Se/S ratio was not that different: 0.04 next to *A. bisulcatus*, 0.13 next to *S. pinnata*, and 0.03 next to nonhyperaccumulators. The hyperaccumulators may affect bioavailability as well as the form of Se in their surrounding soil. For instance, because hyperaccumulators accumulate mainly methyl-SeCys [24], litter deposition may over time change the predominant form of Se in soil surrounding hyperaccumulators from inorganic Se (e.g., selenate) to more organic Se such as methyl-SeCys, which may be more readily taken up by neighbors [9].

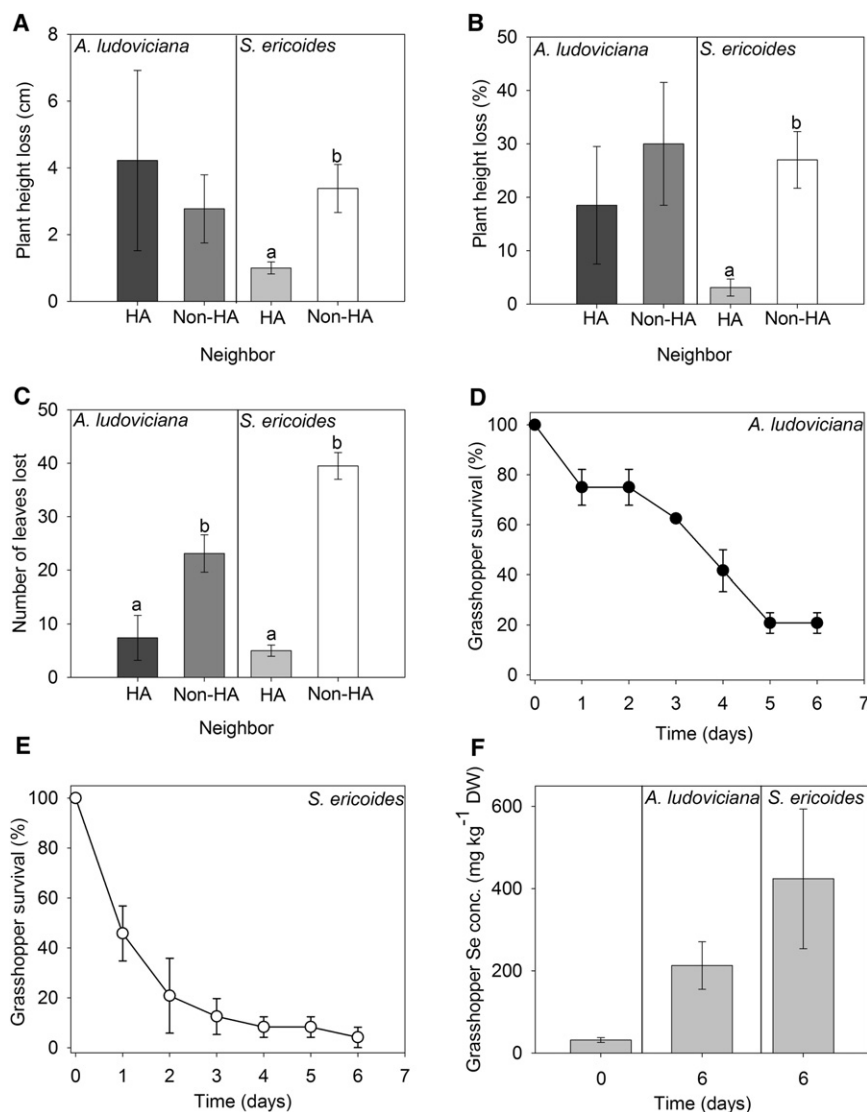


Figure 5. Choice Experiment Comparing Herbivory, Survival, and Selenium Accumulation of Grasshoppers Given the Choice to Feed on *A. ludoviciana* or *S. ericoides* Plants Collected Either Next to Hyperaccumulator *A. bisulcatus* or Next to Nonhyperaccumulators

