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Arsenic hyperaccumulation in the Chinese brake fern (*Pteris vittata*) deters grasshopper (*Schistocerca americana*) herbivory

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Summary

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- Brake fern, *Pteris vittata*, not only tolerates arsenic but also hyperaccumulates it in the frond. The hypothesis that arsenic hyperaccumulation in this fern could function as a defense against insect herbivory was tested.
- Fronds from control and arsenic-treated ferns were presented to nymphs of the grasshopper *Schistocerca americana*. Feeding damage was recorded by visual observation and quantification of the fresh weight of frond left uneaten and number of fecal pellets produced over a 2-d period. Grasshopper weight was determined before and after 5 d of feeding.
- Grasshoppers consumed significantly greater amounts of the frond tissue, produced more fecal pellets and had increased body weight on control plants compared with grasshoppers fed arsenic-treated ferns. Very little or none of the arsenic-treated ferns were consumed indicating feeding deterrence. In a feeding deterrent experiment with lettuce, sodium arsenite at 1.0 mM deterred grasshoppers from feeding whereas 0.1 mM did not. In a choice experiment, grasshoppers preferred to feed on lettuce dipped in water compared with lettuce dipped in 1.0 mM sodium arsenite.
- Our results show that arsenic hyperaccumulation in brake fern is an elemental defense against grasshopper herbivory.

Key words: arsenite, arsenic hyperaccumulation, elemental defense, herbivory, grasshopper, pteridophytes, *Pteris vittata*, heavy metals.

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Introduction

Arsenic (As) is widely distributed in nature and is associated with the ores of metals such as copper, lead and gold. It also enters into the environment as a result of anthropogenic activities such as the use of arsenicals as pesticides, dyes and chemical weapons (Oremland & Stolz, 2003). Because of its carcinogenicity, As contamination of soil and water is an environmental health issue of global proportions (Smith *et al.*, 1992). Ma *et al.* (2001) reported that the Chinese brake fern,

Pteris vittata, hyperaccumulated As in its frond tissue. Following this report, there was great interest in using this fern as a cost-effective green technology for the remediation of As-contaminated soil and water (Tu *et al.*, 2002; Rathinasabapathi *et al.*, 2006a). Several other ferns have also been reported to be capable of As hyperaccumulation, including *Pityrogramma calomelanos*, *Pteris cretica*, *P. longifolia* and *P. umbrosa* (Visoottiviseth *et al.*, 2002; Zhao *et al.*, 2002) but no fern accumulates such high levels of As as *P. vittata* (up to c. 2% of their dry weight). Work in several laboratories is beginning to

unravel the mechanisms of plant tolerance of As and its hyperaccumulation in *P. vittata* (Wang *et al.* 2002; Poynton *et al.*, 2004; Srivastava *et al.*, 2005a; Ellis *et al.*, 2006; Rathinasabapathi *et al.*, 2006b; Singh *et al.*, 2006).

Despite the potential utility of As hyperaccumulating ferns and their unusual biochemistry, the reason(s) why certain ferns evolved As hyperaccumulation is not known. Meharg (2002) suggested that plant tolerance to As and hyperaccumulation could be primitive traits in early land plants that evolved in As-rich environments and were retained because of a selective advantage (Meharg, 2002). Alternatively, As hyperaccumulation might have evolved late in different fern taxa as a convergent adaptation (Meharg, 2002). Although an ecological advantage for As hyperaccumulation has not been demonstrated in ferns, Martens & Boyd (1994) proposed that hyperaccumulated metals can provide elemental defense against herbivores and pathogens. Elemental defenses differ from secondary chemical defenses because their toxic principle is an element taken up from the soil rather than one derived from photosynthate (Boyd, 2004). Studies on nickel (Ni), zinc (Zn) and selenium (Se) hyperaccumulating plants showed that these hyperaccumulated elements could have roles in plant defense, either by toxicity or deterrence or both (Pollard & Baker, 1997; Boyd *et al.*, 2002; Vickerman *et al.*, 2002; Boyd, 2004; Hanson *et al.*, 2004; Boyd & Jhee, 2005; Jhee *et al.*, 2005; Noret *et al.*, 2007).

Our objective was to test the hypothesis that As hyperaccumulation in *P. vittata* has a role in defense against insect herbivory. We chose the grasshopper *Schistocerca americana* for this study because of its wide host range and preliminary observations indicated that it could eat *P. vittata* fronds.

