

Cardenolides, induced responses, and interactions between above- and belowground herbivores of milkweed (*Asclepias* spp.)

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Abstract. Theory has long predicted allocation patterns for plant defense against herbivory, but only recently have both above- and belowground plant defenses been considered simultaneously. Milkweeds in the genus *Asclepias* are a classic chemically defended clade of plants with toxic cardenolides (cardiac glycosides) and pressurized latex employed as anti-herbivore weapons. Here we combine a comparative approach to investigate broadscale patterns in allocation to root vs. shoot defenses across species with a species-specific experimental approach to identify the consequences of defense allocational shifts on a specialist herbivore. Our results show phylogenetic conservatism for inducibility of shoot cardenolides by an aboveground herbivore, with only four closely related tropical species showing significant induction; the eight temperate species examined were not inducible. Allocation to root and shoot cardenolides was positively correlated across species, and this relationship was maintained after accounting for phylogenetic nonindependence. In contrast to long-standing theoretical predictions, we found no evidence for a trade-off between constitutive and induced cardenolides; indeed the two were positively correlated across species in both roots and shoots. Finally, specialist root and shoot herbivores of common milkweed (*A. syriaca*) had opposing effects on latex production, and these effects had consequences for caterpillar growth consistent with latex providing resistance. Although cardenolides were not affected by our treatments, *A. syriaca* allocated 40% more cardenolides to shoots over roots. We conclude that constitutive and inducible defenses are not trading off across plant species, and shoots of *Asclepias* are more inducible than roots. Phylogenetic conservatism cannot explain the observed patterns of cardenolide levels across species, but inducibility per se was conserved in a tropical clade. Finally, given that above- and belowground herbivores can systemically alter the defensive phenotype of plants, we concur with recent calls for a whole-plant perspective in testing models of plant defense allocation.

Key words: allocation trade-offs; *Asclepias syriaca*; cardenolides; constitutive vs. inducible resistance; *Danaus plexippus*; latex; monarch butterfly; plant defense theory; plant–insect interactions; root herbivory; root vs. shoot; *Tetraopes tetraophthalmus*.

INTRODUCTION

Plant traits such as secondary metabolites help to resist herbivory and may be expressed constitutively or induced upon attack. Constitutive resistance is expressed independent of injury, whereas induced resistance is activated or enhanced after the plant has been damaged (Karban and Baldwin 1997). Both of these modes of resistance have been shown to be costly in terms of plant fitness (Strauss et al. 2002) and theory has long predicted that constitutive and inducible resistances should trade off (McKey 1974, Rhoades 1979, Brody and Karban 1992, Thaler and Karban 1997, Koricheva et al. 2004, Morris et al. 2006). It has been proposed that species will rely more strongly on a single mode of defense, depending on the risk of herbivory (Zangerl and Bazzaz 1992). For example, inducibility may be unnecessary for

species that are heavily attacked by herbivores and that invest predominantly in constitutive resistance.

Types of defenses differ not only among species or genotypes, but also between plant organs such as roots and shoots within a single species. Thus, we must take both above- and belowground organs into account to fully understand the allocation patterns of defensive chemicals, as well as the constraints on their expression (Agrawal 2004, Bezemer and van Dam 2005, De Deyn and Van der Putten 2005, Rasmann and Agrawal 2008, van Dam et al. 2009). Zangerl and Rutledge (1996) were the first to meld ideas about evolutionary trade-offs with patterns of defense allocation in plants. They showed that flowers and leaves of wild parsnip (*Pastinaca sativa* L.) were the most likely to experience herbivory and contained the highest constitutive concentration of a toxic compound (xanthotoxin). Parsnip roots, however, which experienced less predictable herbivory, had low constitutive defenses but highly inducible xanthotoxin compared to the aboveground parts of the plant.

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Recent surveys of root chemical defenses showed no consistent pattern of allocation toward roots vs. shoots across taxa (Kaplan et al. 2008b, Rasmann and Agrawal 2008, van Dam et al. 2009). The concentration of constitutive secondary compounds in roots and shoots depended on plant family, species, and genotype (Collantes et al. 1998, 1999, Agerbirk et al. 2003, Hol et al. 2004), plant age (Hara et al. 2000, Frolich et al. 2006), and ontogenetic stage of the tissues sampled (Ohnmeiss and Baldwin 2000, Walls et al. 2005). Induction of the same secondary compounds also showed no consistent pattern. For example, after above- or belowground herbivory, root and shoot defenses can be up- (Baldwin 1998, Schmelz et al. 1999, Bezemer et al. 2004) or down-regulated (van Dam et al. 2004, Rasmann and Turlings 2007).

To address allocation patterns of constitutive and induced chemical defenses in shoots and roots, and potential interactions between above- and belowground herbivores, we have been studying milkweeds (*Asclepias* spp.) and their well-known cardenolide defense system. Studying defense expression across different species (i.e., taking a comparative approach) should reveal common mechanisms or constraints that have persisted through the macroevolutionary process (Agrawal 2007). A comparative study of allocation patterns of secondary metabolites across *Asclepias* spp. should also help to disentangle general patterns of defense allocation to roots and shoots. However, a closer examination of the ecological impacts of root and shoot herbivory in specific species can reveal functional and ecological interactions not visible from larger scale patterns. For example, many chemically mediated plant–herbivore interactions are species specific, and may therefore be difficult to predict simply from measures of chemistry (Blau et al. 1978, De Moraes et al. 1998, Hagele and Rowell-Rahier 2000).

In this study we sought to combine a comparative survey with a more experimental approach on a target species. We asked three specific questions. (1) Within a monophyletic clade of 12 milkweeds, how inducible are cardenolides in the shoots and roots of plants damaged aboveground by monarch caterpillars (*Danaus plexippus*)? (2) Is there a trade-off between constitutive and induced cardenolides, or between allocation to above- vs. belowground defense across the 12 species? (3) In a single species of milkweed (*Asclepias syriaca*), is the performance of specialist aboveground herbivores (monarch caterpillars) affected by a specialist root feeder (*Tetraopes tetraophthalmus*), and what plant traits mediate this interaction?

MATERIAL AND METHODS

Natural history

The milkweeds (*Asclepias* spp., Apocynaceae) are a monophyletic group distinct from their closest relatives, which belong to the African genus *Gomphocarpus* (M. Fishbein, *personal communication*). *Asclepias* includes

about 130 species in North America, including Mesoamerica and the Caribbean, and some 20 species in South America (Woodson 1954, Fishbein et al., *in press*). The monarch butterfly (*Danaus plexippus*, Lepidoptera, Danaidae) is a broadly distributed migratory herbivore that feeds on most milkweed species (Ackery and Vane-Wright 1984, Haribal and Renwick 1998, Agrawal and Fishbein 2006). Other insect herbivores, such as the cerambycid beetles in the genus *Tetraopes* have radiated with milkweeds; each of the 24 *Tetraopes* species is primarily associated with one or a few milkweed species (Farrell and Mitter 1998, Farrell 2001); see Fig. 1.

Milkweed herbivores have to cope with a powerful arsenal of defenses present in the plant. Probably the most potent of these are cardiac glycosides (cardenolides) and latex (Agrawal 2005, Agrawal and Fishbein 2006). Cardenolides disrupt the sodium and potassium flux in cells, and occur in all milkweed tissues, including latex (Malcolm 1991). Sticky white latex is exuded immediately after milkweed plants are damaged and is delivered via specialized canals (laticifers) to most plant parts, serving as a physical and chemical barrier to the herbivores (Dussourd 1999, Agrawal et al. 2008). However, we did not find any latex exudation after mechanical or insect herbivore damage to the roots of *A. syriaca* and this is discussed later in the paper.

Above- and belowground induced cardenolides in 12 *Asclepias* species

To study cardenolide induction in the roots and shoots of milkweeds, we used 12 *Asclepias* species from the subclade Incarnatae (Woodson 1954), emended by Fishbein et al. (*in press*) (see Fig. 2). The series Incarnatae is a monophyletic group composed of approximately 15 species from North and Central America, including the Caribbean, and 7 species from South America. We grew eight north-temperate species and four tropical species.

The 12 milkweed species ($n = 114$ plants, 6–12 plants per species) were germinated at room temperature after stratifying the seeds at 4°C on moist filter paper for two weeks. One seedling per pot (10 cm diameter pots) was transplanted in potting soil (Metro-Mix Sun Gro Horticulture Canada CM Ltd., Vancouver, British Columbia, Canada), and grown in a single growth chamber (12 h daylight, 26°C day:20°C night) for six weeks before harvesting. Plants were watered weekly ad libitum and fertilized (N:P:K 21:5:20 150 ppm N [$\mu\text{g/g}$]) once 10 days after placement in the growth chamber. After 20 days in the growth chamber, approximately half of the plants ($n = 58$, 3–6 per species) were exposed to first-instar monarch caterpillars that had been reared in the lab. Plants received between one and three caterpillars, depending on plant size. Although we recognize that this variation in the number of herbivores on plants is suboptimal, we were able to remove all herbivores from plants after 5 days, thereby reducing



FIG. 1. Larva of the root-specialist longhorn milkweed beetle *Tetraopes tetraophthalmus* (Coleoptera: Cerambycidae) boring through roots of milkweed, *Asclepias syriaca*. Photo credit: S. Rasmann.

any bias due to variation in the amount of time needed to impose a fixed proportion (25%) of leaf damage. The other plants ($n = 56$, 3–7 per species) remained undamaged. Ten days after cessation of the herbivory treatment, all plants were harvested.

Above- and belowground tissues were separated, roots were washed carefully with water, and all plant material was frozen overnight before being dried fully in a drying oven at 40°C. We measured cardenolide concentrations with a spectrophotometric assay developed by Brower et al. (1972) and Nelson (1993), adapted to enable the use of a microplate reader. Roots ($n = 129$, 2–6 plants/species) were ground using a MM300 Retsch grinder (Retsch GmbH, Haan, Germany) in 10-mL steel grinding vessels at 27 Hz for 2 min. We weighed 50 mg of dry root tissue into 2 mL boil-proof microcentrifuge tubes and added 1.9 mL of 95% ethanol. Tubes were vortexed, floated in a sonicating water bath (65°C) for 10 min, and centrifuged at 5000 rpm for 5 min at room temperature. Two 45- μ L aliquots of the supernatant from each tube were pipetted into the wells (active sample and blank) of a 96-well plate. We then added 90 μ L of ethanol to the blanks and 90 μ L of 0.15% 2,204,40-tetranitrodiphenyl (TNDP) in ethanol to the active samples. Finally, 70 μ L of 0.1 mol/L aqueous NaOH was added to all wells to make the solutions basic and to catalyze the colorimetric reaction. After 18

minutes, all wells in the plate were read at 620 nm using a microplate reader. On each plate, we devoted six wells to known concentrations of digitoxin (Sigma, CAS No. 71-63-6) in order to generate a standard curve and convert absorbance values to concentrations of cardenolides. We then employed one-way ANOVA to compare the relative allocation to root cardenolides among the species.

To validate our spectrophotometric method to measure cardenolides, we simultaneously analyzed cardenolides from a subset of five *Asclepias* species (three samples from each species) with the above methods, and by injecting the same samples (after drying and dissolving in methanol), with digitoxin as the internal standard, in a HPLC, and characterized at 218 nm. The two methods were strongly correlated ($n = 15$, $r = 0.82$, $P < 0.001$)

To assess variation in the induced responses of the 12 species, we employed a factorial three-way ANOVA with species, herbivory treatment, and plant part as the main effects. ANOVAs were then performed separately on root and shoot tissues for simplicity of interpretation. We then assessed allocation of cardenolides to roots and shoots by performing Pearson correlations between species means for cardenolides in the absence and presence of herbivory. To gain insight into the macroevolution of root and shoot defense, we estimated

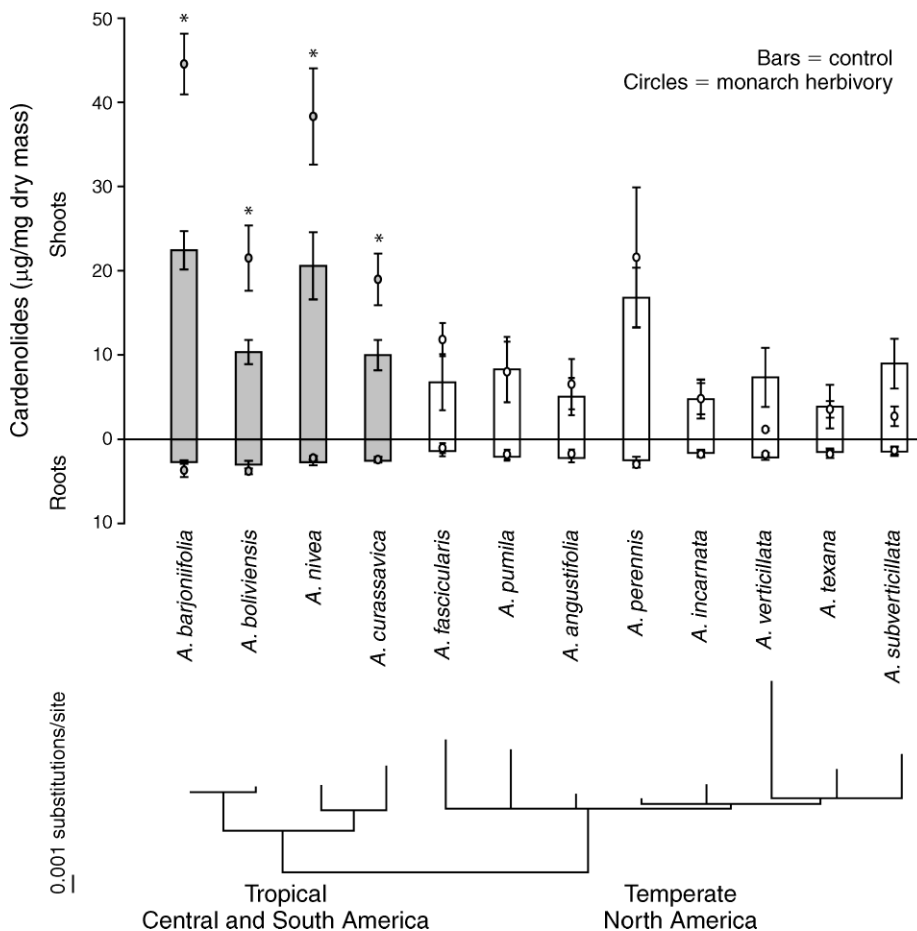


FIG. 2. Root and shoot cardenolide concentrations (mean \pm SE) of 12 *Asclepias* species (series Incarnatae) in undamaged control plants (bars) and plants damaged by monarch caterpillars (circles); $n = 2-7$ plants/species. Asterisks above circles indicate significant increases in shoot cardenolide concentrations after induction ($P < 0.05$). Below the data is the corresponding phylogram for the 12 species pruned from a comprehensive phylogeny of *Asclepias* (Agrawal and Fishbein 2008); the left-hand vertical scale indicates base pair substitutions. Tropical species are represented with solid bars and circles, and temperate species with open bars and circles.

the extent of phylogenetic conservatism (i.e., trait similarity due to shared ancestry) of induced and constitutive cardenolides. Phylogenetic relationships were assessed using a pruned phylogeny of *Asclepias*, which was the 50% majority rule consensus of trees sampled from the stationary distribution of a Bayesian analysis of three non-coding cpDNA regions sampled from 145 taxa (Agrawal and Fishbein 2008).

To assess phylogenetic conservatism, we used Pagel's λ , estimated in BayesTraits (Pagel 1999, 2007, Freckleton et al. 2002, Pagel and Meade 2004, 2007). This parameter is estimated using maximum likelihood (ML) and a generalized least squares (GLS) framework; this value is compared statistically to models where it is set to either zero or 1. A lambda value of 1 indicates phylogenetic conservatism consistent with the tree topology and a random walk model (i.e., trait similarity is directly proportional to the extent of shared evolutionary history). A lambda value of 0 indicates no

influence of shared ancestry on trait values (i.e., phylogenetic independence). Values slightly above 1 are possible and indicate that the phenotypes of closely related species are more similar than is predicted from their evolutionary divergence (M. Pagel, *personal communication*). Models are compared using a likelihood ratio (LR) test, where $LR = 2[\log\text{-likelihood of the better fitting model} - \log\text{-likelihood of the worse fitting model}]$. Here the LR statistic should be χ^2 distributed with one degree of freedom (as long as a single parameter is altered between the two models) (Pagel 2007). In addition, phylogenetically independent correlations were performed on these same data using the *Continuous* module in BayesTraits (Pagel 2007, Pagel and Meade 2007).

To assess negative correlations (trade-offs) in constitutive vs. induced allocation to shoot cardenolides among the 12 species, we employed the test outlined by Morris et al. (2006). This statistical approach

accounts for several issues that have apparently confounded previous attempts to assess a trade-off between constitutive and induced resistance (Morris et al. 2006). Specifically, this approach uses the difference in mean resistance between damaged and control plants as an optimal metric for induced resistance measurements, and uses a modified Monte Carlo procedure that takes into account sampling variation due to limited sample size, measurement error from environmental and genetic differences, and induced susceptibility, i.e., lower resistance in damaged than in undamaged plants (Morris et al. 2006). The correlation between cardenolide allocation to above- and belowground tissues was also assessed.

*Induced resistance above- and belowground
in A. syriaca*

Here we used the common milkweed (*Asclepias syriaca*) and two specialist herbivores, monarch butterfly caterpillars (*Danaus plexippus*) and larvae of the longhorn milkweed beetle (*Tetraopes tetraophthalmus*; Coleoptera, Cerambycidae), to address more mechanistically the root–shoot interactions mediated by herbivores and plant defense. The common milkweed is a long-lived, native weedy plant that occurs throughout eastern North America in open habitats such as roadsides, pastures, and old fields. It is the major food source for migrating monarch butterflies (Seiber et al. 1986, Malcolm et al. 1989) and the specialist longhorn beetle *T. tetraophthalmus*, whose adults feed on leaves and flowers and whose larvae feed on milkweed roots (Agrawal 2004).

For these experiments, all insects and seeds were from common milkweed patches naturally found around Ithaca, New York, USA. *T. tetraophthalmus* adults were kept in large plastic tubs in the laboratory, fed fresh milkweed leaves daily, and given dried grass stems as oviposition sites; see Agrawal (2004). The oviposition substrate was removed from the rearing cages every two days and incubated in the dark at 30°C for 7–10 days. Newly hatched larvae were kept without food in large petri dishes (10 cm diameter) on moist filter paper for up to 24 h before they were placed on the experimental plants. Adult monarchs (10–15 individuals) were kept as a rearing colony in 50 × 50 × 50 cm cages and provided butterfly nectar daily (The Birding Company, Yarmouth, Maine, USA). Females were provided with potted milkweed (*A. syriaca*) plants for oviposition. Eggs were removed with a paint brush, hatched, and reared on fresh leaves overnight before larvae were placed on the experimental plants. *A. syriaca* seeds were germinated, planted in 10 cm diameter pots and grown for 6 weeks in a growth chamber (12:12 h light:dark 26:20°C day:night). Two weeks before the experiment, 84 plants were transplanted to bigger pots (20 cm diameter) and randomized in the growth chamber.

To test how belowground herbivory affects the development of monarchs and the defensive traits in

A. syriaca, a fully factorial experiment with four treatments was established ($n = 15$ plants/treatment): one *D. plexippus* caterpillar (treatment “Monarch”), one *D. plexippus* and four *T. tetraophthalmus* larvae (treatment “Monarch and *Tetraopes*”), four *T. tetraophthalmus* (treatment “*Tetraopes*”), and no herbivores (control). *D. plexippus* caterpillars were placed on the leaves of the plants and *T. tetraophthalmus* larva were placed ~1 cm deep into the soil, near the emerging plant stem. Insects were allowed to feed for seven days, after which, *D. plexippus* caterpillars were removed and weighed on a microbalance. An additional 12 plants in each of “Monarch” and “Monarch and *Tetraopes*” treatments were manipulated as before, but kept untouched in the chamber for three additional days before *D. plexippus* caterpillars were harvested and weighed. Because the growth and feeding rates of both monarchs and *Tetraopes* are exponential, this second sampling date was employed to uncover a possible delayed effect of the belowground herbivory on monarchs.

Two plant traits, latex exudation and cardenolide concentration, were sampled at the seven-day harvest. Latex was collected by punching a 6 mm diameter hole through the tip of the youngest, fully expanded (and if possible, undamaged) leaf of each plant. The latex was collected until no more exudation was observed (about 10 s after damage) on a previously weighted 1 cm diameter piece of filter paper. Cardenolides were measured from 50 mg of dried root and shoot material following the same procedure as previously described. The effect of aboveground herbivory (*D. plexippus*) and belowground herbivory (*T. tetraophthalmus*) on latex was analyzed with a factorial two-way ANOVA. For analyses of cardenolides, we employed a four-way model to assess the impact of *Tetraopes*, monarchs, plant part (roots vs. shoots), and all interactions. Here root and shoot tissues are not completely independent, and thus a fourth term “plant” (nested within the *Tetraopes* × Monarch interaction) was included in the model to account for the true number of replicates ($n = 56$).

RESULTS

*Induced cardenolides above and below ground
in 12 Asclepias species*

Overall, shoots showed fivefold higher cardenolide concentrations compared to roots (Fig. 2). In the absence of herbivory, the 12 species showed fivefold variation in shoot cardenolides. In the presence of monarch damage, however, there was nearly 13-fold variation in shoot cardenolides among the same 12 species (Appendix A: Table A1). Across species, monarch herbivory increased shoot cardenolides by 46%, but there was substantial variation among *Asclepias* species (Appendix A). Roots showed more modest variation in cardenolides, with 2–3 fold variation across the species in the presence or absence of monarch herbivory. Despite apparent species-specific trends for

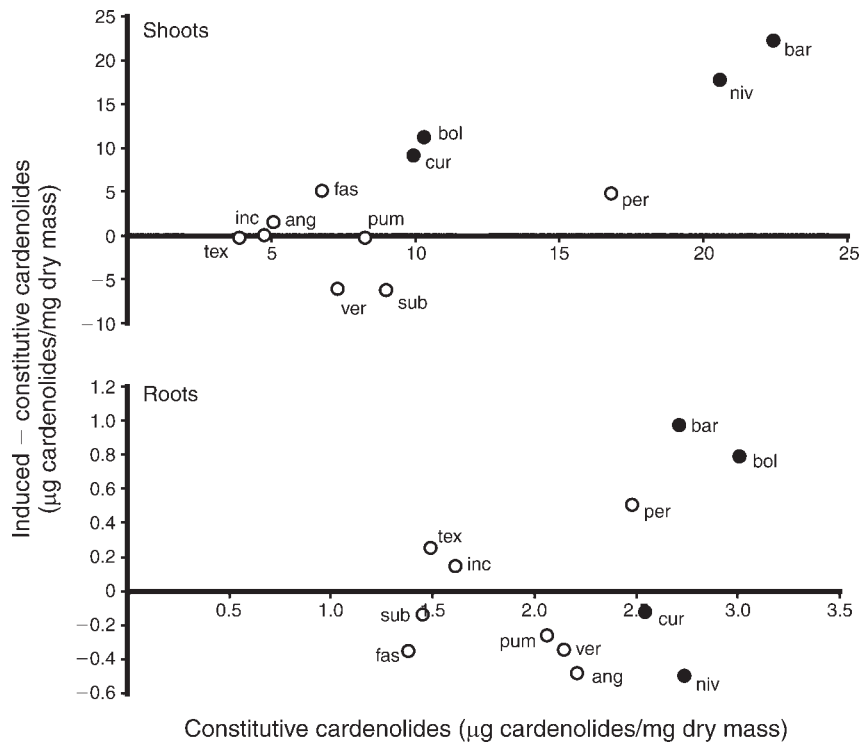


FIG. 3. Raw data for mean investment of constitutive vs. induced (control minus herbivory) cardenolides in shoots and roots for the 12 species of *Asclepias* in the Incarnatae series. Black circles represent species of the tropical clade; open circles represent species of the temperate zone. Three-letter codes refer to the first three letters of species names (see Fig. 2). Both relationships (roots and shoots) had statistically significant regression slopes (after statistical corrections, see *Results: Induced cardenolides above- and belowground in 12 Asclepias species*).

up- and down-regulation of cardenolides after shoot herbivory, neither the treatment nor the interaction term was significant for effects on root cardenolides (Appendix A: Table A1).

We found a correlation between above- and belowground cardenolide concentrations, both without ($n = 12$, $r^2 = 0.45$, $P < 0.017$; independent contrast $\lambda = 0.206$, LR = 4.305 [CI 4.3–4.317], $P = 0.038$) and with aboveground herbivory ($n = 12$, $r^2 = 0.51$, $P < 0.009$; independent contrast $\lambda = 0.393$, LR = 4.357 [CI 4.351–4.63], $P = 0.037$), showing a concerted investment of cardenolide production above and below ground. We found the same result in a survey of 34 milkweed species (Appendix B).

We next used the phylogeny of this group to map similarities in above- and belowground cardenolides of the 12 species (Figs. 2 and 3). For neither root nor shoot cardenolides were we able to estimate a level of phylogenetic conservatism (λ) different from 0 or 1. Our maximum likelihood estimate of λ was 0.188 for shoot cardenolides and 0.566 for root cardenolides (all P s > 0.1 for differences between the estimated λ and set values of 0 or 1, based on the likelihood ratio test). Quite remarkably, however, our measure of shoot-induced response to monarch caterpillars showed a substantial level of phylogenetic conservatism. All four representatives of the tropical clade showed significant induced

cardenolides, whereas none of the north-temperate species showed significant induction: $\lambda = 0.648$ (Figs. 2 and 3). This value was different from 0 (LH = 6.44 [CI 6.433–6.448], $P = 0.011$) and from 1 (LR = 12.004 [CI 12.0–12.013], $P < 0.001$).

Induction was apparently strongest in the species with the highest constitutive levels (the tropical clade; Fig. 3). The exception here is *A. perennis*, which showed high levels of constitutive investment in cardenolides, and no induction (Fig. 3).

To formally test for a relationship between constitutive and induced cardenolides, we ran the Monte Carlo procedure developed by Morris et al. (2006). For shoot data, we found strong evidence for a positive association (the opposite of a trade-off) between constitutive and induced allocation to cardenolides. The observed correlation between induced (damage minus control) and constitutive (control) cardenolides was high ($r = 0.792$, lower 5th percentile of the bootstrap distribution of the correlation coefficient = -0.7413), and the observed slope of the relationship between damaged vs. control species means was positive (2.127, lower 5th percentile = -0.1501). In only 0.33% of the randomized data sets did we find a correlation greater than that observed; thus the probability of seeing a correlation as large (or larger) if there was no association between constitutive and induced resistance is 0.0033.

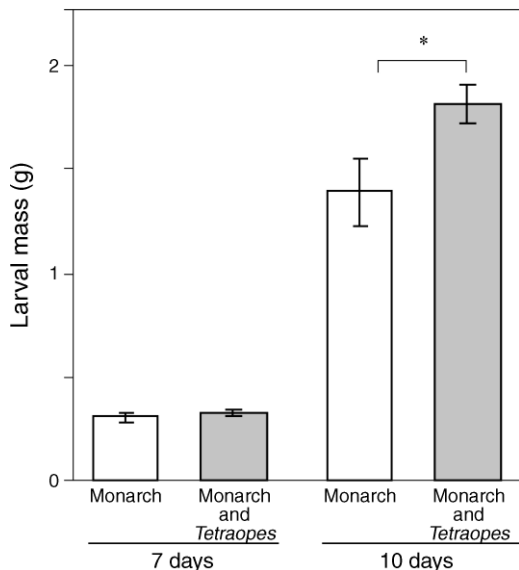


FIG. 4. Mass of monarch caterpillars (mean \pm SE) after seven and 10 days feeding. The asterisk indicates a significant difference ($P < 0.05$) in the 10-day trial.

The same pattern was found for root data: the positive correlation between induced and constitutive cardenolides ($r = 0.377$, lower 5th percentile of the bootstrap distribution of the correlation coefficient = -0.781) and slope (1.33, lower 5th percentile = -0.078). The probability of finding a correlation as large or larger by chance is 0.014. We note that this positive correlation between root constitutive and induced cardenolides exists even though we did not detect a significant effect of aboveground herbivory on root chemistry (i.e., this indicates that there was enough variation to estimate induction, and this correlated with the constitutive state).

Resistance in above- and belowground attacked *A. syriaca*

After 7 days, monarch larvae feeding on plants alone or with *Tetraopes* larvae attacking the roots had similar masses ($F_{1,23} = 0.612$, $P = 0.442$) (Fig. 4). After 10 days of feeding, however, monarchs were 30% heavier on plants attacked simultaneously by the root herbivore compared to caterpillars feeding on plants without *Tetraopes* ($F_{1,13} = 5.324$, $P = 0.038$).

In contrast, we found that latex exudation in the leaves was more than doubled by the feeding of monarchs when compared to the control treatment, but that the effect of monarch herbivory was completely attenuated when plants were attacked belowground by *Tetraopes* larvae (Fig. 5A, two-way ANOVA, monarch $F_{1,56} = 4.683$, $P = 0.035$; *Tetraopes* $F_{1,56} = 1.172$, $P = 0.284$; interaction: $F_{1,56} = 4.942$, $P = 0.030$).

Cardenolide concentrations were unaffected by our treatments in the overall analysis, but shoots had 27%

more cardenolides than roots (Fig. 5B; two-way ANOVA: treatment, $F_{3,104} = 0.719$, $P = 0.543$; plant part, $F_{1,104} = 23.895$, $P < 0.0001$; interaction, $F_{3,104} = 0.693$, $P = 0.558$). Moreover, in analyses of root–shoot cardenolide allocation separated by the four treatments, we found differences between the roots and shoots only in treatments with monarch damage (Fig. 5B; paired t tests: treatment Monarch, difference = -5.254 , $t_{14} < 0.0001$; Monarch and *Tetraopes*, difference = -3.52 , $t_{14} = 0.004$; *Tetraopes*, difference = -1.729 , $t_{15} = 0.106$; control, difference = -1.354 , $t_{13} = 0.2$). We cautiously interpret this result as monarch feeding increasing allocation of cardenolides from roots to shoots.

DISCUSSION

Phylogenetic analyses of plant defense complement more mechanistic studies of within-species variation and selection (Becerra 1997, Wink 2003, Pelsner et al. 2005, Agrawal 2007). Here we took a two-sided approach, starting with a comparative survey and concluding with experiments on *Asclepias syriaca* attacked by specialist herbivores above and below ground. Our results suggest phylogenetic conservatism for inducibility of shoot

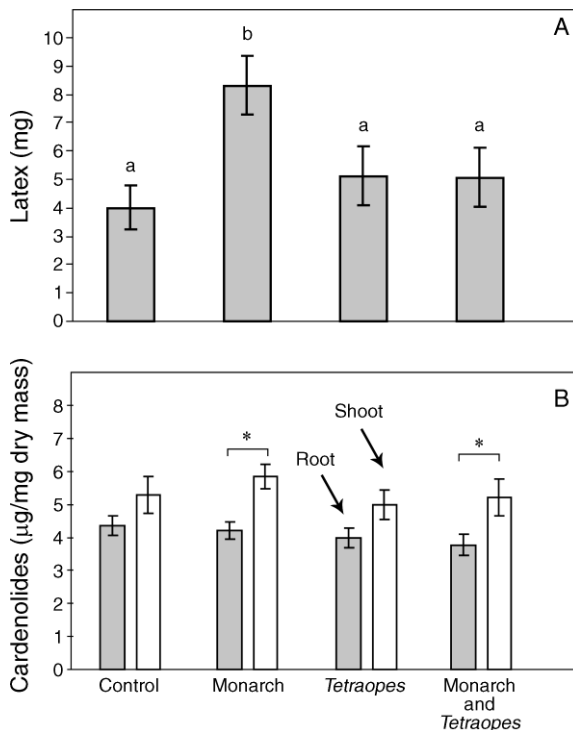


FIG. 5. Defenses in *Asclepias syriaca*: (A) latex exudation from leaves and (B) cardenolide concentration in roots (gray bars) and shoots (open bars); values are means \pm SE. Latex was collected by punching a 6 mm diameter hole through the tip of the youngest, fully expanded (and if possible, undamaged) leaf of each plant and collecting until no more exudation was observed. Different letters above bars in (A) represent significant differences ($P < 0.05$), and asterisks in (B) represent significant differences ($P < 0.05$) between root and shoot cardenolides from paired t tests.

cardenolides. In contrast to long-standing theoretical predictions, we found no evidence for a trade-off between constitutive and induced cardenolides; indeed allocation to the two was strongly positively correlated in both shoots and roots. In addition, allocation to above- and belowground organs was concerted, and these values were positively correlated across species in phylogenetically independent contrasts from two different experiments. Finally, cardenolides were preferentially allocated to shoots over roots in *A. syriaca*, and both root and shoot herbivores affected latex production. These effects had consequences for monarch caterpillar growth.

Evolution of cardenolide defense in roots and shoots

Our estimates of phylogenetic conservatism for the Incarnatae clade of *Asclepias* (12 species) were not distinguishable from either 1 or 0 (in both roots and shoots). In larger data sets we have found some evidence for phylogenetic conservatism in root and shoot cardenolides (Appendix B; Agrawal et al. 2009). Nonetheless, this analysis of 12 species revealed a substantial level of conservatism for shoot inducibility. The interpretation of this result ($\lambda = 0.648$, significantly different than both 0 and 1) is that differences in inducibility between species are phylogenetically constrained, but show less similarity among relatives than would be predicted from their genetic divergence (Freckleton et al. 2002). Remarkably, these patterns of inducibility follow a pattern of both phylogenetic and biogeographic constraint: only the tropical species showed significant levels of induction (Figs. 2 and 3).

A recent literature survey of belowground constitutive defenses showed a full panoply of chemicals present in roots that are typically measured in leaves (Kaplan et al. 2008a, Rasmann and Agrawal 2008). However, there was no consistent pattern of greater or lesser allocation of defenses to roots compared to shoots across taxa (e.g., Zangerl and Rutledge 1996, Adler and Wink 2001). Our results with *Asclepias* showed correlated evolution (i.e., convergence) in allocation to roots and shoots; species that allocate to one organ proportionally allocate to the other. Nonetheless, we found in our analysis of the 12 Incarnatae species and *A. syriaca* that shoots had consistently higher levels of cardenolides than roots.

Both genotype and the environment can modify the levels of defense in roots and shoots. For example, different plant genotypes receiving the same treatment can show different patterns of chemical induction. Root and shoot pyrrolizidine alkaloids in *Senecio jacobea* were genotypically variable following root or shoot herbivory (Hol et al. 2004). The same genotype induced by different treatments (herbivory, mechanical damage, or phytohormones) also can show different patterns of induction, as was demonstrated for glucosinolates in *Brassica* spp. (van Dam et al. 2004, van Dam and Raaijmakers 2006), and phytoecdysteroids in *Spinacia*

oleracea (Schmelz et al. 1999, 2002). Here we have shown that aboveground herbivory can enhance latex exudation in *A. syriaca*, yet the presence of a belowground herbivore can attenuate the induction of latex exudation down to control levels. This result agrees with previous work on latex induction that showed specificity in the strength of the response to different species of herbivore damaging the plant (Van Zandt and Agrawal 2004).

Trade-offs between constitutive and induced responses

It is generally assumed that anti-herbivore defenses are costly for plants (Karban and Myers 1989, Zangerl and Bazzaz 1992, Karban and Baldwin 1997). For example, it has long been predicted that constitutive and induced defenses should trade off (Koricheva et al. 2004). Recently, however, Morris et al. (2006), have argued that tests of this hypothesis have been statistically flawed because traditional methods to detect trade-offs have not accounted for limited sample size, errors in measuring resistance, and issues of induced susceptibility (i.e., a decrease in resistance traits after damage). In our analyses employing the methods of Morris et al. (2006), we found no evidence for a trade-off; on the contrary we observed positive correlations between constitutive and induced cardenolides in the shoots and roots of *Asclepias*. However, we note that this result was driven partly by phylogenetic nonindependence (i.e., four closely related tropical species showed high constitutive cardenolides and high inducibility) (Fig. 4). At the current juncture, we cannot combine phylogenetically independent analyses with the statistically correct tests of trade-offs. However, all other correlations examined in this study were robust to phylogenetic correction, and it appears that there has been positive correlated evolution of root and shoot cardenolide allocation and constitutive and induced responses in *Asclepias*.

Although there is an apparent general trend for trade-offs between constitutive and induced resistance from previous analyses (which have not employed the corrections introduced by Morris et al. [2006], reviewed by Koricheva [2004]), our result of a positive association is not unprecedented (e.g., Zangerl and Berenbaum 1990). Only two other studies have taken a comparative approach across taxa to address this question. Thaler and Karban (1997) also reported a positive correlation between constitutive and induced resistance across 21 wild *Gossypium* (cotton) species, and suggested that constitutive resistance was the ancestral state. Heil et al. (2004), on the other hand, showed the opposite pattern. Facultative myrmecophytic *Acacias* had highly inducible defenses, but derived species with obligate associations with ants had evolved constitutive traits. Thus, constitutively low species showed strong induction and constitutively high species showed no induction, suggesting a trade-off between these two modes of defense.

More complex patterns of constitutive and induced resistance have also been suggested. For example, Zangerl and Rutledge (1996) highlighted differential inducibility between organs in wild parsnip (*Pastinaca sativa*). They showed that flowering parts, which were more likely to be under herbivore attack, contained the highest constitutive concentration of furanocoumarins, while roots, which are exposed to less herbivory, were strongly inducible. Although the relative magnitudes of attack on different plant parts are unknown for *Asclepias* species, aboveground herbivory and root herbivores could both act as potent agents of selection (Wilbur 1976, Price and Willson 1979, Zalucki and Kitching 1982, Ackery and Vane-Wright 1984, Zalucki et al. 1990, Farrell and Mitter 1998, Farrell 2001, Agrawal 2004, 2005). The highly variable investment in root and shoot defense we saw in our survey data provides testable hypotheses for the probability of attack on these plant organs among the *Asclepias* species.

*Manipulation of resistance traits
with above- and belowground herbivory*

We observed that in previously undamaged plants, shoot herbivory increased latex exudation by 48%, but root herbivory reduced it to comparable control levels (Fig. 5). This effect of the root herbivore *Tetraopes* on latex production was reflected in monarch larval mass, which increased in response to root damage (Fig. 4). Although we did not establish a causal correlation between latex exudation and larval mass in this study, this relationship has been studied in detail, showing that latex exudation reduces larval survival and growth (Dussourd and Eisner 1987, Zalucki et al. 2001, Van Zandt and Agrawal 2004).

Other studies have shown that root herbivory can benefit aboveground herbivores (Gange and Brown 1989, Masters et al. 1993), increase preference for damaged plants (Masters et al. 2001), and even reduce the attractiveness of the plant to predators or parasitoids of herbivores (Rasmann and Turlings 2007). However, the reverse has also been shown: foliar herbivores were affected negatively by enhanced levels of terpenoid aldehydes in the leaves of cotton plants after root herbivory (Bezemer et al. 2002). Agrawal (2004) found that root herbivory in *A. syriaca* plants reduced the abundance of leaf-mining flies by 40%, but only for milkweeds that experienced plant competition.

The specificity of associations between above- and belowground organisms sharing a common resource raises two general predictions. First, the intimate interactions between a herbivore and its host plant seem to be dependent on the presence of other herbivores exploiting the plant. Second, the evolution of plant defenses may involve an intricate combination of above- and belowground selection pressures. Recent work confirms the former prediction (Kaplan et al. 2008a). More work is required to elucidate possible patterns in interactions between above- and below-

ground systems that may shape the evolution of plant defense strategies.

Although roots are often similarly (e.g., Wainhouse et al. 1998, Marak et al. 2002, Agerbirk et al. 2003), or even more chemically protected than shoots (e.g., Bezemer et al. 2004, Jorgensen et al. 2005, Walls et al. 2005), we found latex exudation only in the aboveground parts of *A. syriaca*. This is a relatively rare example of strong segregation of defenses. Trichomes and spines may be a similarly segregated form of physical defense. The segregation of latex may result from anatomical or developmental constraints in the production of laticifers (i.e., the canals responsible for latex transportation and exudation), or the fitness consequences for *A. syriaca* of maintaining such traits below ground. Although none of these hypotheses has been investigated, it is clear that belowground herbivory can be substantial for *A. syriaca*, and thus there is potentially strong selection for root defense (Agrawal 2004). Other latex-bearing species (e.g., several Asteraceae and *Ipomoea*) do contain latex in the roots (Farrell et al. 1991, Snook et al. 1994; *personal observations*).

Previous work has shown that cardenolides are inducible by 33% in the aboveground tissues of *A. syriaca* that are attacked by monarch caterpillars or treated with jasmonic acid (Mooney et al. 2008). Thus, it was somewhat surprising that we found no direct evidence for monarch-induced cardenolides in *A. syriaca* in this study, although we did find monarch-inducible cardenolides in four other species (Fig. 2). Our work here, both on *A. syriaca* and 12 other *Asclepias* species, however, shows no impact of shoot or root herbivory on root cardenolides. Thus, if there are inducible responses to *Tetraopes* herbivory, they do not appear to be in the form of known direct defenses (i.e., no latex in roots and cardenolides were not affected by root herbivory). We are currently investigating induced indirect defense in milkweed roots; preliminary work shows that entomopathogenic nematodes (*Heterorhabditis bacteriophora*) are extremely deadly to *Tetraopes* larvae and are attracted by *Tetraopes*-infested plants, as previously shown in other systems (Rasmann et al. 2005). Assessing the relative importance of direct defenses (cardenolides) and indirect defense (nematodes) below ground will help to clarify patterns of defense allocation and induction following above- and belowground herbivory.

CONCLUSIONS

We speculate that a combination of selection by herbivores, abiotic factors, plant physiological constraints, and phylogenetic constraints have led to the patterns of defense allocation that we see above and below ground in the milkweeds. Less than one-third of the *Asclepias* species are attacked by specialist *Tetraopes*, and the determinants of this specificity are currently under investigation. Perhaps some conditions (e.g., wetlands or shaded habitats) are inhospitable for

root herbivores. Nonetheless, root defenses are not independent from shoot defenses, as the two show positive correlated evolution. Although trade-offs are certainly important in the evolution of defense strategies (e.g., Fine et al. 2004), trade-offs are not universal and concerted patterns of defense allocation appear to be the rule (Agrawal and Fishbein 2006, Agrawal et al. 2008). Finally, phylogenetic conservatism appears to be less constraining in the evolution of defense chemistry itself, but inducibility is more strongly phylogenetically constrained. Future work will assess the role of phytohormonal signaling pathways in the evolution of this induction.

We thus concur with recent calls for the simultaneous study of above- and belowground defense allocation (Van der Putten et al. 2001, van Dam et al. 2003, Bezemer and van Dam 2005, Preisser et al. 2007). Because belowground herbivory is common, and because above- and belowground herbivory result in the manipulation of defense allocation systemically, the time has come for integrating root biology into plant defense theories.

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APPENDIX A

ANOVA table for results of 12 *Asclepias* species (*Ecological Archives* E090-169-A1).

APPENDIX B

Bar graph of root cardenolides in 34 species of *Asclepias* (*Ecological Archives* E090-169-A2).

Ecological Archives E090-169-A1

Sergio Rasmann, Anurag A. Agrawal, Susan C. Cook, and Alexis C. Erwin. 2009.
Cardenolides, induced responses, and interactions between above- and belowground herbivores of milkweed (*Asclepias* spp.). *Ecology* 90:2393–2404.

Appendix A. Three-way ANOVAs (and separate and two-way analyses on root and shoot data independently) for effects of species and monarch herbivory on cardenolides of 12 *Asclepias* species.

Analysis	Source	df	<i>F</i> ratio	<i>P</i> value
Root and shoot	plant part (P)	1, 170	216.735	<0.0001
	treatment (T)	1, 170	11.363	0.0009
	species (S)	11, 170	16.641	<0.0001
	P × T	1, 170	11.031	0.001
	P × S	11, 170	13.573	<0.0001
	T × S	11, 170	2.939	0.001
	P × T × S	11, 170	2.741	0.003
Root analysis	T	1, 84	0.035	0.853
	S	11, 84	4.613	<0.0001
	T × S	11, 84	0.54	0.871
Shoot analysis	T	1, 84	11.579	0.001
	S	11, 84	15.573	<0.0001
	T × S	11, 84	2.895	0.003

Ecological Archives E090-169-A2

Sergio Rasmann, Anurag A. Agrawal, Susan C. Cook, and Alexis C. Erwin. 2009. Cardenolides, induced responses, and interactions between above- and belowground herbivores of milkweed (*Asclepias* spp.). *Ecology* 90:2393–2404.

Appendix B. Bar graph of root cardenolides in 34 species of *Asclepias*.

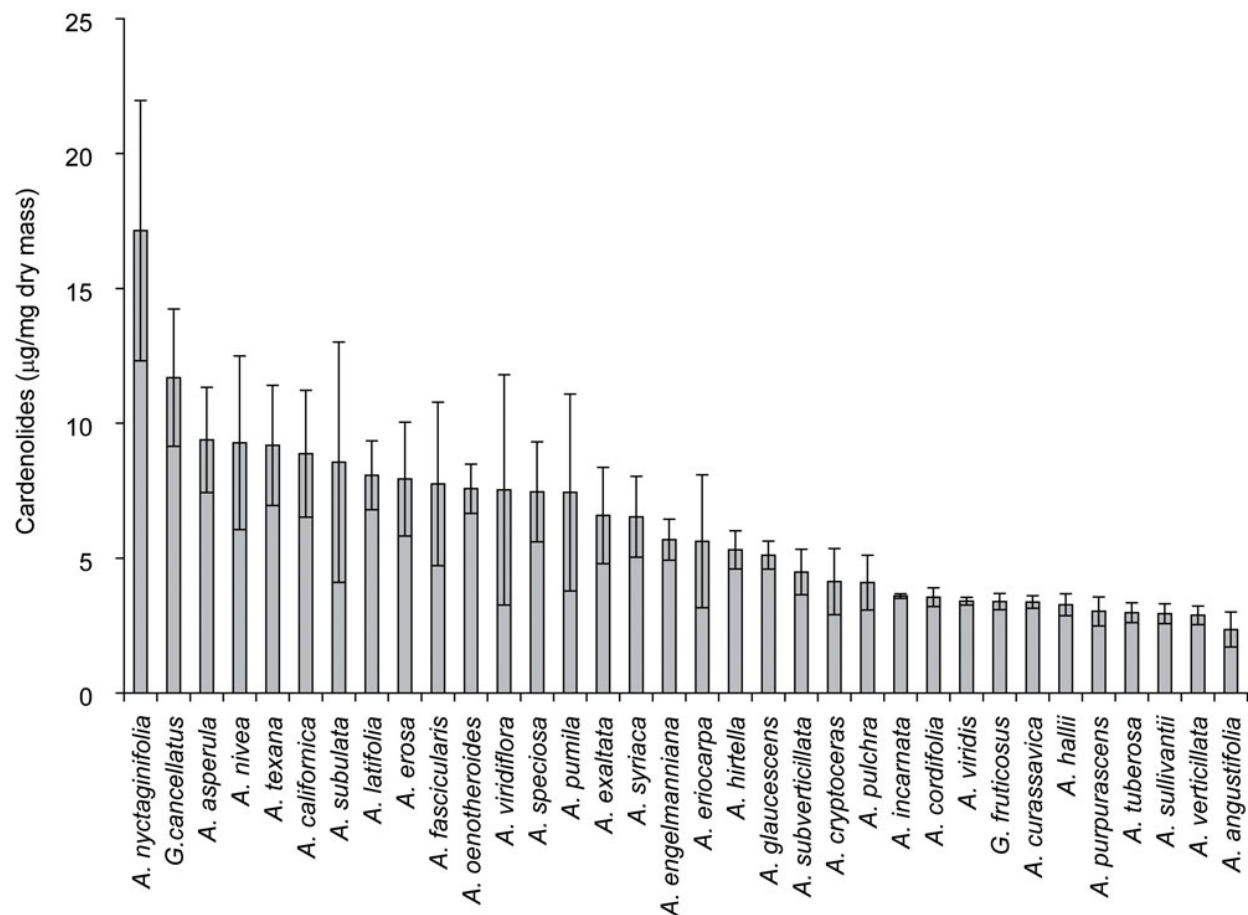


FIG. B1. Cardenolide concentration ($\mu\text{g}/\text{mg}$ of dry tissue, mean \pm SE) in the roots of 32 *Asclepias* and two *Gomphocarpus* species. *A. pulchra* is a subspecies of *A. incarnata*. ($n = 2\text{--}6$ plants/species, ANOVA, $F_{33,122} = 3.35$, $P < 0.0001$). Root cardenolides were compared to a previously published data set of shoot cardenolides from the same set of plants (Agrawal and Fishbein 2008) in order to assess allocation to root and shoot cardenolides. We found that root and shoot cardenolides were positively correlated ($n = 34$, $r = 0.348$, $P = 0.044$), and this was corroborated when using a phylogenetically corrected analysis ($\lambda = 0.709$, LH = 10.263 [CI 10.249–10.286], $P < 0.001$, see text for explanation of statistical analysis).

LITERATURE CITED

Agrawal, A. A., and M. Fishbein. 2008. Phylogenetic escalation and decline of plant defense strategies. *Proceedings of the National Academy of Sciences of the United States of America* 105:10057–10060.