Plant Ecology (2006) 183:91–104
DOI 10.1007/s11258-005-9009-z

Nickel hyperaccumulation by *Streptanthus polygaloides* protects against the folivore *Plutella xylostella* (Lepidoptera: Plutellidae)

Edward M. Jhee[1], Robert S. Boyd[1,*], Micky D. Eubanks[2] and Micheal A. Davis[3]
*[1]Department of Biological Sciences, Auburn University, AL, 36849-5407, USA; [2]Department of Entomology and Plant Pathology, Auburn University, AL, 36849-5413, USA; [3]Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS, 39406-5018, USA; *Author for correspondence (e-mail: boydrob@auburn.edu)*

Received 07 April 2005; accepted in revised form 22 June 2005

Key words: Elemental defence, Glucosinolates, Herbivory, Oviposition

Abstract

We determined the effectiveness of Ni as an elemental defence of *Streptanthus polygaloides* (Brassicaceae) against a crucifer specialist folivore, diamondback moth (DBM), *Plutella xylostella*. An oviposition experiment used arrays of *S. polygaloides* grown on Ni-amended (high-Ni) soil interspersed with plants grown on unamended (low-Ni) soil and eggs were allowed to hatch and larvae fed freely among plants in the arrays. We also explored oviposition preference by allowing moths to oviposit on foil sheets coated with high- or low-Ni plant extract. This was followed by an experiment using low-Ni plant extract to which varying amounts of Ni had been added and an experiment using sheets coated with sinigrin (allyl glucosinolate) as an oviposition stimulant. Diamondback moths laid 2.5-fold more eggs on low-Ni plants than on high-Ni plants and larval feeding was greater on low-Ni plants. High-Ni plants grew twice as tall, produced more leaves, and produced almost 3.5-fold more flowers. Low-Ni plants contained more allyl glucosinolate than high-Ni plants and moths preferred to oviposit on foil sheets dipped in low-Ni plant extract. Moths showed no preference when Ni concentration of low-Ni extract was varied and overwhelmingly preferred sinigrin coated sheets. We conclude that Ni hyperaccumulation is an effective elemental defence against this herbivore, increasing plant fitness through a combination of toxicity to DBM larvae and decreased oviposition by adults.

Introduction

Plants that accumulate high concentrations of elements in their tissues have been termed hyperaccumulators (Brooks et al. 1977). Approximately 418 hyperaccumulator taxa have been discovered and most of these taxa (318, or 76%) hyperaccumulate Ni (Reeves and Baker 2000). Elements reported to be hyperaccumulated include Al, As, B, Cd, Co, Cu, Mn, Ni, Pb, Se and Zn (Reeves and Baker 2000; Jensen et al. 2002; Babaoglu et al. 2004).

Several functions of elemental hyperaccumulation (especially, metal hyperaccumulation) have been proposed (Boyd and Martens 1992), including plant defence (Boyd and Martens 1992; Boyd 1998). Hyperaccumulated metals have been shown to defend plants against crucifer specialist herbivores by: (1) deterrence or selective feeding on low metal plants when presented a choice of

high- or low-metal plants (Pollard and Baker 1997; Jhee et al. 1999); (2) delaying larval development via sublethal effects (Martens and Boyd 1994; Boyd and Moar 1999); and/or (3) causing acute toxicity to feeding larvae (Martens and Boyd 1994; Boyd and Moar 1999).

A plant defence is a characteristic that increases resistance or tolerance against the attack of herbivores (Levin 1976; Mauricio and Rausher 1997; Gatehouse 2002). Resistance is a plant characteristic that influences the amount of damage inflicted by an herbivore, whereas tolerance is a plant's ability to experience damage with no decrease in fitness (Rausher 1992). Many plant characteristics reported to have herbivore-resistant effects are organic compounds produced either primarily (Berenbaum 1995) or secondarily (Fraenkel 1959; Levin 1976) by a plant's photosynthetic machinery. The common role of these compounds is that they can act either at the behavioral level through antixenosis (e.g., reduced oviposition or feeding: Dethier 1954; Whittaker and Feeny 1971; Rosenthal and Janzen 1979; Rausher 1992; Walling 2000) or they may be antibiotic (toxic) to an herbivore (Rausher 1992). Prior studies have shown that hyperaccumulated Ni can cause both types of effects (Boyd and Martens 1994; Martens and Boyd 1994; Boyd and Moar 1999).

Several studies have identified certain elements as having a defensive function against herbivory, such as Si (McNaughton and Tarrants 1983), Ca (Hay et al. 1994), F (Twigg and King 1991), Se (Hanson et al. 2004), and metals such as Cd (Jiang et al. 2005), Ni (Boyd and Martens 1994) and Zn (Pollard and Baker 1997; Jhee et al. 1999; Behmer et al. 2005). These "elemental" chemical defences (Martens and Boyd 1994) differ from organic chemical defences because elemental defences function from elements taken up from soil and sequestered, rather than being produced from photosynthate via metabolic pathways. Additionally, unlike most organic chemicals, elements with toxic effects (such as many heavy metals) cannot be degraded into less toxic components (Hopkin 1989).

Most tests of the defensive function of hyperaccumulated metals are herbivore feeding studies, often using Lepidoptera larvae (Boyd and Martens 1994; Pollard and Baker 1997; Boyd and Moar 1999; Jhee et al. 1999). For many crucifer specialist herbivores, such as certain Lepidoptera larvae, selection of an oviposition site by a female is critical to host choice (Singer 1986). If newly hatched larvae are not capable of searching for additional hosts, they must feed on the plant chosen by the female (Singer 1986). In a review by Singer (1986), oviposition preference is synonymous with behavioral terms such as "choosing, selecting, preferring and discriminating." The term "preference" can therefore be defined as divergence from random behavior, where random behavior has no relationship to variation among plants encountered (Singer 1986). Oviposition preference by phytophagous insects may be important to the potential role of hyperaccumulated metals as a plant defence. To our knowledge, only one study of metal hyperaccumulating plants (Martens and Boyd 1994) has investigated oviposition preference, that of the crucifer specialist herbivore, *Pieris rapae* (Lepidoptera: Pieridae). However, that study found no difference in oviposition amount between high- and low-Ni plants of the Ni hyperaccumulator *Streptanthus polygaloides*. Martens and Boyd (1994) did find significantly greater survival and significantly larger plant biomass for high-Ni plants compared to low-Ni plants, which were almost completely defoliated by larvae. Therefore, in that case, the defensive effect of Ni hyperaccumulation was limited to its effect on larvae rather than influencing oviposition by adults.

In this study, we investigate the defensive function of Ni hyperaccumulation by *Streptanthus polygaloides* Gray (Brassicaceae) and its effect on life cycle stages of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae). For the adult stage, the experiments are designed to test the oviposition preference of diamondback moth (DBM) females based on the distribution of eggs as a composite of female preference. For the larval stage, we ask if hyperaccumulated Ni affects feeding by larvae. We also document the fitness benefit of hyperaccumulation for plants in a mixed population of hyperaccumulating and non-hyperaccumulating *S. polygaloides*. Specifically, we ask: (1) Can female moths of a generalist crucifer herbivore (DBM) discriminate between high- and low-Ni *S. polygaloides*? (2) If there is discrimination, what chemical cues are involved? (3) Is there a difference in relative damage when herbivores are allowed to oviposit and feed on a mixed population

of high- and low-Ni plants? (4) What is the overall effect of herbivory on growth and reproductive effort of high- and low-Ni plants? and (5) Can an artificial diet experiment demonstrate that Ni is probably responsible for the decreased herbivore damage to Ni hyperaccumulating plants?