(A) shows absolute plant height loss, (B) shows relative plant height loss, (C) shows number of leaves lost, (D) shows grasshopper survival on *A. ludoviciana*, (E) shows grasshopper survival on *S. ericoides*, and (F) shows grasshopper Se concentration in animals from the field at day 0 and in animals collected from *A. ludoviciana* or *S. ericoides* after 6 days of cocultivation. Values shown represent means  $\pm$  SEM ( $n = 9$  for A–C;  $n = 3$  for D and E;  $n = 6$ –8 for F). Different lowercase letters above bars indicate significantly different means ( $p < 0.05$ ).

rock with low soil depth, but the climate is very dry (average annual precipitation 374 mm per year), with frequent high winds, cold winters (average  $-10^{\circ}\text{C}$  minimum temperature in January), and hot summers (average  $30^{\circ}\text{C}$  maximum temperature in July). In that sense, the results from this study fit the pattern observed for facilitation. The novelty of the study presented here is that it is the first to show how phytoenrichment with a nonessential element can facilitate growth in neighboring plants in an ecologically relevant setting. Earlier studies with metal hyperaccumulators have only been carried out in phytoremediation settings, which are not very ecologically relevant. Moreover, in those studies, neighbors of hyperaccumulators did show facilitated growth in several cases, but that was due to lower levels of the toxic metal, rather than higher levels as shown here for Se.

Nonprotein amino acids such as methyl-SeCys have been reported to commonly occur in soils, and their importance in ecological and physiological processes is becoming increasingly clear, e.g., via antiherbivory, antimicrobial, and allelochemical activity, or protection from stress [32]. It is also feasible that the bioavailability of Se around hyperaccumulators is affected via the excretion of chelators [33]. In future studies, it will be interesting to analyze the composition and activity of hyperaccumulator exudates, particularly with respect to the presence of Se chelators and selenocompounds. Yet another possible explanation for the finding that *S. ericoides* and *A. ludoviciana* beneficiaries were particularly enriched in Se relative to their soil may be that these neighboring plants are connected directly with their hyperaccumulator neighbors via mycorrhizae and derive selenocompounds via this access. It is known that mycorrhizal fungi are often not host-specific, and one fungal individual can interconnect neighboring plants of different species, distributing resources and facilitating plant growth [34].

Facilitation is thought to be especially important in extreme, harsh environments [35]. The seleniferous site studied here fits that description well. Not only is the soil seleniferous shale

Another very interesting aspect of our current study is that the same process, enrichment by hyperaccumulators of neighboring plants with Se, can at the same time have a competitive effect on one class of neighbors (Se-sensitive plants) and a facilitating effect on another class of neighbors (Se-tolerant plants).

The finding that hyperaccumulators have a negative effect on Se-sensitive ecological partners but offer a niche that may benefit Se-tolerant ecological partners is a recurring theme in our studies of the ecology of Se hyperaccumulators. Whereas Se-sensitive plants may suffer toxicity when growing on the high-Se soil next to hyperaccumulators, Se-tolerant plants benefit from the associated elevated Se levels because it protects them from herbivores. Earlier, we found that whereas Se-sensitive herbivores are deterred by hyperaccumulators and suffer toxicity when forced to feed on them, a Se-tolerant diamondback moth thrives on hyperaccumulator *S. pinnata* [24]. Additional leaf and seed herbivores have been found to occupy this and other hyperaccumulator species (C.F.Q. and E.A.H.P.-S., unpublished data). Similarly, whereas Se-sensitive fungal pathogens were less successful in colonizing high-Se than low-Se plants, Se-tolerant fungi were

