

# Selenium accumulation protects *Brassica juncea* from invertebrate herbivory and fungal infection

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## Summary

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- Certain plant species hyperaccumulate selenium (Se) up to 0.6% of their dry weight. It is not known whether Se hyperaccumulation offers the plants any advantage. In this study the hypothesis was tested that Se can protect plants from invertebrate herbivory or fungal infection.
- Indian mustard (*Brassica juncea*) plants grown with or without Se were subjected to herbivory by caterpillars (*Pieris rapae*) and snails (*Mesodon ferrissi*), or to fungal infection by a root/stem pathogen (*Fusarium* sp.) and a leaf pathogen (*Alternaria brassicicola*).
- When given a choice between leaves with or without Se (0.1% Se of leaf d. wt), the caterpillars strongly preferred leaves without Se ( $P < 0.01$ ), while the snails preferred leaves containing Se ( $P < 0.015$ ). When consumed, the Se leaves were lethal to the caterpillars. The snails showed no toxicity symptoms, even though their tissue Se concentrations were comparable with the caterpillars. Se-containing plants were less susceptible to infection by both fungi.
- In conclusion, Se was shown to protect Indian mustard plants from fungal infection and from herbivory by caterpillars, but not by snails.

**Key words:** Selenium, hyperaccumulation, herbivory, *Brassica juncea*, *Pieris rapae*, *Mesodon ferrissi*, *Alternaria brassicicola*, *Fusarium*.

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## Introduction

Selenium (Se) is both an essential nutrient for many organisms but also toxic at higher levels. As a nutrient, Se is an essential component of certain seleno-enzymes and tRNAs (Stadtman, 1990; Fu *et al.*, 2002). The toxicity of Se at higher concentrations is thought to be due to its chemical similarity to sulfur, leading to nonspecific replacement of S by Se in proteins and other sulfur compounds (Stadtman, 1990). The difference between the amount of Se required as a nutrient and the amount that is toxic is small; as a consequence both Se deficiency and toxicity are common problems worldwide (Terry *et al.*, 2000). Selenium is naturally present in alkaline soils derived from Cretaceous shales or seleniferous rocks (Kabata-Pendias, 1998), and agricultural use of such soils can lead to Se accumulation to lethal levels in livestock or wildlife

(Draize & Beath, 1935; Rosenfeld & Beath, 1964; Wilber, 1980; Ohlendorf *et al.*, 1986; Harris, 1991; von Vleet & Ferrans, 1992; Skorupa, 1998).

Certain plant species are known to accumulate Se to levels far beyond those observed in other species, even from low-Se soils. Their Se levels can reach up to 0.6% of their d. wt without any signs of toxicity, and are commonly in the 0.1% range (Byers, 1936; Brown & Shrift, 1982; Feist & Parker, 2001). These plants are known as Se hyperaccumulators and include species in the genera *Astragalus* (Fabaceae), *Stanleya* (Brassicaceae), *Oenopsis* (Asteraceae), and *Xylorhiza* (Asteraceae) (Beath *et al.*, 1939; Brown & Shrift, 1982; Reeves & Baker, 2000). Selenium hyperaccumulator species are found predominantly on seleniferous soils, where they occur together with nonaccumulator species (Beath *et al.*, 1939). The Se hyperaccumulator plants pose a threat to livestock: their

ingestion continues to cause substantial losses of sheep, cattle and horses due to alkali disease and 'blind staggers' (Draize & Beath, 1935; Rosenfeld & Beath, 1964; Wilber, 1980). Selenium hyperaccumulator plants not only hyperaccumulate Se but are also very tolerant to Se.

Plants are thought to take up and metabolize Se via the sulfur assimilation pathway, resulting in Se accumulation in plant tissue and production of (low-toxic) volatile dimethylselenide (Lewis *et al.*, 1966; Anderson, 1993; Pilon-Smits *et al.*, 1999). This capacity of plants can be used for phytoremediation of Se-contaminated soils and waters (Bañuelos & Meek, 1990; Hansen *et al.*, 1998). Plant accumulation and volatilization of Se tends to correlate with the capacity to accumulate and volatilize sulfur; an example of an active Se/S accumulator and volatilizer is *Brassica juncea* (Indian mustard), one of the most popular species for Se phytoremediation. Selenium hyperaccumulator species also show high levels of Se volatilization (Terry *et al.*, 2000). The mechanism of Se tolerance in Se hyperaccumulator plants is thought to be methylation of selenocysteine (SeCys) by a specific SeCys methyltransferase, which prevents nonspecific translational SeCys incorporation into proteins (Neuhierl & Böck, 1996; Neuhierl *et al.*, 1999; Persans & Salt, 2000; Terry *et al.*, 2000). The mechanism of Se hyperaccumulation is not quite clear, but may be related to SeCys methyltransferase activity as well.

Selenium hyperaccumulation appears to be a constitutive trait, although there can be genetic variation among populations of a species with respect to the degree of Se accumulation, as was shown for *Stanleya pinnata* (Feist & Parker, 2001). In view of the occurrence of Se hyperaccumulation in some, but not all, species from different families, Se hyperaccumulation probably evolved more than once in higher plants (Peterson, 1983). A polyphyletic origin of Se hyperaccumulation suggests that Se hyperaccumulation and hypertolerance can develop fairly easily in these families. Indeed, Se tolerance in the nonaccumulator species *Astragalus cicer* could be acquired by selection in cell culture, and the tolerant cell lines were shown to newly produce methyl-selenocysteine (Wang *et al.*, 1999).

