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Characterization and benefits of selenium uptake by an *Astragalus* hyperaccumulator and a non-accumulator

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Abstract

Background and Aims We characterized the relationship between soil and leaf concentrations of selenium in a hyperaccumulator and a non-accumulator to test the hypothesis that hyperaccumulators take up selenium while non-accumulators exclude it. We examined plant performance metrics and the ability of selenium to protect against herbivory by spider mites.

Methods Known hyperaccumulator and non-accumulator species within the genus *Astragalus* were grown under a range of selenium concentrations and measured for tissue selenium, extent of herbivory, and vigor.

Results Both hyperaccumulators and non-accumulators either failed to meet even the lenient threshold or exceeded even the strict threshold for hyperaccumulation depending on soil concentration. Both had decreased herbivory with increasing leaf selenium, and both grew larger at higher levels of selenium regardless of herbivory, despite a negative impact of higher relative uptake.

Conclusions The relationships between selenium dosage and tissue concentrations matched only some model predictions. Under these conditions, the bioconcentration factor was a better delimiter between species than the absolute tissue concentration. We provide evidence that despite the apparent cost of uptake, selenium can enhance the growth of hyperaccumulators even when herbivory is not a significant factor. We propose the term "elemental stimulation" for this phenomenon.

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Introduction

Hyperaccumulators are plants that take up metals or other trace elements from the soil and concentrate them in aboveground tissues at hundreds or thousands of times background levels. Hyperaccumulation as a phenomenon has been recognized for eight decades (e.g. Beath et al. 1934), even though the term was not coined until much later (see Brooks et al. 1977). Hyperaccumulation is widespread in terms of the number of taxa that accumulate, the life histories of accumulators, and the variety of elements that are accumulated (Krämer 2010; van der Ent et al. 2013; Pollard et al. 2014), and its study has broad phytotechnical (Barillas et al. 2011), ecological (Boyd

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and Martens 1998; Maestri et al. 2010), and evolutionary (Broadley et al. 2001) implications.

Tissue concentration thresholds to determine whether a species is a hyperaccumulator have been established for a range of elements. For the element selenium, plants are considered hyperaccumulators if, when grown on native soil, their leaves contain more than 1000 mg/kg selenium dry weight (Boyd 2007), although some authors argue that the threshold should be as low as 100 mg/kg (van der Ent et al. 2013). Determining whether a plant actually meets those criteria though is more complicated than it may appear (Boyd 2007; Rascio and Navari-Izzo 2011). *Astragalus bisulcatus*, a widely studied, obligate selenium hyperaccumulator known to accumulate more than 10,000 mg/kg (Shrift 1969, Sors et al. 2009), has been collected from its native habitat with tissue concentrations as low as 10 mg/kg, with the median concentration being less than 300 mg/kg (Shrift 1969). A more recent study had similar results, with leaf concentrations at field sites ranging from 95 to just 160 mg/kg (Sors et al. 2009), far below the 1000 mg/kg hyperaccumulator threshold, and with two of three sites containing no individuals above the 100 mg/kg threshold.

The huge range in observed concentrations may be due to individual variation, but it is likely due to variation in soil as well. Native soil concentrations of selenium are far from uniform, with reports from "seleniferous soils" ranging from near zero up to 212 mg/kg (Beath et al. 1937). Most "normal" soils, meanwhile, contain less than 2 mg/kg (Oldfield 2002). It is also often unclear how much selenium in soil is bioavailable, given that there is a poor correlation between total and bioavailable selenium, with the bioavailable portion ranging at least from 0.2 % to 81 % of total soil selenium (Statwick, unpublished data).

A conceptual model of hyperaccumulation predicts that hyperaccumulators should increase tissue concentrations as a function of soil availability until they plateau due to saturation of the uptake pathways, negative feedbacks, or both. Non-accumulators are not expected to take up selenium at all until a concentration that results in rapid toxicity and death (van der Ent et al. 2013). While these model predictions are intended to describe the metal uptake of plants in the field, the degree to which these uptake rates are actually a reflection of species' abilities and not other factors is difficult to verify in an uncontrolled, observational field setting. Climate (Bhatia et al. 2005), soil chemistry (Cakmak 2007), rhizosphere (Lindblom et al. 2013), local genotypes (Roosens et al. 2003), and other factors can all affect the uptake of metals, and could all potentially covary with available soil metal content, thus making it nearly impossible to determine uptake in the field as a function of availability alone. Perhaps more importantly, hyperaccumulators and non-accumulators generally have little or no overlap in natural habitat, making paired comparisons impossible in the field. Thus, to determine the extent to which the relationship between availability and uptake is due to species capacity and not other factors, we compared the response of a congeneric hyperaccumulator and non-accumulator to a range of selenium concentrations that could be encountered by a plant in the field, using a greenhouse-based dose response design that allowed us to hold other variables such as soil texture and moisture constant. We chose to use spiked potting soil instead of field-collected soils in order to a) control for soil variability and b) avoid depleting the finite amount of selenium in a potted volume of field soil (Goolsby and Mason 2015). Given the model above, we expected our hyperaccumulator to rapidly accumulate selenium at low soil concentrations, but to plateau at higher concentrations. We predicted that our non-accumulator would act as a "normal" plant, maintaining a constant low tissue concentration of selenium until some threshold, followed by rapid toxicity and death.

We also tracked plant performance metrics to investigate the hypothesis that selenium, although non-essential to plants (Novoselov et al. 2002; Fu et al. 2002; Lobanov et al. 2009) and easily toxic (Brown and Shrift 1982), can actually enhance the growth of hyperaccumulators, even at extreme concentrations, and even in the absence of ecological stressors. Early literature includes both support (Trelease and Trelease 1938, 1939; Davis 1972) and criticism (Broyer et al. 1972a, b), of this hypothesis, but trace elements stimulating growth directly has not appeared in reviews of hypotheses for adaptive value of hyperaccumulation (e.g. Boyd and Martens 1998; Rascio and Navari-Izzo 2011) until very recently (e.g. Cappa and Pilon-Smits 2014).

It is well established in the fields of pharmacology and toxicology that very low doses of toxic compounds can have stimulatory effects on organisms due to the overcompensation of bioprotective response pathways, in a phenomenon known as hormesis (Calabrese et al. 2007; Mattson 2008). These benefits are characteristically inversely U-shaped, with a narrow range of

concentrations that are beneficial, followed by detrimental toxic effects (Stebbing 1982; Calabrese and Baldwin 2001). Many plants show this pattern, benefiting from fortification with small amounts of trace elements such as zinc and selenium, which become toxic at higher concentrations (Xue et al. 2001; Cakmak 2007; Yao et al. 2009). Nonetheless, while hormetic responses are adaptive, it is typically not the stressor itself that is beneficial, but rather the downstream cellular responses to that stressor (Mattson 2008). This would imply that selection on or upregulation of hormetic pathways should improve stress tolerance generally, but not necessarily the response to an individual stressor specifically (Stebbing 1982). Indeed, it is often the case that exposure to low levels of one stressor (e.g., heat) can reduce the damage done by an entirely different stressor (e.g., cyanide) (Mattson 2008).

Yet, there is a growing body of evidence that hyperaccumulators show positive responses to specific toxic elements (rather than toxic elements generally) at concentrations far higher than those that stunt the growth of normal plants (Küpper et al. 2001; El Mehdawi et al. 2012; Ghasemi et al. 2014; Pollard et al. 2014, Kazemi-Dinan et al. 2015). Hyperaccumulators also appear to benefit across ranges of concentrations that are far broader (e.g. >1000-5000fold (Küpper et al. 2001; Pollard et al. 2014)) than the 10-20fold range typical of a hormetic response (Calabrese et al. 2007). This implies that the direct benefits of hyperaccumulation are mechanistically different from hormesis, and could conceivably be acted upon by selection in such a way as to promote or maintain hyperaccumulation through evolutionary time.

Of the more commonly explored adaptive reasons for hyperaccumulation, only "elemental defense", or the idea that trace elements protect plants from natural enemies such as herbivores and pathogens, has been examined in any depth (Rascio and Navari-Izzo 2011). Elemental defense has been well supported in a wide range of systems (for review, see Boyd 2007), and has become the primary adaptive justification for hyperaccumulation. However, it is not implausible or even improbable that other evolutionary drivers, including a direct benefit of trace elements themselves, exist in concert with elemental defense (Trumble and Sorensen 2008). If trace elements can indeed enhance the growth of hyperaccumulators, even in the absence of natural enemies, we would predict that *Astragalus* selenium hyperaccumulators would grow larger with increasing selenium while non-accumulators would be negatively

impacted by selenium, as it is toxic to most organisms at relatively low concentrations.

We designed an experiment to test the effects of soil selenium in the absence of other environmental stress, however, during our treatment period and despite control efforts, there was an unplanned and persistent infestation of two-spotted spider mite (*Tetranychus urticae*), a generalist cell-disruptor herbivore. Although this confounded our original intent, it gave us the opportunity to investigate the relationship between tissue selenium concentration and herbivory, and allowed us to examine the impact of both elemental defense and selenium dosage on plant performance. Because selenium in *A. bisulcatus* has been previously shown to deter spider mites (Quinn et al. 2010), we predicted that selenium would reduce herbivory in hyperaccumulators, but that its concentration would be too low to protect non-accumulators.

Methods

Study species

We chose two species of *Astragalus* to investigate the hyperaccumulation of selenium. This genus is often thought of as broadly seleniferous, in part because of a substantial but unknown number of species that exhibit at least mild selenium tolerance (Davis 1972; Wang et al. 1999; Moreno Rodriguez et al. 2005; Sors et al. 2009). In fact, there are only 25 known species (<1 % of the genus) classified as true hyperaccumulators (Barneby 1964; Welsh 1985). This gives us the opportunity to test the response to selenium of congeneric species with different a priori tolerance. *Astragalus bisulcatus* (Hook) A. Gray is a fairly widespread hyperaccumulator native to the western United States, and is the most commonly used model hyperaccumulator in the genus (Trelease and Trelease 1938; Freeman et al. 2006; Sors et al. 2009). It is known only to inhabit seleniferous soils in the wild (Barneby 1964). *Astragalus cicer* L. is an Old World species that has been introduced to the United States as a forage crop, in part due to its lack of seleniferous habit and broad tolerance of edaphic and climatic conditions (Acharya et al. 2006). The "Oxley" ecotype used in this study that was first collected in the former USSR and introduced to the United States in 1971 (Acharya et al. 2006). *Astrasgalus cicer* has been shown to accumulate little or no selenium in controlled greenhouse

experiments (Davis 1972), although in tissue culture its cells can be artificially selected to tolerate limited quantities of selenium (Wang et al. 1999).

Greenhouse setup

We planted 98 seeds each of *A. cicer*, the non-accumulator ("Oxley" ecotype, propagated - Granite Seed, Denver, CO), *A. bisulcatus*, the hyperaccumulator (wild collected - Western Native Seed, Coaldale, CO), after physical scarification, on April 26, 2013 at Denver Botanic Gardens (DBG). After one month, we repotted plants, most of which had 1–2 true leaves, in 3.5 in. square pots in soil that was 3 parts Fafard® 4P Mix and one part Turface™. When plants were four months old, three plants of each species were randomly assigned to 12 treatments and arranged in a Latin Square Design. We dosed plants with sodium selenate solutions because selenate is readily bioavailable to hyperaccumulators (Shrift and Ulrich 1969), and because it is the most common bioavailable form of selenium that hyperaccumulators might encounter in the field (Oldfield 2002). Serial dilutions of sodium selenate in tap water were prepared (*w/v*) such that each dilution contained 30–33 % of the concentration of the previous dose, resulting in 12 different treatments from 100 mg/L to 1 µg/L sodium selenate, (i.e., 100 mg/L, 30 mg/L, 10 mg/L, 3 mg/L, 1 mg/L, 300 µg/L, 100 µg/L, 30 µg/L, 10 µg/L, 3 µg/L b, 1 µg/L, and tap water control). Sodium selenate is 41.8 % elemental selenium by mass, such that the doses ranged from 41.8 mg/L (529 µM) to 0.418 µg/L (5.29 n*M*) of elemental selenium. Plants were watered exclusively with their treatment solution for the duration of the experiment, in order to resupply selenium lost by uptake or gradual chemical reduction to unavailable forms (Lu et al. 2009). All plants were regularly watered generously to saturation and allowed to drain freely into hazardous waste containers, in order to elute any excess selenate buildup.

Tissue concentration via ICP-MS

After drying and massing the plants, we removed approximately 25 mg of dried young whole leaf tissue from each plant and pulverized it in a ball mill. We precisely massed between 1 and 10 mg of powdered tissue from each sample and added 750 µl concentrated nitric acid, 250 µl concentrated hydrochloric acid, 100 µl concentrated hydrofluoric acid, and 100 µl concentrated hydrogen peroxide, all of which were trace metal grade (Thermo Fisher Scientific). Samples were then high-pressure digested in a Milestone Ethos EZ (Shelton, CT) microwave digester at 210 °C for 21 min. Samples were then diluted to 15 ml with >18.0 MΩ cm water and analyzed via Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) with an Aglient 7700× (Santa Clara, CA). Microwave digested acids with no plant material and the National Institute of Standards and Technology's Standard Reference Material 1570a "Trace Elements in Spinach Leaves" were used as negative and positive controls, respectively. The method detection limit for the ICP-MS analyses was 0.53 µg Se L^{-1}, and samples ranged from 0.72–716 µg Se L^{-1}.

Plant performance

Plant leaf number and stem length were measured weekly during the treatment period until the final measurement on November 8, 2013 when plants were 7 months old and, by our stem and leaf measurements, no longer appeared to be growing substantially. None had flowered. After the treatment period ended, plants were removed from soil, cleaned, and dried. Aboveground and belowground parts were separated and massed. To account for pre-treatment differences in size, we analyzed the net leaf proportion and net stem growth of plants by subtracting the initial value of leaf number and stem length, respectively, from the final values and then dividing by the initial values.

Herbivory

Spider mites are common greenhouse pests that are typically well controlled by overhead watering, but spread quickly in xeric plants or plants watered at the base. Due to our hand watering, we had a persistent spider mite (*Tetranychus urticae*) infestation during the treatment period. Because DBG is primarily a propagation greenhouse – not an experimental greenhouse – we were not permitted to let the infestation proceed unchecked. We attempted control by periodically inverting each plant and spraying the shoots and leaves with water. The entire greenhouse was fumigated twice during the growing period with the biological insecticides BotaniGard® ES and Aza-Direct, for additional arthropod control.

To quantify degree of herbivory, on November 1, one week before the final harvest, we took digital images of the youngest fully expanded leaf on the main stem of each plant (representing approximately 5–20 % of total leaf area, on average). Because spider mites are cell disruptor herbivores which cause yellowed spots wherever they have fed, we used ImageJ v1.48 and the color threshold tool to calculate the proportion of each leaf that was damaged by herbivory. Images were anonymized and randomized before analysis to eliminate experimenter bias. Images with ambiguous damage/senescence were excluded.

Statistical analysis

All data sets were analyzed via JMP v11. The two species were analyzed together with species as a model effect. To investigate how species and soil Se dosage predict tissue Se concentration and how species and tissue Se concentration predict herbivory, we used ANCOVA with Se dosage and tissue Se as the covariate, respectively. For both of these tests, $\log_{10} + 0.0001$ transformations were applied to both independent and dependent variables to improve normality, since these data sets were highly right-skewed, contained zeros, and spanned several orders of magnitude. We chose this transformation rather than the more standard $\log_{10} + 1$ transformation because our datasets contain values both greater than and less than 1, and a $\log_{10} + 1$ transformation artificially compresses values less than 1 relative to values greater than 1, resulting in a dataset that remains right-skewed. To investigate the ability of the three factors, species, herbivory, and soil dosage, to predict plant performance metrics, we ran two separate ANCOVAs, since herbivory and soil dosage are highly correlated. In both instances, species was the categorical model effect and either herbivory or soil dosage was analyzed as the covariate. For these two analyses we left both independent and dependent variables untransformed, since the dependant variables were approximately normal. We also tested the effects of tissue Se and bioconcentration factor (the ratio of soil dosage to leaf concentration) on these same plant performance metrics. We ran two additional ANCOVAs using species as the categorical model effect and either $\log_{10} + 0.0001$ transformed tissue Se or $\log_{10} + 0.0001$ transformed bioconcentration factor as the covariate. Because of our uncertainty about background levels of selenium in our materials, and thus the accuracy of the bioconcentration factors for the lowest dosages, we excluded plants dosed with less than 0.1 mg/kg Se for the bioconcentration factor analysis.

Results

Soil dosage versus tissue concentration

Both *A. cicer* and *A. bisulcatus* accumulated substantial amounts of selenium. *A. cicer*, the non-accumulator, accumulated as much as 1052 mg/kg of selenium in its dry mass. *A. bisulcatus*, the hyperaccumulator, generally accumulated at least an order of magnitude more than *A. cicer*, ending the treatment period with Se representing as much as 10,000 mg/kg or more of total dry mass. There was a high degree of individual variability within treatments, with as much as 5-fold differences between *A. cicer* individuals and 10-fold differences between *A. bisulcatus* individuals at the same dose (Fig. 1, Fig. S1). 3 % of *A. cicer* individuals surpassed the 1000 mg/kg threshold, 30 % surpassed only the 100 mg/kg threshold, while 67 % surpassed neither. For *A. bisulcatus*, 25 % surpassed the 1000 mg/kg threshold, 19 % surpassed only the 100 mg/kg threshold, and fully 56 % failed to surpass either – a distribution that is strikingly similar to that of wild-collected *A. bisulcatus* plants at ~20 %, ~30 % and ~50 %, respectively (Shrift 1969).

By ANCOVAs, both *A. cicer* and *A. bisulcatus* had leaf concentrations of selenium that, although different from one another with means of 4.0 mg/kg and 20.2 mg/kg, respectively, each remained flat when dosed with between 0 and 100 µg/L of sodium selenate (species $P < 0.001$, log dosage $P = 0.11$, interaction $P = 0.49$, $n = 36$, $R^2 = 0.52$) (Fig. 1). Between 100 µg/L and 10 mg/L sodium selenate, however, the concentration of selenium in both species rose rapidly, up to an average of 394.6 mg/kg for *A. cicer* and 4287.3 mg/kg for *A. bisulcatus* (species $P < 0.001$, log dosage $P < 0.001$, interaction $P = 0.86$, $n = 30$, $R^2 = 0.80$) (Fig. 1). Notably, in this range, which represents the vast majority of seleniferous native soils (Oldfield 2002), the leaf selenium content of the two species was not distinguishable by simple T-test ($P = 0.18$, $n = 30$, df = 1). However, bioconcentration factor, i.e. the ratio of leaf selenium to bioavailable soil selenium, was significantly greater for *A. bisulcatus* (805:1) than
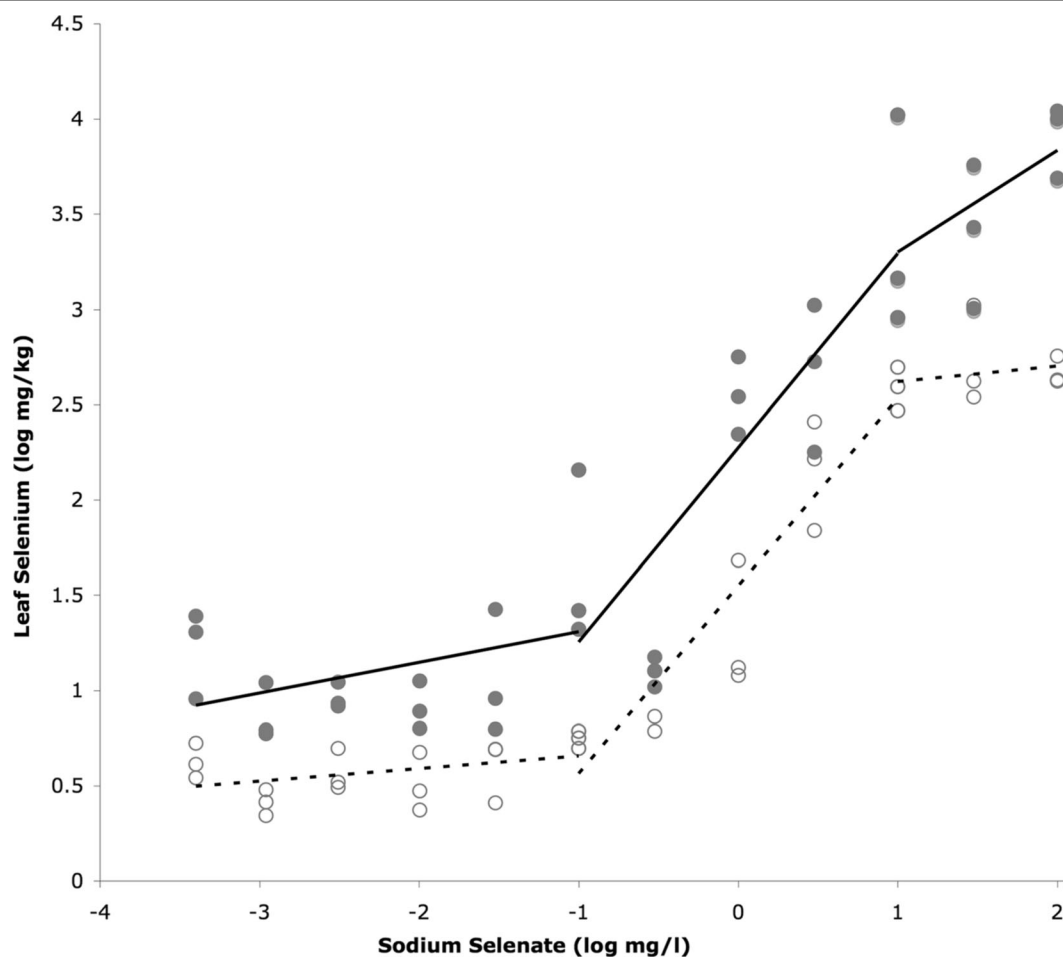
Fig. 1 Accumulation curves of log-transformed whole leaf selenium concentration versus log-transformed soil dosage of sodium selenate. Filled circles are hyperaccumulator *A. bisulcatus* individuals and open circles are non-accumulator *A. cicer* individuals. Linear regression analyses were broken into three segments, 0– 0.1 mg/l, 0.1-10 mg/l, and 10-100 mg/l Solid lines represent best fit lines for *A. bisulcatus* and dashed lines represent best fit lines for *A. cicer*. Species was a significant factor in all three segments, but dosage was only significant for the 0.1-10 mg/l segment

for *A. cicer* (97:1) ($P < 0.01$, $n = 30$, df = 1). From 10 mg/L to 100 mg/L, the accumulation of selenium in both species flattened out once more at an average leaf concentration of 490.7 mg/kg in *A. cicer* and 5356.4 mg/kg in *A. bisulcatus* (species $P < 0.001$, log dosage $P = 0.10$, interaction $P = 0.21$, $n = 24$, $R^2 = 0.71$) (Fig. 1).

Tissue concentration versus herbivory

There was a significant interaction between species and tissue concentration of selenium in predicting herbivory (ANCOVA, species $P < 0.001$, log tissue Se $P < 0.001$, interaction $P < 0.001$, $n = 59$, $R^2 = 0.60$). In other words, although both species experienced declining herbivory with increasing tissue concentrations of selenium, *A. bisulcatus*, the hyperaccumulator, was relatively well protected from herbivory across all leaf concentrations, while *A. cicer*, the non-accumulator, was poorly protected from herbivory at low leaf concentrations but well protected at the higher concentrations (Fig. 2).

Plant performance

Because of a strong colinearity between selenium variables and herbivory, we ran separate analyses to evaluate the ability of each variable to predict plant performance metrics.
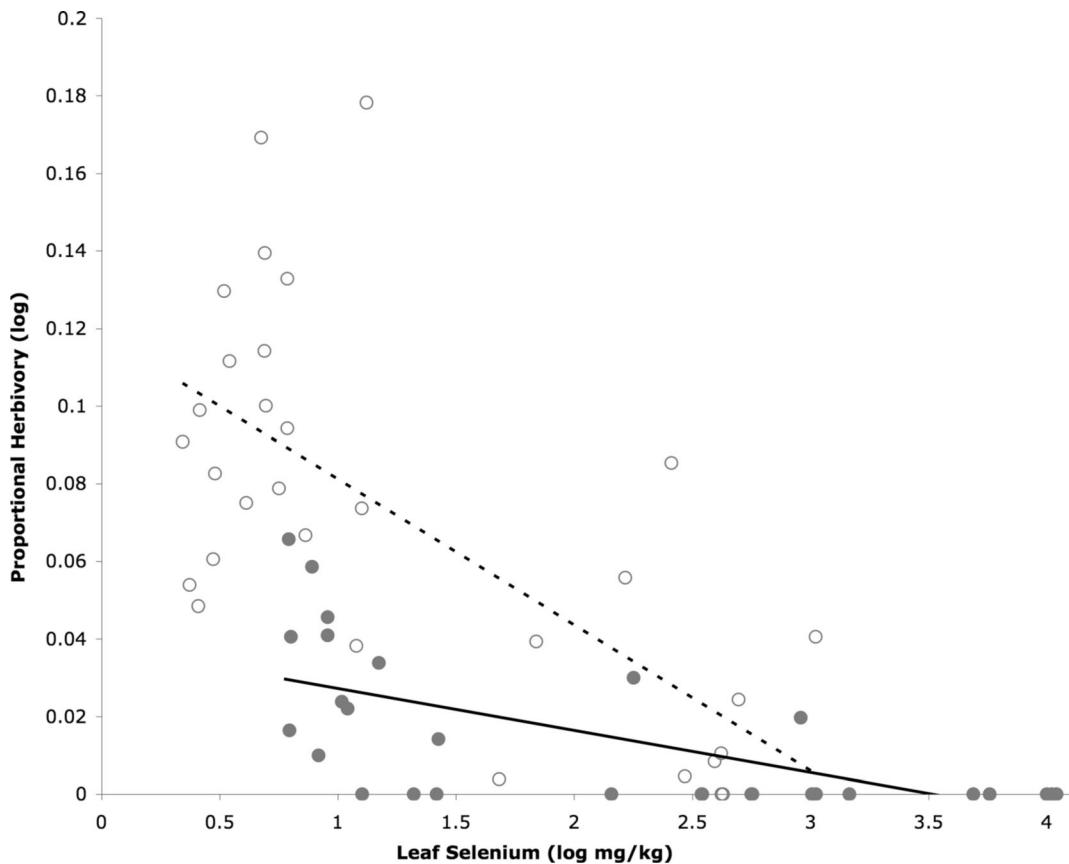
Fig. 2 Plot of log-transformed herbivory (proportion of leaf area damaged) versus total log-transformed leaf selenium concentration. Filled circles are hyperaccumulator *A. bisulcatus* individuals and open circles are non-accumulator *A. cicer* individuals. Solid lines represent best fit lines for *A. bisulcatus* and dashed lines represent best fit lines for *A. cicer*. There was a significant interaction between species and leaf concentration in predicting herbivory, although both species had significant declines in herbivory with increasing tissue selenium

Herbivory versus plant performance

There were no significant effects of herbivory or interactions between herbivory and species on any plant performance metric (ANCOVA, Table 1), although some metrics appeared to be trending toward significance.

Soil dosage versus plant performance

There were significant effects of selenium dosage and/or dosage by species interactions for all plant performance metrics investigated (ANCOVA, Table 2). Although *A. cicer*, the non-accumulator, had greater aboveground biomass across all treatment groups than *A. bisulcatus*, the hyperaccumulator, both species grew larger with increasing selenium dosage (Fig. 3a). However, there was an interaction between species and dosage in the

effect on belowground biomass, with *A. cicer* having no change in root biomass but *A. bisulcatus* having increased root biomass at higher concentrations of soil selenium (Fig. 3b). Consequently, there was also an interaction between species and dosage in predicting root/shoot ratio. *A. cicer* showed a significant decline in root/shoot ratio with increasing selenium while *A. bisulcatus* showed no change (Fig. 3c).

Similarly, there were interaction effects for both net leaf proportion and net stem growth. While nearly all plants had a net loss of leaves over the treatment period, *A. cicer* plants at higher doses of selenium lost more leaves than those at lower doses, while the opposite was true for *A. bisulcatus* (Fig. 3d). *A. bisulcatus* plants at higher doses of selenium had more stem growth than those at lower doses, but there was no difference across *A. cicer* plants.

Table 1 ANCOVA table of species and dosage on plant performance metrics. Non-significant p-values are grayed

	Species	Dosage	Species by Dosage Interaction	n	R^2 adj.
Aboveground Mass	$P < 0.001$	$P < 0.001$	$P = 0.48$	72	0.58
Belowground Mass	$P < 0.001$	$P = 0.90$	$P < 0.05$	72	0.49
Root/Shoot Ratio	$P < 0.001$	$P < 0.05$	$P < 0.01$	72	0.17
Net Leaf Proportion	$P < 0.001$	$P < 0.001$	$P < 0.001$	72	0.60
Net Stem Growth	$P = 0.05$	$P = 0.07$	$P < 0.05$	72	0.11

Most notably, no *A. cicer* plants at any concentration died during the treatment period. None of the three *A. cicer* plants at the highest concentration of sodium selenate (100 mg/L) showed evidence of herbivory, but all displayed apparent stress, as evidenced by a reddish leaflet margin and rachis. This was not necessarily associated with a high leaf selenium concentration, as some plants at lower soil concentrations had similar or higher leaf concentrations but no red margin.

Thus, the substrate generalist *A. cicer* showed positive, neutral, and negative responses to selenium, depending on the performance metric, while the hyperaccumulator *A. bisulcatus* grew better with increasing selenium by every metric.

Leaf selenium and bioconcentration factor versus plant performance

The effects of log-transformed leaf selenium concentrations on plant performance metrics were qualitatively and quantitatively similar to those of soil dosage (ANCOVA, Table S1, Fig. 3e). Log-transformed bioconcentration factor, on the other hand, had nearly the opposite effect on plant performance (ANCOVA, Table 3). It was not a significant predictor leaf proportion, stem length, or belowground biomass, but aboveground biomass declined sharply and significantly for both species with increasing bioconcentration factor (Fig. 3f). In fact, bioconcentration factor was a stronger

predictor (by adjusted R^2) of aboveground biomass than either soil or leaf concentrations of selenium. The interaction between bioconcentration factor and species was significant, meaning that *A. cicer*, the non-accumulator, is more negatively impacted by increasing bioconcentration factor than is *A. bisulcatus*, the hyperaccumulator. Driven by the loss of aboveground biomass, the root/shoot ratio increased significantly for both species with increasing bioconcentration factor, and the interaction effect again indicates a stronger negative impact on *A. cicer*.

It should be noted that there is substantial overlap between species in leaf tissue Se concentrations (Fig. 3e), while there is very little overlap between species in bioconcentration factor (Fig. 3f).

Discussion

Tissue concentration

We were surprised to find that the accumulation curves of both non-accumulators and hyperaccumulators differed from the predictions of the conceptual model (van der Ent et al. 2013). Both plant species had a logistic pattern of accumulation, with no change in tissue concentration across the lowest dosages, a rapid rise at intermediate dosages, and plateauing concentrations at high dosages. This differed from both the logarithmic

Table 2 ANCOVA table of species and herbivory on plant performance metrics. Non-significant p-values are grayed

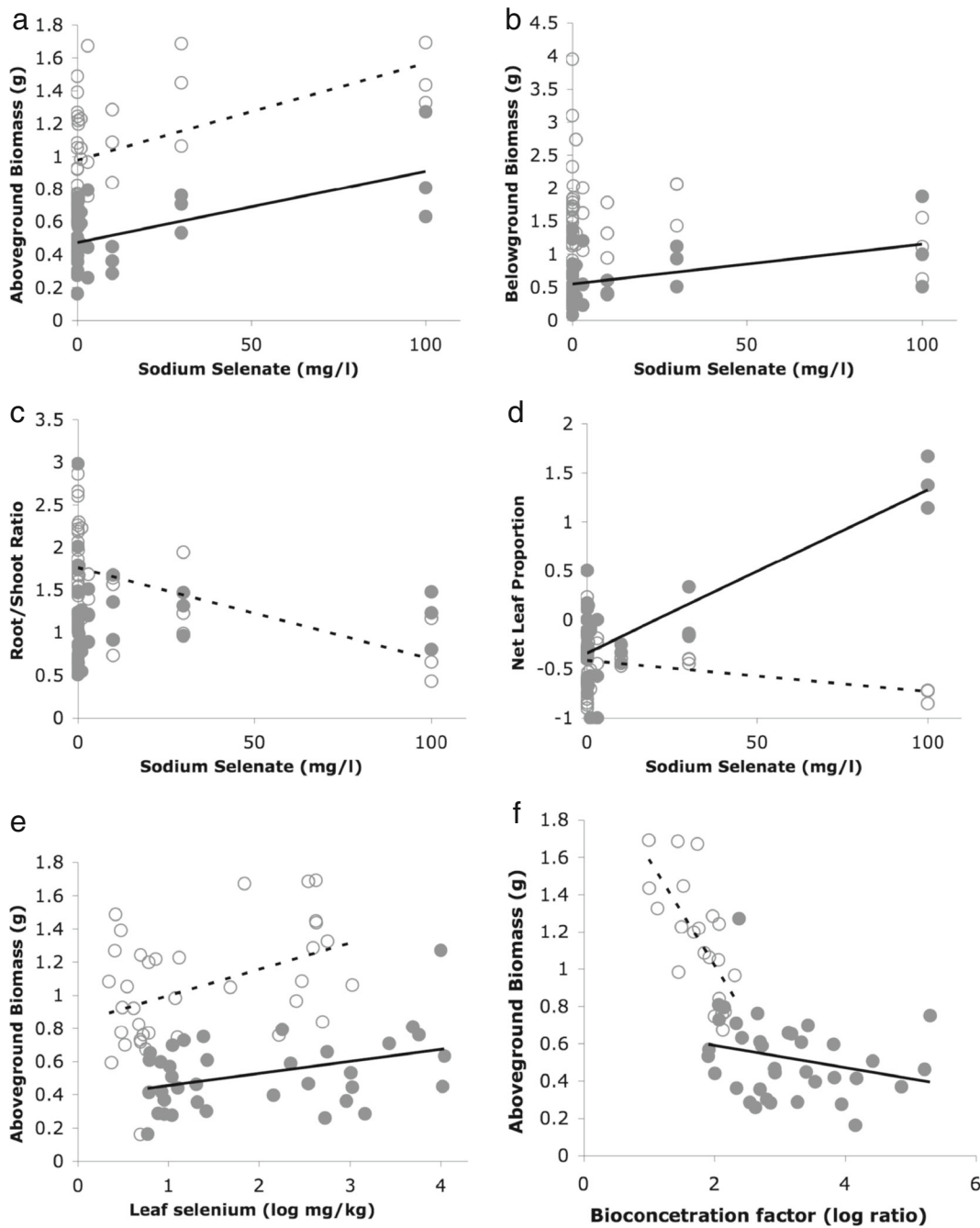|	Species	Herbivory	Species by Herbivory Interaction	n	R^2 adj.
Aboveground Mass	$P < 0.001$	$P = 0.07$	$P = 0.86$	60	0.51
Belowground Mass	$P < 0.001$	$P = 0.98$	$P = 0.28$	60	0.43
Root/Shoot Ratio	$P = 0.10$	$P = 0.16$	$P = 0.17$	60	0.36
Net Leaf Proportion	$P = 0.44$	$P = 0.10$	$P = 0.08$	60	0.15
Net Stem Growth	$P = 0.99$	$P = 0.08$	$P = 0.37$	60	0.07

Fig. 3 Plant performance plots for *A. cicer* and *A. bisulcatus*. Filled circles are hyperaccumulator *A. bisulcatus* individuals and open circles are non-accumulator *A. cicer* individuals. Untransformed sodium selenate dosage is plotted against the untransformed variables **a** aboveground biomass, **b** belowground biomass, **c** root/shoot ratio, and **d** net leaf proportion. **e** Log-transformed whole leaf selenium and **f** log-transformed bioconcentration factor are both plotted against untransformed aboveground biomass. Trendlines are included only for significant relationships

accumulation expected for hyperaccumulators and the flat exclusion of selenium expected for non-accumulators. The flat uptake at low levels of added Se may have

several explanations. Our lowest treatment levels of Se may not have exceeded the soil's binding capacity, such that the Se was not biologically available until after the

Table 3 ANCOVA table of species and log-transformed bioconcentration factor on plant performance metrics. Non-significant p-values are grayed

	Species	Bioconcentration	Species by Bioconcentration Interaction	n	R^2 adj.
Aboveground Mass	$P < 0.01$	$P < 0.001$	$P < 0.05$	42	0.68
Belowground Mass	$P < 0.001$	$P = 0.72$	$P = 0.21$	42	0.52
Root/Shoot Ratio	$P < 0.01$	$P < 0.05$	$P < 0.01$	42	0.29
Net Leaf Proportion	$P = 0.10$	$P = 0.93$	$P = 0.08$	42	0.11
Net Stem Growth	$P < 0.05$	$P = 0.46$	$P = 0.73$	42	0.08

point when the soil became saturated. Alternatively, the background levels of selenium in our materials may have masked any changes in uptake at these low treatment levels. No solid potting media or municipal tap water can be entirely devoid of selenium, and hyperaccumulator seeds alone can contain more than 2000 mg/kg selenium (Trelease and Trelease 1938) (unless perhaps grown for several generations in ultrapure hydroponic solutions, as done for nickel by Brown et al. 1987). As such, it is possible that both species theoretically do have a logarithmic accumulation curve that we did not see for methodological reasons. It is also possible that differences in Se concentration at this small a scale (<100 μg/L) are simply not biologically relevant. Still, we feel that the rapid rise and subsequent plateau for the hyperaccumulator *A. bisulcatus* are largely compatible with the model predictions.

In our non-accumulator, *A. cicer*, however, our results deviated dramatically from predictions. *A. cicer* was expected to maintain a consistently low concentration of selenium in its tissues across all soil dosages by actively preventing the uptake and transport of selenium until some threshold at which acute toxicity stunted or killed the plant (Rascio and Navari-Izzo 2011). Instead, no *A. cicer* plants died, and the accumulation curve for *A. cicer* had the shape expected for a hyperaccumulator, albeit at a lower magnitude. One *A. cicer* individual even exceeded the 1000 mg/kg level typically cited as the hyperaccumulator threshold for selenium (Boyd 2007), and eleven individuals exceeded the more lenient 100 mg/kg threshold (van der Ent et al. 2013), although we grant that such thresholds are only considered valid for plants growing on native soils (Rascio and Navari-Izzo 2011).

Still, we feel strongly that *A. cicer* is indeed a true non-accumulator - not a cryptic hyperaccumulator. True field hyperaccumulation in *Astragalus* is limited two closely related clades containing 25 species (including *A. bisulcatus*) nested well within the monophyletic new world group known as Neo-Astragalus (Barneby 1964; Wojciechowski 2005). *A. cicer,* as an Old World species, is well separated from the hyperaccumulators by several hundred non-accumulating sister species and at least 4.4 ± 0.8 million years of divergence (Wojciechowski et al. 1999, Wojciechowski 2005). Meanwhile, a broad taxonomic range of *Astragalus* non-accumulators have been shown to accumulate more than 200 mg/kg when dosed in the greenhouse with as little as 1.6 mg/L (20 μM) selenium as selenate (Sors et al. 2005). Even the model plant *Arabidopsis thaliana* can accumulate as much as 1000 mg/kg when dosed with just 4 mg/L (50 μM) selenate (Zhang et al. 2007). Indeed, many, if not most, plants seem to behave this way (Pollard et al. 2014). Clearly, the phenomenon of induced accumulation in metaliferous soil is not unique to *A. cicer*.

There are several possible reasons *A. cicer* and other non-accumulators do not actively exclude selenium in greenhouse studies as had been predicted. First, non-accumulators, particularly those that are naïve to metaliferous environments, have little adaptive incentive to evolve active metal exclusion mechanisms. We might expect that only non-accumulating metalophytes (plants that habitually live on metaliferous soils) such as *Silene vulgaris* (termed "excluders" in the model of van der Ent et al. 2013) would adaptively benefit from such mechanisms. Second, perhaps hyperaccumulation ability may not be as bimodal as the admittedly arbitrary concentration thresholds make it appear. Some argue that hyperaccumulation, at least for certain taxa, may not be a physiologically distinct phenomenon, but rather just the right tail of a lognormal distribution of uptake (van der Ent et al. 2013). Although much more in-depth sampling of a range of species would need to be done to confirm or refute that hypothesis (similar to the

methodology of White et al., 2007, with more conge-neric comparisons), the similar curve shapes between species and unexpectedly high tolerance of *A. cicer* do seem to provide some preliminary support for the idea. Finally, it may be the case that non-accumulators do indeed act as excluders in the field, but not in the greenhouse, for reasons that are not yet clear, but could include differences soil texture, soil chemistry, selenium speciation, or the rhizosphere. Either way, the model prediction for "normal" plants may need to be revised from flat exclusion to sigmoidal or logarithmic uptake if common garden experiments and wild plant censuses find similar results.

Herbivory and elemental defense

Since the elemental defense hypothesis is already very well supported (Boyd 2007), and has even been demon-strated specifically for *A. bisulcatus* and spider mites (Quinn et al. 2010), we were not surprised to find spider mite herbivory on *A. bisulcatus* decrease with increasing tissue concentrations of selenium. It is worth noting, however, that the significant negative relationship be-tween tissue selenium and herbivory in *A. cicer* shows that selenium as an elemental defense can be effective even in generalist non-accumulator plants growing in relatively typical soil concentrations. It has been well documented that metals deter most herbivores even at relatively low concentrations in artificial diets (Coleman et al. 2005; Cheruiyot et al. 2013), in accumulators and hyperaccumulators (Hanson et al. 2003, 2004; Behmer et al. 2005, Quinn et al. 2010), and in excluder metalophytes (Ernst et al. 1990). However, since gener-alist plants seem to store different chemical forms of metals than metal-adapted plants (Sors et al. 2005) it is apparent from our results that even low concentrations of the less volatile inorganic compounds characteristic of generalists can be an effective defense. Thus, our results support the "defensive enhancement hypothesis", or the idea that the first generalist plants capable of colonizing toxic substrates could have received an immediate, albeit small, defense against herbivory, even before the evolu-tion of true hyperaccumulation or metalliferous habit (Boyd 2012).

Perhaps the more unexpected finding is that for a given leaf concentration of selenium, *A. bisulcatus* has less herbivory than *A. cicer*, at least up until about 1000 mg/kg, when the two converge near zero herbivory (Fig. 2). This finding has at least two possible explanations: either the hyperaccumulators had additional defenses that the non-accumulators did not, or the hyperaccumulators stored Se in forms (e.g., methylselenosysteine, selenomethionine, selenosystathionine, etc.) that caused stronger deterrence than those in non-accumulators.

The first of these explanations seems unlikely, given that neither of the most common organic defenses found in *Astragalus* (aliphatic nitro and indolizidine alkaloids (Rios and Waterman 1997)) have been found in *A. bisulcatus* (Williams and Barneby 1977). Although 263 (52 %) of the North American *Astragalus* species tested contained detectable amounts of these com-pounds, only 1 of the 24 hyperaccumulator species tested contained them. This is not surprising, given inorganic/organic defense trade-offs: cheap, abundant elemental defenses might mean that hyperaccumulators can eschew more costly organic defenses (Boyd 2007). However, this would put them at a disadvantage on selenium-poor soils, where they would be relatively undefended, and indeed, do not naturally occur. *A. cicer*, meanwhile, has been found to contain at least some toxic alkaloids (Rios and Waterman 1997). It is still possible, however, that *A. bisulcatus* is better defended due to leaf toughness, C/N ratio, moisture content, or some other factor that could create differen-tial herbivory between species.

While these factors may contribute to defense in *A. bisulcatus*, we feel that the difference in herbivory is more likely related to selenium uptake. Selenium hyperaccumulators cause taste and odor aversion in mammals and insects, likely due to volatile organic selenium compounds, including dimethylselenide and others (Hanson et al. 2003, Freeman et al. 2007, Freeman et al. 2009; Pfister et al. 2010). Since *A. bisulcatus* and *A. cicer* differ drastically in their ratios of organic selenium metabolites to total selenium (Sors et al. 2005), it is likely that for a given total tissue concentration of total selenium, the higher proportion of organic compounds in *A. bisulcatus* would cause stronger aversion, even though the higher proportion of inorganic selenium in *A. cicer* may actually be more toxic (Pickering et al. 2003).

Plant performance

Contrary to our predictions and the findings of other studies (Trelease and Trelease 1938; Broyer et al. 1972a; El Mehdawi et al. 2012), we found that both *A. cicer* and *A. bisulcatus* responded positively to selenium dosage

by at least one plant performance metric. We initially thought this was due to elemental defense, but herbivory did not play a statistically significant role in the performance of the plants, so other mechanisms are likely at play. While herbivory was a near-significant predictor of several plant performance metrics (Table 2), we believe this is more likely an artifact of the strong correlation between soil dosage and herbivory ($R^2 = 0.57$) than an indication of type II error. Future 2-factor designs that vary dosage and herbivory independently should be performed to separate these effects.

For *A. cicer*, the seemingly contradictory responses to selenium (loss of leaves, no change in root mass or stem length, increase in aboveground mass) may be the result of a complex interplay between herbivory, toxicity, and other physiological responses. For *A. bisculatus*, since selenium dosage was a statistically significant predictor of all performance metrics and herbivory was not a significant predictor of any performance metric, we feel that elemental defense alone is insufficient to explain the adaptive benefit of hyperaccumulation. While elemental defense by selenium is clearly an important driving factor for hyperaccumulator growth and distribution in the field (Galeas et al. 2008), we have demonstrated that it is likely not the only factor, and it may not even be the primary factor in instances when herbivory is not limiting fitness (Trumble and Sorensen 2008).

Of the five evolutionary hypotheses for hyperaccumulation other than elemental defense, as summarized by Boyd and Martens (1998), three (tolerance by sequestration, disposal from the body via deciduous organs, and nonadaptive inadvertent uptake) do not predict improved growth with increasing selenium, and in fact, may predict the opposite. One hypothesis (interference, also called elemental allelopathy) only predicts improved growth when plants are grown in competition. The remaining hypothesis, drought resistance via increased osmotic potential, seems inadequate given that plants were regularly watered to saturation, and our preliminary data suggest that selenium provides no advantage to these species when drought stressed (Statwick, unpublished data). Thus, only physiological benefit to the plant (Cappa and Pilon-Smits 2014) appears sufficient to explain the improved growth with increasing selenium.

Still, although we found a positive relationship between soil or leaf selenium and biomass, we found a negative relationship between bioconcentration factor and biomass. This implies that despite the benefits to a plant of possessing selenium, there are costs to actively concentrating it in tissues, particularly for non-accumulators. Thus, depending on the environmental context, the physiological and ecological benefits of possessing selenium at high concentrations may or may not offset the metabolic costs of uptake, which perhaps helps explain some of the distribution and accumulation patterns of wild plants.

Concluding remarks

One must be cautious when applying the results of controlled studies to models of ecological dynamics in natural settings, since the advantage of being able to hold environmental variables constant is also a limitation. Although a greenhouse can effectively determine whether plants have the physiological capacity for certain behaviors, such findings are not necessarily ecologically relevant, especially if the conditions in the greenhouse are never encountered by the plant in the field. Keeping this caveat in mind, we suggest that the value of these data lies primarily in characterizing the physiological potential of hyperaccumulators and non-accumulators under idealized conditions.

Given the large range of leaf concentrations we measured in this experiment, both across and within treatments, and the substantial degree of overlap between species, we found that the tissue concentration threshold definition of hyperaccumulation was only marginal at delineating hyperaccumulators from non-accumulators. In this experiment, more than half of the hyperaccumulator individuals failed to meet even the most lenient tissue threshold for hyperaccumulation. Only 16 hyperaccumulator individuals exceeded 100 ppm, yet 11 non-accumulator individuals also exceeded that same threshold. Of course, our greenhouse conditions may not accurately represent field conditions; ecological correlates may make the distinction between hyperaccumulators and non-accumulators more discreet in the wild. Still, given that previous studies of wild collected *A. bisulcatus* have found that as many as 40–60 % of individuals have tissue concentrations less than 100 mg/kg (Shrift 1969; Sors et al. 2009), we feel that a more thorough sampling of wild hyperaccumulators and their soils is warranted.

In this experiment, the bioconcentration factor (the ratio of tissue trace elements to bioavailable substrate trace elements), performed better than the absolute concentration threshold in delineating hyperaccumulators

from non-accumulators. For example, during the period of rapid linear increase in leaf concentration for our hyperaccumulator and non-accumulator species (100 µg/kg-10 mg/kg sodium selenate, representing the bulk of "native soil" concentrations), the mean leaf concentration was not significantly different between our hyperaccumulator and our non-accumulator. The bioconcentration factors, on the other hand, remained similar across dosages and were significantly different between species. *A. cicer* averaged a 97:1 ratio, while *A. bisulcatus* averaged an 805:1 ratio.

Some authors advocate that the bioconcentration factor, while not without some technical issues (see van der Ent et al. 2013), may be a better indicator of hyperaccumulation ability than the absolute threshold (Hobbs and Streit 1986; Zayed et al. 1998). It may even be a more ecologically relevant standard than a tissue concentration threshold, since concentration thresholds only measure metal tolerance, not necessarily accumulation ability per se. A potential limitation of the threshold definition is that there are many metallophytes which tolerate extraordinarily high tissue concentrations of metals, but are certainly not hyperaccumulators (Hobbs and Streit 1986; Ernst et al. 1990; McGrath and Zhao 2003; Rascio and Navari-Izzo 2011; Pollard et al. 2014; Goolsby and Mason 2015). Instead, hyperaccumulators are physiologically united by their ability to actively (and apparently at some cost) take up metals through their roots and actively translocate those metals from their roots to their shoots (Rascio and Navari-Izzo 2011, Cappa and Pilon-Smits 2014). Both of these pathways, by definition, lead to an increased bioconcentration factor as compared to a non-accumulating plant – tolerant or otherwise. However, since our own study was greenhouse based and thus not necessarily representative of field conditions, we propose that a field-based comparison of these two definitions of hyperaccumulation should be conducted.

The Se/S ratio also has been proposed as a better discriminator between selenium hyperaccumulators and non-accumulators than the absolute tissue threshold. Due to strong molecular similarity between selenium and sulfur and the enhanced ability of selenium hyperaccumulators to discriminate between the two, selenium hyperaccumulators should have a substantially elevated Se/S ratio as compared to non-accumulators (White et al., 2007). However, we were unable to test this hypothesis due to inherent limitations of sulfur detection by ICP-MS. This definition may also be of limited applicability to hyperaccumulators of other elements, as it is unclear what elemental ratios would be analogous.

Finally, we suggest that Broyer et al. (1972a)'s skepticism of selenium as a growth promoting element, coupled with limited historical collaboration between researchers of seleniferous and serpentine systems (compare Brown and Shrift 1982 vs Baker and Brooks 1989 and sources therein), has led to little serious consideration of the idea that hyperaccumulated elements can provide a direct benefit to plant growth. We grant that it has not yet been satisfactorily demonstrated that trace elements benefits plants directly rather than indirectly, such as through chelation of other toxic elements, facilitation of mycorrhizae, etc. (but see Broyer et al. 1972a; Lindblom et al. 2013). However, our study and recent others like it suggest that selenium (El Mehdawi et al. 2012), nickel (Küpper et al. 2001; Ghasemi et al. 2014; Pollard et al. 2014; Kazemi-Dinan et al. 2015), and perhaps even cadmium (Roosens et al. 2003; Kazemi-Dinan et al. 2015) can benefit hyperaccumulators of these elements across concentration ranges that span three or more orders of magnitude and reach well into the acutely toxic range for most organisms. We therefore suggest the new label "elemental stimulation" for this phenomenon, and believe it should be more thoroughly investigated.

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