Methods

Study species

The Ni hyperaccumulator *Streptanthus polygaloides* Gray (Brassicaceae) is an annual herb endemic to serpentine chaparral of the western foothills of California's Sierra Nevada (Reeves et al. 1981; Kruckeberg 1984). Reeves et al. (1981) reported that Ni hyperaccumulation occurred in all herbarium specimens from the nine natural populations they examined. Nickel measurements in above-ground tissues of these specimens ranged from 1100 to 16,400 μg g^{-1} dry mass while root tissue Ni concentrations ranged from 2000 to 2460 μg g^{-1} dry mass (Reeves et al. 1981). Seeds for our experiment were collected from an ultramafic outcrop on the west shore of Pine Flat Reservoir, King's River, Fresno County, California (Kruckeberg 1984).

The diamondback moth (DBM), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), is an oligophagous herbivore of plants in the Brassicaceae (Fraenkel 1959; Talekar and Shelton 1993). Reputed to be the most widespread lepidopteran on Earth (Talekar and Shelton 1993), it is a pest of crucifer crops in California but it is not known if it attacks *S. polygaloides*. A survey of arthropods associated with one *S. polygaloides* population (Wall and Boyd 2002) did not report finding *P. xylostella*. Diamondback moths were used as bioassay herbivores for oviposition and artificial diet experiments. A laboratory colony was established using eggs obtained from a colony at Cornell University. Founder moths for the Cornell colony were collected from the wild in Geneva, NY, USA. After the colony was established at Auburn University (Harvey 2002), it was supplemented with wild individuals collected from Auburn, Lee County, Alabama, USA. An artificial diet (BioServe: Frenchtown, New Jersey, USA) was used to maintain the colony similar to the colony maintenance procedures of Harvey (2002). We used the following protocol for colony maintenance: Sheets of DBM eggs obtained from the established colony were sterilized in a 10% bleach solution for 20 s, rinsed in deionized water for 1 min and allowed to dry. Dried egg sheets were cut into strips containing approximately 300–400 eggs per strip and each strip was placed in a 250 ml paperboard cup with about 1 cm of congealed artificial diet covering the bottom. The cups of diet and eggs were placed in an incubator at 37 °C and 30–50% relative humidity until they hatched and the first-instar larvae began to feed (approximately 60 h after eggs were laid). Egg sheets were then removed and larvae allowed to feed until pupation (approximately 11–12 days after hatching). Pupae on the top half of the cups were placed into screen cages. Pupae were incubated at room temperature (23 °C), 30–50% relative humidity, and 16 h day:8 h night photoperiod. Adults emerged, mated, and laid eggs on scored aluminium foil sheets dipped in sterilized collard juice. Scoring of the foil (making grooves in its surface) stimulates egg laying by DBM, which avoids ovipositing on smooth leaf surfaces (Talekar and Shelton 1993).

Oviposition and larval feeding experiment

An experiment to determine if hyperaccumulated Ni deters oviposition by DBM was conducted in cages within a greenhouse at the Plant Science Research Center (PSRC) at Auburn University. Cages measured $100 \times 60 \times 60$ cm and were constructed of polyvinyl chloride (PVC) piping enclosed by standard mosquito netting (Recreational Equipment Incorporated: Sumner, Washington, USA). Plants were grown on greenhouse soil (Pro-Mix: Premier Horticulture, Red Hill, Pennsylvania, USA) amended to approximately 800 μg Ni g^{-1} dry mass by adding dried powdered $NiCl_2$ (Sigma: St. Louis, Missouri, USA). Low-Ni plants were grown on unamended soil. Approximately 25–30 seeds were sown in 8×8 cm pots filled with soil and topped with a 1 cm layer of perlite. Plants were grown approximately 1.5 mo in a greenhouse in Auburn University under a 16 h light:8 h dark photoperiod and 30 °C:22 °C thermoperiod. Plants were watered twice daily. After 1 mo pots were thinned to four plants per pot so that each pot contained plants of similar height and leaf morphological characteristics (leaf lobing).

Five pots of four-week-old high-Ni *S. polygaloides* plants and five pots of low-Ni plants were alternately arranged in a 2×5 array, so the pots touched, and were enclosed within a single cage. Two open containers of DBM pupae (approximately 100 pupae) were placed next to the array of plants. Sucrose dissolved in water (10% w/v) with a single drop of yellow food coloring added (McCormick and Co. Inc.: Hunt Valley, MD, USA) was placed within a 100 ml beaker as a food supply for eclosing moths. Wicks (Mohawk Dental Supply: Syracuse, NY, USA) were placed into the beaker to provide access to the sucrose solution by moths. A total of four replicate cages were used in this experiment.

Diamondback moths were allowed to eclose and mate, and female moths laid eggs on plants within the cages. The experiment used a 16 h light:8 h dark photoperiod and 30 °C:22 °C thermoperiod. Plants were watered twice daily. After 9 days, moths were removed from cages and the number of eggs was counted on individual plants in each pot within each cage.

Initial plant height was measured (from soil to the apical meristem) and number of leaves was counted on plants. We expressed oviposition preference as the mean number of eggs per cm stem and mean number of eggs per leaf on each plant type in each cage. Because we were not investigating DBM host specificity (Singer 1986), but rather the defensive benefit of hyperaccumulated Ni, we used the distribution of eggs (on day 9) as a composite of female DBM preference. Oviposition data, expressed as eggs per cm stem height and eggs per leaf, were analyzed with JMP IN 5.1 (SAS Institute 2005) using Wilcoxon 2-Sample tests. Differences in mean values were considered significant at $\alpha \leq 0.05$.

Larvae were allowed to freely migrate and feed among the arrays of pots within each cage. To determine the effect of hyperaccumulated Ni on numbers of hatched larvae, we added numbers of larvae and pupae counted on each plant on day 18 (9 days after ovipositing adults were removed). The numbers of larvae and pupae were counted on each plant within each pot for each cage. This allowed us to compare mean numbers of larvae and pupae per plant calculated for each treatment within each cage. Each cage was considered a replicate ($n = 4$). Numbers of larvae and pupae on high-Ni compared to low-Ni plants within a cage were analyzed with a Wilcoxon 2-Sample test using JMP IN 5.1 (SAS Institute 2005). Differences in numbers of larvae and pupae between high- and low-Ni plants were considered significant at $\alpha \leq 0.05$.

We measured change in plant height and change in number of leaves to compare resistance effects of high- and low-Ni plants to DBM larvae based on changes in plant size. We measured final height and number of leaves for each plant after all remaining larvae underwent pupation (day 24). For ease of counting, leaves with at least 50% tissue remaining were counted as whole leaves. Change in plant height and change in number of leaves were analyzed using Wilcoxon 2-Sample tests with JMP IN 5.1 (SAS Institute 2005). Differences between high- and low-Ni plants were considered significant at $\alpha \leq 0.05$.

Because plant size change combines both growth and herbivore damage, we also measured herbivore damage to plants on day 24. A subjective damage assessment was made for each plant, using a scale of 1–11. The scale was as follows: (1) No damage; (2) Small holes present and <10 visible trenches from first instar larvae; (3) Small holes present, tips of leaves with small chew marks, >10 trenches; (4) Plant 30–39% defoliated; (5) 40–49% defoliation; (6) 50–59% defoliation; (7) 60–69% defoliation; (8) 70–79% defoliation; (9) 80–89% defoliation; (10) 90–99% defoliation; (11) All leaves eaten (only the stem remaining). For this assessment, defoliation was an overall estimate of total leaf tissue removed from each plant. Each plant within each pot was assigned a damage level, the mean damage level for plants in each pot within a cage was calculated and means for each pot treatment (high- and low-Ni) then were calculated for the entire cage. Damage levels of high- and low-Ni plants from all cages were compared using a Wilcoxon 2-Sample test at $\alpha \leq 0.05$ using JMP IN 5.1 (SAS Institute 2005).