If Se hyperaccumulation has evolved multiple times in history, a common selection pressure may have driven the evolution of this trait. Several possible biological roles have been suggested for hyperaccumulation of the other elements Ni and Zn: to confer metal tolerance, to increase drought resistance, to give competitive advantage over other plants via allelopathic interactions, or to give protection from herbivory or infection (Reeves *et al.*, 1981; Boyd & Martens, 1993; Pollard *et al.*, 2000). Selenium may play similar roles in Se hyperaccumulator plants. In the present study, the hypothesis is tested that Se can protect plants from invertebrate herbivory and fungal infection. Indian mustard was chosen as a model species, because it is well-characterized, fast growing, tolerant to Se and a good Se accumulator (up to 0.1% of DW without

toxicity symptoms), and its invertebrate herbivores and fungal pathogens are available. The white cabbage butterfly (*Pieris rapae*) was chosen as a representative *Brassica*-specific herbivore, and the land snail *Mesodon ferrissi* as a representative general herbivore. Two phytopathogenic fungi were chosen: the *Brassica*-specific leaf pathogen *Alternaria brassicicola*, and a general root/stem pathogen *Fusarium* species isolated from the rhizosphere of a nonaccumulator Brassicaceae species growing on Se-rich soil.

## Materials and Methods

### Biological material

*Brassica juncea* (L.) Czerniak seeds (Indian mustard, accession no. 173874) were obtained from the North Central Regional Plant Introduction Station (Ames, IA, USA). The seeds were germinated on coarse sand. After 1 wk the seedlings were transferred to a hydroponic setup consisting of aerated 4-l containers with half-strength Hoagland's solution (Hoagland & Arnon, 1938). The plants were grown under glasshouse conditions at 25°C and a 16/8 L/D photoperiod. The nutrient solution was replaced every week. After 2 wk in hydroponics, half of the plants were supplied with 20 µM sodium selenate in half-strength Hoagland's solution while the other half was kept without Se as a control. The duration of the Se treatment varied per experiment and is indicated in the text.

White cabbage butterfly eggs (*Pieris rapae* Linnaeus (L.)) and adult land snails (*Mesodon ferrissi* Pilsbry) were obtained commercially (Carolina Biological Supply Co., 2700 York Road, Burlington, NC 27215, USA). White cabbage butterfly occurs worldwide; its larvae feed exclusively on *Brassica* species. *Mesodon ferrissi* snails are generalist herbivores. Their natural habitat is the Smoky Mountains (USA), an area that is not particularly Se rich.

The *Alternaria brassicicola* (Schweinitz) Wiltshire strain used (ATCC34622) is part of the strain collection of Chris Lawrence (Colorado State University). The phytopathogenic *Fusarium* sp. used was isolated from roots of *Abyssum minus* (Brassicaceae), a nonSe accumulator growing on Se-rich soil (2 mg kg<sup>-1</sup> d. wt) in Fort Collins, CO, USA (Pine Ridge Nature Area, South Overland Trail). The plant roots were washed in sterile water and 1 cm root sections were placed onto half-strength malt extract agar (MEA) plates (0.75% w/v malt extract broth, 1.5% v/w bactoagar). One of the resulting fungal cultures growing on the root was isolated, hyphal tipped, recultured and identified as a chlamyospore-forming *Fusarium* sp.

### Choice feeding experiments

To determine whether *Pieris rapae* caterpillars or *Mesodon ferrissi* snails have any feeding preference when given the choice between high- and low-Se leaves, 250 ml aerated

plastic containers were used each containing one animal and two *B. juncea* leaf pieces of  $2.5 \times 2.5$  cm, one from a high-Se plant and one from a low-Se plant ( $n = 8$  containers per experiment, plus one control without animal). There were no visible differences between the  $\pm$ Se plants or leaf sections used. The Se levels in the plants used for each experiment are shown in the figures and text. The choice feeding experiments were carried out over either 6 h (caterpillar experiments) or 4 d (snail experiments), to allow for sufficient leaf consumption. The leaf sections were weighed at the beginning and end of the experiment to determine leaf f. wt loss as a measure of animal feeding, and the animal fresh weight was determined to allow calculation of feeding rate per gram animal. Leaf material comparable with that used for the experiment, or material not eaten during the experiment was used to determine the Se concentration in the leaves, as described below ( $n = 3$  samples per experiment, pooled from three leaves each).

### Nonchoice feeding experiments

Nonchoice feeding experiments were performed to determine whether the Se-rich leaves used were toxic to the animals. For each experiment, leaf samples were taken to determine leaf Se concentrations, as described below.

Two types of experiment were performed with the caterpillars, both using intact plants in a hydroponic setup grown with or without Se as described above. In the first, newly hatched larvae (3–6 mm) were placed on the plants ( $n = 6$  per treatment) and left for 9 d, after which they were weighed and visually examined. In the second, 9-d-old caterpillars (c. 30 mm, 4th instar, cultivated from eggs on nonSe *B. juncea* plants) were weighed, placed on the plants ( $n = 3$  per treatment) and left for 2 d; the f. wt of the animals was examined daily. The animals and their frass were analyzed for tissue Se concentration as described below.

