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LETTER

Latitudinal patterns in plant defense: evolution of cardenolides, their toxicity and induction following herbivory

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Abstract

Attempts over the past 50 years to explain variation in the abundance, distribution and diversity of plant secondary compounds gave rise to theories of plant defense. Remarkably, few phylogenetically robust tests of these long-standing theories have been conducted. Using >50 species of milkweed (*Asclepias* spp.), we show that variation among plant species in the induction of toxic cardenolides is explained by latitude, with higher inducibility evolving more frequently at lower latitudes. We also found that: (1) the production of cardenolides showed positive-correlated evolution with the diversity of cardenolides, (2) greater cardenolide investment by a species is accompanied by an increase in an estimate of toxicity (measured as chemical polarity) and (3) instead of trading off, constitutive and induced cardenolides were positively correlated. Analyses of root and shoot cardenolides showed concordant patterns. Thus, milkweed species from lower latitudes are better defended with higher inducibility, greater diversity and added toxicity of cardenolides.

Keywords

above- and belowground defenses, herbivory, induced defense, milkweeds, monarch butterfly, phytochemical diversity, root, shoot, tradeoffs, tropical defense hypothesis.

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INTRODUCTION

Widespread literature suggests that plants from lower latitudes experience stronger biotic interactions (Pennings *et al.* 2009; Schemske *et al.* 2009) and therefore should invest in higher levels of defenses (Coley & Aide 1991). Indeed, tropical plants are more likely to produce toxic alkaloids (Levin & York 1978), latex (Lewinsohn 1991) and typically have tougher leaves of lower nutritional quality than temperate species (Coley & Aide 1991). In marine systems, tropical algae tend to have a greater diversity of secondary metabolites, to be better defended, and to have stronger deterrent properties than temperate algae (Bolser & Hay 1996). A comprehensive recent review, however, indicates that only six of 16 studies found higher levels of herbivory at lower latitudes (Moles *et al.* 2011). For plant defenses, despite the strong patterns described above, screens of other secondary metabolites such as tannins and flavonoids showed mixed results, and apparently less than a quarter of studies showed increased production of toxic secondary metabolites at lower latitudes (Moles *et al.* 2011).

Although most of the early studies cited above were comparative in nature, phylogenetically rigorous tests that account for evolutionary history were lacking. Addressing evolutionary history is important, however, because patterns in defense may be confounded with phylogenetic patterns. For example, directional latitudinal trends in defense may be caused by the high diversity of particular tropical lineages. By controlling for phylogenetic history, we can ask whether higher levels of defense in the tropics are the result of repeated independent evolution, which would suggest that higher defenses are adaptive.

A potential cause of the lack of resolution about large-scale patterns in plant defense comes from the fact that it is still unclear whether the evolution of defense proceeds via changes in the amount of particular compounds, phytochemical diversity or other aspects of chemical toxicity (Table 1). Debate around the evolutionary and ecological significance of defense compounds has centred on why there is such a diversity of secondary compounds, even within a species. Are all of the secondary metabolites in a plant biologically active or does phytochemical diversity serve little role other than increasing the probability of producing a few biologically active compounds when ecological circumstances requiring defense arise (Jones & Firn 1991; Berenbaum & Zangerl 1996; Rasmann & Agrawal 2009)? Phylogenetic analyses of phytochemical diversity showed that some plants tend to increase defence during evolutionary history by increasing chemical structural complexity or other aspects of toxicity (Becerra *et al.* 2009). A survey of >30 *Asclepias* species, on the contrary, showed a directional macroevolutionary trend of decreasing total cardenolides in favour of increased investment in plant tolerance to damage (Agrawal & Fishbein 2008). Mixtures of secondary compounds can also have a synergistic ecological effect, causing greater negative impacts on herbivores than when compared with equal amounts of single compounds (Berenbaum & Zangerl 1996).

Inducibility, or the ability to increase defensive traits after herbivore attack, has classically been viewed as a way for plants to cope with high resource demands and the unpredictability of herbivore attack (Zangerl & Bazzaz 1992; Karban *et al.* 1999). Theory predicts that because constitutive and induced defences ought to compete for resources, they should trade off among genotypes or species (Zangerl & Bazzaz 1992; Agrawal *et al.* 2010; Rasmann *et al.* 2011), nonetheless, evidence for tradeoffs between constitutive and induced defense is mixed, (Morris *et al.* 2006; Bingham & Agrawal 2010; Rasmann *et al.* 2011).

Milkweeds in the Pan-American genus *Asclepias* are optimal candidates to investigate classic plant defence theories. They have a well-characterized defensive arsenal (including toxic cardenolides and latex) (Zalucki *et al.* 2001), tremendous variation among species (Agrawal & Fishbein 2008), and known phylogenetic relationships

Table 1 Predictors of plant chemical toxicity and evidence for cardenolides. In particular, the toxic effects of cardenolides may increase with (1) overall amount, (2) phytochemical diversity, (3) more lipid-soluble (and thus more membrane-permeable) compounds and (4) higher inducibility.

Chemical measure	Why toxic?	Evidence for cardenolides
Amount (μg mg^{-1} dry tissue)	Dose dependent effects	Cohen 1985 Rasmann *et al.* 2009b Rasmann *et al.* 2011
Diversity (number of distinct peaks per sample)	Difficult to deal with chemical mixtures	No tests with cardenolides, but evidence from Berenbaum & Zangerl 1996
Polarity (estimated by HPLC retention time)	Less polar compounds are more able to cross membranes	Malcolm & Zalucki 1996 Rasmann *et al.* 2009b de Roode *et al.* 2008
Inducibility (absolute increase following herbivory)	Dose dependent effects, variability in food quality	Karban *et al.* 1997 Rasmann *et al.* 2011

(Fishbein *et al.* in press). Cardenolides (or cardiac glycosides) disrupt the sodium and potassium flux in cells, and occur in all milkweed tissues and in latex (Malcolm 1991). Despite insect behavioural and physiological adaptations to reduce cardenolide exposure and toxicity (Dussourd & Eisner 1987; Holzinger & Wink 1996), several lines of evidence show that cardenolides continue to be detrimental to both above and belowground herbivores (Zalucki *et al.* 1990; Agrawal 2005; Rasmann *et al.* 2011). However, there is an indication that not all cardenolides are equally toxic. The core aglycone steroidal structures of cardenolides can be decorated with many structural sugar groups (the 'glycoside' of cardiac glycosides), which alter the physico-chemical properties of the molecule. Cardenolides with different polarities are absorbed at different rates through the insect gut, with non-polar cardenolides being more readily absorbed than polar ones (Malcolm 1991) and therefore, more detrimental to the herbivores (Scudder & Meredith 1982; Malcolm 1991; Petschenka & Dobler 2009; Rasmann *et al.* 2009b).