Our final measure of plant performance was total reproductive effort. After all surviving larvae pupated (by day 24), pupae were removed and plants were allowed to continue growth in a greenhouse at the PSRC. Plants were maintained with twice daily watering at a 16 h light:8 h dark photoperiod and a 30 °C:22 °C thermoperiod. The number of flowers present per pot was counted 42, 51, 62, 79, and 90 days after oviposition. Total reproductive output, defined as mean number of

flowers per pot of each treatment type (high-Ni or low-Ni) per cage, was analyzed using a Wilcoxon 2-Sample test on day 90 data using JMP IN 5.1 (SAS Institute 2005) with significant differences determined at $\alpha \leq 0.05$.

Foil oviposition experiments

Because we observed significantly more eggs per cm stem and eggs per leaf on low-Ni plants in oviposition trials (see Results section), we proceeded with a series of experiments to determine if hyperaccumulated Ni or glucosinolates were likely responsible for the difference in DBM oviposition behavior. To remove the influence of morphology and focus on chemical differences between high- and low-Ni plants, plant extracts applied to scored aluminium foil sheets were used to test oviposition choice.

High- and low-Ni plants were grown similar to the procedure for the oviposition and larval feeding experiment described above. We harvested 26 g fresh weight of high-Ni and 26 g fresh weight of low-Ni aboveground plant material. Plant material of each plant treatment was boiled for 10 min in 250 ml water, and we then blended the plant material and water with a food blender (Sunbeam/Oster: Boca Raton, FL, USA). The mixture from each plant type was strained using a colander and the plant extracts used to coat 4×6 cm scored aluminium foil sheets by dipping the sheets in an 18×14×4 cm pan. Foil sheets were allowed to dry for 24 h at room temperature. One pair of high-Ni and low-Ni extract foil sheets was hung from thread within each of five oviposition cages (cages described above for oviposition and larval feeding experiment). Approximately 100 pupae were allowed to eclose and oviposit for 5 days. A moth food source was supplied as described above for the plant oviposition experiment. This experiment was conduced in a greenhouse at the PSRC under a 16 h light:8 h dark photoperiod and a 30 °C:22 °C thermoperiod. After 5 days, foil sheets were replaced and moths continued to oviposit for three additional days. The total number of eggs on each type of foil sheet was counted for each cage over the 8 day period and converted to counts per day. Mean numbers of eggs on high- vs. low-Ni plant extract foil sheets were compared with a Wilcoxon 2-Sample test using JMP IN 5.1 (SAS Institute 2005) and differences were considered statistically significant at $\alpha \leq 0.05$.

Since DBM oviposited significantly more on low-Ni extract sheets (see Results section), we proceeded by using low-Ni *S. polygaloides* plant extracts to determine whether oviposition by DBM was affected by extract Ni concentration. Low-Ni *S. polygaloides* extracts, made using the procedure outlined above, were used so that concentrations of organic chemicals that may serve as oviposition stimulants, such as glucosinolates (Talekar and Shelton 1993), would be uniformly present in all treatments. We varied Ni concentrations by adding $NiCl_2$ (stock solution = 2 M Ni) to separate batches of plant extract to produce extract containing concentrations of 1, 5, 10 and 50 mM Ni. Five scored foil sheets (4×6 cm) were dipped into each solution and a control of low-Ni plant extract was also used to coat five foil sheets. Foil sheets were allowed to dry at room temperature for 24 h.

This experiment was conducted in a greenhouse at the PSRC under a 16 h light:8 h dark photoperiod and a 30 °C:22 °C thermoperiod. The oviposition cages described above were used for the experiment. One sheet of each Ni concentration (0, 1, 5, 10, 50 mM Ni) was placed in each cage. Each foil sheet was supported upright by a paper clip embedded within a 16 cm diameter sheet of polyethylene foam resting on the bottom of the cage, so that both sides of the foil sheet would be available for oviposition. Foil sheets were arranged in a circle so that all sheets were evenly spaced with one side facing inward toward a food source for the adult DBM. The food source was supplied as described above for the plant oviposition experiment. Approximately 100 pupae were placed on the center of the foam sheet and moths allowed to eclose, mate and oviposit for 5 days. Foil sheets were replaced for one additional day. The total number of eggs laid on foil sheets of each Ni concentration was counted for each cage and mean numbers of eggs laid per day were analyzed using JMP IN 5.1 (SAS Institute 2005) with a Kruskal–Wallis test with significance at $\alpha \leq 0.05$.

We used sinigrin (allyl glucosinolate) to determine whether differences in glucosinolate concentration may have influenced oviposition preference of DBM. Sinigrin is one of several glucosinolates known to stimulate DBM oviposition (Talekar

and Shelton 1993). This experiment was conducted in a greenhouse at the PSRC under a 16 h light:8 h dark photoperiod and a 30 °C:22 °C thermoperiod. We used scored aluminium foil sheets coated with a 1% sinigrin monohydrate (Sigma: St. Louis, MO, USA) solution. Powdered sinigrin monohydrate (250 mg) was dissolved in 100 ml deionized water and then diluted to a 1% solution to dip 4×6 cm foil sheets. Uncoated foil sheets were used for comparison. A pair of foil sheets (one uncoated and one sinigrin-coated) was hung in each of four oviposition cages (described above). A food source was supplied as described above for the plant oviposition experiment and approximately 100 pupae were placed in each cage and allowed to eclose, mate and oviposit. Adults were allowed to oviposit for 5 days and the number of eggs per sheet was counted. We calculated mean number of eggs per sheet per day for each treatment and analyzed the data using a Wilcoxon 2-Sample test with JMP IN 5.1 (SAS Institute 2005). Differences were considered significant at $\alpha \leq 0.05$.

Plant chemical analysis

Additional pots of plants, sown at the same time as the experimental plants and using the same soil treatments as the oviposition and larval feeding experiment, were grown for 4 weeks and harvested for analysis of plant chemical composition. Aboveground biomass was dried in an oven at 60 °C for 10 days. To determine plant element concentrations, samples were ground with mortar and pestle and 0.5 g dry mass subsamples were wet digested using 10 ml of acid mix (700 ml concentrated HNO_3 + 300 ml concentrated $HClO_4$) within 250 ml glass digestion tubes for 24 h. Tubes then were heated on a block digester within a perchloric acid fume hood at 190 °C until digestion was complete. Once the tubes cooled, 2.5 ml HCl was added to each tube and contents were transferred to 25 ml volumetric flasks. Contents of the volumetric flasks were brought up to 25 ml by adding deionized water and transferred to 100 ml plastic storage bottles (Nalgene: Rochester, New York, USA). Element concentrations were determined using an inductively coupled argon plasma (ICP-AE) spectrophotometer (SPECTRO CIROS CCD: Kleve, Germany).

Total C and N (%) of high- and low-Ni *S. polygaloides* samples were also measured to determine C/N ratios. Ten samples of four-week-old plants for each treatment were dried in an oven at 60°C and ground with mortar and pestle. Carbon and N% were determined by combusting 0.5 g plant samples at 1050 °C using a LECO CN-2000 Analyzer (LECO Corporation: St. Joseph, MO, USA).

Concentrations of all elements, as well as C/N ratios, were compared between high- and low-Ni plant samples by Wilcoxon 2-Sample tests using JMP IN 5.1 (SAS Institute 2005). Differences between high- and low-Ni plant samples were considered significant at $\alpha \leq 0.05$.

Additional pots of high- and low-Ni *S. polygaloides* were grown for 4 weeks and harvested for analysis of plant glucosinolate composition. Samples were frozen immediately after harvest at −80 °C and freeze-dried (Vertis Research Equipment: Gardiner, NY, USA). A subsample (0.5 g) from each sample was analyzed via Gas Chromatography (GC) by modification of a High Pressure Liquid Chromatography (HPLC) analysis method (ISO 9167–1:1992) published by the International Organization for Standardization. The GC modifications were that, after eluting from the ion exchange column, glucosinolates were dried at 60 °C under nitrogen and derivatized with methylimidazole, *N*-Methyltrimethylsilyltrifluoroacetamide (MSTFA) and trimethylchlorosilane (TMCS) before injecting into the column. Gas chromatography theoretical response factors were used instead of experimental response factors as for HPLC. A canola seed reference sample (obtained from the Commission of European Communities and certified using all methods of glucosinolate analysis) was included as an internal check. Glucosinolates were quantified as micromoles g^{-1} dry mass. Quantities of each glucosinolate, as well as total glucosinolate concentration, were compared between high- and low-Ni plants using a Wilcoxon 2-Sample test (SAS Institute 2005) for each comparison at $\alpha \leq 0.05$.