The nonchoice snail feeding experiments were performed either over a period of 8 d using intact plants grown in a hydroponic setup with or without Se as described above ( $n = 6$  per treatment), or over a period of 16 d in 250-ml containers using detached leaves from similar plants ( $n = 8$  per treatment). The f. wt of the animals was measured at the beginning and end of the experiment, and their appearance was examined visually. The animals were collected at the end of the experiment for determination of tissue Se concentration ( $n = 3$  per treatment).

### Fungal infection and Se tolerance

For infection with the leaf pathogen *Alternaria brassicicola*, Indian mustard plants were grown with or without Se as described above. Intact 5-wk-old-plants treated  $\pm$ Se ( $n = 3$  plants per treatment with 10 leaves each) were sprayed with *A. brassicicola* spore suspension ( $5.10^4$  spores  $\text{ml}^{-1}$ ), and the

number of resulting lesions per leaf were measured after 4 d.

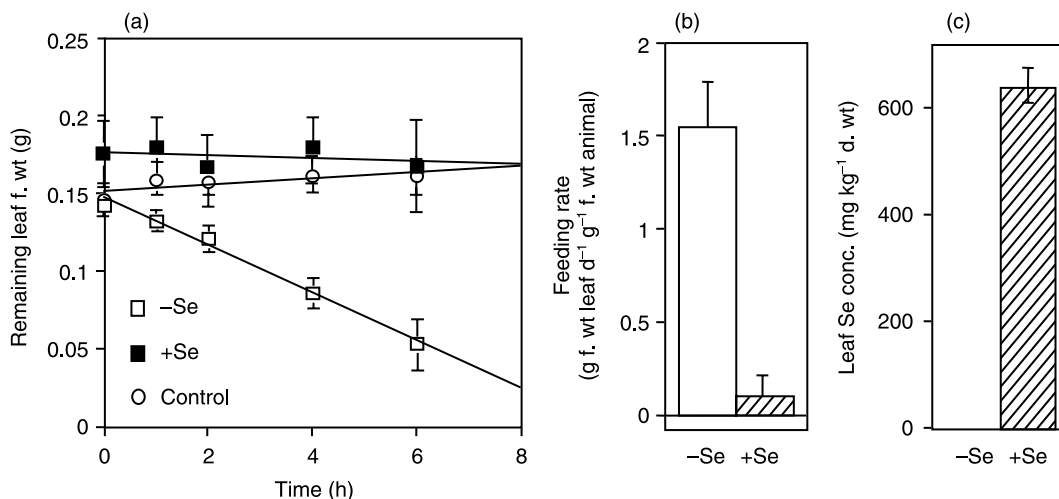
For infection with the root/stem pathogen *Fusarium* sp., Indian mustard seedlings were grown for 7 d on Murashige and Skoog (1962) agar medium with or without  $100 \mu\text{M}$  sodium selenate ( $25^\circ\text{C}$ , 16/8 L/D). The seedlings ( $n = 20$  per treatment) were weighed, submerged briefly into fungal spore suspension ( $5.10^6$  spores  $\text{ml}^{-1}$ ) and placed with their roots between two moist  $10 \times 40$  cm filter paper strips (3 mm thick), which were rolled up and placed in a 1-l beaker containing 200 ml of half-strength Hoagland's nutrient solution (Hoagland & Arnon, 1938). Control seedlings without fungi were placed in separate beakers. After 7 d under the same growth conditions, the f. wt of the seedlings was measured again and they were visually examined. Leaf samples ( $n = 3$ ) were collected from the control treatments  $\pm$ Se for Se analysis.

To determine the Se tolerance of the *Fusarium* and *Alternaria* isolates used for the plant infection experiments, the fungi were inoculated onto half-strength MEA plates containing a range of sodium selenate concentrations (one plate per concentration per species). A  $2 \times 2$  mm agar plug from an earlier culture (grown without Se) was placed in the center of each plate and after 10 or 17 d the diameter of the fungal mycelium was measured. The  $\text{EC}_{50}$  values (50% reduction in diameter after 17 d) were estimated from the graphs shown in Figs 5c and 6c.

### Elemental and statistical analyses

Leaf samples for elemental analysis were dried for 2 d at  $70^\circ\text{C}$ . Animals were first frozen, then dried for 2 d at  $70^\circ\text{C}$ . Where indicated the snails were separated into soft tissue and shell. The biological material was acid-digested as described by Zarcinas *et al.*, 1987) in order to extract the Se. The Se concentration in the digests was quantified by inductively coupled plasma atomic emission spectrometry (ICP-AES & Fassel, 1978). The detection limit of this method is around  $0.05 \text{ mg kg}^{-1}$  d. wt.

For statistical analyses the software package JMP-IN version 3.2.6 was used (SAS Institute, Cary, NC, USA). The averages and standard errors of the mean are shown in the figures. Note: in some cases the standard error was too small to be recognized by the graphing program; in those cases no error bar is shown. Pairs of means were compared statistically using *t*-tests. In addition, for the *Fusarium* experiment a 2-way ANOVA was performed to examine the interaction between Se and fungus treatments. All data were first examined to determine if they met the assumptions of normality and homoscedasticity. Data that did not meet these assumptions were transformed as appropriate (either arcsine square root or  $\ln + 1$ ) before analysis (Sokal & Rohlf, 1981). Statistically significant differences ( $\alpha = 0.05$ ) are described in the text and figure legends.