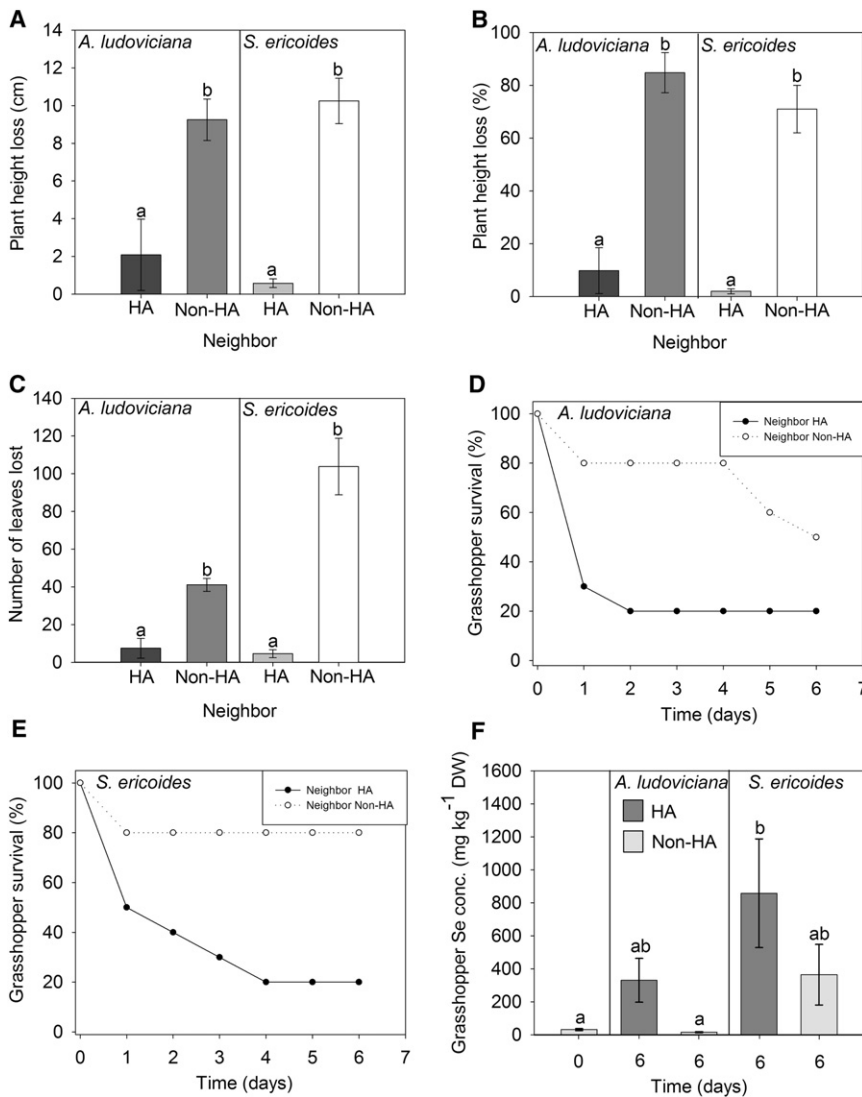


Figure 6. Nonchoice Experiment Comparing Herbivory, Survival, and Se Accumulation of Grasshoppers Fed *A. ludoviciana* or *S. ericoides* Plants Collected Next to Hyperaccumulator *A. bisulcatus* or Next to Nonhyperaccumulators

(A) shows absolute plant height loss, (B) shows relative plant height loss, (C) shows number of leaves lost, (D) shows grasshopper survival on *A. ludoviciana*, (E) shows grasshopper survival on *S. ericoides*, and (F) shows grasshopper Se concentration in animals from the field at day 0 and in animals collected after 6 days of cocultivation with *A. ludoviciana* or *S. ericoides*. Values shown represent means  $\pm$  SEM ( $n = 6$  for A–C;  $n = 1$  for D and E;  $n = 6$ –8 for F). Different lower case letters above bars indicate significantly different means ( $p < 0.05$ ).