Effect of Ni on DBM: artificial diet experiment

The effect of Ni on larval mortality was determined by raising larvae on artificial diet amended with varying concentrations of Ni. Artificial diet

(BioServe: Frenchtown, NJ, USA) was amended with $NiCl_2$. Separate batches of diet were amended with $NiCl_2$ to 0.75, 1, 1.5 and 2 mM Ni concentrations. A control treatment of unamended diet was included. For each concentration, 100 ml of diet was made and divided into 12 separate 30 ml plastic cups each containing approximately 2–3 ml of diet. An additional cup was filled with diet for later analysis of its Ni concentration on a dry mass basis. A single foil strip of DBM eggs (approximately 100) was added to each cup of diet. Diet cups were placed into an incubator at 37 °C and approximately 30–50% humidity. Egg sheets were removed from cups after larvae hatched and had begun to feed (approximately 60 h after the egg sheets were collected). When egg sheets were removed, we counted the number of first instar larvae in each cup. The number of live larvae was counted every 2–3 days thereafter. Counting of live individuals in all cups within an experiment was ended when adults began to eclose from the control treatments (16 days after eggs were collected). Percent mortality on day 16 was calculated based on the number of moths, pupae and live larvae compared to the initial number of larvae. Percent pupation, defined as percent of initial larvae forming pupae on day 14, was calculated to determine whether Ni may have a sublethal effect by delaying larval development. Data were analyzed by one-way ANOVA using JMP IN 5.1 (SAS Institute 2005) with multiple comparisons by Student's-*t* least significant difference test. Prior to analysis, data were arcsine square root transformed to better fit the assumptions of ANOVA (Zar 1996). Differences in % mortality and % pupation were considered significant at $\alpha \leq 0.05$.

Diet samples were analyzed to determine Ni concentrations in μg Ni g^{-1} dry mass of diet to provide measurements in units comparable to those generated by plant tissue analyses. Four diet samples of each treatment were dried in an oven at 60 °C for 7 days and ground with mortar and pestle. Subsamples (0.5 g dry mass) were wet digested in the same manner as plant biomass used for plant element concentration analysis described above. Nickel concentrations in the digests were measured using an inductively coupled argon plasma (ICP-AE) spectrophotometer (SPECTRO CIROS CCD: Kleve, Germany).

Results

Plant chemical analysis

Streptanthus polygaloides grown on high-Ni soil hyperaccumulated Ni and plants grown on unamended soil did not. High-Ni plants contained significantly more Ni (243-fold more) than low-Ni plants (Table 1). Elemental analysis showed no significant differences between high- and low-Ni plants in concentrations of B, Ca, Cd, Co, Cu, Fe, Mg, Se, or Zn (Table 1). The C/N ratios also did not significantly differ (Table 1).

Four glucosinolates were found in concentrations > 0.05 μmoles g^{-1} (Table 2). Allyl glucosinolate (sinigrin) was present in greatest concentration, followed by methylthio-pentenyl, 4-OH-benzyl, and 4-OH-3-methyl-indolyl glucosinolates, respectively. Total glucosinolate concentration did not significantly differ between high- and low-Ni plants but low-Ni plants produced significantly more of the dominant glucosinolate (allyl glucosinolate) compared to high-Ni plants (Table 2). Methylthio-pentenyl and 4-OH-benzyl glucosinolate levels did not significantly differ (Table 2), whereas 4-OH-methyl-indolyl glucosinolate levels were significantly greater in high-Ni plants compared to low-Ni plants (Table 2).

Table 1. Element and plant nutritional quality analysis of aboveground portions of *Streptanthus polygaloides*.

Parameter measured	Soil treatment	
	High-Ni	Low-Ni
Boron ($\mu g\ g^{-1}$)	76 ± 9.5	69 ± 5.1
Cadmium ($\mu g\ g^{-1}$)	0.086 ± 0.092	0.088 ± 0.052
Calcium ($\mu g\ g^{-1}$)	28,500 ± 1170	26,700 ± 880
Cobalt ($\mu g\ g^{-1}$)	0.50 ± 0.15	0.46 ± 0.16
Copper ($\mu g\ g^{-1}$)	24 ± 6.8	22 ± 5.3
Iron ($\mu g\ g^{-1}$)	77 ± 13	84 ± 6.6
Magnesium ($\mu g\ g^{-1}$)	3490 ± 530	3480 ± 580
Nickel ($\mu g\ g^{-1}$)*	6830 ± 60	28.4 ± 1.3
Selenium ($\mu g\ g^{-1}$)	0.99 ± 0.092	0.99 ± 0.13
Zinc ($\mu g\ g^{-1}$)	721 ± 21	720 ± 44
C/N ratio	10.3 ± 0.90	10.6 ± 0.68

Data are means ± SE. For elemental analysis, $n = 4$ for each soil treatment. For C/N ratio, $n = 10$ for each soil treatment.*Indicates differences were significant based on Wilcoxon 2-Sample tests at $\alpha < 0.05$.

Table 2. Glucosinolate concentrations (μmol g^{-1} dry mass) of high- and low-Ni *Streptanthus polygaloides* as means ± SE ($n = 18$ for high-Ni samples, $n = 16$ for low-Ni samples).

Glucosinolate	Treatment		*Z* Score
	High-Ni	Low-Ni	
Allyl (sinigrin)	1.0 ± 0.12	1.2 ± 0.09	1.93*
Methylthio-pentenyl	0.58 ± 0.074	0.40 ± 0.042	1.57
4-OH-benzyl	0.43 ± 0.059	0.26 ± 0.027	1.90
4-OH-3-methyl-indolyl	0.11 ± 0.004	0.096 ± 0.005	1.99*
Total	2.1 ± 0.24	2.0 ± 0.14	0.19

*Indicates $\alpha < 0.05$ based on Wilcoxon 2-Sample tests.

Oviposition and larval feeding experiment

Initial measurements showed that high- and low-Ni plants did not differ in size at the start of the plant oviposition experiments. Neither height nor leaf number significantly differed between high- and low-Ni plants (Table 3). Diamondback moths laid 2.7-fold more eggs on low-Ni plants than high-Ni plants when data were scaled to plant size using stem length and 2.5-fold more on low-Ni plants than high-Ni plants when data were scaled to numbers of leaves (Table 3). Low- and high-Ni plants differed in numbers of larvae and pupae after 9 days of feeding. Low-Ni plants had significantly more larvae and pupae (83-fold more) compared to high-Ni plants (Table 3). Damage scores also showed significantly more damage to low-Ni plants compared to high-Ni plants (Table 3). As a result of higher herbivore load and increased damage to low-Ni plants, high-Ni plants grew twice as much as low-Ni plants and increased in number of leaves, whereas low-Ni plants lost leaves due to herbivory (Table 3).

We compared the total number of flowers produced by high- and low-Ni plants as an estimate of plant fitness. On each date of measurement (days 42, 51, 62, 79, and 90) high-Ni plants produced consistently more flowers compared to low-Ni plants (Figure 1). By day 90, high-Ni plants had produced significantly more flowers (> 3-fold more) than low-Ni plants (Figure 1) (Wilcoxon 2-Sample test: $Z = 2.17$, $\alpha < 0.05$).