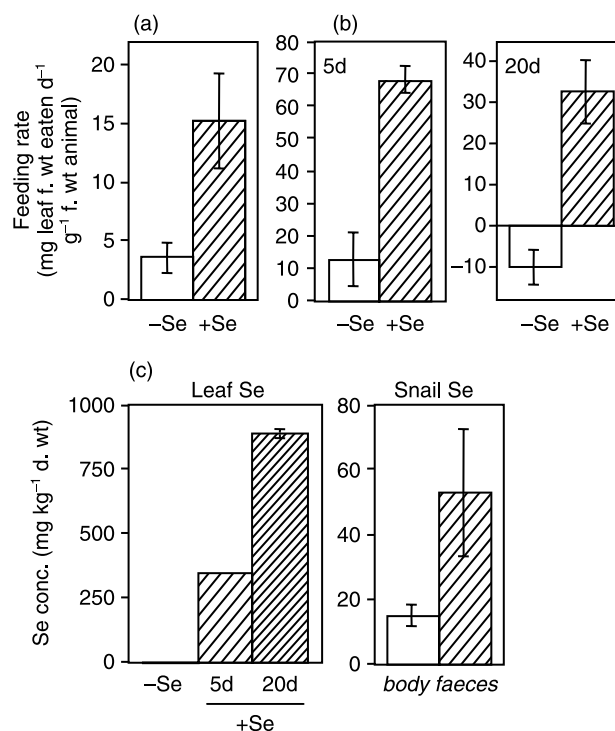
**Fig. 1** Caterpillar choice feeding experiment, comparing feeding by *Pieris rapae* of *Brassica juncea* leaf sections with or without Se. Control experiments contained no caterpillar. Shown are means  $\pm$  SE. (a) Leaf f. wt over the course of the feeding experiment. (b) Rate of feeding on  $\pm$  SE leaves, calculated from (a);  $P = 0.0013$ . (c) Se concentration in the leaf sections used. Animal mortality was 0% during the experiment.

## Results

### Choice feeding experiments

To determine whether leaf Se acts as a feeding deterrent for invertebrate herbivores, choice feeding experiments were performed in which individual animals were presented with a choice between two leaf sections, one containing Se and one without Se. Caterpillars of the *Brassica*-specific herbivore *Pieris rapae* could distinguish between leaves with and without Se, and preferred to feed on leaf sections without Se. In fact, the leaf sections without Se were eaten at a *c.* 15-fold higher rate than the leaf sections containing Se (Fig. 1a,b,  $P < 0.01$ ). Although feeding rates on +Se leaves were much lower, the leaves were not untouched, suggesting the caterpillars tasted them and subsequently stopped feeding on them. The average Se concentration of the Se-containing leaf sections was  $600 \text{ mg kg}^{-1} \text{ d. wt}$  (Fig. 1c) or 0.06% Se on a d. wt basis. While the results shown were from commercially available *P. rapae*, similar results were obtained using *P. rapae* caterpillars collected from a Colorado State University experimental cabbage field near Fort Collins, CO, USA ( $P < 0.05$ , results not shown).

Surprisingly, the snail *Mesodon ferrissi*, a generalist herbivore, preferentially fed on leaves containing Se and ignored the -Se leaves (Fig. 2a,  $P < 0.05$ ). The snail choice experiments were repeated using leaves with different Se concentrations, and similar results were obtained in all cases (Fig. 2b,c,  $P < 0.05$ ). Thus, it appears that *M. ferrissi* can distinguish between Se-containing and noncontaining leaves and preferentially feed on leaves containing Se. There was no apparent acute toxicity of the Se in the leaves to the snails during the choice feeding experiment, because none of the snails died in



**Fig. 2** Snail (*Mesodon ferrissi*) choice feeding experiment over 4d, comparing feeding on *Brassica juncea* leaf sections with or without Se. Shown are means  $\pm$  SE. (a) Experiment I, using leaves from plants grown from seed with or without Se. (b,c) Experiment II, using leaves from plants treated with Se for 5 or 20 d. (a,b): the rate of feeding on  $\pm$  Se leaves ( $P < 0.015$ ). (c) Se concentration in the leaves used for experiment II (left panel) and in snail body (soft tissue plus shell) and faeces (right panel). Animal mortality rate was 0% for both experiments. Note: the -Se leaves in (b) gained some f. wt from the moist filter paper they were on.