Materials and Methods

Plants

Uniform 2-month-old *P. vittata* L. plants with four to six fronds each were grown hydroponically in half-strength Hoagland nutrient solution in a controlled environment room as described in Tu *et al.* (2004). Plants treated with As were supplied with 0.16 mM (50 mg kg⁻¹) sodium arsenate in the nutrient solution for 1 wk before harvesting of the fronds. Fronds were used immediately after harvest.

Arsenic determination

Samples of arsenic-treated and untreated control fern tissue (*c.* 0.5 g each, *n* = 3) were homogenized three times in 3 × 1 ml of 50% (v : v) methanol in water, using a glass rod. The pooled extract from each sample was clarified by centrifugation at 10 000 g for 5 min. The supernatant was analysed for total arsenic and arsenite. Arsenate and arsenite were separated using an As speciation cartridge (Metal Soft Center, Highland Park, NJ, USA), which retains arsenate (Meng *et al.*, 2001).

Total arsenic (AsV and AsIII) and arsenite (AsIII) were determined by using an atomic absorption spectrometer (Varian 240Z Zeeman; Varian, Walnut Creek, CA, USA) (Chen & Ma, 1998).

Grasshoppers

A laboratory colony of grasshopper (*Schistocerca americana* (Drury)) was started from a field collection made at Dade City (FL, USA) in 1991. Nymphs were maintained on a diet of Romaine lettuce (*Lactuca sativa*) in a cage at 30°C and 80% relative humidity. Before their use in the experiment with fern leaves, third instar grasshopper nymphs were starved for 24 h and randomly assigned to treatments. Preliminary observations indicated that grasshoppers ate fern leaves well when starved. Grasshopper nymphs used in the two lettuce experiments were not starved.

Grasshopper feeding on fern – no-choice experiment

For each replicate, a starved nymph was placed inside a transparent circular plastic box (17 cm diameter and 7.5 cm high) closed with a lid containing two wire mesh (1 × 1 mm mesh size) circular windows of 3 cm diameter each. A layer of moist paper towel was laid on the floor of the box. A glass vial with a small amount of wet cotton holding a frond from either a control or arsenic-treated *P. vittata* was placed in the plastic box. Fronds and grasshoppers were weighed before the experiment. The experiment was carried out at 27–30°C. Every 24 h for 2 d the number of fecal pellets per insect and the weight of the tissue left uneaten were measured. These same grasshoppers were confined for an additional 3 d on either untreated or treated plants and their mass at the end of the 5-d period was measured.

Sodium arsenite for grasshopper feeding deterrence – no-choice experiment

Romaine lettuce leaf segments (of 50–60 cm²), weighing between 6 g and 9 g fresh weight each, were dipped for 10 s in water (control) or sodium arsenite in water at 0.1 mM or 1.0 mM. The treated lettuce leaf segment was placed on a wet paper towel and presented to a starved nymph in a plastic box as described above for 48 h. The amount of leaf area eaten was scored using a scale of 1–10, with 10 representing 100% of the surface area of the leaf segment consumed. The number of fecal pellets was also recorded 24 and 48 h after the beginning of the experiment. Unlike the experiments with ferns where the weight of the tissue uneaten was recorded, the surface area of lettuce eaten was quantified here because this was more accurate than the wet weight, which was affected by the moisture because of the dipping treatment. Arsenite in lettuce dipped in 0.1 mM and 1.0 mM sodium arsenite solutions was determined following extraction of the tissue in 50% methanol.

Sodium arsenite for grasshopper feeding deterrence – choice experiment

This experiment was conducted similarly to the no-choice experiment except that two pieces of lettuce, one dipped in water and another dipped in 1.0 mM sodium arsenite, were provided in each of the boxes. The amount of tissue eaten from both of these treatments was estimated using the 1–10 scale described earlier. Both the experiments using lettuce were done for 48 h.

Experimental design and statistical analyses

For all experiments a completely randomized design was followed. Data were analysed using PROC GLM (SAS Institute, 1997) and means were separated using Student's *t*-test or Tukey's Studentized range test when treatment and control means were compared or Duncan's multiple range test when more than two means were compared at a significance level of $\alpha = 0.05$.