Foil oviposition experiments

The significantly greater number of eggs laid on low-Ni plants led us to use a series of foil experiments to determine whether DBM could detect Ni or if an organic chemical (such as glucosinolates) was influencing oviposition choice. Diamondback moths laid significantly more eggs (2-fold more) on foil sheets coated with low-Ni plant extract than on those coated with high-Ni plant extract (Table 4). However, DBM could not detect Ni as egg numbers did not vary significantly when Ni was added to low-Ni plant extracts, even at 50 mM Ni (Table 4). In a final foil experiment, we used a 1% w/v sinigrin monohydrate solution to determine if this chemical served as an oviposition stimulant for DBM. Diamondback moths laid significantly more eggs (37-fold more) on foil sheets coated with sinigrin compared to uncoated foil sheets (Table 4).

Table 3. Plant size data from plant oviposition experiments.

Parameters measured	Treatment		*Z* score
	High-Ni	Low-Ni	
Plant data			
Pre-oviposition height	3.7 ± 0.06 cm	3.4 ± 0.15 cm	1.78
Pre-oviposition number of leaves	12 ± 0.39 leaves	11 ± 0.30 leaves	1.16
Change in height after 24 days	12 ± 0.56 cm	6.4 ± 1.1 cm	2.17*
Change in number of leaves after 24 days	7.3 ± 0.29 leaves	−6.5 ± 1.9 leaves	2.17*
DBM response data			
Eggs per cm stem	33.6 ± 17.1	89.1 ± 17.1	2.17*
Eggs per leaf	10.4 ± 4.59	26.0 ± 4.59	2.17*
Larvae + pupae per pot after 18 days	0.050 ± 0.667	4.15 ± 0.677	2.23*
Plant damage scores after 24 days (11 as maximum damage)	1.2 ± 0.067 (out of 11)	8.3 ± 0.94 (out of 11)	2.18*

Parameters measured are presented as means ± SE ($n = 4$).*Indicates $\alpha < 0.05$ based on Wilcoxon 2-Sample tests.

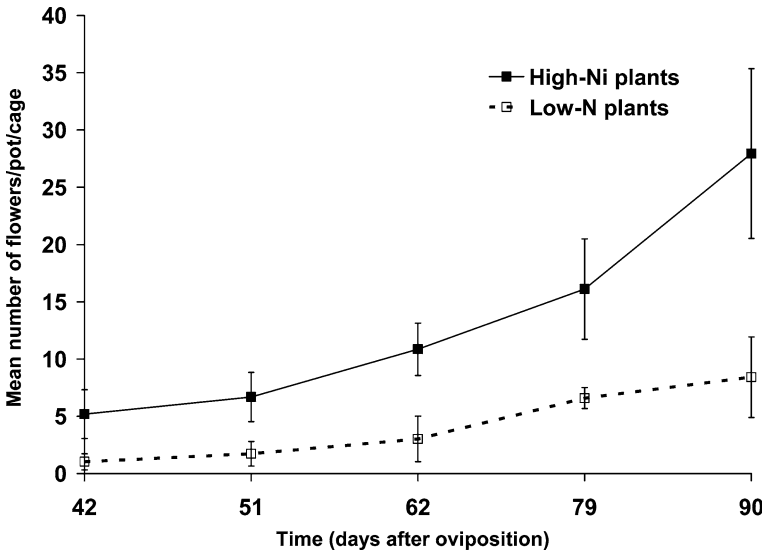

Figure 1. Flower production of *Streptanthus polygaloides* after herbivory by *Plutella xylostella*. Vertical lines are ±SE for each mean.

Table 4. Results from foil oviposition experiments to determine if *Plutella xylostella*: (1) can discriminate between high- and low-Ni *S. polygaloides* plant extracts; (2) responds to Ni concentration in *S. polygaloides* plant extracts; and (3) responds to sinigrin as an oviposition stimulant.

Experiment	Treatment	Mean eggs per sheet/day ± SE	Statistic	df	*p* value
(1) High- and low-Ni plant extract	High-Ni plant	660 ± 90	$Z = 2.30$	1	< 0.05
	Low-Ni plant	1200 ± 120			
(2) Low-Ni plant extract with $NiCl_2$added	Control (no Ni)	250 ± 44	$X^2 = 7.64$	5	0.18
	1 mM	240 ± 31			
	5 mM	150 ± 31			
	10 mM	180 ± 60			
	20 mM	250 ± 35			
	50 mM	230 ± 22			
(3) Sinigrin solution or uncoated	1% Sinigrin	810 ± 81	$Z = 2.18$	1	< 0.05
	Uncoated	22 ± 11			

Effect of Ni on DBM: artificial diet experiment

Samples of diet were analyzed by ICP-AE analysis to convert Ni concentrations from mM Ni to μg Ni g^{-1} mass so that we could compare directly diet Ni toxicity to plant tissue Ni concentrations (the latter in Table 1). Nickel concentrations for the diet treatments were (mean ± SE): 8.8 ± 0.69 μg Ni g^{-1} (0 mM Ni control diet), 100 ± 1.2 μg Ni g^{-1} (0.75 mM), 140 ± 2.6 μg Ni g^{-1} (1.0 mM), 200 ± 0.75 μg Ni g^{-1} (1.5 mM) and 260 ± 3.75 μg Ni g^{-1} (2 mM).

The artificial diet experiment showed that Ni toxicity was sufficient to account for the lethal response observed for DBM larvae feeding on high-Ni plants. Larval mortality significantly increased with increasing $NiCl_2$ concentration (Figure 2) (ANOVA: $F_{4,55} = 9.93$; $p < 0.0001$). Student's-*t* least significance test showed significantly increased mortality of DBM larvae fed diet Ni concentrations of 140 μg Ni g^{-1} or greater (compared to control diet, Figure 2). We also observed a sublethal effect of Ni on pupation rates in the artificial diet experiment. Pupation rates were

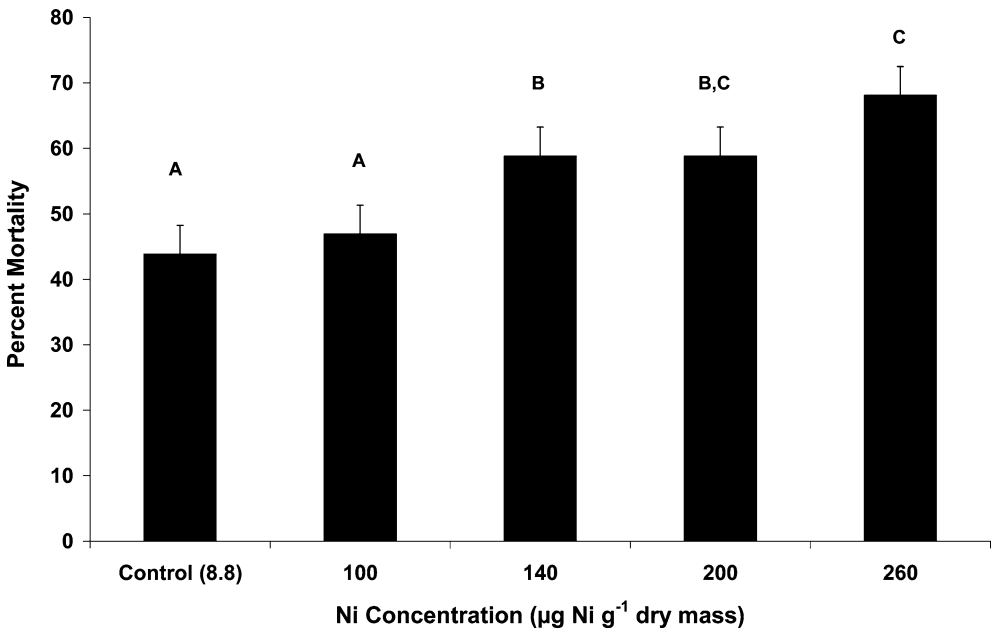

Figure 2. Mean mortality of *Plutella xylostella* larvae on day 16 of the artificial diet experiment. Vertical lines are + SE for each mean. Means with the same letter are not statistically different from one another based on Student's-*t* least significant difference test at $p \leq 0.05$.

significantly greater for the control treatment than for all other treatments based on Student's-*t* LSD test ($t = 2.00$, $p < 0.05$) (Figure 3). Increases in diet Ni concentrations led to still lower pupation rates (Figure 3).

