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Developmental and induced responses of nickel-based and organic defences of the nickel-hyperaccumulating shrub, *Psychotria douarrei*

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Summary

- Developmental and inducible changes in metal-based (nickel (Ni)) and organic defences (phenolics) are compared in the Ni-hyperaccumulating shrub, *Psychotria douarrei*.
- Young and old leaves of *P. douarrei* shrubs, subjected to different degrees of simulated herbivory, were analyzed for metals, tannins, macronutrients and total carbon, and compared with a co-occuring nonhyperaccumulator shrub, *Ficus webbiana*.
- Leaf age affected both nickel Ni-based and organic defences in *P. douarrei*; foliar metal concentrations were higher in mature leaves, whereas organic defences were higher in young leaves. Neither metal-based nor organic defences were increased by simulated herbivore damage, implying noninducibility, although some organic defence compounds were significantly reduced. *P. douarrei* had a greater percentage of total phenolics, condensed tannins and protein precipitation ability than *F. webbianai*. Since total carbon content did not differ between species, *Psychotria* invests more of its leaf carbon budget in organic defences than does *Ficus*.
- Data suggest that *P. douarrei* foliage is well protected by Ni, but tannins have multiple functions. The high concentrations of tannins in *Psychotria* leaves might function as a detoxification mechanism for elevated cytoplasmic metal concentrations, in addition to providing defensive benefits.

Key words: Ni hyperaccumulation, serpentine, herbivory, elemental defence, *Psychotria*.

Introduction

Plants that take up relatively large amounts of heavy metals from the soil and sequester them in their tissues are termed metal hyperaccumulators (Brooks *et al.*, 1977). The threshold metal content used to define a hyperaccumulator depends on the particular metal accumulated. For instance, nickel (Ni) hyperaccumulators, which comprise more taxa than any other type of metal hyperaccumulator, contain more than 1000 µg Ni g^{-1} dry tissue (Brooks *et al.*, 1977). Boyd & Martens (1992) summarized several hypotheses that have been advanced to explain the evolution of hyperaccumulation; however, it is the metal defence hypothesis that has garnered the most supporting evidence (Boyd & Martens, 1998a). Metals in plant tissues deter feeding (Pollard & Baker, 1997; Jhee *et al.*, 1999), delay larval development (Martens & Boyd, 1994; Boyd & Moar, 1999) and are acutely toxic to certain insect herbivores (Boyd & Martens, 1994; Martens & Boyd, 1994; Boyd & Moar, 1999) and plant pathogens (Boyd *et al.*, 1994; Ghaderian *et al.*, 2000).

Because metals are complexed with common metabolic products such as citrate (Sagner *et al.*, 1998), malate (Mathys, 1977; Gabbrielli *et al.*, 1991), amino acids (Krämer *et al.*, 1996), or oxalate (Mathys, 1977), translocation and compartmentalization may represent the only direct metabolic costs for metal-based defences. Thus, the metabolic cost for metal-based defences is presumed to be low compared with the costs of constructing and maintaining carbon-based defences (Martens & Boyd, 1994). For example, Gulmon & Mooney (1986) estimated the cost of synthesis of phenolics to be 2.6 g of assimilated CO_2 g^{-1} of phenolic compound, whereas the cost for leaf production was 1.9–2.7 g CO_2 g^{-1} of leaf tissue, thus the carbon cost of tannin production may rival the cost of producing new leaves. Sequestration of toxic heavy metals may provide defence (benefit) against insects and pathogens and reduce the need (cost) to produce expensive carbon-based defences. Although the 'basal allocation' (*sensu* McDonald *et al.*, 1999) of metabolic resources toward defence may be genetically fixed, further allocation of resources to defensive chemistry should occur only when the benefits of avoiding herbivory exceed defence costs (Bazzaz *et al.*, 1987). Therefore, we predict that plants defended by metals will invest fewer resources into other types of organic defenses.

Certain elemental defences (e.g. silicification) have been shown to be inducible (McNaughton & Tarrants, 1983). However, since metal hyperaccumulation is a constitutive trait (Boyd & Martens, 1998b) and may have functions other than defence (Boyd & Martens, 1992; Boyd & Martens, 1998a), it is not likely to be affected by the presence or absence of herbivore damage. To date, the inducibility of metal-based defences has only been investigated in one species, the Ni hyperaccumulator, *Streptanthus polygaloides* Gray (Davis & Boyd, 2000). In that study, foliar Ni levels of *S. polygaloides* were unaffected by the presence of either simulated herbivore damage or live herbivores, whereas levels of organic defences (glucosinolates) were increased (Davis & Boyd, 2000).

Leaf maturity also influences leaf defensive characteristics. In tropical forest understories, immature leaves of woody plants experience up to 100 times as much damage from herbivory and disease compared to older leaves (Coley & Aide, 1991). Up to 70% of lifetime damage can occur during leaf expansion (Coley & Kursar, 1996). Young leaves are attractive to herbivores because they are less tough (Nichols-Orians & Schultz, 1990) and have higher nitrogen and water contents than mature leaves (Nichols-Orians & Schultz, 1990; Kursar & Coley, 1991). To reduce damage from herbivory, young leaves of woody tropical plants have higher concentrations of phenolic compounds than mature leaves (Coley & Aide, 1991; Coley & Kursar, 1996). Although Ni levels can be higher in mature leaves than in expanding leaves (Boyd *et al.*, 1999), it is not known if temporal distribution patterns of Ni affect levels of carbon-based defences.

Few studies have examined interactions between defences, including the interaction between organic and elemental chemical defences (Pennings, 1996; Pennings *et al.*, 1998). The purpose of this study was to characterize constitutive and induced levels of a carbon-based defence (phenolics) and a metal-based defence (Ni) in a Ni hyperaccumulator species. Specifically, we wanted to: determine the effects of simulated herbivory on levels of Ni-based defences; characterize developmental patterns of Ni-based defences; contrast the developmental and induced responses of Ni-based and organic defences; and compare these responses with a co-occuring nonhyperaccumulator shrub. Plants that are well defended by foliar metal should invest less carbon towards the construction of organic defences than plants that do not utilize a metal-based defence. Therefore, we expected to find reduced tannin content in the foliage of a hyperaccumulator when compared to leaves of a nonhyperaccumulator species.

Materials and Methods

Study site and species

The experiment was conducted in December 1996 in the Parc de la Rivière Bleue on Grand Terre, the largest island of New Caledonia. Grand Terre is unique in that almost one third (*c.* 5500 km^2) of the island is covered by ultramafic (serpentine) rock (Brooks, 1987). The study site was located in a protected area of humid tropical forest, near study plots used by Jaffré & Veillon (1990) and Boyd *et al.* (1999) at the Kauri Géant along Rivière Bleue. This site is on serpentine soil, and Ni hyperaccumulation was well represented by at least six co-occuring species of Ni hyperaccumulators. The understory contained the Ni hyperaccumulator shrubs *Psychotria douarrei* (Beauvis.) Däniker, *Hybanthus austrocaledonicus* (Veill.) Schinz & Guillamin *ex* Melchior, and *Casearia silvana* Schltr. The overstory contained *Homalium guillaumii* (Viell.) Briq., *Geissois hirsuta* Brongn, and *Sebertia acuminata* Pierre *ex* Baillon. The Ni hyperaccumulator used for this study, *P. douarrei* (Rubiaceae), has one of the highest known leaf Ni contents (up to 47 000 μg Ni g^{-1} or 4.7% of d. wt) (Jaffré & Schmid, 1974). *Ficus webbiana* (Miq.) Miq. (Moraceae), a co-occuring, nonhyperaccumulating shrub, was chosen as a comparative species based on similarities with *P. douarrei* in geographical location, position in forest stratum, growth habit, leaf morphology, leaf phenology and population density.

Damage treatments were applied to leaves of different ages on shrubs of both species. For each species, 20 shrubs of similar size were arbitrarily chosen, and five branches on each shrub were randomly assigned a damage treatment. Damage treatments, designed to simulate different levels of herbivore damage, were defined as no damage, moderate damage (*c.* 33% of leaf blade removed) and severe damage (*c.* 66% of leaf blade removed). Damage was inflicted by using scissors to remove the distal portion of the leaf at a right angle to its main axis. For each shrub, moderate and severe damage treatments were randomly assigned to leaves on each of two branches, while leaves on one branch were left undamaged. Two sets of leaves were damaged on each shrub so that enough biomass would remain for analyzes. For each branch, treatments were applied to both young and old leaves. Young leaves were defined as the first fully expanded leaves on a branch and old leaves were defined as the two leaves most proximal to the main trunk. For each branch, two leaves of each age category received the appropriate damage treatment and a colored wire was wrapped around the petiole of each leaf to identify treatment and age. This resulted in six treatments per shrub: young leaf, no damage (YN); young leaf, moderate damage (YM); young leaf, severe damage (YS); old leaf, no damage (ON); old leaf, moderate damage (OM); and old leaf, severe damage (OS). Leaves were harvested two weeks after damage treatments were applied and were air-dried at room temperature. All leaves of the same treatment from an individual shrub were combined to increase the biomass available for analysis. Dried leaves were transported to Auburn University, AL, USA, for laboratory analyzes. Before analyzes were performed, dried leaves were ground in a Retsch grinder (F. Kurt Retsch GmbH and Co.KG, Haan, Germany) to pass through a 0.2-mm sieve.

Elemental analysis

Elemental contents of leaves were determined by dry-ashing 0.1 g of sample in a muffle furnace at 450°C for 4.5 h followed by serial digestions with 1 M HNO_3 and 1 M HCl. Samples were filtered and analyzed for Ni by atomic absorption spectrometry, $\lambda = 720$ nm (IL 251, Instrumentation Laboratory, Lexington, MA, USA). The same digestates also were analyzed for Ca, Co, Cr, K, Mg, Mn, P, Pb and Zn using an inductively coupled argon plasma spectrometer (ICAP 9000, Jarrell-Ash, Franklin, MA, USA).

Total phenolics assay

Total foliar phenolic content was determined using a modified Folin-Denis method (Pritchard *et al.*, 1997). For each sample, 40 mg tissue was extracted for 30 min in 600 µl 70 : 30 acetone : distilled water. Samples were sonicated during extraction to reduce extraction time. After extraction, samples were centrifuged for 1 min to remove particulates, and 15 µl supernatant was diluted in 5 mL dH_2O. Folin-Denis reagent (2 ml) (prepared as in Pritchard *et al.*, 1997) was added to 2 ml of diluted sample and mixed thoroughly. After 3 min, 2 ml Na_2CO_3 solution (106 g l^{-1}) was added. Two hours later, absorbances at $\lambda = 725$ nm were recorded using a Spectronic 21 spectrophotometer (Milton Roy, Rochester, NY, USA).

Radial diffusion assay

The biological activity of tannins in leaf samples was measured by their protein-precipitation ability. We used a BSA protein-binding assay (Hagerman, 1987). Agarose plates were prepared by methods outlined in Waterman & Mole (1994). Briefly, agarose was dissolved in buffer (50 mM acetic acid, 60 µM ascorbic acid, pH 5.0) to make a 1% (w/v) solution. The solution was brought to boil, allowed to cool to 45°C, and BSA was added to make a 0.1% (w/v) solution. After the BSA dissolved, 9.5 ml aliquots of the agarose-BSA solution were added to 9.5 cm diameter Petri dishes. After the solution cooled, six 4 mm diameter wells were bored into the agarose gel in each plate. Tissue was extracted and centrifuged as for the total phenolics assay and three 8 µl aliquots of supernatant were loaded into each well in a plate. Each of the six treatments per shrub was represented by a sample in the wells on the same plate. Allocation of the treatments to wells was randomized and four plates were prepared from samples of each shrub to reduce possible effects of well depth variation among plates. Plates were sealed with Parafilm (American National Can. Inc., Menasha, WI, USA) and placed in a 30°C oven for 96 h. As the tannins in each sample diffused outward from a well, a whitish-opaque ring formed. Perpendicular diameters of diffusion rings were measured for each treatment, averaged for the four plates and used to obtain treatment means for each shrub.

Condensed tannins

Condensed tannin content was determined using the BuOH-HCl technique (Mole & Waterman, 1987). For each sample, 20 mg tissue was extracted with 1.3 ml 70% methanol. After centrifugation, 500 µl supernatant was added to 7.0 mL BuOH-HCl reagent (0.7 g $FeSO_4{*}7H_2O$ in 25 mlconc HCl, brought up to 1 l with *n*-butanol) and placed in a 98°C oven for 40 min. After samples cooled, absorbances were read at $\lambda = 550$ nm.

C and N analysis

Total carbon and nitrogen contents of dried, ground samples were determined using a Fisons NA 1500 NCS Analyzer (Fisons Instruments, Milan, Italy) following the methods outlined in Torbert *et al.* (1998).

Statistical analysis

Data were analyzed with a two-way ANOVA with leaf age and extent of simulated herbivore damage as main factors. Since the study species were taxonomically unrelated, data for each species were analyzed separately. However, some comparisons were made between species (one-way ANOVA with species as the main factor), but these were limited to overall characterization of levels of metals and organic defences.

The radial diffusion assay and colorimetric assays (i.e. Folin-Denis and BuOH-HCl) are useful for determining relationships between treatments, but are of limited value for determining quantitative amounts of tannins (A. Hagerman, pers. comm.). Therefore, we did not attempt to convert those data into quantitative measures of tannins. Radial diffusion data were analyzed as precipitation ring areas and total phenolics (Folin-Denis) and condensed tannins (BuOH-HCl) data were analyzed as raw absorbances (A. Hagerman, pers. comm.).

Analyzes were performed using Statview 5.0 (SAS Institute, 1998). Pairwise comparisons of individual treatment means were made using Fisher's Protected Least Significant Difference (PLSD) test (SAS Institute, 1998). Statistical comparisons were considered significant at $\alpha \leq 0.05$.

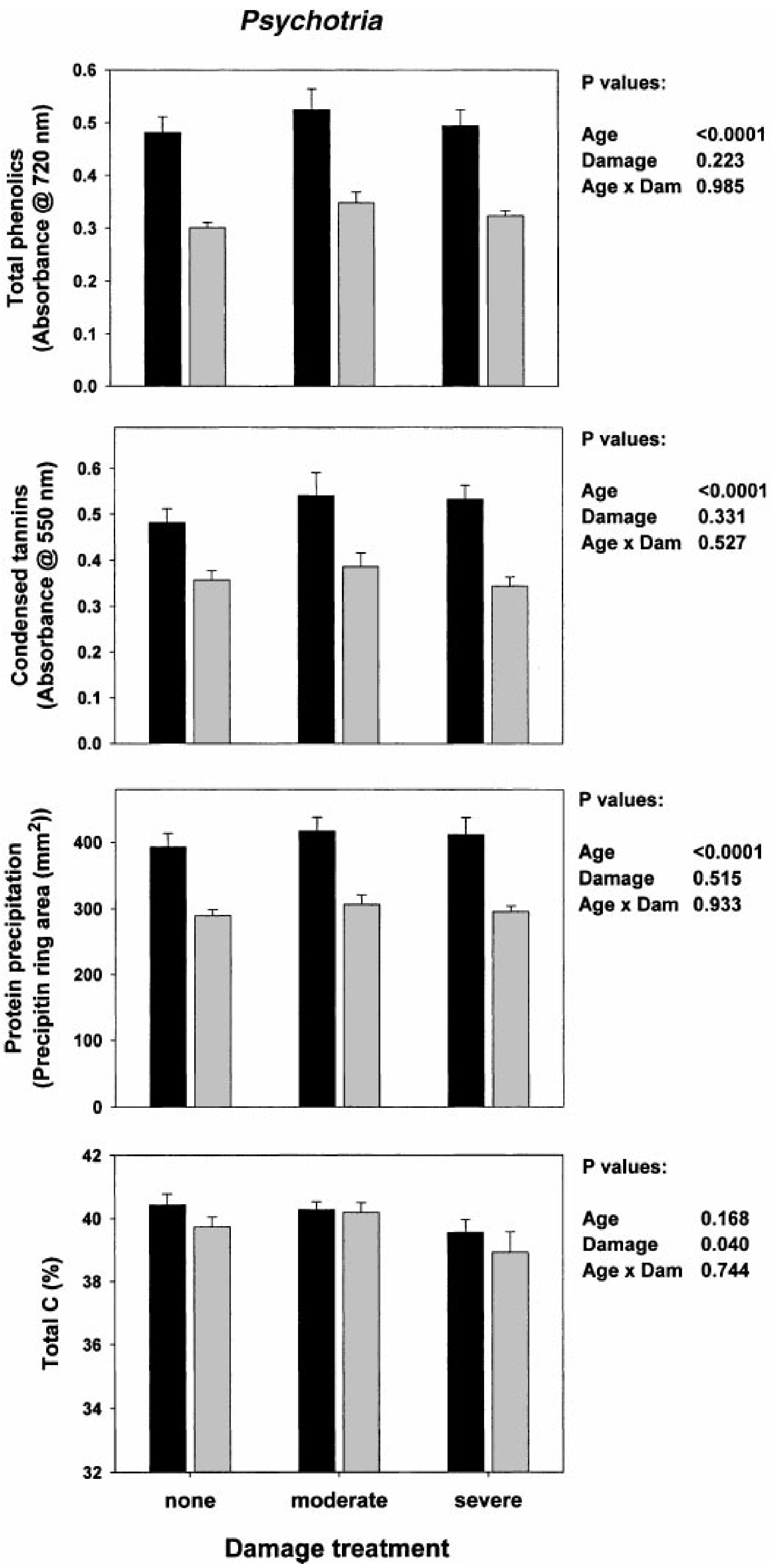

Fig. 1 Measurements of total carbon (%) and carbon-based defensive compounds in young foliage (black columns) and old foliage (gray columns) of *Psychotria douarrei* subjected to different levels of simulated herbivory: none (no damage), moderate (*c.* one-third of leaf blade removed), severe (*c.* two-thirds of leaf blade removed). Total phenolics and condensed tannin data are presented as raw absorbances; protein precipitation data are presented as precipitation ring areas (mm^2). Bars represent means ± SE; $n = 20$.

Results

Organic defences

All three organic defence parameters exhibited similar age responses for each species. Total phenolics, condensed tannins and protein precipitation ability were higher in young leaves of both species (Figs 1, 2). No significant damage effects were observed for any organic defences for *Psychotria*, whereas damage significantly reduced total phenolic content ($P = 0.009$) and condensed tannins ($P = 0.009$) in *Ficus* leaves. Total phenolics, condensed tannins and protein precipitation ability in severely damaged *Ficus* leaves were lower than in moderately damaged leaves ($P = 0.005$, $P = 0.002$, and $P = 0.035$, respectively, Fisher's PLSD). Overall, total phenolics, condensed tannins, and protein precipitation ability were significantly higher for *Psychotria* leaves than *Ficus* leaves (Table 1).

Elemental analyzes – carbon

Total carbon content (%) of *Psychotria* and *Ficus* foliage exhibited different age responses, but shared similar damage responses. Young and mature *Psychotria* foliage did not differ in carbon content (Fig. 1), whereas foliar carbon content was significantly lower in mature *Ficus* leaves ($P < 0.0001$, Fig. 2). Damage reduced foliar carbon contents for both species ($P = 0.040$ for *Psychotria* and $P = 0.044$ for *Ficus*). Severely damaged *Psychotria* leaves contained less carbon than both undamaged leaves ($P = 0.022$, Fisher's PLSD) and moderately damaged leaves ($P = 0.039$, Fisher's PLSD). Similarly, severely damaged *Ficus* leaves had less carbon than undamaged leaves ($P = 0.022$, Fisher's PLSD). Total leaf carbon content (%) did not differ between species (Table 1).

Elemental analyzes – macronutrients

For each species, macronutrients differed among treatments for some, but not all of the elements measured (Tables 1, 2, and 3). Young leaves of both species had higher levels of N, P and K than mature leaves ($P = 0.051$, $P < 0.0001$, and $P < 0.0001$, respectively, for *Psychotria* foliage; $P < 0.0001$ for N, P and K for *Ficus* foliage). Levels of Ca and Mg were higher in mature foliage for both species ($P = 0.026$ and $P = 0.095$, respectively, for *Psychotria* foliage; $P = 0.002$ and $P < 0.0001$, respectively, for *Ficus* foliage). No significant responses to damage occured for any macronutrients for either species. *Psychotria* leaves had higher levels of N ($P < 0.0001$) and P ($P = 0.066$) and lower levels of Ca ($P < 0.0001$) than *Ficus* leaves. Levels of K did not differ between species.

Elemental analyzes – metals

In general, metal concentrations were higher in mature leaves for most metals (Tables 2 and 3). Foliar Zn did not differ with respect to age for either species. Damage did not affect metal concentrations of either species for any of the metals measured. *Psychotria* leaves had significantly higher metal contents for five (Ni, Zn, Cr, Pb, and Co) of the seven metals measured (Table 1).

Discussion

Serpentine soils are typically characterized by a paucity of certain nutrients (e.g. Ca, K, P) and relatively high levels of Ni, Mg, Co, and Cr (Brooks, 1987). These harsh conditions often result in serpentine floras that are specifically adapted to ultramafic soils, although it is unclear if nutrient limitations (Kruckeberg, 1984; Nagy & Proctor, 1997) or other factors such as drought and soil depth (Kruckeberg, 1984) are responsible for these unique communities. Jaffré (1980) estimated that 60% of the 1500 plant species found on New Caledonian serpentine soils are endemic to their ultramafic substrates. Within the serpentine flora (but not always endemic to serpentine soils) is an even smaller 'flora', the metal hyperaccumulators. This distinct trait has been the focus of many studies, but the metal-based plant defence hypothesis has received the most experimental support among the current hypotheses seeking to explain the evolution of hyperaccumulation (Boyd & Martens, 1998a).

Martens & Boyd (1994) suggested that one benefit of a metal-based defence is the decreased need to invest resources (i.e. C and N) into organic defences. Thus, species that are putatively protected by metals should not invest as much carbon into organic defences as nonhyperaccumulators. Our data did not support this prediction; the Ni-hyperaccumulator, *P. douarrei*, had 12% more total phenolics, 49% more condensed tannins, and 73% higher protein precipitation ability than the nonhyperaccumulator, *F. webbiana* (Table 1). Since total carbon content (%) did not differ between species, it appears that *Psychotria* invests more of its leaf carbon budget toward organic defences than does *Ficus*.

Why would *Psychotria* divert cellular resources into synthesis of organic defences if its foliage is already defended by Ni? Both Ni and tannins may have multiple functions, including defence. Nickel hyperaccumulation may be involved in drought or metal tolerance (Boyd & Martens, 1992) and may not have evolved solely as an herbivore defence for *Psychotria*. Likewise, Bernays (1981) has suggested that tannins have cellular and ecological functions that do not involve defence. For example, tannins may simply be repositories for excess carbon when plant growth occurs under nutrient-limited conditions (Bryant, 1987). For hyperaccumulators, tannins may function as metal-binding compounds. Tannins have many hydroxyl radicals which endow them with a strong affinity for many cations, including metals. Tannins are used industrially as metal chelators to remove Fe ions from water used in food industries (Matsuo *et al.*, 1995) and extract Cu, Pb, Cd, Cr, and Hg from industrial waste waters (Randall *et al.*, 1974). Neumann *et al.* (1995) found that much of the Cu in the Cu tolerant

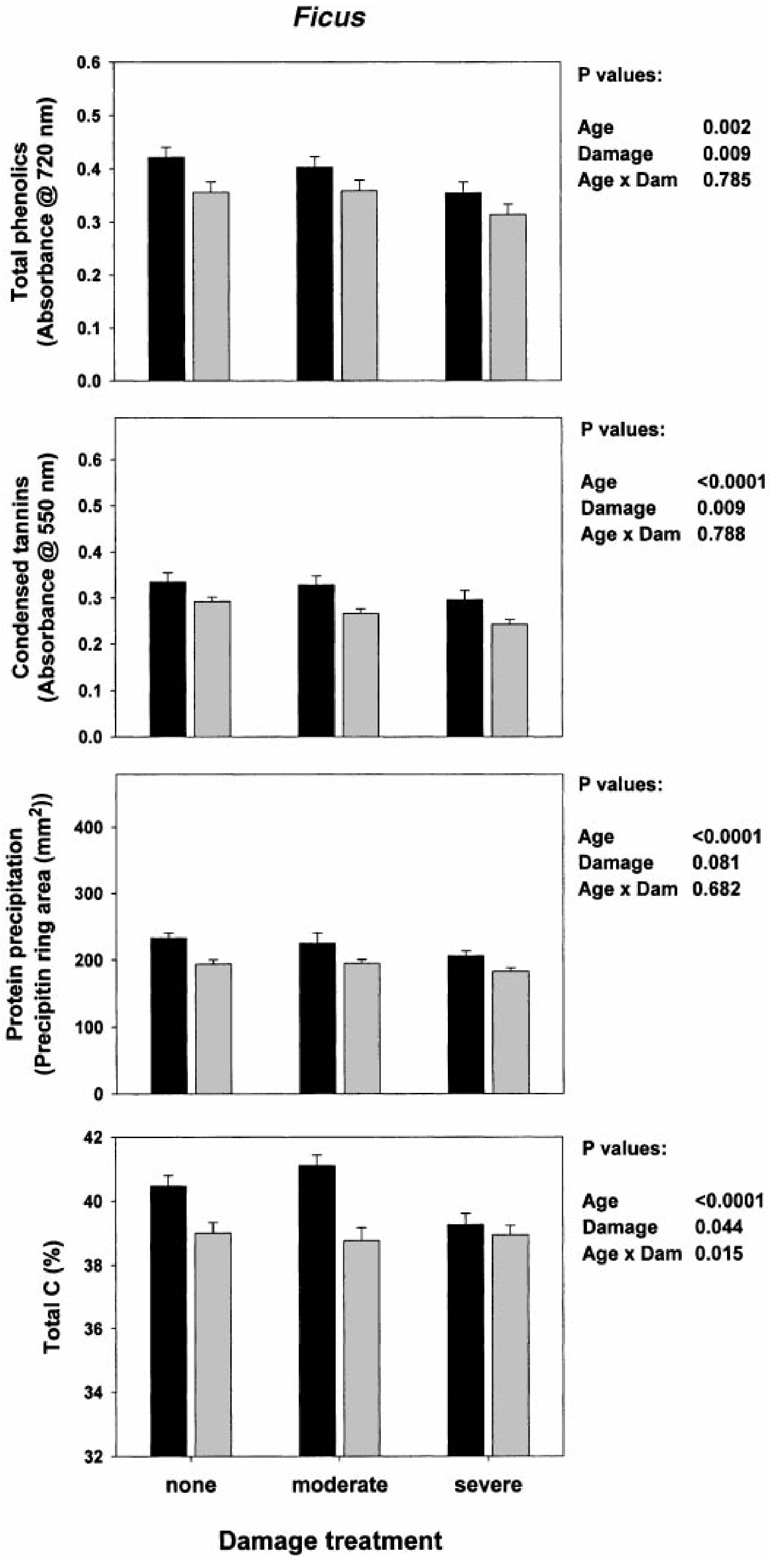

Fig. 2 Measurements of total carbon (%) and carbon-based defensive compounds in young foliage (black columns) and old foliage (gray columns) of *Ficus webbiana* subjected to different levels of simulated herbivory: none (no damage), moderate (*c.* one-third of leaf blade removed), severe (*c.* two-thirds of leaf blade removed). Total phenolics and condensed tannin data are presented as raw absorbances; protein precipitation data are presented as precipitation ring areas (mm^2). Bars represent means ± SE; $n = 20$.

Table 1 Interspecific comparisons of foliar organic defences, macronutrients, and metals between the Ni hyperaccumulator shrub, *Psychotria douarrei*, and the nonhyperaccumulator shrub, *Ficus webbiana*

	Psychotria douarrei	*Ficus webbiana*	P values
Organic defences			
Total phenolics	0.41 (± 0.01)	0.37 (± 0.01)	0.006