In this study, we revisit classic hypotheses of plant defense by assaying aboveground tissues in 51 species of milkweeds and root tissues in 18 species, using phylogenetically controlled analyses. Specifically we asked: (1) Are there latitudinal gradients in plant defenses? (2) What is the relationship between different axes of cardenolide defense? (Table 1) and (3) Are there tradeoffs between constitutive defense and its inducibility?

MATERIALS AND METHODS

Milkweed biogeography

The *Asclepias* is a monophyletic group of about 130 species in North America, Mesoamerica and the Caribbean, with six additional species endemic to South America (Woodson 1954; Bollwinkel 1969; Fishbein *et al.* in press). *Asclepias* species are found from southern Canada to central Argentina. To map the distribution of the 51 species used herein, we recorded the maximum, minimum and mean latitude for each of the species' distributions using Woodson (1954), Bollwinkel (1969), available online data (http://plants.usda.gov) and personal comments from M. Fishbein.

Aboveground cardenolides collection

Seeds of 51 species (49 *Asclepias* species and two species from the African sister genus *Gomphocarpus*) were collected by the authors and their colleagues or purchased from native plant nurseries (sources are given in the Acknowledgements) (Table S1 Supporting information). All plants (6–12 plants per species) were germinated in a warm (28 °C), dark chamber after stratifying the seeds at 4 °C on moist filter paper for 2 weeks. One seedling per pot (10 cm diameter pots) was transplanted into potting soil (Metro-Mix Sun Gro Horticulture Canada CM Ltd., Vancouver, British Columbia, Canada) and grown in a growth chamber (12 h daylight, 26 °C day : 20 °C night) for 4 weeks before harvesting. Plants were watered *ad libitum* and fertilized [N : P : K 21 : 5 : 20 150 ppm N (g/g)] once every week. To test for aboveground inducibility of cardenolides, after 30 days of growth, leaves of approximately half of the plants (3–6 per species) were exposed to one-first-instar monarch butterfly caterpillar (*Danaus plexippus* L.), a species that feeds almost exclusively on *Asclepias* spp. The other plants (3–7 per species) remained undamaged. Three days after cessation of the herbivory treatment (*c.* 10% leaf damage per plant), all plants were harvested and aboveground tissue was flash frozen before being dried in a drying oven at 40 °C.

Root cardenolide collection

To assess root cardenolide production, we additionally germinated and grew 18 species of *Asclepias* (n = 10 replicates per plant species), which comprised a subset of the previous 51 species (Table S1). Plants were grown using the methods above, except that plants were placed out of doors (on a rooftop patio). After a month of growth, plants were exposed to five, first-instar larvae of the specialist cerambycid beetle *Tetraopes tetraophthalmus*, by placing the larvae about 1 cm deep within the rhizosphere of the plant. *Tetraopes tetraophthalmus* is nested within the New World clade of *Tetraopes* (Farrell & Mitter 1998). Adults feed on leaves and flowers, whereas larvae feed exclusively on roots of the milkweeds. *Tetraopes tetraophathalmus* adults were collected on naturally occurring *A. syriaca* patches around Ithaca, NY, USA and kept in large ventilated containers (30 × 20 × 15 cm) in the laboratory. Males and females were provided with fresh milkweed leaves and oviposition sites (dried grass stems). The oviposition substrate was removed from the rearing boxes every third day and incubated in the dark at 27 °C for 7–10 days. Newly hatched larvae were kept in large petri dishes (10 cm diameter) on moist filter paper for a maximum of 24 h before adding them to the roots of the experimental plants. Plants and herbivores were then left to grow for an additional month before harvesting root material, which was flash frozen in liquid nitrogen and oven-dried at 40 °C for 3 days. Root and shoot cardenolides were

subsequently extracted in methanol and amount was determined by UV absorption using HPLC according to Rasmann *et al.* (2009b).

Cardenolide diversity analyses

Leaf and root cardenolides of each species were aligned along the time axis of the HPLC chromatogram. Based on our protocol and column type, early-eluting peaks are considered to be more polar than later-eluting peaks (Rasmann *et al.* 2009b). Each species was recorded with the total number of cardenolide peaks (richness) and the total amount (the sum of all individual peaks). We arbitrarily considered species to have the same type of cardenolide when there was less than 0.2 min difference between peak retention times in a total run of 35 min. In addition to cardenolide richness and amount, we sought to assess relative distribution of cardenolides across the time range for each species. Specifically, we borrowed evenness measures from the biodiversity literature to measure peak diversity indices for each species using the Shannon–Wiener index $H = -\text{sum}(P_i \log[P_i])$, where P_i is the relative amount of a given cardenolide peak in a species divided by the total amount of cardenolides in that species. Finally, to provide a measure of cardenolide polarity, we developed a mean polarity index $P = \text{sum}(P_i RT_i)$, where RT_i is the retention time of the ith peak in the species, weighted by each peak's relative amount Pi.

Statistical analysis

A comprehensive phylogeny was pruned with the retention of branch lengths to create a phylogram for the 51 and 18 species experiments, recording shoot and root cardenolides respectively (Fishbein *et al.* in press). We used simple Pearson's correlations to test for the relationship between mean latitude of species and total cardenolide production, diversity and polarity indices, both aboveground and belowground.

In addition to raw correlations, we tested for the effect of shared evolutionary history in a maximum likelihood phylogenetic generalized least squares (PGLS) framework using Pagel's Continuous, implemented in BayesTraits (Pagel 1999). Using PGLS, models of trait evolution differing in complexity can be compared using a likelihood-ratio test (LR), in which LR = −2[log-likelihood of the better-fitting model – log-likelihood of the worse-fitting model]. Under the assumption of model equivalence, the LR statistic should be chi-square distributed with one degree of freedom. (when a single parameter is altered between the models compared). We first estimated phylogenetic signal of all continuous traits using Pagel's lambda (λ) in Continuous (Pagel 1999). The estimated λ is compared statistically to models, where λ is forced to either 0 or 1. A λ-value of 1 indicates phylogenetic signal consistent with a random-walk model (i.e. trait similarity is directly proportional to the extent of shared evolutionary history). A λ-value approaching 0 indicates that the data fit a model where there is no relationship between shared ancestry and trait values (i.e. phylogenetic independence). For the phylogenetic independent correlation analyses, the LR parameter was estimated from a random-walk model (λ = 1) with and without an estimated covariance. When the raw and the PGLS analysis gave the same result, only the latter is presented, otherwise, both analyses are presented.

To test for potential negative correlations between constitutive cardenolides (total amount, diversity and polarity) and their inducibility (i.e. the difference in mean phenotype values for each species between control and damaged plants), we conducted tests to control for potential spurious correlations in such analyses. Specifically, we employed the test outlined by Morris *et al.*(2006) developed in Matlab (version 7.5.0.342 – R2007b, MathWorks Inc., USA). This statistical approach accounts for several issues that have apparently confounded previous attempts to assess a tradeoff between constitutive and induced resistance (Morris *et al.* 2006). Specifically, it 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). In addition, we performed phylogenetically controlled analyses to test for correlations between mean constitutive cardenolide levels and their inducibility.

RESULTS

Diversity of cardenolides and phylogenetic signal

Across 49 species of *Asclepias* and two species of the sister genus *Gomphocarpus,* we found 86 unique cardenolide peaks in the leaves, with a maximum of 33 peaks for *A. incarnata* ssp. *pulchra* and a minimum of two peaks for *A. tuberosa*. In the roots of 18 species, we found 47 unique peaks with a maximum of 19 peaks in *A. asperula* and a minimum of three in *A. subverticillata* (Table S1). In addition, we found a positive relationship between species ranking of total root vs. shoot cardenolide amount (here species are ranked to control for potential heterogeneity among two different growing environments) ($n = 18$ species, PGLS analysis, likelihood ratio (LR) = 8.608, $P = 0.003$).

We then estimated phylogenetic signal for each trait (Table S2). Both inducibility of shoot cardenolides and species mean latitude showed phylogenetic signal consistent with Brownian motion evolution (i.e. λ = 1) (Table S2). Phylogenetic signal for constitutive cardenolide levels, their absolute number and the diversity index in shoots was estimated at less than 1 (λ = 0.646, 0.408, 0.464 respectively), but their interpretation is uncertain because none of these values was statistically distinguishable from 0 to 1. Root cardenolides also carried some phylogenetic signal (λ = 0.709 for constitutive and 0.682 for inducibility), which was significantly different from 0 to 1 (Table S2). All other traits, including our index of cardenolide polarity showed little or no phylogenetic signal.

Biogeography of milkweed defenses

Are there latitudinal gradients in plant defenses?

Across 51 species, constitutive foliar cardenolides, and more so, their inducibility, declined when moving away from the equator (Fig. 1, Table S3, raw correlation for shoot constitutive cardenolides $r = -0.277$, $P = 0.049$, PGLS LR = 0.562, $P = 0.453$ and their inducibility, PGLS; $r = -0.329$, LR = 4.47, $P = 0.034$). Root cardenolides also showed a negative relationship between latitude and constitutive cardenolide levels, but only before accounting for phylogenetic non-independence (Table S3, raw correlation for root constitutive cardenolides, $r = 0.479$, $P = 0.044$, PGLS LR = 1.506, $P = 0.219$) and not for root inducibility (PGLS LR = 0.006,

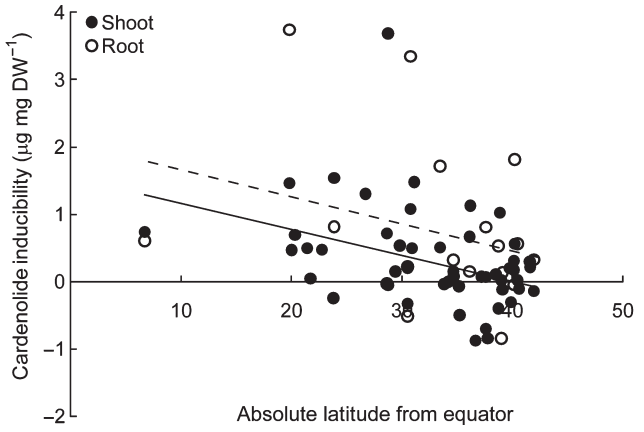

Figure 1 Negative relationship between shoot and root inducibility of cardenolides (change in amount between damaged and control plants) as a function of their latitudinal distribution. For shoots (black dots, solid line, $n = 51$ milkweeds species), monarch (*Danaus plexippus*) caterpillars were used as the inducing agent and this relationship holds true after accounting for shared phylogenetic history. For roots (open dots, dashed line, $n = 18$ milkweeds species) *Tetraopes tetraophthalmus* was used as the root herbivore, and this relationship was only significant in a raw correlation.

$P = 0.938$). Total number of cardenolide peaks and diversity also decreased in the shoots of more temperate milkweeds (Table S3, raw correlations, $r = -0.298$, $P = 0.034$ and $r = -0.290$, $P = 0.039$ for peaks and diversity, respectively, but not when accounting for phylogenetic non-independence, for peaks LR = 0.074, $P = 0.786$ and for diversity, LR = 0.538, $P = 0.463$). Finally, polarity of peaks tended to decrease when moving away from the equator, although this result was not significant (Table S3, $r = -0.137$, $P = 0.336$ and LR = 3.002, $P = 0.083$).

Since previous analyses had shown a phylogenetic reduction in cardenolide investment across 51 species of milkweeds (Agrawal *et al.* 2009), we sought to disentangle the potential confounding effect of geographic location (latitude) and that more derived species are also the ones growing further away from the equator (Woodson 1954; Farrell & Mitter 1998). Our strongest result above, that inducibility of foliar cardenolides decreases away from the tropics, persisted even when including node depth of each *Asclepias* spp. as predictor of cardenolide inducibility (multiple regression analysis; LR estimated as the difference between the full model and the model lacking latitude = 5.700, $P = 0.017$ and LR estimated as the difference between the full model and the model lacking node depth = 0.014, $P = 0.906$).

Evolution of cardenolide toxicity

What is the relationship between different axes of cardenolide defense?

To assess how cardenolides have evolved along multiple axes of defense (Table 1), we assessed the relationship between the amounts of cardenolides above and belowground with their diversity, polarity and inducibility. Since all diversity index values (in the constitutive state, damaged state and the inducibility) strongly correlated positively with cardenolide richness (Table S4), we only analysed the total number of cardenolide peaks as our measure of chemical diversity. Also, because trait values in the damaged (induced) state always positively correlated with trait values in the constitutive state (data in Table S4), we only present trait values for the constitutive state and inducibility (i.e. the difference between damaged and control plants).

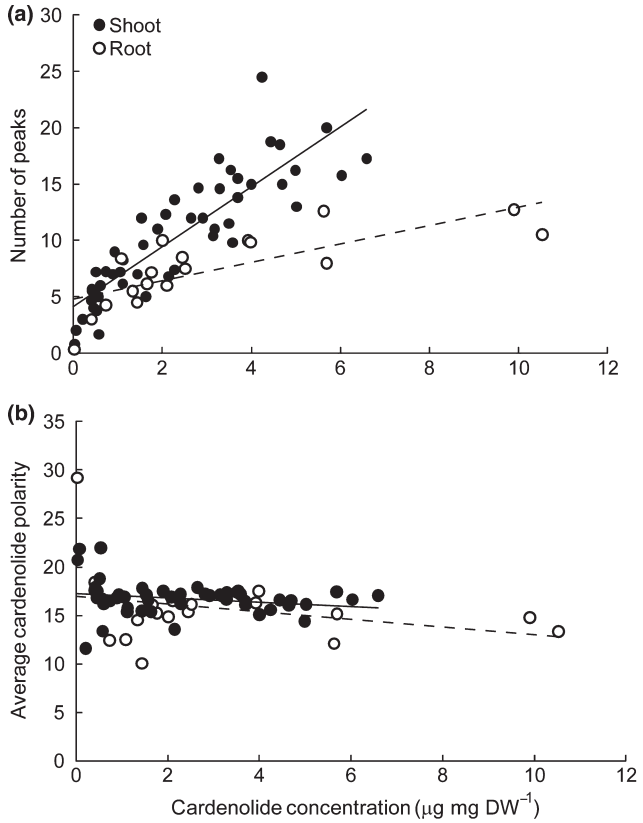

Figure 2 Relationship between constitutive cardenolide amount and (a) the number of individual cardenolide peaks and (b) average constitutive peak polarity. Black dots/solid line represents shoot data, and open dots/dashed line represent root data of 51 and 18 milkweed species respectively. In the top panel (a), the slopes of the regression lines are significantly different from each other ($t_{65} = 6.572$, $P < 0.0001$).

We found that a species' total investment in cardenolides was highly and positively predictive of the number of distinct cardenolides produced (Fig. 2a, Table S4; for shoots: $r = 0.860$, LR = 68.74, $P < 0.0001$; and roots: $r = 0.483$, LR = 4.784, $P = 0.028$). However, we saw differential investment of peak production for the same amount of cardenolides in roots vs. shoots (see different slopes of the regression lines in Fig. 2a), suggesting that roots tend to invest more in an abundance of one or a few cardenolides rather than on higher numbers of peaks. The relationship between inducibility (damaged – constitutive) cardenolide amount and the inducibility of peak number held positive for shoots ($r = 0.592$, LR = 23.278, $P < 0.0001$), but not for roots ($r = 0.354$, LR = 2.404, $P = 0.121$), again indicating that induction in the roots strongly favours the increase of the peaks already present instead of increasing the diversity of cardenolides.

Average peak polarity of cardenolides in shoots was negatively correlated with total amount in the constitutive state (Fig. 2b, Table S4; shoot polarity vs. cardenolides: $r = -0.190$, LR = 7.374, $P = 0.007$), but not for roots ($r = -0.271$, LR = 1.114, $P = 0.291$) and not for inducibility (shoots; $r = -0.081$, LR = 1.636, $P = 0.201$ and for roots; $r = -0.099$, LR = 2.108, $P = 0.147$). This result for shoot cardenolides indicates that as total amounts increase among species, so too does the predicted toxicity of the cardenolides (less polar compounds are more toxic).

Are there tradeoffs between constitutive defenses and their inducibility?

To answer this question we assessed the relationship between constitutive investment and inducibility for all cardenolide traits (total amount, polarity and number of peaks) using two complementary analyses (spurious error corrected correlations and phylogenetically corrected correlations). Since we assume that correlations that are corrected for spurious error are more critical in assessing tradeoffs among constitutive trait values and inducibility (Morris *et al.* 2006; Rasmann *et al.* 2009a), we only pursued the phylogenetic analysis if the first analysis was significant. Contrary to predictions, instead of tradeoffs, we found positive relationships between constitutive cardenolides and their inducibility in both shoots and roots (Fig. 3a, for shoots, correlation corrected for spurious correlations: $r = 0.484$, $P = 0.007$ and PGLS LR = 11.886, $P = 0.005$; roots, correlation corrected for spurious correlations: $r = 0.794$, $P = 0.006$ and PGLS LR = 6.262, $P = 0.012$).

We further found some evidence for a relationship between constitutive polarity and its inducibility in shoots (Fig. 3b, corrected for spurious correlations: $r = -0.3249$, $P = 0.064$, PGLS: LR = 21.774, $P < 0.0001$) and roots (Fig. 3b, corrected for spurious correlations: $r = -0.541$, $P = 0.02$; PGLS: LR = 35.74, $P < 0.0001$). This latter result for roots is, however, driven by one species (*A. tuberosa*, see outlier in Fig. 3b for root data), which has very few peaks, and all very polar (Table S1, spurious corrected-correlation without *A. tuberosa*, $r = -0.275$, $P = 0.214$). Thus, for the shoot data, plant species with more polar constitutive cardenolides tended to induce more non-polar forms. Finally, we did not find a relationship between constitutive number of individual peaks and their inducibility for shoots (Fig. 3c; corrected for spurious correlations: $r = 0.038$, $P = 0.244$) or roots (Fig. 3c corrected for spurious correlations: $r = -0.314$, $P = 0.266$).

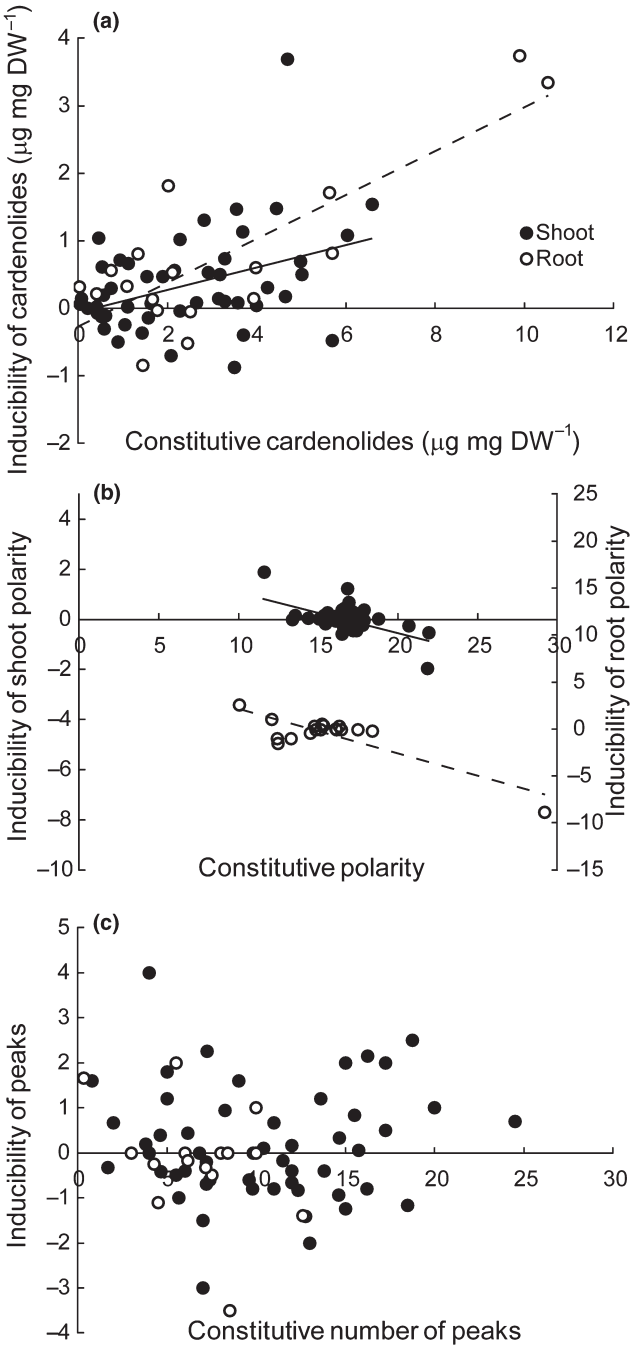

Figure 3 Relationships between constitutive levels and the inducibility (damaged – control) of (a) total cardenolides, (b) index of polarity for cardenolides and (c) number of peaks. Black dots/solid line represent shoots and open dots/dashed line represents roots of 51 and 18 species of milkweed, respectively. In the middle panel (b), a secondary *y*-axis for root cardenolides is shown on the right side of the graph for ease of representation.

DISCUSSION

In accordance with biogeographic hypotheses about variation in biotic interactions and the evolution of plant defense, we found that milkweed species from lower latitudes invest more in defense against herbivory than more temperate species, and this was primarily driven by increasing inducibility of cardenolides in lower latitude species. Although latitude itself is not the ultimate cause of this effect, it is concordant with hypotheses about biotic selection pressures being the strongest at lower latitudes (Pennings *et al.* 2009; Schemske *et al.* 2009). We additionally found that species with higher levels of constitutive cardenolide amounts also produced a higher diversity of cardenolide types, more non-polar (and likely more toxic) forms, and showed greater induction following attack by monarch butterfly caterpillars. These relationships, which hold true after accounting for the phylogenetic non-independence of the species, strongly support the notion of positive-correlated evolution along multiple axes of cardenolide defense.

Why so many types of cardenolides?

There are two major hypotheses for why individual plant species produce many different types of closely related secondary compounds. First, increasing the diversity of compounds (especially those that are not costly) may simply increase the probability that one compound is highly active against a specific consumer (Jones & Firn 1991). Second, chemical diversity *per se* enhances plant resistance against the wide variety of organisms interacting with the plant (Berenbaum *et al.* 1991). Berenbaum & Zangerl (1996) tested the idea that diversity of secondary metabolites primarily functions to increase the likelihood of at least one being highly defensive. Contrary to expectations, they found that furanocoumarins in *Pastinaca sativa* are equally and effectively toxic to a wide variety of herbivores. In contrast, however, there exists a large number of natural products with no known or very low activity [e.g. only a few of the 100-plus gibberellins have a known

biological activity, but those few that are active are potent at nanomolar amounts (Fischbach & Clardy 2007)]. Additional to the single compound activity, the production of some chemical mixtures can synergistically improve the activity of compounds when compared with the sum of each individual compound individually (Berenbaum *et al.* 1991; Duffey & Stout 1996; Steppuhn & Baldwin 2007; Rasmann & Agrawal 2009). To our knowledge, nobody has tested for synergism between various cardenolides, but our pattern of correlated evolution of increasing diversity and amounts of cardenolides is consistent with the synergism hypothesis.

There is evidence indicating that some biosynthetic pathways leading to the accumulation of plant natural products are not fully active, because of suppression of gene expression (Degenhardt *et al.* 2010). Indeed, roots and shoots of our 18 milkweed species showed striking differences in cardenolide profiles, suggesting high tissue specificity for gene expression and/or production of these toxic molecules. In fact, roots may experience totally different guilds of herbivores compared with aboveground tissues, leading to differential expression of defenses. For example, milkweed roots are primarily subject to feeding by specialized *Tetraopes* spp. larvae (Farrell & Mitter 1998). Shoot cardenolides, although effective against some herbivores such as monarch larvae, were ineffective against adult *Tetraopes* (Agrawal 2005). Whatever the ultimate reason, it is clear that investment in cardenolides is somewhat differentiated between roots and shoots of *Asclepias* spp. but they also show correlated evolution (i.e. species with low overall shoot cardenolides also tended to have low root cardenolides, etc.). Total above- and belowground cardenolide production was positively associated with the diversity of cardenolide forms (Table S4). Contrary to models of the macroevolution of defenses against herbivores, we previously showed that total cardenolides decreased during the diversification of *Asclepias* and thus, along with total cardenolide amounts, there may be directional selection for reduced cardenolide diversity as chemical defense becomes less important than other strategies (Agrawal & Fishbein 2008).

Chemical polarity and toxicity

In addition to the evolutionary correlation between amount and diversity of cardenolides discussed above, we also reported that species with higher levels of total cardenolides had more non-polar cardenolides (Fig. 2b). This suggests that high cardenolide species also have more toxic forms (Malcolm 1991). Indeed, monarch caterpillars feeding on leaves painted with a less polar cardenolide (digitoxin) grew less than when feeding on leaves painted with a more polar compound (ouabain) (Rasmann *et al.* 2009b). Generally, cardenolide activity inhibits Na^+ K^+-ATPases and amino acid changes in this sodium-potassium pump render it less sensitive to cardenolides (Holzinger & Wink 1996). This is, however, not the only mechanism for cardenolide insensitivity in animals. Some insects prevent toxicity of ingested cardenolides by simply preventing them from passing the gut membrane (Malcolm 1991). For example, while the Na^+ K^+-ATPases of the oleander hawk moth, *Daphnis nerii,* are highly sensitive to the cardenolide ouabain when it is injected directly into the moth's body, the low permeability of the gut membrane for such compounds prevents its toxicity when ingested normally (Petschenka & Dobler 2009).

Others have suggested that toxicity of secondary metabolites against herbivores may evolve by increases in molecular complexity (Berenbaum & Feeny 1981). Furanocoumarins in the Apiaceae have evolved increased molecular complexity (from linear form to angular forms) in an apparently escalating coevolutionary arm race against specialist herbivores (Berenbaum & Feeny 1981). Farrell & Mitter (1998) suggested that diversification and reciprocal adaptation to specialist root herbivores spurred the production of high levels and more complex cardenolides in milkweeds; i.e. from the simpler calotropogenin type to a more complex labriniformin type. We found here that milkweeds investing in high levels of cardenolides, as well as higher levels of inducibility, were also investing in less polar forms and because it is unclear how polarity corresponds to the chemical types discussed by Farrell & Mitter (1998), future research will need to further investigate the structure-function relationship of cardenolides. Thus, it would appear that given the evolutionary relationships previously shown (Agrawal *et al.* 2009) and the ones described herein, there was a general decrease of total cardenolides, along with a decrease of their diversity and an increase in polarity during diversification of *Asclepias*. We expect that the net impact of these traits is declining plant resistance, but this remains to be tested with bioassays.

Tradeoffs between constitutive and induced defense?

Contrary to general predictions, we found a positive relationship between constitutive cardenolide production and their induction (Fig. 3a). Interestingly, shoot results are in accordance with results for root cardenolides and with a previous analysis that examined only 12 species of *Asclepias* (Rasmann *et al.* 2009a). In contrast to the present comparison among species, we have found a tradeoff between constitutive and induced cardenolide production across genotypes of a single species (*A. syriaca*) in both the roots (Rasmann *et al.* 2011) and shoots (Bingham & Agrawal 2010). Investment in constitutive levels should be favoured in more predictable environments, whereas inducibility should be favoured in places where herbivore presence is less constant (Zangerl & Rutledge 1996). Nonetheless, across >50 milkweed species, we find greater constitutive cardenolides evolving with greater induction of the same compounds (Fig. 3a). Our result of a positive association is not unprecedented. Two other studies have taken a comparative approach across taxa to address this question. Thaler & Karban (1997) reported a positive correlation between constitutive and induced resistance across 21 wild cotton (*Gossypium*) 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 tradeoff between these two modes of defense.

We consider our findings a result of repeated evolution independent of phylogenetic history and the geography of diversification. Milkweeds seem to have originated in the tropics and then diversified preferentially northward into temperate regions (Woodson 1954; Farrell & Mitter 1998). Nonetheless, even when accounting for this directional radiation into the temperate zone, we still found consistently stronger inducibility in species that inhabit lower latitudes (Fig. 1).

Concluding speculation

If plant defense against herbivores, like most adaptations, is composed of multiple traits, and those traits act additively or synergistically, then there may be positive-correlated evolution among traits. Here, all

evidence points towards a scenario where expansion of *Asclepias* into higher latitudes was accompanied with a decrease both aboveground and belowground in the multimodal cardenolide defense (i.e. less abundant, less diverse, less inducible and more polar). Indeed, root defenses are not independent from shoot defenses, as the two show positive-correlated evolution. At the current juncture, it is impossible to disentangle the effect of herbivore pressure vs. habitat in shaping patterns of cardenolide production, although there is indication that specialist herbivores such as monarch butterflies and *Tetraopes* have a tropical origin and later expanded pole wards following *Asclepias* (Farrell & Mitter 1998; Brower & Jeansonne 2004). Although tradeoffs are certainly important in the evolution of defense strategies, tradeoffs are not universal and concerted patterns of defense allocation appear to be common. We advocate including biogeography and induced defense as additional dimensions of the plant defense syndrome hypothesis (Kursar & Coley 2003; Agrawal & Fishbein 2006).

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Table S1 Trait values for 49 *Asclepias* and two *Gomphocarpus* milkweed species.

Table S2 Estimated phylogenetic signal for shoot and root cardenolide-related traits.

Table S3 Phylogenetic analyses of cardenolide traits against average *Asclepias* spp. latitudinal distribution.

Table S4 Pairwise interactions between cardenolide-related traits.

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Table S1. Trait values for 49 *Asclepias* and 2 *Gomphocarpus* milkweed species. Shown are number of diverging nodes for each species in the phylogeny, average latitude in absolute values (i.e. measuring the average deviation from the equator), total shoot cardenolide amount (μg/mg dry weight), number of cardenolide peaks, peak diversity, and average peak polarity in the constitutive and in the damaged state for all milkweeds, and for 18 milkweed species same root traits.

Species	**Nodes**	**Latitude**	**Organ**	**Consitutive**				**Damaged**			
				Total conc.	#peaks	Diversity	Polarity	Total conc.	#peaks	Diversity	Polarity
A. amplexicaulis	16	36.125	Shoot (S)	1.120	8.250	1.756	15.770	1.786	9.200	1.781	15.840
			Root (R)	3.925	10.000	0.845	16.304	4.073	11.000	0.825	16.558
A. angustifolia	11	23.808	S	1.050	7.200	1.825	16.919	0.805	7.000	1.893	16.940
A. arenaria	14	37.203	S	1.633	5.000	1.602	15.401	1.704	6.200	1.576	15.361
A. asperula	15	30.791	S	6.026	15.750	2.341	16.628	7.105	15.800	2.378	16.459
			R	10.528	10.500	0.949	13.316	13.875	11.500	1.053	12.247
A. barjoniifolia	11	20.337	S	4.988	16.200	2.521	14.385	5.680	15.400	2.488	14.419
A. boliviensis	12	22.781	S	1.899	11.000	2.023	17.522	2.373	10.200	2.005	17.533
A. brachystephana	13	26.725	S	2.813	14.667	2.391	17.215	4.116	15.000	2.216	16.819
A. californica	7	40.169	S	4.644	18.500	2.575	16.041	4.817	17.333	2.429	15.998
A. candida	10	21.731	S	3.997	15.000	2.468	15.087	4.039	13.750	2.333	15.114
A. cordifolia	6	38.746	S	3.693	13.800	2.483	16.111	3.292	13.400	2.506	16.051
			R	2.107	6.000	0.628	16.474	2.638	6.000	0.638	16.415
A. cryptoceras	10	41.637	S	0.456	4.000	1.710	16.846	1.492	8.000	1.718	18.065
A. curassavica	9	6.733	S	3.276	17.250	2.243	16.648	4.014	19.250	2.444	16.679
			R	3.981	9.833	0.914	17.511	4.585	9.833	0.913	17.461
A. engelmanniana	13	34.191	S	0.210	3.000	0.807	11.599	0.206	3.000	0.966	13.476
A. eriocarpa	13	36.225	S	3.690	15.500	2.228	16.478	4.818	16.333	2.365	15.877
A. erosa	8	33.833	S	2.274	7.400	1.456	17.204	2.235	6.800	1.432	17.499
A. exaltata	18	41.591	S	0.735	7.250	1.462	16.483	1.030	9.500	1.785	16.861

A. fascicularis	10	39.742	S	0.566	5.000	1.429	16.645	0.764	6.800	1.739	17.081
A. hallii	17	37.608	S	2.076	12.333	2.083	16.909	1.378	11.500	2.212	17.589
			R	1.334	5.500	0.705	14.513	2.143	7.500	0.813	14.061
A. hirtella	13	38.508	S	3.289	14.600	2.234	17.364	3.392	13.667	2.108	17.341
A. humistrata	9	30.933	S	5.019	13.000	2.180	16.140	5.518	11.000	2.106	16.262
A. incarnata incarnata	13	39.117	S	0.511	7.200	1.752	18.775	0.389	6.500	1.725	18.785
			R	1.656	6.167	0.685	16.109	1.792	6.000	0.649	16.101
A. incarnata pulchra	13	40.175	S	4.240	24.500	2.618	15.573	4.550	25.200	2.635	15.848
			R	2.517	7.500	0.727	16.150	2.467	7.000	0.694	16.097
A. lanceolata	16	28.758	S	0.418	5.667	1.626	17.884	0.361	4.667	1.607	18.259
A. latifolia	15	34.700	S	3.582	9.800	1.683	17.200	3.660	9.000	1.655	17.184
A. lemmonii	13	29.423	S	3.140	10.400	1.885	17.134	3.287	10.500	1.861	17.076
A. linaria	6	23.875	S	6.588	17.250	2.437	17.029	8.126	17.750	2.437	16.909
			R	5.690	8.000	0.825	15.147	6.504	8.000	0.804	15.065
A. longifolia	15	29.825	S	2.913	12.000	1.976	17.026	3.446	11.333	2.041	16.734
A. mexicana	11	21.442	S	0.537	3.800	1.170	21.960	1.152	4.000	1.204	21.432
A. nivea	9	19.808	S	3.539	16.250	2.473	17.478	5.004	18.400	2.607	17.435
			R	9.898	12.750	1.088	14.774	13.638	11.333	1.032	15.089
A. obovata	21	30.533	S	0.453	4.000	1.033	17.497	0.653	4.000	1.048	17.473
			R	2.449	8.500	0.861	15.351	1.931	5.000	0.766	15.668
A. oenotheroides	16	20.033	S	1.532	12.000	2.145	17.140	2.000	11.600	2.081	17.154
A. perennis	12	31.117	S	4.439	18.750	2.626	16.571	5.917	21.250	2.660	16.278
A. pumila	11	39.908	S	0.574	1.667	0.494	13.383	0.268	1.333	0.435	13.367
A. purpurascens	17	39.000	S	0.407	4.667	1.171	17.580	0.426	4.250	1.190	17.717
			R	1.437	4.500	0.486	10.047	0.594	3.400	0.587	12.619
A. quadrifolia	19	40.625	S	0.606	6.000	1.587	16.171	0.496	5.600	1.609	16.284
A. rubra	22	35.167	S	0.426	5.500	1.635	17.584	0.355	5.000	1.452	17.285
A. solanoana	15	38.900	S	2.279	13.600	2.175	16.235	3.299	14.800	2.210	16.256
A. speciosa	17	40.508	S	1.112	6.167	1.628	15.372	1.129	6.600	1.644	15.521
			R	0.732	4.250	0.620	12.433	1.293	4.000	0.628	11.419

A. subulata	9	28.758	S	4.690	15.000	2.268	16.537	8.377	17.000	2.470	16.562
A. subverticillata	14	30.525	S	1.434	4.600	1.594	15.465	1.065	5.000	1.538	15.276
			R	0.412	3.000	0.437	18.412	0.630	3.000	0.413	18.157
A. sullivantii	12	40.233	S	2.149	6.800	1.830	13.566	2.711	6.800	1.741	13.719
			R	2.009	10.000	0.948	14.833	3.824	10.000	0.957	14.722
A. syriaca	22	41.950	S	1.573	9.600	1.875	16.553	1.434	9.000	2.021	16.591
			R	1.082	8.400	0.928	12.497	1.406	8.400	1.008	10.945
A. texana	13	28.633	S	0.929	9.000	2.149	17.168	1.641	10.600	2.048	16.721
			R	1.762	7.167	0.787	15.223	1.728	6.833	0.778	15.735
A. tuberosa	22	34.667	S	0.070	2.000	0.693	21.851	0.218	2.667	1.068	19.876
			R	0.028	0.333	0.000	29.214	0.349	2.000	0.548	20.334
A. variegata	17	35.242	S	0.887	7.000	1.620	16.847	0.387	5.500	1.352	17.087
A. verticillata	13	37.625	S	0.031	0.800	1.060	20.710	0.097	2.400	1.568	20.462
A. vestita	9	36.667	S	3.502	11.500	2.155	17.389	2.625	11.333	2.143	17.386
A. viridiflora	12	37.775	S	1.444	7.000	1.958	17.808	0.599	4.000	1.849	17.568
A. viridis	14	33.467	S	3.175	11.000	2.091	17.003	3.678	11.667	2.095	16.799
			R	5.627	12.600	1.089	12.087	7.342	11.200	1.078	13.091
G. cancellatus	7	34.142	S	5.687	20.000	2.448	17.422	5.211	21.000	2.561	16.979
G. fruticosus	6	29.058	S	2.647	12.000	2.048	17.894	2.734	12.167	2.086	17.849

Table S2. Estimated phylogenetic signal for shoot and root cardenolide related traits. Shown are constitutive levels of cardenolides, peak diversity, polarity, and their inducibility (damage – control), as well as for each species center of distribution (latitude). Models of estimated λ are compared to models v where λ is fixed at either 1 or 0. Asterisks indicate significant differences in likelihood ratios (LR) between the estimated model and the constrained model under chi-square distribution ($p < 0.05$).