Discussion

Our results support the "elemental defence hypothesis" of Ni hyperaccumulation. A plant defence increases resistance or tolerance against the attack of herbivores (Levin 1976; Mauricio and Rausher 1997; Walling 2000). In our experiment, high-Ni plants grew taller, produced more leaves, and produced more flowers compared to low-Ni plants. Thus, high-Ni plants were better defended against DBM than low-Ni plants. A critical question for the elemental defence hypothesis (see Huitson and Macnair 2003; Macnair 2003) is whether the defensive effect is due to Ni or to another trait that correlates with hyperaccumulation status. Although we cannot completely rule out other correlated traits (but can address glucosinolate concentrations: see below), our artificial diet experiment did explore the toxicity of Ni to DBM. That experiment clearly showed a significant toxic Ni effect at a concentration of 140 μg Ni g^{-1}, far less than the 6830 μg Ni g^{-1} measured in our high-Ni *S. polygaloides*. This strongly supports our conclusion that Ni is the toxic substance in high-Ni plants. Thus, we have shown that hyperaccumulated Ni can defend *S. polygaloides* against a crucifer specialist (such as DBM).

We also note that the least toxic dose of Ni in the artificial diet experiment is far less than the 1000 μg Ni g^{-1} threshold used to define Ni hyperaccumulation. At 140 μg Ni g^{-1}, it is just above the level (100 μg Ni g^{-1}) used to define Ni accumulation in plants (Reeves and Baker 2000). Furthermore, we found a negative effect of even lower levels of Ni (100 μg Ni g^{-1}) on pupation rate. These results suggest that Ni may have a defensive effect at concentrations below hyperaccumulator levels. If further research confirms this suggestion, then elemental defences may be more widespread and ecologically important than heretofore supposed (Boyd 2004). Boyd (1998) suggested that hyperaccumulation may have evolved from accumulation via a stepwise increase in metal concentration driven by the selective value of Ni as a

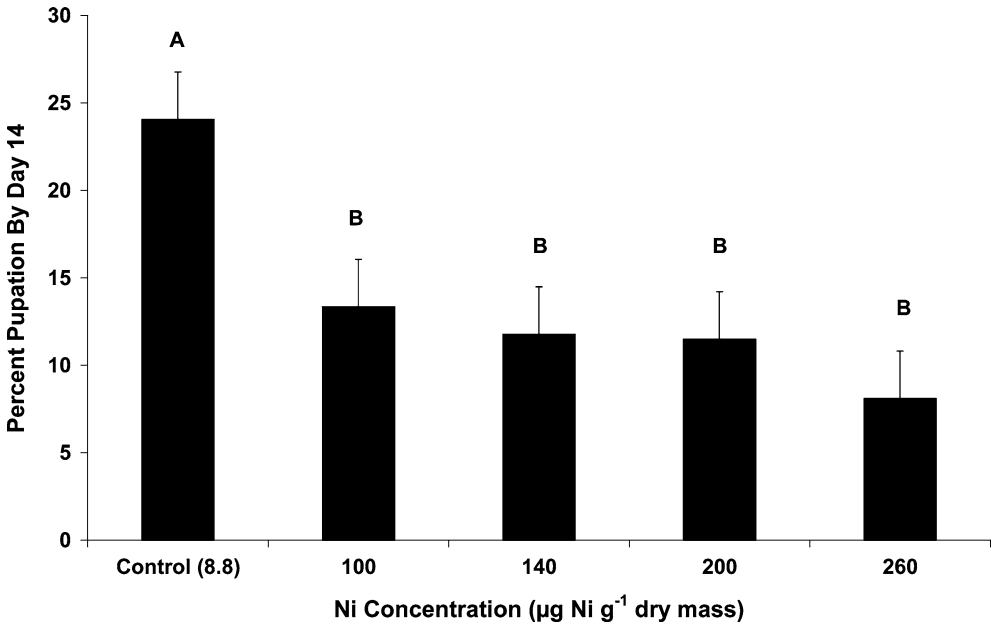

Figure 3. Mean percent pupation by *Plutella xylostella* larvae on day 14 of the artificial diet experiment. Vertical lines are +SE for each mean. Means with the same letter do not statistically differ from one another based on Student's-*t* least significant difference test at $p \leq 0.05$.

plant defence. The toxicity to DBM of accumulator levels of Ni illustrates that this scenario may have occurred, but requires that at least some native herbivores be similarly susceptible to relatively low concentrations of Ni. Field studies are greatly needed to test these ideas.

This is the second study to test elemental defence of Ni hyperaccumulation using an oviposition experiment, but the first to use reproductive effort to measure fitness. The oviposition experiment of Martens and Boyd (1994) used *Pieris rapae* and *S. polygaloides*, but found no significant difference in number of eggs laid on high- vs. low-Ni plants. Similar to our study, *Pieris rapae* larvae consumed significantly more of the plant biomass of low-Ni plants compared to high-Ni plants. Our study is unique because, along with plant size measurements, we used reproductive effort (flower number) as a measure of plant fitness. Because low-Ni plants suffered greater damage than high-Ni plants (Table 3), they were unable to recover quickly and produced only a third as many flowers as high-Ni plants (Figure 1). Our more direct measure of fitness illustrates the selective advantage of Ni hyperaccumulation in the face of herbivore pressure.

Pollard (2000) suggested that metal hyperaccumulating plants can serve as model systems for the study of coevolution. Several definitions of coevolution maintain that it is a reciprocal series of adaptations between plant traits and insect traits to ensure fitness for both the potential host plant and herbivore (Ehrlich and Raven 1964; Janzen 1980; Futuyma 1983). It is well documented that cruciferous plants utilize organic chemicals, such as glucosinolates, as a broad defence against unadapted herbivores (Feeny 1977; Rosenthal and Janzen 1979; Louda and Mole 1991; Harborne 1993). Shapiro (1981a) reported that the butterfly, *Pieris sisymbrii*, is a specialist herbivore of several *Streptanthus* species, including *S. glandulosus* and *S. breweri*, both of which are found on ultramafic soils in California. He also noted that some populations of *S. polygaloides* may be attacked by *P. sisymbrii* (Shapiro 1981b). Because specialists such as *P. sisymbrii* and DBM can circumvent the toxic properties of glucosinolates (Erikson and Feeny 1974; Levin 1976; Shapiro 1981a; Louda and Mole 1991) to use crucifers as food sources, then Ni may serve as a novel defence (*sensu* Boyd 1998) against crucifer specialists. In the context of the plant/herbivore

"arms race" (Whittaker and Feeny 1971), Ni hyperaccumulation by *S. polygaloides* may be an evolutionary innovation protective against crucifer specialists (as is shown in our study of DBM). Similarly, Ni hyperaccumulation by plants in other families may defend them against herbivores that have evolved tolerance to the organic chemical defences possessed by those plants. For example, a relatively large number of Ni hyperaccumulating taxa have been documented from the Rubiaceae and Euphorbiaceae (Reeves 2003), families well known for putatively defensive alkaloids (Seigler 1998) and diterpene esters (Raffauf 1996), respectively. Certainly these questions deserve further experimental exploration.