benefits associated with elevated Se, i.e., reduced herbivory. Some Se-tolerant neighbors appear to actively forage for Se, judged from preferential root growth toward the hyperaccumulator. Earlier, Se-sensitive plants were shown to be negatively impacted by their elevated Se when growing on soil collected around hyperaccumulators. Thus, the Se deposited by hyperaccumulators likely has both competitive and facilitative effects, which may together affect species composition in seleniferous areas. This study provides the framework for future studies investigating the facilitative effects of hyperaccumulating plants on their neighbors. In future studies, it will be interesting to investigate whether the observed facilitation involves only ecological or also physiological benefits. Furthermore, it will be interesting to investigate whether the hyperaccumulators affect Se speciation in their neighbors and to what

observed to thrive in hyperaccumulator rhizosphere and litter [36, 37]. Moreover, there are indications that native bumblebee pollinators of hyperaccumulators in seleniferous areas are Se tolerant [38]. Taken together, Se appears to be very important for the ecological interactions of Se hyperaccumulator species, and hyperaccumulators may have a profound effect on the overall ecology of seleniferous habitats. The enhanced soil Se levels around hyperaccumulators may have a negative effect on Se-sensitive plant species while facilitating Se-tolerant ones. Via these mechanisms, hyperaccumulators may affect plant species composition and, consequently, higher trophic levels. If this is true, hyperaccumulators may be ecosystem engineers, modifying their local (seleniferous) habitat, influencing community distribution, and altering species abundance [39, 40]. This will be an intriguing question to address in future studies.

## Conclusions

This study is the first to show a facilitating effect by means of enrichment with a nonessential element. Selenium hyperaccumulators enrich their neighbors with Se, and when these neighboring plants are Se-tolerant, they enjoy the ecological

extent the soil phytoenrichment with Se is due to litter decomposition and/or root exudation. Also, species composition and abundance may be surveyed in more detail close to and away from hyperaccumulators in the field, to test the effect of the hyperaccumulators.

## Experimental Procedures

### Study Site

The field site for this study was Pine Ridge Natural Area in Fort Collins, CO (40°32.70N, 105°07.87W). The soil and vegetation properties of this seleniferous area were described in detail in a previous study [18]. For this study, we made use of naturally occurring plant species on the site: the two Se hyperaccumulating species *A. bisulcatus* (two-grooved milkvetch, Fabaceae) and *S. pinnata* (prince's plume, Brassicaceae), as well as two species often found in the vicinity of these hyperaccumulators: *Artemisia ludoviciana* (white sage; Asteraceae) and *Symphyotrichum ericoides* (white heath aster; Asteraceae). Furthermore, in one study, *Medicago sativa* (alfalfa; Fabaceae) was used as a control species.

### Effect of Proximity to Selenium Hyperaccumulators on Neighboring Plant Size and Elemental Concentration

*Artemisia ludoviciana* and *S. ericoides* plants were collected from three locations within the same area: (1) in close proximity (<1 m) to the



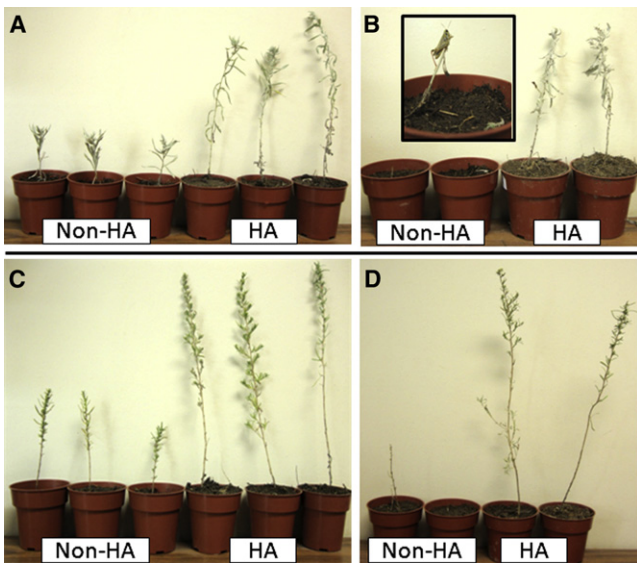


Figure 7. Representative Plants Used in the Laboratory Grasshopper Herbivory Experiments

(A) and (B) show *A. ludoviciana*, and (C) and (D) show *S. ericooides* plants used in the laboratory grasshopper herbivory experiments. The plants were collected in the field next to hyperaccumulator *A. bisulcatus* (HA) or nonhyperaccumulator (nonHA) neighbors. Before exposure to grasshopper herbivory (A and C) and after 6 days of exposure to grasshopper herbivory in the nonchoice experiment (B and D) (data shown in Figure 6). The inset in (B) shows a representative grasshopper at the end of the experiment.

hyperaccumulator *A. bisulcatus* (2) in close proximity to hyperaccumulator *S. pinnata*, and (3) next to nonhyperaccumulator species and >4 m from any hyperaccumulator. The sampling was as follows: four plants from each hyperaccumulator species (*A. bisulcatus* or *S. pinnata*) were selected. The hyperaccumulators selected were at least 8 m from other hyperaccumulators of the same species that were sampled. These plants were selected because they were the largest of their species at the field site, they had the highest concentration of Se, and they had populations of both *A. ludoviciana* and *S. ericooides* growing in close proximity (<1 m). Around each hyperaccumulator plant, four *A. ludoviciana* and four *S. ericooides* plants were collected. For comparison, the same number of *A. ludoviciana* and *S. ericooides* plants were collected from locations at least 4 m away from any hyperaccumulator plant. The *A. ludoviciana* and *S. ericooides* were analyzed for total biomass (dry weight of root plus shoot), stem height, and number of leaves. The shoot Se and S concentration was also determined, as described below. In addition, soil was collected from between the *A. ludoviciana* or *S. ericooides* plants and their neighbors (*A. bisulcatus*, *S. pinnata*, or nonhyperaccumulators). The soil samples were collected from the top 5 cm, after removal of any litter. The soil was sieved, acid-digested, and analyzed for Se and S as described earlier [18].

#### Determination of Root Directional Growth

Four plants from each of the hyperaccumulator species (*A. bisulcatus* or *S. pinnata*) were selected, and around each hyperaccumulator plant, the direction of root growth was determined for ten *A. ludoviciana* and ten *S. ericooides* plants. The root direction was classified as toward the hyperaccumulator when the root was bent horizontally and grew in the direction of the hyperaccumulator neighbor (i.e., in the quarter section of the radius that was closest to the hyperaccumulator). Root direction was classified as neutral when the root grew vertically or when it grew bent horizontally in a direction that was neither toward nor away from the hyperaccumulator neighbor (i.e., in the two quarter sections of the radius that were at intermediate distance from the hyperaccumulator neighbor). Finally, root growth was classified as away from the hyperaccumulator when the root grew bent horizontally, in a direction pointing away from the hyperaccumulator (i.e., in the quarter section of the radius that was furthest from the hyperaccumulator). The same experimental procedure was followed for the control species *M. sativa*, except that only four *A. ludoviciana* and four *S. ericooides*

plants were analyzed around each of the selected *M. sativa* individuals, and only three *M. sativa* plants were chosen. The reason for this lower number was that there were fewer *A. ludoviciana* and *S. ericooides* plants around *M. sativa*.

#### Field Arthropod and Herbivory Survey

The same *A. ludoviciana* and *S. ericooides* individuals, whose collection is described above under the heading “Effect of Proximity to Se Hyperaccumulators on Neighboring Plant Size and Elemental Concentration,” were surveyed for the number of arthropods they harbored in the field at the time of collection, as well as for their number of leaves with signs of herbivory. The arthropod collection was carried out as described earlier [41]. In short, this was done by shaking the plant vigorously inside a bucket and using an aspirator to collect the resulting animals. Leaves were classified as showing herbivory when part of the leaf or leaf margin was missing or when there was a hole in the leaf; necrotic spots were not counted as herbivory. Because *S. ericooides* showed two different types of leaves (small and large), herbivory was scored separately for both leaf types, and the Se levels in both leaf types were measured.

#### Laboratory Herbivory Experiments

Entire *A. ludoviciana* and *S. ericooides* plants were dug out in the field and placed in 10 cm diameter pots in their own field soil. For each species, two categories of plants were collected: (1) in close proximity to *A. bisulcatus* and (2) next to a nonhyperaccumulator and >4 m away from any hyperaccumulator. There were not enough *A. ludoviciana* and *S. ericooides* plants left in the field next to *S. pinnata* to look at the effect of that hyperaccumulator as well. The plants were taken to the lab, and in preparation for controlled herbivory experiments, the stem height of each individual plant was measured and the number of leaves counted; in addition, a leaf sample was collected for Se analysis. Grasshoppers were collected in bulk from the same field site, using a sweep net. Earlier [14], a similar sweep on this site yielded 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.

#### Choice Feeding Experiment

For each of the two species *A. ludoviciana* and *S. ericooides*, three aquaria were prepared, each containing three (high-Se) plants collected next to *A. bisulcatus* and three (low-Se) plants collected next to nonhyperaccumulator neighbors. Each of these plants had been analyzed for height, number of leaves, and Se concentration as described above. Eight grasshoppers were added to each aquarium, making sure that for each aquarium, animals of similar size were used, and the aquaria were covered. Over the subsequent 6 days, the plants were watered every 2 days, and grasshopper survival was counted daily. At the end of the 6-day herbivory trial, the remaining stem height and number of leaves of each plant were measured, and plant height and leaf loss were calculated from the difference between the initial and final numbers. Furthermore, the live and dead grasshoppers were collected and analyzed for Se as described below.

#### Nonchoice Feeding Experiment

For each of the two species *A. ludoviciana* and *S. ericooides*, two aquaria were prepared, each containing six plants: one aquarium contained (high-Se) plants collected next to *A. bisulcatus*, and the other aquarium (low-Se) plants collected next to nonhyperaccumulator neighbors. Ten grasshoppers were added to each aquarium, and herbivory and grasshopper survival were monitored over 6 days as described above.

#### Elemental Analysis

Leaves, soil, and animals collected as described above were acid-digested and analyzed for Se and S as described earlier [6]. In short, the samples were dried at 50°C for 48 hr, weighed, and digested in nitric acid as described [42]. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used as described by [43] to determine each digest’s elemental composition.

#### Statistical Analysis

The software programs JMP-IN (version 3.2.6) and SAS (both from the SAS Institute, Cary, NC) were used for statistical data analysis. Table S1 shows the results of all statistical analyses. A Student’s t test was used to compare differences between two means. Analysis of variance (ANOVA) followed by a post hoc Tukey-Kramer test was used when comparing multiple means.



A Brown-Forsythe test for unequal variances and a normal quantile plot was determined to ensure that the data conformed to the assumptions of our statistical analyses. In cases where distribution was not normal, a data transformation was performed. Specifically, an arcsine transformation was performed for the *S. ericoides* data in Figure 5B and a log transformation for the *A. ludoviciana* S levels in Figure 2C. In cases where transformations still did not yield normal distribution, different nonparametric tests were performed (Wilcoxon [Kruskal-Wallis], Median, Van der Waerden). All nonparametric tests showed the same results; the Van der Waerden results are shown in Table S1. For comparing direction of root growth (Figure 3), a chi-square test of association, a Z-test, and Fisher's exact test were used. The first null hypothesis tested (in the test of association) was that root growth of the two neighboring species (*A. ludoviciana* and *S. ericoides*) was not different in direction between the three species *A. bisulcatus*, *S. pinnata*, and *M. sativa*. The second null hypothesis tested (for the Z-test) was that the proportion of roots growing toward the central plant was not different between hyperaccumulator and *M. sativa* versus a one-sided alternative that the proportion of roots growing toward the hyperaccumulator was higher than the proportion growing toward *M. sativa*. To alleviate the problem that in some cases the expected frequency was less than 5, we performed the Fisher's exact test. For the grasshopper Se comparisons (Figure 5; Figure 6), the live and dead animals did not show significantly different Se levels and therefore the data were pooled. For grasshopper survival percentages, the data are the percentage of total grasshoppers that survived during the experiment; therefore no statistical analysis could be carried out on those data (Figures 6D and 6E).

#### Supplemental Information

Supplemental Information includes two figures and one table and can be found with this article online at doi:10.1016/j.cub.2011.07.033.

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