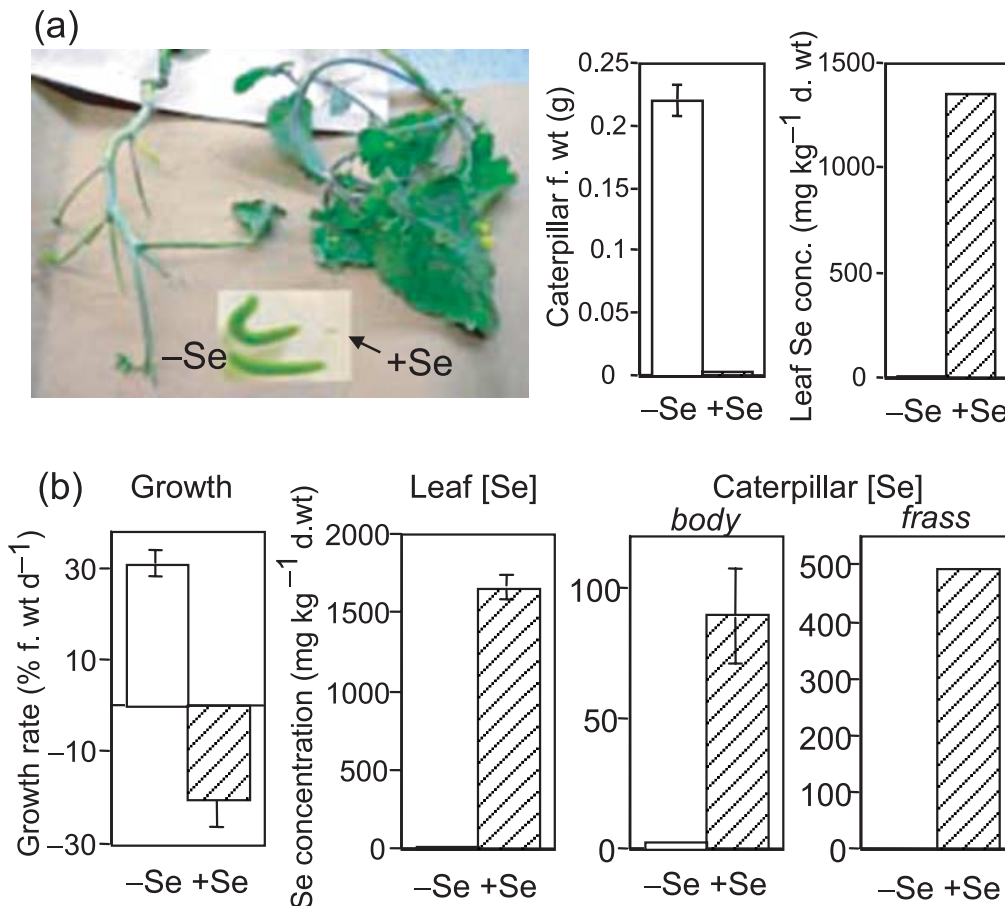
spite of eating predominantly Se-rich leaves for 4 d, resulting in 20 mg Se kg<sup>-1</sup> snail d. wt (combined body and shell), and 50 mg kg<sup>-1</sup> d. wt in snail faeces (Fig. 2C).

### Nonchoice feeding experiments

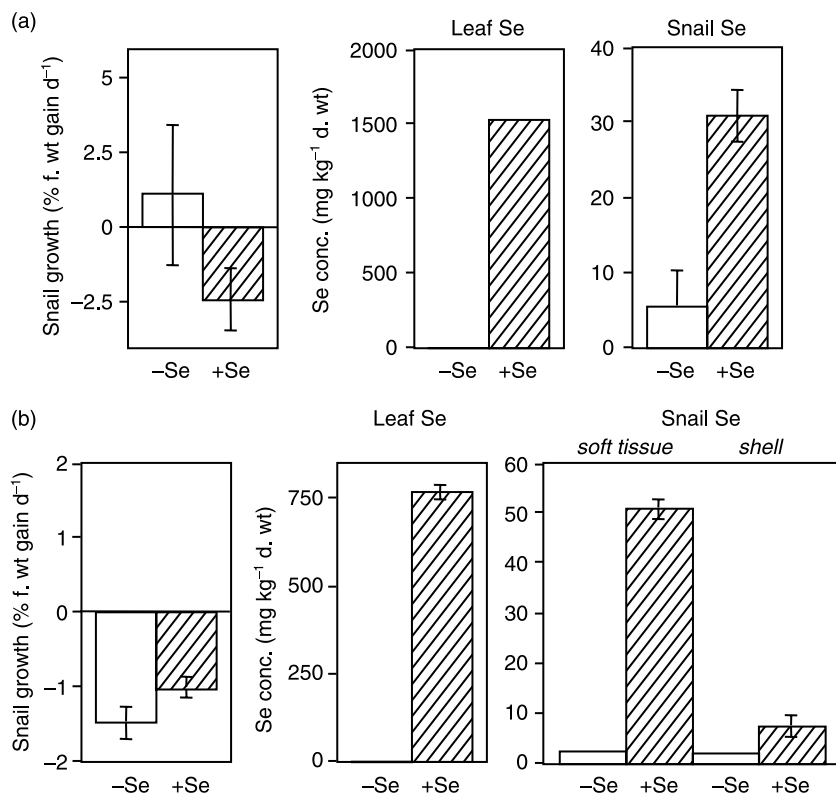
The Se-containing *B. juncea* plants were acutely toxic to the caterpillar larvae, as was shown in nonchoice feeding experiments. When newly hatched larvae were placed on plants with or without Se, the larvae did not grow on the Se-containing plants (containing 1300 mg Se kg<sup>-1</sup> d. wt, Fig. 3a), but all died within 9 d without visible feeding damage on the plants (Fig. 3a). By contrast, on plants without Se the caterpillars ate all of the leaf material in 9 d and grew substantially (Fig. 3a). Similarly, when 9-d-old caterpillars were placed on plants containing Se (1600 mg kg<sup>-1</sup> d. wt) they lost 20% of their f. wt in the first day, while caterpillars placed on plants without Se gained 30% in the same period (Fig. 3b,  $P < 0.01$ ). After one more day, all caterpillars on the Se-rich plants had died,

while all animals on the plants without Se survived. The average Se concentration in the caterpillars fed for 2 d on the Se leaves was 90 mg kg<sup>-1</sup> d. wt, while in the caterpillar frass it was 500 mg kg<sup>-1</sup> d. wt (Fig. 3b).