Plants often use a combination of defences to reduce herbivory (Berenbaum and Neal 1985; Hay et al. 1994; Scott et al. 2002; Dyer et al. 2003). It has been suggested that, for hyperaccumulators, a trade-off may exist so that plants defended by elemental defences can invest less carbon in the construction of organic defences (Martens and Boyd 1994; Boyd 1998, 2004). Little research documents organic defence concentrations in hyperaccumulators to support this trade-off hypothesis. Davis and Boyd (2000) found that a potential trade-off of organic plant defences and elemental defences may exist for *S. polygaloides*. In a comparison between Ni hyperaccumulating *S. polygaloides* and a non-hyperaccumulating species, *S. insignis*, Davis and Boyd (2000) found glucosinolate concentrations to be significantly less in the hyperaccumulator. However, they did not find a significant difference in glucosinolate concentration when they compared plants of the hyperaccumulator grown on high-Ni soil vs. low-Ni soil (Davis and Boyd 2000). Another investigation of glucosinolate concentration (Tolrà et al. 2001) was conducted for the Zn hyperaccumulator, *Thlaspi caerulescens* (Brassicaceae). That study supported the trade-off between metal hyperaccumulation and organic defences, since plants that hyperaccumulated Zn possessed significantly lower concentrations of glucosinolates than low-Zn plants (Tolrà et al. 2001). A more recent study of *T. caerulescens* (Noret et al. 2005) confirmed this trade-off between Zn and glucosinolate concentrations in this species.

Confirming the earlier work of Davis and Boyd (2000) but using a more comprehensive technique, we did not find reduced organic defences in hyperaccumulating *S. polygaloides*, as we found no significant difference in total glucosinolate concentration between high- and low-Ni plants (Table 2). However, there were differences (or at least trends) in concentration for specific glucosinolates (Table 2). Specifically, allyl glucosinolate (sinigrin) was reduced in high-Ni plants. This reduced level may have led to significantly fewer eggs laid on high-Ni plants by female DBM (Table 4) because sinigrin stimulates DBM oviposition (Justus and Mitchell 1996). The decreased egg load of high-Ni plants is a unique result of our study and points to a previously undocumented defensive consequence of Ni hyperaccumulation. Shapiro (1981c) pointed out that larvae of pierid butterflies can have large negative fitness effects on single *Streptanthus* plants and that some *Streptanthus* species have evolved egg mimics to deter oviposition (Shapiro 1981b). Our results suggest that a shift in the composition of glucosinolates of Ni hyperaccumulating *S. polygaloides* may serve a similar function by reducing the level of allyl glucosinolate (sinigrin), an oviposition stimulant for DBM and at least some pierid butterfly species (Huang and Renwick 1994).

A complete explanation for the oviposition preference by female DBM moths for low-Ni *S. polygaloides* is unclear. The quantitative difference in allyl glucosinolate seems insufficient to account for the striking response by female moths. However, physical cues may play a role in oviposition choice (Bernays and Chapman 1987) and so morphological factors may have influenced oviposition choice. For DBM, a recent study (Justus et al. 2000) has shown that physical cues can influence oviposition. No studies have yet examined morphological differences between high- and low-Ni plants of *S. polygaloides* and future research should investigate possible morphological differences between hyperaccumulating and non-hyperaccumulating plants.

Nickel hyperaccumulation by *S. polygaloides* provides more insight into the evolutionary "arms race" between plants and insect herbivores. Many Ni hyperaccumulators (25%) belong to the Brassicaceae (Reeves and Baker 2000), and many possess organic defences (such as glucosinolates) that are common to that family (Erickson and Feeny 1974; Ernst 1990; Louda and Mole 1991; Harborne 1993). Nickel hyperaccumulation may provide *S. polygaloides* and other Ni hyperaccumulator members of the Brassicaceae with a defence against crucifer specialist herbivores. Our study has shown

that this defence functions at two life history stages. First, ovipositing females lay fewer eggs on hyperaccumulating plants. Second, hyperaccumulating plants contain levels of Ni that are far above the toxic dose for larvae. The result is a fitness benefit (shown by differential flower production) of >3-fold. While our study is the first to show a reproductive benefit of hyperaccumulation when plants are attacked by an unadapted herbivore, it will remain unclear if this defensive benefit is an adaptation or exaptation (Gould and Verba 1982; Boyd 2004) without an extensive phylogenetic study of hyperaccumulating plants and their herbivores.

Acknowledgements

The authors wish to thank Dr. John Odom for assistance with ICP analysis and Bill Wills for assisting with carbon and nitrogen analysis. Glucosinolate analysis was performed by POS Pilot Plant Corporation of Saskatoon, Canada. The authors express gratitude to Dr. Debbie Folkerts, Dr. Mark MacKenzie and two anonymous reviewers for critically reviewing this manuscript.

References

Babaoglu M., Gezgin S., Topal A., Sade B. and Dural H. 2004. *Gypsophila sphaerocephala* Fenzl ex Tchihat.: a boron hyperaccumulator plant species that may phytoremediate soils with toxic B levels. Turkish J. Bot. 28: 273–278.

Behmer S.T., Lloyd C.M., Raubenheimer D., Stewart-Clark J., Knight J., Leighton R.S., Harper F.A. and Smith J.A.C. 2005. Metal hyperaccumulation in plants: mechanisms of defence against insect herbivores. Funct. Ecol. 19: 55–66.

Berenbaum M.R. 1995. Turnabout is fair play: secondary roles for primary compounds. J. Chem. Ecol. 21: 925–940.

Berenbaum M.R. and Neal J.J. 1985. Synergism between myristicin and xanthotoxin, a naturally cooccurring plant toxicant. J. Chem. Ecol. 11: 1349–1358.

Bernays E.A. and Chapman R.F. 1987. Evolution of deterrent responses in phytophagous insects. In: Chapman R.F., Bernays E.A. and Stoffolano J.G. (eds.), Perspectives in Chemoreception and Behavior, Springer-Verlag, New York, pp. 159–174.

Boyd R.S. 1998. Hyperaccumulation as a plant defensive strategy. In: Brooks R.R. (ed.), Plants that Hyperaccumulate Heavy Metals, CAB International, Wallingford Oxon, pp. 181–201.

Boyd R.S. 2004. Ecology of metal hyperaccumulation. New Phytol. 162: 563–567.

Boyd R.S. and Martens S.N. 1992. The raison d'être for metal hyperaccumulation in plants. In: Baker A.J.M., Proctor J. and Reeves R.D. (eds.), The Vegetation of Ultramafic (Serpentine) Soils, Intercept, Andover, pp. 279–289.

Boyd R.S. and Martens S.N. 1994. Nickel hyperaccumulated by *Thlaspi montanum* var *montanum* is acutely toxic to an insect herbivore. Oikos 70: 21–25.

Boyd R.S. and Moar W.J. 1999. The defensive function of Ni in plants: response of the polyphagous herbivore *Spodoptera exigua* (Lepidoptera: Noctuidae) to hyperaccumulator and accumulator species of *Streptanthus* (Brassicaceae). Oecologia 118: 218–224.

Brooks R.R., Lee J., Reeves R.D. and Jaffré T. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. J. Geochem. Explor. 7: 49–57.

Davis M.A. and Boyd R.S. 2000. Dynamics of Ni-based defense and organic defences in the Ni-hyperaccumulator, *Streptanthus polygaloides* (Brassicaceae). New Phytol. 146: 211–217.

Dethier V.G. 1954. Evolution of feeding preferences in phytophagous insects. Evolution 8: 33–54.

Dyer L.A., Dodson C.D., Stireman J.O., Tobler M.A., Smilanich A.M., Fincher R.M. and Letourneau D.K. 2003. Synergistic effects of three piper amides on generalist and specialist herbivores. J. Chem. Ecol. 29: 2499–2514.

Ehrlich P.R. and Raven P.H. 1964. Butterflies and plants: a study in coevolution. Evolution 18: 586–608.

Erickson J.M. and Feeny P. 1974. Sinigrin: a chemical barrier to the black swallowtail butterfly, *Papilio polyxenes*. Ecology 55: 103–111.

Ernst W.H.O. 1990. Ecological aspects of sulfur metabolism. In: Rennenberg H., Brunold C., De Kok L.J. and Stulen I. (eds.), Sulfur Nutrition and Sulfur Assimilation in Higher Plants, SPB Academic Publishing, The Hague, pp. 131–144.

Feeny P. 1977. Defensive ecology of the cruciferae. Annl Mo Bot. Garden 64: 221–234.