Results

Pteris vittata plants grown in hydroponic solution took up arsenate that was supplied in the medium. Fronds of arsenic-treated plants had $46.4 \pm 3.2 \text{ mg kg}^{-1}$ arsenic compared with $2.9 \pm 1.6 \text{ mg kg}^{-1}$ in the fronds of control plants, the values indicating mean and standard error of the mean for three replicates. These means were significantly different from each other at $\alpha = 0.05$. Of this arsenic $83 \pm 0.03\%$ of the arsenic found in the arsenic-treated plant was in the form of As(III) whereas only $65 \pm 0.13\%$ was in this form in the control plants ($n = 3$). Starved grasshoppers ate fronds from control plants (Fig. 1a), damaging the green pinnules. Occasionally, however, the insects also ate the rachis portion of the frond (data not shown). When observed at 24 h and 48 h after the start, each insect had consumed, on average, $100 \text{ mg tissue d}^{-1}$ and produced 10–13 fecal pellets per day (Fig. 2a,b). By contrast, the insects fed with arsenic-treated ferns did not eat the leaves except for minor damage to leaf margins in one or two cases (Fig. 1b). This feeding deterrence was corroborated by data on the amount of leaf eaten and the fecal pellets produced per insect per day; both variables being significantly lower for insects feeding on fronds from arsenic-treated plants than for insects feeding on control plants (Fig. 2a,b).

In the longer-duration feeding study, the insects fed control fronds for 5 d increased in mass by $11.1 \pm 2.7 \text{ mg}$ (mean \pm SE, $n = 5$). Insects fed arsenic-treated fern decreased in weight, losing on average $7.7 \pm 3.9 \text{ mg}$ (mean \pm SE, $n = 5$), with no obvious signs of food consumption except occasional minor damages to frond margins. These insect weight changes were significantly different when analysed using Tukey's Studentized range test at $\alpha = 0.05$.

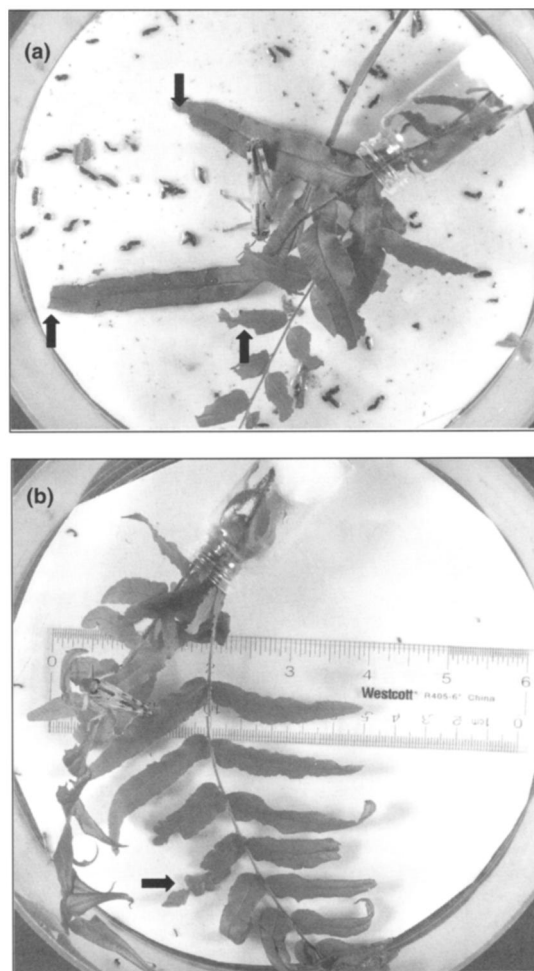


Fig. 1 Feeding damage on *Pteris vittata* fronds from either control (a) or arsenic-treated plants (b) by grasshopper *Schistocerca americana* nymphs. The arrows indicate examples of damage on the frond. Photographs were taken 48 h after releasing the insects onto the test fronds.

When analysed for arsenite, lettuce dipped in 0.1 mM and 1.0 mM arsenite solutions contained 2.3 ± 0.2 and $46.14 \pm 22 \text{ mg kg}^{-1}$ FW (mean \pm SE, $n = 3$), respectively. The amount of leaf eaten was measured and the fecal pellets were counted after 24 h and 48 h. Insects consumed significantly more of the leaf dipped in water or 0.1 mM arsenite (Fig. 3a) than those dipped in 1.0 mM arsenite, which almost completely deterred insect feeding (Fig. 3a). This was also corroborated by the number of fecal pellets, which were significantly less in the 1.0 mM arsenite treatment on day 1 and were less, but not significantly on day 2 (Fig. 3b). Figure 4 illustrates the feeding damage of grasshoppers on lettuce dipped in water (a), 0.1 mM arsenite (b) or 1.0 mM arsenite (c) 24 h after beginning the experiment.

In a choice experiment, where grasshoppers were presented with lettuce dipped in water and lettuce dipped in 1.0 mM arsenite in the same plastic box, the grasshoppers preferred to consume more control lettuce than arsenite-treated lettuce, as

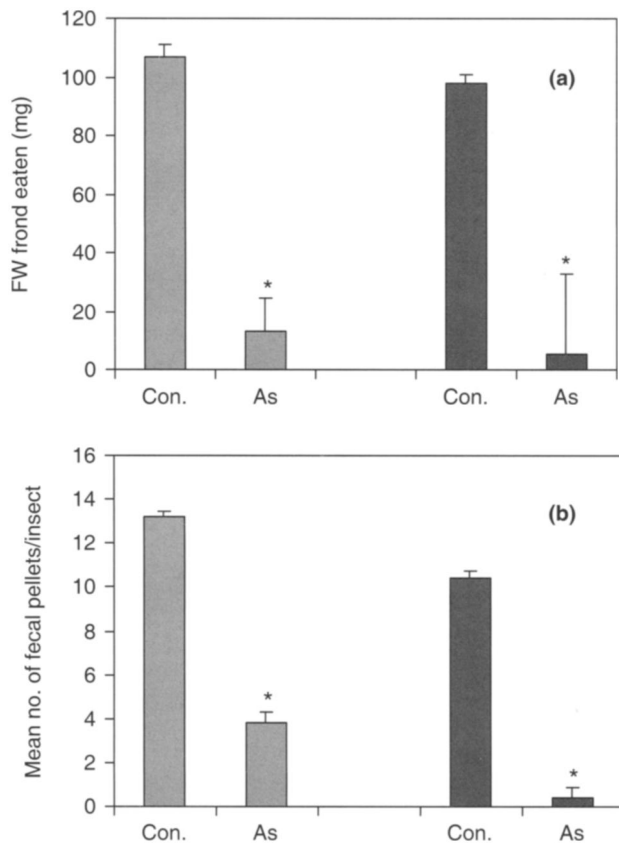


Fig. 2 Feeding deterrence of grasshopper (*Schistocerca americana*) by arsenic-hyperaccumulating fern (*Pteris vittata*) fronds. (a) Fresh weight (FW) of frond eaten per day per insect and (b) the mean number of fecal pellets produced per day per insect when fronds from control (Con.) and arsenic-treated (As) plants were supplied to *Schistocerca americana*. Observations are for 24 h (tinted bars) and 48 h (closed bars) from the beginning of the experiment. Bars denoted by asterisks were significant different by the mean separation test at $\alpha = 0.05$, $n = 5$ for each treatment.

shown by the amount of leaf eaten between the two treatments. Insects consumed a significantly greater area of the control leaf than the treated leaf (Fig. 5).

Discussion

Our results show that As accumulated in *P. vittata* fronds at the concentration averaging 46 mg kg^{-1} was sufficient to deter grasshoppers from feeding. While this could be the concentration consumed by the insect, the exact concentration of As is not known because of limited knowledge of subcellular and tissue-specific accumulation of arsenite in the fern. The X-ray absorption spectroscopy and imaging studies indicated that arsenic was found in the upper and lower epidermal layers of the frond, probably in the vacuoles (Lombi *et al.*, 2002; Pickering *et al.*, 2006). Such distribution would be ideal for arsenic to play a role in insect feeding deterrence.