No apparent toxicity of Se-rich leaves to snails was observed in nonchoice feeding experiments. When snails were placed on intact plants with or without Se (1500 mg Se kg<sup>-1</sup> d. wt, Fig. 4a) there was no significant difference in snail f. wt gain between the two treatments over 8 d (Fig. 4a), nor was there any apparent mortality over the course of the experiment, despite snail Se concentrations of 30 mg kg<sup>-1</sup> d. wt (body + shell, Fig. 4a) in the +Se animals. Similarly, when snails were fed detached leaves from plants treated with or without Se for 16 d (leading to 750 mg Se kg<sup>-1</sup> d. wt in the Se-treated plants, Fig. 4b) there were no differences in growth or mortality between snails feeding on leaves with or without Se (Fig. 4b), despite Se concentrations as high as 50 and 7.5 mg Se kg<sup>-1</sup> d. wt in soft tissue and shell, respectively, in Se-supplied snails (Fig. 4b).



**Fig. 3** Caterpillar (*Pieris rapae*) nonchoice feeding experiment on intact plants with or without Se. (a) Experiment over 9 d starting with newly hatched larvae. Shown is the appearance of representative plants and caterpillars after 9 d, animal f. wt, and leaf Se concentration in the plants. Survival rate -Se was 83%, +Se 0%. (b) Experiment over 2 d starting with 9-d-old caterpillars, measuring caterpillar growth on a fresh weight basis, Se concentrations in the plants, in caterpillar tissue, and in caterpillar frass. Survival rate -Se was 100%, +Se 0%. Shown are means  $\pm$  SE;  $P < 0.01$  for growth and mortality  $\pm$  SE.



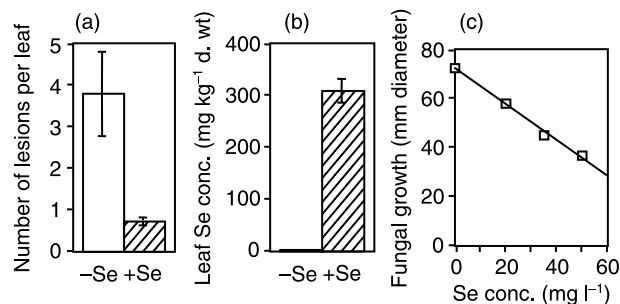
**Fig. 4** Snail (*Mesodon ferrissi*) nonchoice feeding experiments. (a) using intact mature plants treated with or without Se, over a feeding period of 8 d. (b) using detached leaves from mature plants treated with or without Se, over a feeding period of 16 d. Shown are snail growth, and snail and leaf Se concentrations. Snails were separated into soft tissue and shell before Se analysis. Values shown represent the means and standard errors;  $P > 0.05$  for animal growth and mortality  $\pm$  Se.

### Fungal infection and Se tolerance

To explore whether Se accumulation in *B. juncea* can protect the plant from infection by phytopathogenic fungi, two fungal species were tested: the *Brassica*-specific leaf pathogen *Alternaria brassicicola*, and a general stem/root pathogen, *Fusarium* sp., isolated from the rhizosphere of the nonaccumulator species *Alyssum minus* (Brassicaceae) growing on Se-rich soil.

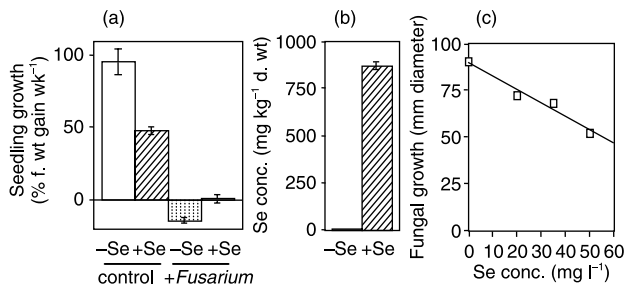
To investigate Se-dependent susceptibility of *B. juncea* to *A. brassicicola*, plants treated with or without Se were sprayed with a spore suspension and the numbers of resulting lesions were counted after 4 d. The number of lesions per leaf on the Se-containing plants was 5-fold smaller than the number of lesions on the plants that did not contain Se ( $P < 0.01$ , Fig. 5a). Thus, Se appeared to protect the *B. juncea* plants from infection by *A. brassicicola*. The Se concentrations in the plants used for these experiments (Fig. 5b) were *c.* 300 mg kg<sup>-1</sup> d. wt. To determine whether this tissue Se concentration is likely to be toxic to this *Alternaria* species, the fungus was grown on agar plates with varying Se concentrations. The EC<sub>50</sub> (Se concentration resulting in 50% growth inhibition) was *c.* 55 mg l<sup>-1</sup> Se (Fig. 5c). Thus, it is feasible that the Se concentrations in the leaves, 300 mg kg<sup>-1</sup> d. wt corresponding with *c.* 60 mg kg<sup>-1</sup> f. wt, indeed inhibit *A. brassicicola* growth.

To test whether Se can protect *B. juncea* from infection by the stem/root pathogen *Fusarium*, sterile seedlings grown on agar medium with or without Se were inoculated by briefly



**Fig. 5** Susceptibility of 5-wk-old *Brassica juncea* plants to fungal infection by *Alternaria brassicicola*. (a) Number of infection lesions per leaf on *B. juncea* plants treated  $\pm$ Se, as measured 4 d after infection with *A. brassicicola* ( $P < 0.01$ ). (b) Leaf Se concentrations in the plants used. (c) Growth of *A. brassicicola* after 10 d on MEA agar plates containing different Se concentrations.

immersing them in a *Fusarium* spore suspension. Both the Se-treated and the control seedlings were successfully infected by the *Fusarium* isolate, as indicated by brown spots on stem and root, developing into lesions. Although there were no obvious visual differences in the severity of infection between seedlings treated with or without Se, the seedlings that did not contain Se had lost 14% of their f. wt after 7 d, while the Se-containing seedlings gained 1% (Fig. 6a,  $P < 0.05$ ). Control seedlings grown in the absence of the fungus gained 50% (+Se) and 100% (-Se) in f. wt, respectively (Fig. 6a,  $P < 0.05$ ). Thus,



**Fig. 6** Susceptibility of 7-d-old *Brassica juncea* seedlings to fungal infection by a phytopathogenic *Fusarium* sp. (a) Seedling growth  $\pm$ Se and  $\pm$  *Fusarium* ( $P < 0.05$  between  $\pm$ Se for both treatments). (b) Shoot Se concentration in the seedlings. C: Growth of the *Fusarium* isolate after 17 d on MEA agar plates containing different Se concentrations.

the Se-treated seedlings grew less well than the control seedlings in the absence of the pathogen (presumably due to the Se in the tissue, which was 800 mg Se kg<sup>-1</sup> d. wt in the Se-treated plants, Fig. 6b), but inoculation with the fungus affected the f. wt of the Se-treated seedlings less than that of control seedlings (interaction  $F_{1,70} = 27.73$ ,  $P < 0.0005$ ).

To determine whether the observed 800 mg Se kg<sup>-1</sup> d. wt in the seedling tissue was likely to be toxic to this *Fusarium* species, the fungus was grown on agar plates with varying Se concentrations. The EC<sub>50</sub> was around 60 mg l<sup>-1</sup> Se (Fig. 6c). Therefore, 800 mg Se kg<sup>-1</sup> d. wt plant tissue (i.e. *c.* 80 mg Se kg<sup>-1</sup> f. wt) is indeed likely to inhibit the growth of this *Fusarium* isolate.

## Discussion

From the results presented here it appears that Se accumulation can protect *B. juncea* plants from herbivory by caterpillars from the *Brassica*-specific herbivore *P. rapae*. These caterpillars avoided Se-rich *B. juncea* leaves when presented with the choice between  $\pm$ Se leaves. Not only did Se deter feeding by *P. rapae*, but when fed with leaves containing Se in the *c.* 0.1% d. wt range the caterpillars suffered acute toxicity, both in the newly hatched stage and at the 4th instar stage. Although the possibility that this toxicity was due to the presence of Se-induced toxins cannot be excluded, the toxicity was most probably due to the Se itself in the leaves. Trumble *et al.* (1998) showed that for *Sporoptera exigua* caterpillars the LC<sub>50</sub> for various forms of Se in artificial diet were below 50 mg kg<sup>-1</sup> d. wt feed, that is 20-fold lower than the Se concentration in the leaves used here. Interestingly, the *Sporoptera exigua* caterpillars showed a preference to feed on (artificial) diet without Se rather than a diet containing Se (Vickerman & Trumble, 1999), indicating that feeding deterrence by Se may be a common phenomenon in caterpillars. These results are in agreement with a recent publication by Bañuelos *et al.* (2002) reporting that the cabbage looper (*Trichoplusia ni*) preferred

to feed on *B. juncea* plants without Se rather than on plants containing Se at 465 mg kg<sup>-1</sup> d. wt.

The tissue Se concentration in the plants used here, *c.* 0.1% of d. wt, is common for Se hyperaccumulator plants in the field such as *Astragalus bisulcatus* and *Stanleya pinnata* (Rosenfeld & Beath, 1964; Feist & Parker, 2001). Moreover, the most common form of Se in mature leaves of *B. juncea* and *A. bisulcatus* is the same, that is selenate (de Souza *et al.*, 1998; Pickering *et al.*, 2000). Thus, if these levels of Se protected *B. juncea*, it is feasible that the Se in hyperaccumulator plants can protect them from herbivory by caterpillars in the field. It remains to be tested at which threshold concentration in plant tissue Se becomes protective – Se may even be able to protect plants at Se concentrations found in nonhyperaccumulator species. Interesting in this respect is the report by Vickerman *et al.* (2002) that varieties of the nonaccumulator *Atriplex* that accumulated more Se showed reduced insect growth and survival of *Spodoptera exigua*.

Other elements besides Se were also able to protect hyperaccumulator plants from caterpillar herbivory. For instance, in the presence of caterpillars, *Streptanthus polygaloides* plants on Ni soil grew and survived better than plants on nonNi soil, and Ni-containing plant material was toxic to the animals (Martens & Boyd, 1994). Nickel-containing *Thlaspi montanum* material was also acutely toxic to *Pieris rapae*: the larvae did not grow and suffered 100% mortality (Boyd & Martens, 1994). Nickel at high levels was also shown to protect *Streptanthus* species from herbivory by caterpillars from *Spodoptera exigua* (Boyd & Moar, 1999). These results obtained in lab experiments were in agreement with a field experiment for the Ni hyperaccumulator *Streptanthus polygaloides*, showing protection from herbivory by high Ni (Martens & Boyd, 2002). The heavy metal Zn was shown to protect *Thlaspi caerulescens* from herbivory by caterpillars (*Pieris brassicae*) and locusts (*Schistocerca gregaria*): in a choice experiment both preferred to eat low-Zn leaves (Pollard & Baker, 1997; Jhee *et al.*, 1999). Thus, accumulation of toxic elements by plants appears to be an effective way to deter insect herbivory and to confer insect toxicity.

The other herbivore tested, the generalist *M. ferrissi*, was not deterred by the Se in *B. juncea* leaves. By contrast, the snails preferentially fed on Se-containing leaves, ignoring the nonSe leaves. The snails actually appeared to be attracted to the smell of the Se-containing leaves: in one situation, 16 snails migrated over a distance of *c.* 20 cm in the course of 2 h, to feed on some detached leaves containing Se, ignoring a similar set of leaves without Se in the same area (pers. obs.). If the snails indeed are attracted to the smell of the Se leaves, the volatile attractant might be dimethylselenide, which Se-supplied plants are known to emit (Lewis *et al.*, 1966).

The Se in the leaves, at levels as high as 1500 mg kg<sup>-1</sup> d. wt, did not appear to be toxic to the snails, because there were no differences in snail growth or mortality between snails fed on  $\pm$  Se leaves. This difference in apparent Se toxicity between

the caterpillars and the snails may in part be caused by the much (*c.* 100-fold) higher rate of leaf consumption by the caterpillars compared to the snails (Fig. 1b and 2a), which in turn is related to the fact that the caterpillars were in the juvenile growing stage, while the snails were adults. Comparison of body Se concentrations showed *c.* 2-fold higher Se levels in the caterpillars than the snails (90 compared with 50 mg Se kg<sup>-1</sup> d. wt). Although it may be possible that the toxicity threshold level is between 50 and 90 mg Se kg<sup>-1</sup> d. wt in both animals, it appears more likely that the snails are more Se tolerant, perhaps owing to metabolic detoxification. Other experiments with molluscs, in the context of metal hyperaccumulation, showed opposite feeding preferences compared with those reported here for Se. In one study, Ni was shown to defend *Senecio coronatus* (Asteraceae) from herbivory by the brown garden snail *Helix aspersa* (Boyd *et al.*, 2002). In another study, Zn was shown to protect *Thlaspi caerulescens* from herbivory by slugs (*Deroceras caruanae*): the slugs preferred to eat low-Zn leaves when given the choice (Pollard & Baker, 1997). Thus, although heavy metal hyperaccumulation may offer plants protection from herbivory by snails, Se hyperaccumulation does not appear to offer a similar protection, at least not from the species tested here.

Selenium was shown to protect *B. juncea* from fungal infection, both from the *Brassica*-specific leaf pathogen *A. brassicicola* and the general stem/root pathogen *Fusarium* sp. The Se concentrations in the plant material used indeed appeared to be high enough to inhibit the growth of the fungi used, as judged from fungal growth on Se-containing agar plates. Another hyperaccumulated element, Ni, was shown earlier to also be able to protect plants from microbial infections. Seedlings of Ni hyperaccumulator *Alyssum* spp. showed enhanced resistance to fungal infection by a *Pythium* isolate; the degree of resistance was correlated with plant Ni-concentration (Ghaderian *et al.*, 2000). Also, the Ni hyperaccumulator *Streptanthus polygaloides* showed Ni-related enhanced resistance to fungal infection by the powdery mildew *Erysiphe polygoni* and *Alternaria brassicicola*, as well as to bacterial infection by *Xanthomonas* species (Boyd *et al.*, 1994). Therefore, accumulation of toxic elements appears to be an effective protection against fungal infections.

The finding that Se accumulation offers protection from caterpillar herbivory and fungal infection is of interest in view of the fact that the function of Se hyperaccumulation by plants is unknown. As mentioned above, the tissue Se concentrations in the plants used here are comparable to those found in Se hyperaccumulator plants in the field, and the chemical species of Se in the tissues are the same. Thus, if these levels of Se can protect *B. juncea* from caterpillar herbivory and fungal infection, it is feasible that the Se in hyperaccumulator plants can protect them in a similar fashion in the field. If so, this would be in agreement with a role for caterpillar herbivory and/or fungal infection as selection pressures for the evolution of Se hyperaccumulation. Of course, the results

presented here are preliminary, and many more experiments will need to be performed with hyperaccumulator and nonaccumulator species in lab and field before conclusions can be drawn with any certainty.

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