Fraenkel G. 1959. The raison d'être of secondary plant substances. Science 121: 1466–1470.

Futuyma D.J. 1983. Evolutionary interactions among herbivorous insects and plants. In: Futuyma D.J. and Slatkin M. (eds.), Coevolution, Sinauer Associates Inc., Sunderland, pp. 207–231.

Gatehouse J.A. 2002. Plant resistance towards insect herbivores: a dynamic interaction. New Phytol. 156: 145–169.

Gould S.J. and Verba E.S. 1982. Exaptation – a missing term in the science of form. Paleobiology 8: 4–15.

Hanson B., Lindblom S.D., Loeffler M.L. and Pilon-Smits E.A.H. 2004. Selenium protects plants from phloem-feeding aphids due to both deterrence and toxicity. New Phytol. 162: 655–662.

Harborne J.B. 1993. Introduction to Ecological Biochemistry. Academic Press, London.

Harvey C.T. 2002. The effects of habitat complexity on biological control of herbivores and natural enemy interactions. M.S. Thesis, Auburn University.

Hay M.E., Kappel Q.E. and Fenical W. 1994. Synergisms in plant defenses against herbivores: interactions of chemistry, calcification, and plant quality. Ecology 75: 1714–1726.

Hopkin S.P. 1989. Ecophysiology of Metals in Terrestrial Invertebrates. Elsevier Science, New York.

Huang X. and Renwick J.A.A. 1994. Relative activities of glucosinolates as oviposition stimulants for *Pieris rapae* and *P. napi oleracea*. J. Chem. Ecol. 20: 1025–1037.

Huitson S.B. and Macnair M.R. 2003. Does zinc protect the zinc hyperaccumulator *Arabidopsis halleri* from herbivory by snails? New Phytol. 159: 453–460.

Janzen D.H. 1980. When is it coevolution? Evolution 34: 611–612.

Jiang R.F., Ma D.Y., Zhao F.J. and McGrath S.P. 2005. Cadmium hyperaccumulation protects *Thlaspi caerulescens* from leaf feeding damage by thrips (*Frankliniella occidentalis*). New Phytol. (in press).

Jensen S., Broadley M.R., Robbrecht E. and Smets E. 2002. Aluminum hyperaccumulation in angiosperms: A review of its phylogenetic significance. Bot. Rev. 68: 235–269.

Jhee E.M., Dandridge K.L., Christy A.M. and Pollard A.J. 1999. Selective herbivory on low-zinc phenotypes of the hyperaccumulator *Thlaspi caerulescens* (Brassicaceae). Chemoecology 9: 93–95.

Justus K.A., Dosdall L.M. and Mitchell B.K. 2000. Oviposition by *Plutella xylostella* (Lepidoptera: Plutellidae) and effects of phylloplane waxiness. J. Econ. Entomol. 93: 1152–1159.

Justus K.A. and Mitchell B.K. 1996. Oviposition site selection by the diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae). J. Insect Behav. 9: 887–898.

Kruckeberg A.R. 1984. California Serpentines: Flora, Vegetation, Geology, Soils and Management Problems. University of California Press, Berkeley.

Levin D.A. 1976. The chemical defenses of plants to pathogens and herbivores. Annl. Rev. Ecol. Sys. 7: 121–159.

Louda S. and Mole S. 1991. Glucosinolates: chemistry and ecology. In: Rosenthal G.A. and Berenbaum M.R. (eds.), Herbivores: Their Interactions with Secondary Plant Metabolites, Academic Press, San Diego, pp. 123–164.

Macnair M.R. 2003. The hyperaccumulation of metals by plants. Adv. Bot. Res. 40: 63–105.

Martens S.N. and Boyd R.S. 1994. The ecological significance of nickel hyperaccumulation: a plant chemical defense. Oecologia 98: 379–384.

Mauricio R. and Rausher M.D. 1997. Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. Evolution 51: 1435–1444.

McNaughton S.J. and Tarrants J.L. 1983. Grass leaf silicification: natural selection for an inducible defense against herbivores. Proc. Natl. Acad. Sci. USA 80: 790–791.

Noret N., Meerts P., Tolrà R., Poschenrieder C., Barceló J. and Escarre J. 2005. Palatability of *Thlaspi caerulescens* for snails: influence of zinc and glucosinolates. New Phytol. 165: 763–772.

Pollard A.J. 2000. Metal hyperaccumulation: a model system for coevolutionary studies. New Phytol. 146: 179–181.

Pollard A.J. and Baker A.J.M. 1997. Deterrence of herbivory by zinc hyperaccumulation in *Thlaspi caerulescens* (Brassicaceae). New Phytol. 135: 655–658.

Raffauf R.F. 1996. Plant Alkaloids: A Guide to Their Discovery and Distribution. Food Products Press, New York.

Rausher M.D. 1992. Natural selection and the evolution of plant–animal interactions. In: Roitberg B.D. and Isman M.S. (eds.), Insect Chemical Ecology: An Evolutionary Approach, Chapman and Hall, New York, pp. 20–88.

Reeves R.D., Brooks R.R. and Macfarlane R.M. 1981. Nickel uptake by Californian *Streptanthus* and *Caulanthus* with particular reference to the hyperaccumulator *S. polygaloides* Gray (Brassicaceae). Am. J. Bot. 68: 708–712.

Reeves R.D. 2003. Tropical hyperaccumulators of metals and their potential for phytoextraction. Plant Soil 249: 57–65.

Reeves R.D. and Baker A.J.M. 2000. Metal-accumulating plants. In: Raskin I. and Ensley B.D. (eds.), Phytoremediation of Toxic Metals, John Wiley, New York, pp. 193–229.

Rosenthal G.A. and Janzen D.H. 1979. Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

SAS Institute. 2005. JMP IN 5.1. Thomson – Brooks/Cole, Belmont.

Scott I.M., Puniani E., Durst T., Phelps D., Assabgul R.A., Sanchez-Vindas P., Poveda L., Philogene B.J.R. and Arnason J.T. 2002. Insecticidal activity of *Piper tuberculatum* Jacq. extracts: synergistic interaction of piper amides. Agri. Forest Entomol. 4: 137–144.

Seigler D.S. 1998. Plant Secondary Metabolism. Kluwer, Dordrecht.

Shapiro A.M. 1981a. Egg-mimics of *Streptanthus* (Cruciferae) deter oviposition by *Pieris sisymbrii* (Lepidoptera: Pieridae). Oecologia 48: 142–143.

Shapiro A.M. 1981b. The pierid red-egg syndrome. Am. Nat. 117: 276–294.

Shapiro A.M. 1981c. The pierid fauna of jewel flower at a mid-elevation Sierran locality. J. Lepidopterists' Soc. 35: 322–324.

Singer M.C. 1986. The definition and measurement of oviposition preference in plant-feeding insects. In: Miller J. and Miller T.A. (eds.), Plant-Insect Interactions, Springer-Verlag, Berlin, pp. 65–94.

Talekar N.S. and Shelton A.M. 1993. Biology, ecology, and management of the diamondback moth. Annl. Rev. Entomol. 38: 275–301.

Tolrà R.O., Poschenrieder C., Alonso R., Barceló D. and Barceló J. 2001. Influence of zinc hyperaccumulation on glucosinolates in *Thlaspi caerulescens*. New Phytol. 151: 621–626.

Twigg L.E. and King D.R. 1991. The impact of fluoroacetate-bearing vegetation on native Australian fauna: a review. Oikos 61: 412–430.

Wall M.A. and Boyd R.S. 2002. Nickel accumulation in serpentine arthropods from the Red Hills, California. Pan-Pacific Entomol. 78: 168–176.

Walling L.L. 2000. The myriad plant responses to herbivores. J. Plant Growth Regul. 19: 195–216.

Whittaker R.H. and Feeny P.P. 1971. Allelochemicals: Chemical interactions between species. Science 171: 757–770.

Zar J.H. 1996. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs.