Nickel hyperaccumulation as an elemental defense of *Streptanthus polygaloides* (Brassicaceae): influence of herbivore feeding mode

Edward M. Jhee[1], Robert S. Boyd[1] and Micky D. Eubanks[2]

[1]Department of Biological Sciences and [2]Department of Entomology and Plant Pathology, Auburn University, Auburn, AL 36849-5407, USA

Author for correspondence:
Robert S. Boyd
Tel: +1 334 844 1626
Fax: +1 334 844 1645
Email: boydrob@auburn.edu

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Summary

• No study of a single nickel (Ni) hyperaccumulator species has investigated the impact of hyperaccumulation on herbivores representing a variety of feeding modes.
• *Streptanthus polygaloides* plants were grown on high- or low-Ni soils and a series of no-choice and choice feeding experiments was conducted using eight arthropod herbivores. Herbivores used were two leaf-chewing folivores (the grasshopper *Melanoplus femurrubrum* and the lepidopteran *Evergestis rimosalis*), a dipteran rhizovore (the cabbage maggot *Delia radicum*), a xylem-feeder (the spittlebug *Philaenus spumarius*), two phloem-feeders (the aphid, *Lipaphis erysimi* and the spidermite *Trialeurodes vaporariorum*) and two cell-disruptors (the bug *Lygus lineolaris* and the whitefly *Tetranychus urticae*).
• Hyperaccumulated Ni significantly decreased survival of the leaf-chewers and rhizovore, and significantly reduced population growth of the whitefly cell-disruptor. However, vascular tissue-feeding insects were unaffected by hyperaccumulated Ni, as was the bug cell-disruptor.
• We conclude that Ni can defend against tissue-chewing herbivores but is ineffective against vascular tissue-feeding herbivores. The effects of Ni on cell-disruptors varies, as a result of either variation of insect Ni sensitivity or the location of Ni in *S. polygaloides* cells and tissues.

Key words: aphid, folivore, herbivory, hyperaccumulator, rhizovore, spidermite, spittlebug, *Streptanthus polygaloides*.

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Introduction

Hyperaccumulating plants are defined by the extremely high concentration of metals sequestered within their tissues (Brooks *et al.*, 1977). The threshold metal concentration used to define a hyperaccumulator depends on the particular metal sequestered. For nickel (Ni) hyperaccumulators, plants must contain 1000 μg g^{-1} dry mass or greater (Brooks *et al.*, 1977). At least 318 taxa hyperaccumulate Ni (Reeves & Baker, 2000).

Several functions of metal hyperaccumulation have been proposed (Boyd & Martens, 1992), including plant defense (Boyd & Martens, 1992; Boyd, 1998). A plant defense increases resistance against the attack of herbivores (natural enemies) (Levin, 1976; Mauricio & Rausher, 1997), where resistance is defined as a plant characteristic that reduces damage inflicted by an herbivore (Rausher, 1992). Some elements sequestered by plants and algae may have defensive functions. These include silicon (Si) (McNaughton & Tarrants, 1983), fluorine (F) (Twigg & King, 1991), calcium (Ca) (Hay *et al.*, 1994), cadmium (Cd) (Jiang *et al.*, 2005), Ni (Boyd & Martens, 1994), zinc (Zn) (Pollard & Baker, 1997; Jhee *et al.*, 1999; Behmer *et al.*, 2005) and selenium (Se) (Hanson *et al.*, 2003, 2004). Termed 'elemental' chemical defenses by Martens & Boyd (1994), these defenses differ from organic chemicals because they are elements taken up from soil and sequestered in tissues rather than being produced from photosynthate. Martens & Boyd (1994) also pointed out that, unlike many organic chemicals, toxic elements cannot be degraded into less

toxic components, thus eliminating one possible herbivore counterdefense tactic.

Plants are consumed by a diverse array of herbivores, and plant–herbivore interactions can be influenced by herbivore feeding mode (Strauss, 1991; Karban & Baldwin, 1997; Gavloski & Lamb, 2000). Studies that have compared damage caused by herbivores representing different feeding modes on the same plant species have often found differing impacts (e.g. Moran & Whitham, 1990; Strauss, 1991; Meyer, 1993; Gavloski & Lamb, 2000). For example, Moran & Whitham (1990) found plant biomass and seed set of *Chenopodium album* (Chenopodiaceae) were reduced by a leaf-gall forming aphid but unaffected by a root-feeding aphid. Similarly, Meyer (1993) found that the relative growth rate of *Solidago altissima* (Asteraceae) was decreased to the greatest extent by a xylem-feeding spittlebug, less by a leaf-chewing beetle and not at all by a phloem-feeding aphid. Defenses can also vary in their effectiveness against herbivores of different feeding modes. For example, Karban & Nagasaka (2004) found that defenses induced by damage to *Raphanus sativus* (Brassicaceae) increased plant resistance to leaf-chewing folivores (caterpillars) but decreased resistance to phloem-feeding aphids.

It is unlikely that elemental defenses will provide complete protection against all herbivores because some plant enemies circumvent every plant defense (Gatehouse, 2002; Karban & Agrawal, 2002). Therefore, studies that use herbivores representing various feeding modes are needed to establish the boundaries of elemental defenses. Most studies of defense by hyperaccumulated metals have used folivores and almost all have found defensive effects (e.g. Boyd & Martens, 1994; Pollard & Baker, 1997; Boyd & Moar, 1999; Jhee *et al.*, 1999; but see the exception of Huitson & Macnair, 2003). Only two studies have examined the defensive function of hyperaccumulation using nonfolivore herbivores, with contrasting results. Boyd & Martens (1999) examined the effect of hyperaccumulated Ni by *Streptanthus polygaloides* (Brassicaceae) on the pea aphid *Acyrthosiphon pisum* (Homoptera: Aphididae), and Hanson *et al.* (2004) studied the effect of hyperaccumulated Se on the green peach aphid *Myzus persicae* (Homoptera: Aphididae). Boyd & Martens (1999) reported that Ni hyperaccumulation was ineffective as a plant defense against the pea aphid, but Hanson *et al.* (2004) found that Se hyperaccumulation was toxic to the green peach aphid.

No study has addressed the effect of a metal hyperaccumulated by a single plant species on arthropods representing a variety of feeding modes. In this study we examined the potential defensive role of hyperaccumulated Ni by *S. polygaloides* on arthropods of diverse feeding modes including folivores, a rhizovore, xylem- and phloem-feeding insects, and two cell-disrupting arthropods. Our study included eight arthropod species representing four feeding modes. Folivores used were the red-legged grasshopper *Melanoplus femurrubrum* (Orthoptera: Acrididae) and the cross-striped cabbageworm *Evergestis rimosalis* (Lepidoptera: Pyralidae). We used the cabbage maggot (cabbage root fly) *Delia radicum* (Diptera: Anthomyiidae) as a rhizovore. Vascular tissue-feeding insects were represented by the xylem-feeding meadow spittlebug *Philaenus spumarius* (Homoptera: Cercopidae) and two phloem-feeding insects, the turnip aphid *Lipaphis erysimi* (Homoptera: Aphidae) and the greenhouse whitefly *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). The fourth feeding mode was cell disruption, represented by the tarnished plant bug *Lygus lineolaris* (Heteroptera: Miridae) and two-spotted spidermite *Tetranychus urticae* (Acarina: Tetranychidae). To date, no study of hyperaccumulator defense has examined the effect of metals on dipterans or mites, so our choices allowed us to examine examples from previously unexplored taxonomic groups. Our inclusion of xylem-feeding and rhizovore insects also enabled us to examine these feeding modes for the first time in a study of hyperaccumulator plant herbivory. None of these arthropods has been reported as feeding on *S. polygaloides* in its natural habitat (Wall & Boyd, 2002).