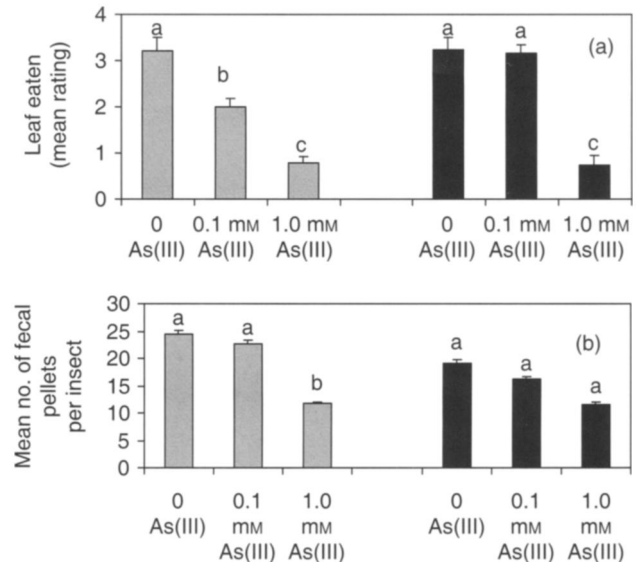


Fig. 3 Feeding of grasshopper (*Schistocerca americana*) on lettuce (*Lactuca sativa*) dipped in 0.1 mM and 1.0 mM arsenite (As) and water control. (a) Amount of leaf eaten per day per insect (scored using a relative scale of 1–10, as described in the Materials and Methods section) and (b) the mean number of fecal pellets produced per day per insect. Observations are for 24 h (tinted bars) and 48 h (closed bars) from the beginning of the experiment. For each day, means shown with different letters indicate significant difference by Duncan's multiple range test at $\alpha = 0.05$ with $n = 5$.

Pteris vittata ferns growing in uncontaminated sites (0.44 – 7.56 mg kg^{-1}) accumulated between 11.8 mg kg^{-1} and 64 mg kg^{-1} in its fronds (Ma *et al.*, 2001). The concentration ranges found in the frond indicates that there is potential for this arsenic concentration to act as a herbivore deterrence.

Data on tissue consumed and production of fecal pellets both indicated that arsenic-treated fern fronds were not consumed by *S. americana* (Figs 1 and 2) in no-choice tests. Like many generalist herbivores, the grasshoppers made many test bites before rejecting the arsenic-accumulated fronds (Pollard & Baker, 1997; Behmer *et al.*, 2005), suggesting that olfaction alone was not sufficient to detect the arsenite or arsenite-induced phytochemicals if any.

Pteris vittata, native to eastern Asia, is widely distributed throughout the tropics and subtropics including South Africa, Madagascar, New Guinea, Australia and parts of North and South Americas (Jones, 1987). *Schistocerca americana* is known to be distributed throughout the eastern USA to the Great Plains and south to Mexico (Thomas, 1991). However, it is highly unlikely that *S. americana* is a natural pest of *P. vittata*. While our experiments suggest that insect damage could possibly have been a selection pressure for the evolution of arsenic hyperaccumulation in ferns, it is not known whether arsenic hyperaccumulation trait has that advantage under natural field conditions. Gould & Vrba (1982) coined the term exaptation to denote adaptations that now enhance fitness of an organism but were not built by natural selection for their

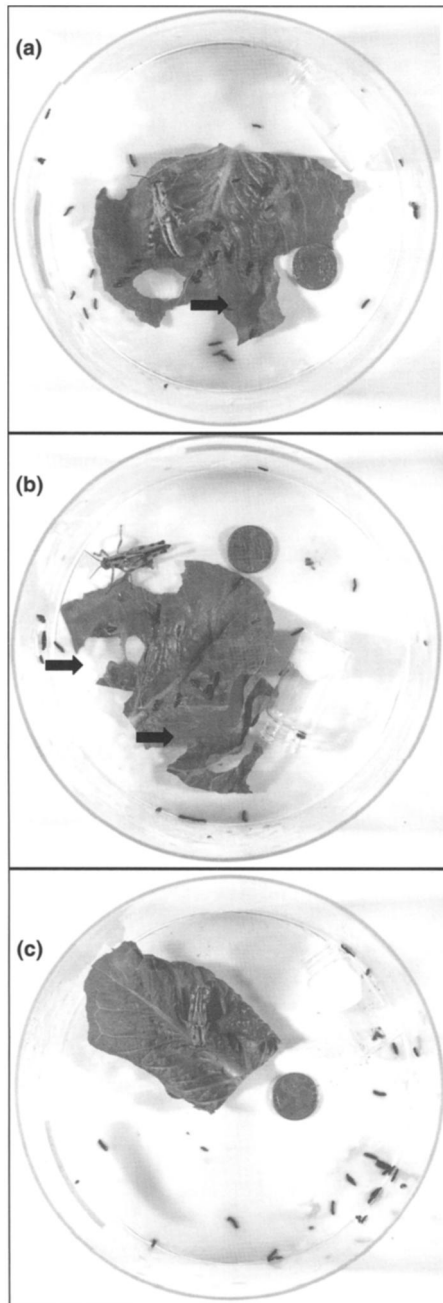


Fig. 4 Feeding deterrence of grasshopper (*Schistocerca americana*) nymphs presented with lettuce (*Lactuca sativa*) dipped into (a) water, (b) 0.1 mM or (c) 1.0 mM sodium arsenite. The arrows indicate examples of damage on the frond. Photographs were taken 24 h after releasing the insects onto the test leaf.

current role. The As hyperaccumulation trait could be an exaptation (Gould & Vrba, 1982) as a character evolved for other currently unknown uses but later 'coopted' for their role in herbivore deterrence. This idea is suggested in 'inadvertent uptake hypothesis' of metal hyperaccumulation wherein metal hyperaccumulation has no selective value but the trait

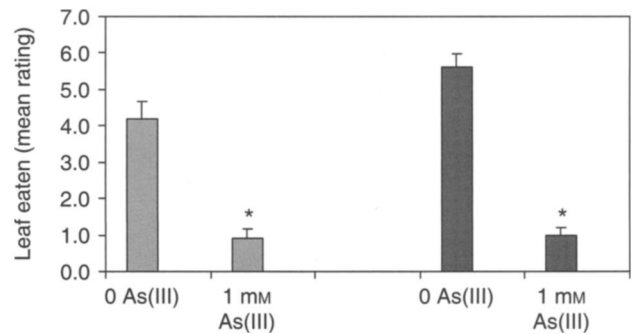


Fig. 5 Preference of grasshopper (*Schistocerca americana*) nymphs for lettuce (*Lactuca sativa*) dipped with 1 mM arsenite and control in a choice test. Amount of leaf eaten was scored based on a relative scale of 1–10 as described in the Materials and Methods section. Observations are for 24 h (tinted bars) and 48 h (closed bars) from the beginning of the experiment. Bars followed by asterisks indicate significant difference between the control and the treatment means by Student's *t*-test at $\alpha = 0.05$ with $n = 5$ for each treatment/control.

is a byproduct of other physiological processes in metal hyperaccumulating plants (Boyd & Martens, 1998).

In a study done in Costa Rica, Rowell *et al.* (1983) reported that forest grasshoppers *Hylopedetes nigrithorax* and *Homomastax dentata* ate ferns and preferred to eat certain species over others. In that study, a number of chemical parameters such as total phenolics, astringency and fiber content of the ferns did not correlate to the grasshoppers' choice (Rowell *et al.*, 1983). Heavy metal hyperaccumulation was not considered as a potential deterrent in that study. Our results here are consistent with As hyperaccumulation having a role in insect deterrence.

It is possible that arsenite itself acts as a deterrent. Alternatively, secondary products induced in response to arsenite treatment might be the feeding deterrent. To distinguish between these possibilities, we tested arsenite solutions for feeding deterrence. Our results showed that sodium arsenite directly acted as a feeding deterrent at 1 mM (Figs 3 and 4). In a choice experiment, grasshoppers preferred lettuce dipped in water over lettuce dipped in 1 mM arsenite (Fig. 5). The concentration of arsenite in lettuce dipped in 1 mM sodium arsenite was comparable with that found in arsenic-treated fern fronds. Our results are consistent with the hypothesis that the grasshoppers sense arsenite during test bites and arsenite itself can act as a deterrent. However, we did not determine if As-induced *P. vittata* natural products also play a role in insect feeding deterrence.

Plant resistance due to allelochemicals and heavy metals other than As has been investigated in great detail (Boyd, 2004; Hanson *et al.*, 2004; Jhee *et al.*, 2005). However, our study is the first example where arsenite accumulated in the plant tissue is shown to have a role in feeding deterrence against a generalist herbivore. This is significant in designing novel insect deterrents because if arsenite is directly sensed by

the chemosensory organs of the insect herbivore, less toxic analogs of arsenite (e.g. phosphite, sulfite, selenite, etc.) may have potential value as feeding deterrents. Alternatively, if *P. vittata* produces specific chemicals in response to arsenic treatment and if they have deterrence effects on herbivores, these chemicals may also be valuable as biopesticides.

There has been a controversy in the literature as to whether ferns receive less feeding damage from insect herbivores than angiosperms (Hendrix & Marquis, 1983). Only certain ferns exhibit arsenic hyperaccumulating abilities while many angiosperms screened lack this trait (Dembitsky & Rezanka, 2003). Ferns are also known to accumulate other heavy metals such as Se (Srivastava *et al.*, 2005b) and lead (Kamachi *et al.*, 2005). Hence, it is possible that both the genotype of fern and their soil environment, which affect their potential to absorb heavy metals, might determine the degree of insect herbivore damage.

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