Organ	Trait	Deployment strategy	λ	LR when compared to λ = 0	LR when compared to λ = 1	Interpretation
	Latitude		0.672	5.962*	2.998	Some phylogenetic signal
Shoots	Total cardenolide amount	Constitutive (C)	0.646	5.896*	18.284*	Significant phylogenetic signal, but less than would be expected by Brownian motion evolution.
		Inducibility (I-C)	0.925	8.218*	1.778	Phylogenetic signal consistent with Brownian motion evolution.
	Number of peaks	C	0.408	3.668*	25.616*	Significant phylogenetic signal, but less than would be expected by Brownian motion evolution
		I-C	0	0	7.542*	No phylogenetic signal

	Diversity index	C	0.464	5.664*	12.35*	Significant phylogenetic signal, but less than would be expected by Brownian motion evolution
		I-C	0	0	20.502*	No phylogenetic signal
	Polarity index	C	0	0	32.370*	No phylogenetic signal
		I-C	0	0	17.760*	No phylogenetic signal
Roots	Total cardenolide amount	Constitutive (C)	0.709	0.62	1.054	Ambiguous, but some signal likely
		Inducibility (I-C)	0.682	0.674	0.86	Ambiguous, but some signal likely
	Number of peaks	C	0	0	6.782*	No phylogenetic signal
		I-C	0	0	21.614*	No phylogenetic signal
	Diversity index	C	0	0	12.558*	No phylogenetic signal
		I-C	0	0	21.212*	No phylogenetic signal
	Polarity index	C	0	0	14.678*	No phylogenetic signal
		I-C	0	0	17.482*	No phylogenetic signal

Table S3. Phylogenetic analyses of cardenolide traits against average *Asclepias* spp. distribution (latitude). Shown are Spearman correlation coefficient (r), and correlation significance levels (p), likelihood ratios LR (calculated as twice the difference in likelihood ratio of the estimated model, and a model where trait co-variance is set to zero) for cardenolide levels, number of peaks, diversity and polarity, across 51 species (shoots) and 18 species (roots) of milkweeds in the constitutive state and for inducibility (damage – control plants). Additionally, we performed multiple regression phylogenetic analyses of average species latitude, and species node depth in the phylogeny, predicting cardenolide levels, diversity and polarity, across 51 species (shoots) and 18 species (roots) of milkweeds that showed significant relationship with latitude in previous analyses. Likelihood ratios LR are here calculated as twice the difference the likelihood values of the full estimated model, and a model where either latitude or node depth was missing. Significance is derived from chi-square distribution (* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.001$).

Organ	Trait	Deployment strategy	r	p_{raw}	LR_{PGLS}	Latitude LR (– lat)	Nodes LR (-node)
Shoots	Cardenolide amount	Constitutive	-0.284	**0.044***	0.626	0.728	2.318
		Inducibility	-0561	**0.016***	**4.954****	**5.700****	0.014
	Number of peaks	Constitutive	-0.298	**0.034***	0.074	0.090	0.484
		Inducibility	-0.035	0.809	0		
	Diversity	Constitutive	-0.290	**0.039***	0.538	0.600	0.910
		Inducibility	0.049	0.730	0.376		
	Polarity	Constitutive	-0.137	0.336	**3.002***	**2.94***	0.764
		Inducibility	0.168	0.237	1.778		
Roots	Cardenolide amount	Constitutive	-0.774	**0.044***	1.506	0.227	0.938
		Inducibility	-0.669	0.238	0.006		
	Number of peaks	Constitutive	-0.353	0.151	0.650		
		Inducibility	0.147	0.561	0.212		
	Diversity	Constitutive	-0.284	0.252	0.638		
		Inducibility	0.164	0.514	0.28		
	Polarity	Constitutive	-0.157	0.535	0.002		
		Inducibility	-0.075	0.766	0.208		

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Table S4. Pairwise interactions between total amount of cardenolides, their diversity and their polarity across the shoots of 51 species and the roots of 18 species of milkweeds. Shown are correlation coefficients (r) in the raw correlations and the differences in likelihood values (LR) between the estimated correlation and the likelihood of the model where co-variation between two variables is forced to be null in phylogenetic corrected analyses (PGLS). In PGLS, significance is derived from chi-square distribution. * = p < 0.1, ** = p < 0.05, *** = p < 0.001.

Organ	Treatment	Variable	By variable	r	LHPGLS
Shoots	Constitutive	Amount	Diversity	0.792***	44.452***
		Amount	Polarity	-0.19	7.374***
		Amount	Peaks	0.86	4.784**
		Polarity	Diversity	-0.137	3.798*
		Amount	Peaks	0.878	75.138***
	Damaged	Amount	Diversity	0.774	48.224***
		Amount	Polarity	-0.23	8.92**
		Amount	Peaks	0.833	60.41***
		Polarity	Diversity	-0.099	3.692*
		Amount	Peaks	0.892	80.846***
	Inducibility	Amount	Diversity	0.159	1.544
		Amount	Polarity	-0.081	1.636
		Amount	Peaks	0.592	23.278***
		Polarity	Diversity	-0.076	1.116

		Amount	Peaks	0.444	11.176**
Roots	Constitutive	Amount	Diversity	0.658	3.246
		Amount	Polarity	-0.271	1.114
		Amount	Peaks	0.483	4.784**
		Polarity	Diversity	-0.651	19.962***
		Amount	Peaks	0.958	45.150***
	Damage	Amount	Diversity	0.736**	4.776**
		Amount	Polarity	-0.263	1.248
		Amount	Peaks	0.538	7.028**
		Polarity	Diversity	-0.551	10.944**
		Amount	Peaks	0.906	30.752***
	Inducibility	Amount	Diversity	0.224	2.824*
		Amount	Polarity	-0.099	3.542*
		Amount	Peaks	0.354	2.404
		Polarity	Diversity	-0.587	22.960***
		Amount	Peaks	0.822	20.832