Our experimental design involved a two-step approach. We first tested each arthropod species for differential survival when fed either high- or low-Ni plant material. These no-choice experiments allowed us to investigate elemental defense by examining herbivore response (survival and population growth) to plant metal concentration. If a defensive effect of Ni was observed in a no-choice experiment, we proceeded to a choice experiment in which herbivores were presented with both high- and low-Ni plant material. Choice experiments allowed us to determine if deterrence or selective feeding occurred, resulting in decreased damage to high-Ni plants. Decreased damage to high-Ni plants relative to low-Ni plants may lead to differential plant fitness and in turn could result in evolution of resistance traits (defenses) within a plant population (Boyd, 2004).

Materials and Methods

Plant species

The nickel 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 hyperaccumulation of Ni from all herbarium specimens of the nine natural populations examined. Ni measurements in above-ground tissues from these specimens ranged from 1100 to 16 400 μg Ni g^{-1} dry mass, while root tissue Ni concentrations ranged from 2000 to 2460 μg Ni g^{-1} dry mass (Reeves *et al.*, 1981).

Seeds were collected from an ultramafic outcrop on the west shore of Pine Flat Reservoir, King's River, Fresno County, California, USA (Kruckeberg, 1984). Approximately 25–30 seeds were sown in 8 × 8 cm pots of glasshouse soil (ProMix from Premier Horticulture, Red Hill, PA, USA) amended with dried powdered $NiCl_2$ (Sigma, St. Louis, MO, USA) to

a Ni concentration of approx. 800 μg Ni g^{-1} dry mass to grow high-Ni plants. Approximately 25–30 seeds per pot were sown on unamended soil to grow low-Ni plants. Plants were grown for approx. 1.5 months in a glasshouse in Auburn, Lee County, Alabama, USA under a 16 h light : 8 h dark photoperiod and a 30°C day : 22°C night thermoperiod. Plants were watered twice daily. After 1 month, pots were thinned so that each pot contained six to eight plants of similar height and leaf morphological characteristics. Pots were thinned again to three or four plants at the beginning of experiments.

For each feeding experiment, 10 additional pots of both high-Ni and low-Ni plants were grown for 1.5 months in the glasshouse for Ni analysis. Above-ground biomass was harvested and dried in an oven at 60°C for 5 d. Biomass samples were combined into four replicates for each soil treatment to obtain adequate quantities for Ni analysis. However, for the rhizovore experiment, cabbage maggots were fed 4-cm-long roots that were harvested from plants grown for 2 months. All roots were used for cabbage maggot feeding trials and none was available for Ni analysis.

Nickel analysis of plant material

Dried plant samples grown for Ni analysis were ground with mortar and pestle and 0.5-g subsamples were wet-ash-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. The next day, tubes 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 of 1 M HCl was added to each tube and contents were transferred to 25-ml volumetric flasks. The contents of the volumetric flasks were brought to 25 ml by adding deionized water and transferred to 100-ml plastic storage bottles (Nalgene, Rochester, New York, NY, USA). Ni concentration was determined using an atomic absorption spectrophotometer (IL 251; Instrumentation Laboratory, Lexington, MA, USA).

Ni concentrations of high- and low-Ni plant samples were compared by *t*-tests using JMP IN 5.1 (SAS Institute, 2005). Differences in Ni concentration between high- and low-Ni plant samples were considered significant at $\alpha \leq 0.05$.

Leaf-chewing insects

Red-legged grasshopper The red-legged grasshopper *Melanoplus femurrubrum* De Geer (Orthoptera: Acrididae) occurs widely in North America and is considered a generalist folivore of herbaceous vegetation (Bailey & Mukerji, 1976). Grasshoppers were collected via sweep netting of pastures at the E. V. Smith Agricultural Research Station, Macon County, Alabama on 6 July 2002.

Individuals were randomly assigned to 60 × 30 × 30 cm steel wire mesh (40 cm^{-1}) cages until five grasshoppers were in each cage. Seven cages each received a single pot of high-Ni *S. polygaloides* and seven cages received a single pot of low-Ni plants. The experiment was conducted on laboratory benches outside of the Plant Science Research Center (PSRC), Auburn University, Lee County, Alabama. The average photoperiod was 16 h light : 8 h dark and the average thermoperiod was 32°C day : 22°C night for the experimental period (6 July to 5 August 2002).

Fresh pots of *S. polygaloides* were added to the cages on days 4, 12, 16, 18, 21, 23, 25 and 30 to allow grasshoppers to feed *ad libitum*. Old pots of plants were removed when fresh plants were added and cages were checked for number of grasshoppers alive when new plant material was provided. Grasshopper survival was analyzed by repeated measures analysis of variance (ANOVA) using JMP IN 5.1 (SAS Institute, 2005).

Because the no-choice experiment showed significantly decreased survival of grasshoppers fed high-Ni plants (see the Results section), a choice experiment was conducted to determine whether grasshoppers would selectively feed on high- or low-Ni plants. Initial plant height was measured from soil level to the apical meristem of each plant in order to standardize the effect height may have on grasshopper feeding. Differences in plant height between high- and low-Ni plants were analyzed using JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test at $\alpha \leq 0.05$.

Grasshoppers were randomly assigned to 60 × 30 × 30 cm steel wire mesh cages (40 cm^{-1}) until five grasshoppers were in each cage. Pairs of *S. polygaloides* pots (one high- and one low-Ni) were enclosed as the food source in each cage. A total of 20 experimental cages were used. Ten cages contained grasshoppers and 10 cages lacked grasshoppers to compare high- and low-Ni soil treatments on plants with and without grasshopper feeding. This experiment was conducted on laboratory benches outside of the PSRC. Plants were watered twice daily. The average photoperiod was 16 h light : 8 h dark and the average thermoperiod was 32°C day : 22°C night for the experimental period (7–18 August 2002).

Grasshoppers were allowed to feed for 10 d and pots were removed from cages to measure final plant height. Change in plant height (cm) was analyzed with StatView 5.0 (SAS Institute, 1998) using a two-way ANOVA to determine whether grasshopper herbivory and/or plant Ni concentration affected change in plant height (including the interaction term). Data were arcsine square root transformed before ANOVA to better satisfy the assumptions of ANOVA (Zar, 1996). Post hoc mean separations were performed using Fisher's protected least significant difference (PLSD) test (SAS Institute, 1998).

Cross-striped cabbageworm The cross-stripped cabbageworm *Evergestis rimosalis* Guenee (Lepidoptera: Pyralidae) is an oligophagous pest of plants belonging to the Brassicaceae (Mays & Kok, 1997). It is considered an opportunistic invader of commercial crops, becoming abundant after more common Lepidopteran pests such as *Pieris rapae* (Lepidoptera: Pieridae), *Trichoplusia ni* (Lepidoptera: Noctuidae) and *Plutella xylostella*

(Lepidoptera: Plutellidae) are reduced or eliminated by insecticide application (Mays & Kok, 1997).

Wild neonate (first instar) larvae were collected from *Brassica rapa* (Brassicaceae) and *Raphanus sativus* (Brassicaceae) growing in 20-cm-diameter pots placed outdoors adjacent to the PSRC during July 2002. Foliar feeding trials were conducted in a laboratory at 24°C under 24-h illumination by fluorescent lighting (General Electric Sunshine Full Spectrum, 5000 °K). A single larva was placed into each of 40 plastic Petri dishes (5-cm-diameter) lined with filter paper (Whatman #40), wetted with water to increase humidity. Twenty of the Petri dishes were randomly selected to receive high-Ni *S. polygaloides* leaves and the remainder received leaves from low-Ni plants. Fresh mature leaves were provided every 1–2 d in sufficient quantity for larvae to feed *ad libitum*. Leftover material was removed when fresh leaves were added. Larval survival was recorded every 2 d for 10 d, at which point the experiment was terminated because all larvae had died. Survival data were analyzed with JMP IN 5.1 (SAS Institute, 2005) by repeated measures ANOVA to determine if plant Ni concentration significantly affected survival.

Because high-Ni leaves significantly reduced larval survival in the no-choice experiment (see the Results section), a follow-up choice experiment determined if larvae would selectively feed on low-Ni leaves when presented with both high- and low-Ni leaves. First instar cross-striped cabbageworm larvae were collected from the above-mentioned *B. rapa* and *R. sativus* plants placed adjacent to the PSRC. Feeding trials were conducted in a laboratory at 24°C under 24-h illumination by fluorescent lighting (General Electric Sunshine Full Spectrum, 5000 °K). A single larva was placed into each of 20 plastic Petri dishes (5-cm-diameter) lined with moistened filter paper (Whatman #40). Each Petri dish received one high-Ni *S. polygaloides* leaf and one low-Ni leaf, matched in size by length (measured from petiole to leaf apex). High- and low-Ni leaves were morphologically similar so that, after leaves were placed into a dish, the Ni concentration in each leaf was unknown to us unless tested for hyperaccumulated Ni using dimethylglyoxime (DMG) paper (filter paper impregnated with DMG). DMG paper has been used as a colorimetric assay for hyperaccumulated Ni in plant tissues (Reeves *et al.*, 1999). Thus, this was a blind experiment, where experimental bias was removed because leaf type (high- or low-Ni) was not known until after testing leaf samples with DMG paper.

Petri dishes were examined daily for the location of each caterpillar on the two leaves. Larval choice data were recorded for each insect that was directly resting on, or feeding on, one of the leaves in each dish. After recording larval choice, both leaves within a dish were tested for Ni concentration by squeezing each leaf onto DMG paper. Fresh pairs of leaves were then placed in the Petri dish. Observations were also made on the presence or absence of chewing damage to leaves. The 40 Petri dishes were checked daily for position of larvae over a 10-d period.

Choice experiment data for caterpillar location were analyzed using contingency table analysis with STATVIEW 5.0 (SAS Institute, 1998). This analysis determined if larvae preferred high-Ni or low-Ni leaves on each day of the experiment by comparing larval location data against a random (50 : 50) expectation.

Rhizovore

We used the cabbage maggot *Delia radicum* L. (Diptera: Anthomyiidae) to represent root-feeding insects in our study of elemental defense. Cabbage maggots are oligophagous agricultural pests of members of the Brassicaceae (Ellis & Hardman, 1975; Ellis *et al.*, 1999). Feeding of cabbage maggot larvae within the root system of plants can weaken the plants and lead to eventual plant death (Fournet *et al.*, 2000).

Eggs were obtained from a laboratory colony (Cornell University, New York State Agricultural Experiment Station, Geneva, New York, NY, USA) used for research on agricultural crops (Jyoti *et al.*, 2001). We conducted no-choice root-feeding trials (28 high-Ni and 28 low-Ni) in a laboratory at 24°C. Experiments were conducted under total darkness within a laboratory bench drawer to simulate natural conditions for these root-feeding larvae. Plastic Petri dishes (5-cm-diameter) were lined with filter paper (Whatman #40) moistened with deionized water. Three to five 2-month-old *S. polygaloides* tap roots, removed from plants and rinsed with deionized water, were cut to a length of 4 cm and placed into each dish.

Fifty cabbage maggot eggs were counted under a dissecting microscope and placed onto the *S. polygaloides* roots in each dish using a fine artist's paintbrush. Dishes were sealed with Parafilm (Modern Biology Inc., West Lafayette, IN, USA) to prevent drying. Eggs were allowed to hatch and larvae to feed. After 7 d, we examined roots under a dissecting microscope with a dissecting needle for number of larvae. Numbers of larvae in each root type were analyzed with JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test to determine whether more larvae survived on high-Ni or on low-Ni roots (at $\alpha = 0.05$).

A root choice feeding experiment was conducted after we observed significantly greater cabbage maggot survival on low-Ni roots than on high-Ni roots (see the Results section). We conducted this trial (40 replicates) in a laboratory bench drawer at 24°C under total darkness. Petri dishes were lined with filter paper (Whatman #40) moistened with deionized water. One mature, 2-month-old high-Ni *S. polygaloides* root (4 cm long) was rinsed with deionized water and placed into a 5-cm-diameter Petri dish, along with a rinsed low-Ni root section of similar size. Roots were placed adjacent to each other along their lengths so that they touched, thereby allowing movement of cabbage maggot larvae between root types. Fifty cabbage maggot eggs were counted under a dissecting microscope and placed onto the junction between the two root pieces using a fine artist's paintbrush. Petri dishes were sealed with Parafilm to prevent drying.

This choice feeding trial used a blind experimental design. Because high- and low-Ni roots were morphologically indistinguishable after roots were placed into Petri dishes, we did not know which root contained hyperaccumulated Ni. After allowing the eggs to hatch and larvae to feed for 7 d, roots were removed and examined under a dissecting microscope with a dissecting needle. After the first root in each Petri dish was examined for larvae, it was pressed against DMG paper to determine whether the root contained hyperaccumulated Ni. On the basis of this result, the treatment of the other root was revealed and recorded, and it was examined for number of larvae. Results were analyzed with JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test to compare numbers of cabbage maggots in high-Ni and low-Ni roots at $\alpha = 0.05$.

Vascular tissue-feeding arthropods (xylem or phloem)

Meadow spittlebug The meadow spittlebug *Philaenus spumarius* L. (Homoptera: Cercopidae) feeds on xylem sap of many plant species (Wiegert, 1964). We selected this insect for a no-choice experiment because initial observations showed that it accepted *S. polygaloides* as a food source. Nymphs for this experiment were collected from spittle-bearing stems of *Rubus* sp. growing in a field in Auburn, Alabama on 18 May 2003.

Pairs of pots of high- and low-Ni *S. polygaloides* were matched according to plant height in order to control for plant size. The height of each plant was measured from the base of the stem to the apical meristem, and height data were analyzed with a Wilcoxon two-sample test using JMP IN 5.1 (SAS Institute, 2005) to ensure equivalent plant height between treatments. Nymphs were assigned to plants by selecting two nymphs, randomly assigning one nymph from a pair to a high-Ni plant within a pot, and placing the other nymph on a low-Ni plant in the corresponding paired pot. This process was repeated until all 62 collected nymphs were placed on plants within 16 pots (eight high-Ni and eight low-Ni pots; three or four nymphs per pot). This experiment was conducted in a laboratory at 24°C and with 24-h illumination by fluorescent plant grow lights (General Electric Sunshine Full Spectrum, 5000 °K). Pots of plants were spaced approx. 10 cm apart under the grow lamps to prevent movement of nymphs from one pot to another. Plants were watered daily with tap water, using a squeeze bottle to direct water at the soil so feeding nymphs would not be disturbed.

The numbers of nymphs alive in each pot were counted daily for 9 d. Survival of nymphs was compared between high- and low-Ni plants to determine if plant metal concentration significantly affected survival using repeated measures ANOVA with JMP IN 5.1 (SAS Institute, 2005). A choice experiment was not conducted for spittlebugs because plant Ni concentration did not significantly affect spittlebug survival (see the Results section).

Greenhouse whitefly The phloem-feeding greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Homoptera: Aleyrodidae) was chosen for this experiment because it accepted *S. polygaloides* as a host plant. The greenhouse whitefly is a polyphagous agricultural pest of economically important crops, such as cotton, and many species of the Brassicaceae (Byrne & Bellows, 1991). These insects are of economic importance because their honeydew secretions can serve as a medium for sooty mold fungi (*Capnodium* spp.) and can discolor parts of plants used for food and fiber (Byrne & Bellows, 1991). Whiteflies can also serve as vectors of economically important plant pathogens (Byrne & Bellows, 1991). Adult whiteflies were captured in a glasshouse used for cotton research at Auburn University using an aspirator. Twenty adult flies were captured into each of 20 plastic vials.

Pots of high- and low-Ni *S. polygaloides* were thinned to three remaining plants of roughly equivalent height and number of leaves. Plant height was recorded at the beginning of the experiment to match equivalent pairs of high- and low-Ni pots and control for effects of plant size on whitefly population growth. Plant height was recorded at day 9 and also was measured at the conclusion of the experiment to determine if whitefly nymph feeding affected growth rates of *S. polygaloides*. Plant height at the beginning of the experiment was analyzed with a Wilcoxon two-sample test using JMP IN 5.1 (SAS Institute, 2005) to ensure equivalent plant height between plant treatments. Change in plant height (cm) was analyzed for significant differences between high- and low-Ni plants with JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test at $\alpha = 0.05$.

Pairs of high- and low-Ni *S. polygaloides* pots, paired according to plant size, were placed in 1.5-l plastic containers that were used as cages. We modified the lids by cutting an 8-cm-diameter hole and gluing standard mosquito netting (Recreational Equipment Inc., Sumner, WA, USA) over it. The container itself was modified by cutting out 8-cm-diameter holes on all four sides and covering the holes with mosquito netting for air flow. Also, another hole was cut and covered with netting on the bottom of the container to allow for water drainage.

Experiments were conducted within a glasshouse at the PSRC under a 16 h light : 8 h dark photoperiod and a 30°C day : 22°C night thermoperiod. Plants were watered twice daily. Each vial of adult whiteflies was randomly assigned to a cage and the whiteflies in the vial released into the cage. Adults were allowed to oviposit for 5 d, after which they were removed with an aspirator and one plant from each cage was randomly removed by cutting it at the soil surface. Plant height was recorded and eggs on the leaves and stem were counted using a dissecting microscope. Nine days after adult removal, a second plant from each pot was randomly selected and removed from each cage. Plant height was measured and the numbers of second instar nymphs present on these plants were counted using a dissecting microscope. Seventeen days after adult removal, pupae on the remaining plant in each pot were counted using a dissecting microscope and the plant height was measured.

Numbers of eggs, nymphs and pupae were each divided by plant height at time of data collection to standardize counts to plant size. Data were analyzed with JMP IN 5.1 (SAS Institute, 2005) using Wilcoxon two-sample tests to compare the effect of plant Ni concentration on eggs cm^{-1} stem, nymphs cm^{-1} stem and pupae cm^{-1} stem at $\alpha = 0.05$. A choice experiment was not conducted because plant Ni concentration did not significantly affect whitefly population growth (see the Results section).

Turnip aphid The turnip aphid *Lipaphis erysimi* Kaltenbach (Homoptera: Aphidae) is an oligophagous pest of commercial members of the Brassicaceae (Dixon, 1998). Wild turnip aphids were collected in July 2002 from plants in four 20-cm-diameter pots of *B. rapa* and *R. sativus* growing outdoors adjacent to the PSRC. Aphids were collected by clipping colonized leaves off of host plants.

Experiments were conducted within a glasshouse at the PSRC under a 16 h light : 8 h dark photoperiod and a 30°C day : 22°C night thermoperiod. Plants were watered daily. Plant height was recorded at the beginning and the conclusion of the experiment to determine if turnip aphid feeding affected growth of *S. polygaloides*. Plant height at the beginning of the experiment was analyzed at $\alpha = 0.05$ with a Wilcoxon two-sample test using JMP IN 5.1 (SAS Institute, 2005) to ensure equivalent plant height between plant treatments. Change in plant height of high- and low-Ni plants also was analyzed using a Wilcoxon two-sample test at the conclusion of the experiment to determine if aphid feeding affected growth ($\alpha = 0.05$).

Ten high- and 10 low-Ni *S. polygaloides* plants were each placed into 1.5-l plastic cages (see the whitefly no-choice experiment for cage description). Fifty adult aphids were placed in each cage and allowed to feed and reproduce. After 14 d, aphids were counted on each plant and the mean number of aphids was calculated for each cage. Differences in mean number of aphids per cm of stem (calculated as total number of aphids divided by total height of all plants in each pot) for high- and low-Ni plants were analyzed with JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test at $\alpha \leq 0.05$.

Cell-disrupting arthropods

Tarnished plant bug The tarnished plant bug *Lygus lineolaris* Palisot de Beauvois (Heteroptera: Miridae) was chosen because it accepted *S. polygaloides* as a host. It is a common agricultural pest that attacks many food and fiber plants (Snodgrass, 1986; Young, 1986). Tarnished plant bugs feed by lacerating plant tissue with their stylets, secreting digestive enzymes and withdrawing liquefied plant material (Tingey & Pillemer, 1977). Tarnished plant bugs can severely damage plants because of the high volume of liquefied plant tissue ingested and because they feed on all plant parts including leaves, meristems, and seed pods (Tingey & Pillemer, 1977). Tarnished plant bug nymphs were obtained from a laboratory colony (Southeastern Insect Management Research Unit, USDA, Stoneville, MS, USA) used in agricultural research (Snodgrass & Scott, 2002).

Plant height was recorded at the beginning of the experiment and at its conclusion. Initial plant height was measured to match pairs of pots and thus control any influence of plant size on tarnished plant bug survival. Plant height was also measured at the conclusion of the experiment to determine whether tarnished plant bugs differentially affected the growth rate of high- or low-Ni *S. polygaloides*. To determine whether tarnished plant bugs influenced plant growth, change in plant height was analyzed for high- and low-Ni plants using JMP IN 5.1 (SAS Institute, 2005) with a Wilcoxon two-sample test ($\alpha \leq 0.05$).

Feeding experiments were conducted within a glasshouse in Auburn, Lee County, Alabama under a 16 h day : 8 h night photoperiod and a 30°C day : 22°C night thermoperiod. Plants were watered twice daily. A single pot of high- and low-Ni plants was placed into 20 1.5-l plastic cages (see the whitefly no-choice experiment for cage description). Nymphs were assigned to plants by selecting two nymphs, randomly assigning one nymph from a pair to a high-Ni plant within a pot, and placing the other nymph onto a low-Ni plant in the corresponding paired pot. This process was repeated until 200 nymphs were placed on plants within 20 pots (10 high-Ni and 10 low-Ni pots; 10 nymphs per pot). Numbers of live insects were counted on day 7 and day 14. Survival data were compared between bugs feeding on high- and low-Ni plants to determine if survival was significantly affected by plant metal concentration. Data were analyzed with repeated measures ANOVA using JMP IN 5.1 (SAS Institute, 2005). Soil treatment, time and interaction significance were determined at $\alpha \leq 0.05$.

Two-spotted spidermite The two-spotted spidermite *Tetranychus urticae* K. (Acari: Tetranychidae) was chosen because it accepted *S. polygaloides* as a host. It is an agricultural pest of a wide variety of food and fiber crops, causing severe injury to plants characterized by a 'burning' of leaves or their abscission (van de Vrie *et al.*, 1972). Spidermites feed primarily on the underside of leaves and can damage mesophyll and bundle sheath cells (van de Vrie *et al.*, 1972). Adult *T. urticae* were obtained from a laboratory colony (Department of Entomology, Kansas State University, Manhattan, KS, USA) used for agricultural research (Li & Margolies, 1993).

Pots of *S. polygaloides* were thinned to three remaining plants of roughly equivalent height. Plant height was recorded at the beginning of the experiment. Each pot was placed on top of a 120-ml plastic cup (Solo Cup Co., Urbana, IL, USA), which was placed within an upright 2-cm-deep and 20-cm-diameter saucer. The upright saucer was filled with water to serve as a moat to prevent spidermites from escaping from each pot (D. Margolies, Kansas State University, pers. comm.). The experiment was conducted at room temperature (24°C) and under 24-h illumination (General Electric Sunshine Full Spectrum, 5000 °K). Air conditioning in the room was turned

off so that air circulation in the room was minimized. This prevented spidermite movement between plants via silking, by which spidermites may disperse in an aerial manner (Smitley & Kennedy, 1985; Li & Margolies, 1993). Approximately 20 adult females and 20 adult males were placed onto each plant using a fine artist's paintbrush. On day 23 after application, spidermites were counted on each plant using a dissecting microscope and the mean number of mites per cm initial plant height was calculated for each pot.

Initial plant height was analyzed for significant differences between high- and low-Ni plants with JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test. Change in plant height was also recorded at the conclusion of the experiment to compare growth rates of high- and low-Ni *S. polygaloides*. Spidermite numbers, expressed per initial cm of stem, were analyzed with JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test at $\alpha \leq 0.05$ to determine whether plant metal concentration affected population size of spidermites.

A choice feeding experiment was conducted because spidermite population size was significantly greater on low-Ni plants than on high-Ni plants in the no-choice experiment (see the Results section). Twelve high-Ni and 12 low-Ni pots of *S. polygaloides* were thinned to three plants per pot and plant height was recorded. Pairs of high- and low-Ni pots of *S. polygaloides* were placed on top of 120-ml plastic cups (Solo Cup Co.), which in turn were placed within upright 2-cm-deep, 20-cm-diameter saucers. The upright saucers were filled with water to serve as moats to prevent spidermites escaping from pots. Pairs of pots were adjacent to each other and plants were interwoven so at least one plant from each pot overlapped a plant in the adjacent pot. This allowed free movement of spidermites from plants of one Ni concentration to the other. The experiment was conducted at room temperature (24°C) and with 24-h illumination (General Electric Sunshine Full Spectrum, 5000 °K), and air conditioning in the room was turned off to prevent spidermite silking.

Fifty adult *T. urticae* were placed on a 2×4 cm piece of soybean leaf, which was then placed on top of the interwoven portions of *S. polygaloides* plants in each pair of pots. As the piece of soybean leaf dried, spidermites moved to either high- or low-Ni plants. On day 19 after spidermite application, pots of plants were gently separated and spidermites were counted on each plant type using a dissecting microscope.

The difference in initial plant height was analyzed between high- and low-Ni plants with JMP IN 5.1 (SAS Institute, 2005) using a Wilcoxon two-sample test. Plant height was also recorded at the conclusion of the experiment so that a Wilcoxon two-sample test could compare growth rates of high- and low-Ni *S. polygaloides*. The mean number of spidermites in each pot was divided by the mean initial height of all plants within a pot. Numbers of spidermites per initial cm of stem were analyzed to determine whether plant Ni concentration influenced spidermite host choice (Wilcoxon two-sample test, $\alpha \leq 0.05$).

Results

Elevated soil Ni resulted in significantly elevated Ni concentration (above the 1000 g Ni g^{-1} threshold for hyperaccumulator status) for *S. polygaloides* above-ground biomass for all experiments (Table 1). High-Ni plants had approx. 150 times the Ni concentration of low-Ni plants.

Leaf-chewing insects

Red-legged grasshopper High-Ni plants significantly affected grasshopper survival in the no-choice experiment. Repeated measures ANOVA yielded significant treatment (plant Ni status:

Table 1 Foliar nickel (Ni) concentrations of *Streptanthus polygaloides* grown on high-Ni or low-Ni soils

		Foliar Ni concentration (μg Ni g^{-1} dry mass)		
Herbivore	Experiment	High-Ni	Low-Ni	*t*-value
Red-legged grasshopper (*Melanoplus femurrubrum*)	Choice	7340 (151)	71.0 (151)	34.0
	No-choice	6290 (162)	22.8 (162)	27.4
Cross-striped cabbageworm (*Evergestis rimosalis*)	Choice	6830 (190)	24.5 (190)	25.3
	No-choice	6830 (190)	24.5 (190)	25.3
Meadow spittlebug (*Philaenus spumarius*)	No-choice	7390 (151)	15.6 (150)	34.5
Turnip aphid (*Lipaphis erysimi*)	No-choice	6830 (190)	24.5 (190)	25.3
Greenhouse whitefly (*Trialeurodes vaporariorum*)	No-choice	5430 (203)	76.5 (203)	18.6
Tarnished plant bug (*Lygus lineolaris*)	No-choice	5270 (204)	30.5 (204)	18.2
Two-spotted spidermite (*Tetranychus urticae*)	Choice	1820 (12.8)	18.8 (12.8)	98.8
	No-choice	7960 (249)	70.1 (249)	22.5

For all experiments, values are mean (standard error); $n = 4$. All *t*-values are significant at $P < 0.0001$.

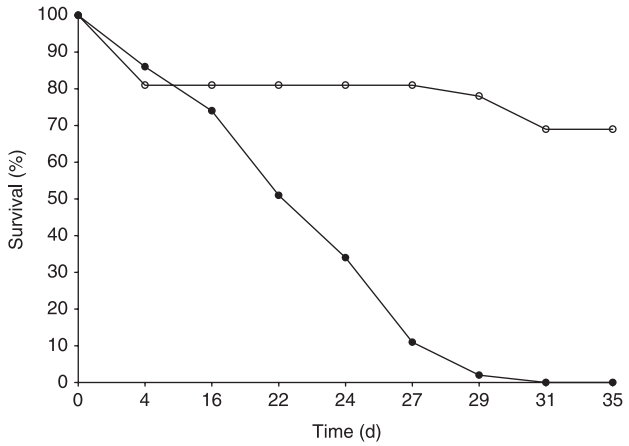

Fig. 1 Survival of red-legged grasshoppers (*Melanoplus femurrubrum*) fed either high-nickel (filled circles) or low-nickel (open circles) leaves of *Streptanthus polygaloides* during a no-choice experiment.

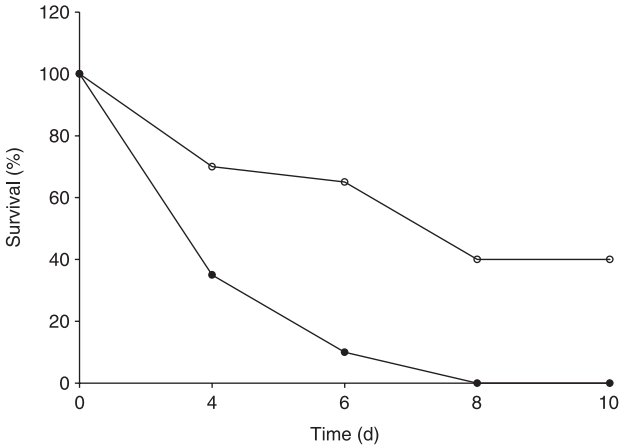

Fig. 2 Survival of cross-striped cabbageworm (*Evergestis rimosalis*) larvae fed high-nickel (filled circles) or low-nickel (open circles) leaves of *Streptanthus polygaloides* during a no-choice experiment.

$F_{1,12}$ = 11.7, P < 0.01), time ($F_{1,12}$ = 15.3, P < 0.01) and interaction ($F_{1,12}$ = 6.46, P < 0.01) terms for grasshopper survival. By day 29 all grasshoppers fed high-Ni leaves had died (Fig. 1), whereas 78% of those fed low-Ni leaves were still alive. At the conclusion of the experiment (day 35) 69% of grasshoppers fed low-Ni leaves survived.

Because the no-choice experiment revealed significantly greater survival of grasshoppers fed low-Ni *S. polygaloides*, we conducted a choice experiment to determine whether grasshoppers would selectively feed on high- or low-Ni plants. Two-way ANOVA yielded significant herbivore ($F_{1,46}$ = 95, P < 0.001), Ni ($F_{1,46}$ = 8.0, P < 0.01) and interaction ($F_{1,46}$ = 20, P < 0.001) terms for change in plant height. Grasshoppers significantly decreased the height of experimental plants compared with plants without grasshoppers (Fisher's PLSD, P < 0.001). When exposed to grasshopper herbivory, high-Ni plants grew 1.2 ± 0.21 cm [mean ± standard error (SE), n = 15] whereas low-Ni plants lost 0.70 ± 0.21 cm of height. Two-way ANOVA also yielded significant herbivore ($F_{1,46}$ = 360, P < 0.001), Ni ($F_{1,46}$ = 60, P < 0.01) and interaction ($F_{1,46}$ = 70, P < 0.001) terms for change in number of leaves. Grasshoppers fed high- and low-Ni plants significantly decreased the leaf number of experimental plants compared with plants without grasshoppers (Fisher's PLSD, P < 0.001). When exposed to grasshopper herbivory, high-Ni plants lost only 1.1 ± 0.46 leaves (mean ± SE) whereas low-Ni plants had 6.5 ± 0.46 leaves eaten.

Cross-striped cabbageworm Foliar Ni concentration significantly affected cross-striped cabbageworm survival. Repeated measures ANOVA showed significant treatment ($F_{1,38}$ = 9.1, P < 0.01), time ($F_{1,38}$ = 160, P < 0.0001) and interaction ($F_{1,38}$ = 2.9, P < 0.05) terms for caterpillar survival. Survival of larvae was greater for those feeding on low-Ni leaves than for those feeding on high-Ni leaves for each day of the experiment. No larvae feeding on high-Ni leaves were alive by day 8, whereas 40% of those feeding on low-Ni plants were still alive (Fig. 2).

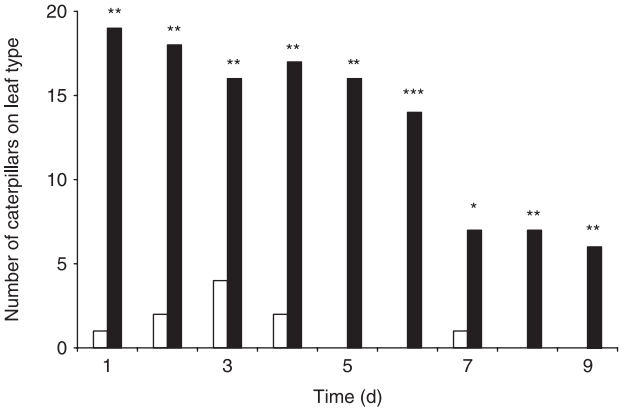

Fig. 3 Cross-striped cabbageworm (*Evergestis rimosalis*) choice of high-nickel (open bars) or low-nickel (filled bars) *Streptanthus polygaloides* leaves. Heights of bars decrease as the experiment progresses because of caterpillar mortality. Based on contingency table analysis: *, P < 0.01; **, P < 0.001; ***, P < 0.0001.

The follow-up choice experiment showed that larvae preferred low-Ni *S. polygaloides* leaves, choosing low-Ni leaves significantly more frequently than high-Ni leaves on each day (Fig. 3). On many days (days 5, 6, 8, 9, 10 and 11) no larvae were found on high-Ni leaves. Although a few caterpillars were resting on high-Ni leaves on some days (days 1, 2, 3, 4 and 7; Fig. 3), we observed no feeding damage to high-Ni leaves at any time. In contrast, feeding damage was seen on at least some low-Ni leaves on each day of the experiment.

Rhizovore

Although 50 cabbage maggot eggs were placed into each Petri dish, few larvae were produced by day 7. All larvae, however,

Table 2 Plant height analyses for no-choice experiments using vascular tissue-feeding and cell-disrupting herbivores

Insect	Response variable (cm)	High-Ni	Low-Ni	*Z*-score	*P*
Vascular tissue-feeding					
Meadow spittlebug (*n* = 16)	Initial height	12.7 (0.32)	12.8 (0.38)	0.474	0.636
(*Philaenus spumarius*)	Change in height	11.1 (0.89)	9.41 (0.69)	1.42	0.156
Greenhouse whitefly (*n* = 20)	Initial height	3.30 (0.17)	3.27 (0.25)	0.342	0.732
(*Trialeurodes vaporariorum*)	Change in height	16.6 (0.68)	16.8 (0.95)	0.644	0.519
Turnip aphid (*n* = 20)	Initial height	7.74 (0.26)	8.20 (0.17)	1.35	0.177
(*Lipaphis erysimi*)	Change in height	8.91 (0.31)	8.75 (0.55)	0.267	0.790
Cell-disrupting					
Tarnished plant bug (*n* = 20)	Initial height	3.45 (0.14)	3.38 (0.12)	0.231	0.817
(*Lygus lineolaris*)	Change in height	17.0 (1.6)	18.0 (0.48)	0.038	0.970
Two-spotted spidermite (*n* = 24)	Initial height	8.81 (0.48)	8.76 (0.59)	0.114	0.909
(*Tetranychus urticae*)	Change in height	6.17 (0.38)	6.41 (0.52)	0.664	0.507

For all experiments, values are mean (standard error).

were found in low-Ni roots. There were 1.0 ± 1.6 (mean ± SE, n = 28) larvae in low-Ni roots and none in high-Ni roots. This difference was statistically significant (Wilcoxon two-sample test: Z = 4.4, P < 0.001).

The follow-up choice experiment determined whether cabbage maggots would preferentially choose high- or low-Ni roots of *S. polygaloides*. After 7 d, no larvae were found in high-Ni roots whereas low-Ni roots had 0.95 ± 0.83 (mean ± SE, n = 20) larvae. This difference in larval choice was significant, based on a Wilcoxon two-sample test (Z = 4.5, P < 0.001).

Vascular tissue-feeding arthropods (xylem or phloem)

No significant difference in height existed between high- and low-Ni plants at the beginning of each vascular-feeding insect experiment (Table 2). Change in plant height was measured at the conclusion of each vascular tissue-feeding insect experiment to compare plant growth. We found no significant difference in growth between high- and low-Ni plants for all three experiments (Table 2), indicating that there was no differential effect of the insects on high- or low-Ni plants.

Meadow spittlebug Elevated Ni concentration in *S. polygaloides* had no effect on spittlebug survival. Survival was about 50% on both high- and low-Ni plants by day 6 and declined to about 40% by day 9 (Fig. 4). Repeated measures ANOVA showed a significant effect of time ($F_{1,14}$ = 25, P < 0.001) but no significant difference attributable to treatment (plant Ni concentration: $F_{1,14}$ = 2.7, P = 0.12). The interaction term also was not significant ($F_{1,14}$ = 3.3, P = 0.09).

Greenhouse whitefly Hyperaccumulated Ni did not affect greenhouse whitefly population growth. Plant Ni concentration did not affect number of eggs laid, number of nymphs or number of pupae on high- or low-Ni *S. polygaloides* (Table 3).

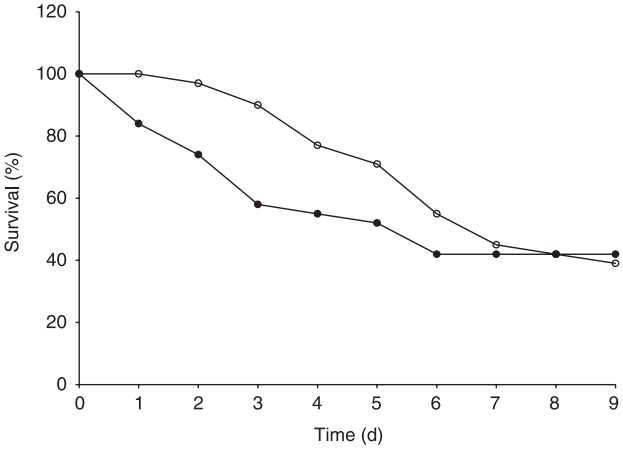

Fig. 4 Survival of meadow spittlebug (*Philaenus spumarius*) nymphs fed high-nickel (filled circles) or low-nickel (open circles) leaves of *Streptanthus polygaloides* during a no-choice experiment.

Table 3 Effect of plant nickel (Ni) concentration on greenhouse whitefly (*Trialeurodes vaporariorum*) population growth

Response variable	High-Ni	Low-Ni	*Z*-score	*P*
Eggs cm^{-1}	31.7 ± 4.54	35.0 ± 5.53	0.415	0.678
Nymphs cm^{-1}	15.4 ± 1.54	15.2 ± 1.05	0.113	0.909
Pupae cm^{-1}	6.10 ± 0.387	5.52 ± 0.455	1.10	0.273

Response variables are calculated as response variable scaled to initial plant height (cm). Values are mean ± standard error.

Turnip aphid Turnip aphids also were unaffected by elevated Ni in *S. polygaloides*. A Wilcoxon two-sample test showed no significant difference between the numbers of aphids per cm of stem on high- and low-Ni *S. polygaloides* (Z = 1.1, P = 0.27)

after 14 d. At this time, high-Ni pots had 22 ± 28 (mean ± SE, n = 10) aphids per cm of stem, while low-Ni plants had 8.2 ± 13 aphids per cm of stem.

Cell-disrupting arthropods

Plant height was not significantly different between high- and low-Ni plants before exposure to herbivores (Table 2). Change in plant height was measured at the conclusion of each vascular tissue-feeding experiment to compare herbivore impact upon plant growth. We found no significant difference in growth for high- and low-Ni plants in the two cell-disruptor feeding mode experiments as well as the spider mite experiment (Table 2).

Tarnished plant bug Hyperaccumulated Ni did not affect tarnished plant bugs. Survival declined quickly over the 2 wk of the experiment but was similar for insects feeding on high- and low-Ni plants (Fig. 5). Repeated measures ANOVA showed that time was significant ($F_{1,18}$ = 98, P < 0.001) but there was no effect of treatment (plant Ni status: $F_{1,18}$ = 0.0003, P = 0.94). The interaction term also was not significant ($F_{1,18}$ = 0.05, P = 0.67).

Two-spotted spidermite Spidermites were negatively affected by hyperaccumulated Ni. Population size by day 23 was significantly greater on low-Ni plants than on high-Ni plants (Wilcoxon two-sample test: Z = 3.7, P < 0.001). Low-Ni plants had 37-fold more spidermites on them: 37 ± 9.5 mites cm^{-1} stem (mean ± SE, n = 10) vs. 0.99 ± 0.53 for high-Ni plants.

The follow-up choice experiment found that spidermites preferentially fed and reproduced on low-Ni *S. polygaloides*. After 19 d, spidermite population size on low-Ni *S. polygaloides* was significantly greater than that on high-Ni plants, based on mean mite abundance on each plant type (Wilcoxon two-sample test: Z = 4.1, P < 0.001). High-Ni plants had 0.05 ± 0.01 mites cm^{-1} stem (mean ± SE, n = 12) and low-Ni plants had 0.19 ± 0.01 mites cm^{-1} stem.

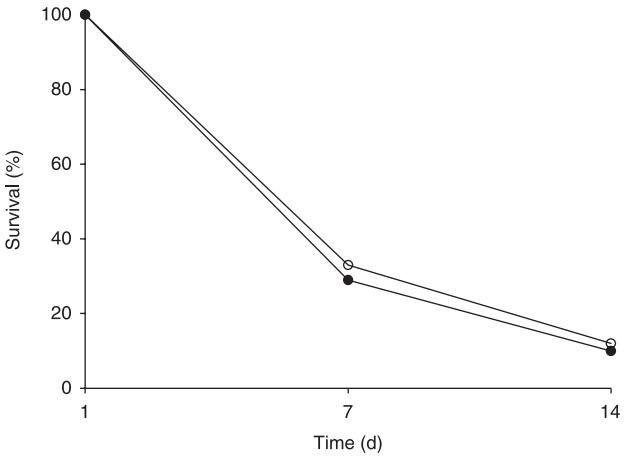

Fig. 5 Survival of tarnished plant bugs (*Lygus lineolaris*) fed high-nickel (filled circles) or low-nickel (open circles) leaves of *Streptanthus polygaloides* during a no-choice experiment.

Discussion

Our experiments tested the elemental defense hypothesis with herbivores representing various feeding modes. Because no-choice experiments showed significantly lower survival or herbivore population growth for cabbage maggots, cross-striped cabbageworms, grasshoppers and two-spotted spidermites on high-Ni plant tissues, we conclude that hyperaccumulated Ni defends *S. polygaloides* against these herbivores (Walling, 2000). Choice experiments showed that Ni hyperaccumulation in *S. polygaloides* confers herbivore resistance upon high-Ni *S. polygaloides* through deterrence. Red-legged grasshoppers fed to a significantly greater extent upon low-Ni plants of *S. polygaloides* than upon high-Ni plants when presented with a choice of the two plant types. Similarly, spidermites had significantly greater population growth on low-Ni plants compared with high-Ni plants when presented with a choice of the two plant types. We also found complete deterrence for cabbage maggot and cross-striped cabbageworm larvae. No cabbage maggots were found in high-Ni roots when a choice was presented, thus suggesting that larvae discriminated strongly between high- and low-Ni roots. Cross-striped cabbageworms were observed to rest on or crawl over high-Ni leaves but we found no visible damage to those leaves. A complete deterrent effect of hyperaccumulated metals is rare. To our knowledge, only Pollard & Baker (1997) have reported it before, for *P. rapae* given a choice of Zn hyperaccumulating or nonhyperaccumulating *Thlaspi caerulescens* (Brassicaceae).

As postulated by Boyd (2004), herbivores with different feeding modes may respond differently to elemental defenses. The present study, as well as our previous work (e.g. Boyd & Martens, 1994; Martens & Boyd, 1994; Boyd & Moar, 1999; Boyd *et al.*, 2002), has shown that leaf-chewing folivores (in the present study, red-legged grasshoppers and cross-striped cabbageworms) are especially susceptible to hyperaccumulated Ni. Although the location of Ni in tissues of *S. polygaloides* is unexplored, other Ni hyperaccumulators store Ni within epidermal and subepidermal tissues (Mesjasz-Przybylowicz *et al.*, 1996; Krämer *et al.*, 1997; Kupper *et al.*, 2001) and that Ni may be complexed with organic acids such as citrate or malate and sequestered within cell vacuoles (Reeves, 1992; Anderson *et al.*, 1997; Krämer *et al.*, 2000; Salt & Krämer, 2000). Leaf-chewing grasshoppers and caterpillars in the present study may have been negatively affected by Ni hyperaccumulated in *S. polygaloides* within subepidermal vacuoles. As tissue-chewing herbivores ruptured those vacuoles the Ni would be liberated and could then be toxic.

We found that high levels of Ni did not affect phloem-feeding herbivores. Experiments with turnip aphids and greenhouse whiteflies (Table 3) showed no defensive effect of hyperaccumulated Ni. Previous experiments with the pea aphid

(*Acyrthosiphon pisum*) found no effect of hyperaccumulated Ni in *S. polygaloides* (Boyd & Martens, 1999). Boyd & Martens (1999) suggested that phloem-feeding insects may be unaffected because phloem tissue of *S. polygaloides* is relatively low in Ni. While xylem sap of Ni hyperaccumulators may contain Ni bound to amino acids (Krämer *et al.*, 1997), little information exists regarding the Ni concentration of phloem fluid. Our conclusion that hyperaccumulated Ni is ineffective against phloem-feeding herbivores may be specific to this element, however, as Hanson *et al.* (2004) showed that Se-hyperaccumulating *Brassica juncea* (Brassicaceae) was both toxic and deterrent to aphids.

Another vascular-feeding insect unaffected by hyperaccumulated Ni was the meadow spittlebug. Hyperaccumulated Ni may be complexed with amino acids within xylem sap (Krämer *et al.*, 1997). Using ^{63}Ni, Anderson *et al.* (1997) demonstrated that Ni absorbed by roots in the South African hyperaccumulator *Berkheya coddii* (Asteraceae) travels through the xylem sap. Spittlebug nymphs produce a mass of bubbles during their instar development, which may aid in water retention and protection from predators (Hamilton & Morales, 1992). Filter paper saturated with DMG has been used as a colorimetric assay for hyperaccumulated Ni in plant tissues based on a pink color change (Reeves *et al.*, 1999). In our experiments, nymphs feeding on high-Ni plants produced spittle that yielded a positive (pink) reaction when tested with DMG paper. Spittle produced by nymphs is excreted from a filtering chamber where some substances may pass through the gut unchanged or unassimilated (Ponder *et al.*, 2002). The complex excretory system of spittlebugs may allow them to tolerate elevated dietary Ni, as it was clear from the DMG test that Ni was present in the xylem sap consumed by spittlebugs.

We also found no significant effect of hyperaccumulated Ni on the cell-disrupting tarnished plant bug *Lygus lineolaris*. A field survey of arthropods associated with *S. polygaloides* (Wall, 1999; Wall & Boyd, 2002) reported several hemipteran herbivores, including *Lygus hesperus*, feeding on this species. These *L. hesperus* contained approx. 31 μg Ni g^{-1} dry mass (Wall & Boyd, 2002) and fed on high-Ni plants with no apparent adverse effects. We suggest that *L. lineolaris* may have been able to feed on hyperaccumulating *S. polygaloides* and tolerate high plant Ni concentrations in the same (unknown) manner as *L. hesperus* in the field. Although the mechanism by which they can feed on high-Ni plants is unknown, we suggest that tarnished plant bugs are relatively tolerant of Ni, as it seems unlikely that this cell-disruptor was able to avoid ingesting Ni when feeding on high-Ni plants. We base this suggestion on two lines of evidence. First, the other cell-disruptor we tested (the two-spotted spidermite) was negatively affected by Ni, indicating that its feeding mode exposed it to elevated Ni. Secondly, field studies of *S. polygaloides* have discovered two other cell-disruptor hemipteran species that have relatively high whole-body Ni concentrations, thus indicating dietary exposure to Ni. One of these, *Melanotrichus boydi* (Miridae), is a specialist herbivore on *S. polygaloides* and was found to contain 800 μg Ni g^{-1} (Schwartz & Wall, 2001). The other, *Coquilletia insignis* (Miridae), contained 500 μg Ni g^{-1} when collected from *S. polygaloides* (Boyd *et al.*, 2004).

Although considerable evidence has now been garnered for the defensive effectiveness of hyperaccumulated Ni (Boyd, 2004), it is unclear how many other hyperaccumulated elements may also have defensive functions. To our knowledge, only Zn, Se and Cd have been explicitly tested to date. Hyperaccumulated Se defends against some folivores (Hanson *et al.*, 2003) and phloem-feeders (Hanson *et al.*, 2004) but results for Zn have been mixed. Pollard & Baker (1997) and Jhee *et al.* (1999) showed high-Zn plants were defended against some folivores, and yet Huitson & Macnair (2003) and Noret *et al.* (2005) found no effect of hyperaccumulated Zn on snails (*Helix aspersa* Müller), suggesting that some folivores are insensitive to Zn. Boyd *et al.* (2002) showed that *H. aspersa* is not unaffected by all hyperaccumulated metals, as they demonstrated toxicity and deterrence for high-Ni leaves of the Ni hyperaccumulator *Senecio coronatus* (Asteraceae). Jiang *et al.* (2005) reported that Cd hyperaccumulation decreased feeding damage caused by the thrip *Frankliniella occidentalis* (Thysanoptera: Thripidae), which feeds by cell disruption. There is some tantalizing evidence, however, that all hyperaccumulated metals can defend plants against at least some herbivores. Coleman *et al.* (2005) used an artificial insect diet amended with eight metals [Cd, cobalt (Co), chromium (Cr), copper (Cu), magnesium (Mn), Ni, lead (Pb) and Zn] hyperaccumulated by plants. They showed that all tested metals were toxic at hyperaccumulator concentrations to larvae of an insect folivore, the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae). Furthermore, toxicity extended far below hyperaccumulator concentrations for all the metals tested, suggesting that plants other than hyperaccumulators may benefit from elemental defenses. Additional studies are needed to reveal the extent and effectiveness of elemental defenses against the myriad natural enemies of plants.

We conclude that Ni in *S. polygaloides* defends against tissue-chewing insect herbivores (leaf-chewing and root-feeding) and some cell-disruptors but is ineffective against vascular tissue-feeding insects. Although the defensive function of hyperaccumulation has been investigated before (see summary in Boyd, 2004), our study is notable in several ways. First, we test the defensive effect of a metal against arthropod species representing a diversity of feeding modes. Besides representatives of the leaf-chewing folivores (Lepidoptera and mollusks) and aphids that have been tested by other studies of elemental defenses (e.g. Boyd & Martens, 1994; Pollard & Baker, 1997; Boyd & Moar, 1999; Jhee *et al.*, 1999; Huitson & Macnair, 2003; Hanson *et al.*, 2004), we used representatives of two previously untested feeding modes. Ours is the first study to use a xylem feeder (meadow spittlebug) and the first to use a rhizovore (cabbage maggot). Secondly, we used two arthropod orders previously untested with metal hyperaccumulating

plants. We demonstrated a defensive effect of Ni hyperaccumulation on a noninsect arthropod, the two-spotted spidermite (Order Acari), as well as the cabbage maggot (Order Diptera). Thirdly, this study broadens our knowledge of the effects of Ni-based defense because the other arthropods used in our experiments were previously untested species from insect orders that have been tested before. Finally, our work helps refine the defense hypothesis by exploring herbivore traits (e.g. feeding mode) that can influence the effectiveness of a resistance trait (in this case Ni hyperaccumulation). This can predict which suite(s) of herbivores is (are) affected by hyperaccumulated elements and guide the design of much-needed field studies of the defensive effects of elemental hyperaccumulation (e.g. Martens & Boyd, 2002).

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