Condensed tannins	0.44 (± 0.01)	0.29 (± 0.01)	<0.0001
Protein precipitation ability	353 (± 8.8)	206 (± 4.0)	<0.0001
Total C (%)	39.8 (± 0.2)	39.6 (± 0.2)	0.273
Macronutrients			
Total N (%)	1.6 (± 0.03)	1.2 (± 0.02)	<0.0001
P	700 (± 20)	650 (± 20)	0.066
K	11400 (± 350)	11300 (± 620)	0.921
Ca	7600 (± 230)	15300 (± 360)	<0.0001
Mg	6200 (± 130)	5900 (± 150)	0.186
Metals			
Ni	16400 (± 530)	90 (± 10)	<0.0001
Zn	87.3 (± 5.7)	35.7 (± 3.9)	<0.0001
Mn	42.1 (± 2.0)	44.6 (± 1.7)	0.322
Cr	11.8 (± 1.0)	7.1 (± 0.5)	<0.0001
Co	17.1 (± 0.5)	1.3 (± 0.1)	<0.0001
Pb	39.9 (± 0.5)	6.2 (± 3.0)	<0.0001

Elemental concentrations are expressed as $\mu g\ g^{-1}$ unless otherwise noted. Total phenolics and condensed tannin data are raw absorbances; λ,725 nm; λ,550 nm, respectively. Protein precipitation data are precipitin ring areas. Data are means ± SE; $n = 20$.

plant, *Armeria maritima* (Mill.) Willd. ssp. *halleri* A. and D. Love, was localized within the vacuoles of tannin idioblasts. We suggest that the high levels of tannins in *Psychotria* leaves may function in the chelation or detoxification of Ni, in addition to providing defensive benefits.

Leaf age can affect levels of foliar organic defence compounds. In tropical understoreys, young foliage of woody shrubs generally has higher levels of organic defences than mature foliage (Coley & Aide, 1991; Coley & Kursar, 1996) since expanding leaves are more palatable to herbivores (i.e. young leaves are less tough and have lower C : N ratios than mature leaves) (Kursar & Coley, 1991). Our data support this prediction for both *Psychotria* and *Ficus*, with *Psychotria* leaves exhibiting the largest contrast. Young *Ficus* leaves had 16% more total phenolics, 13% more condensed tannins, and 14% higher protein precipitation ability than mature leaves, while young *Psychotria* leaves exhibited 30%, 35%, and 27% higher values than mature leaves for the same parameters.

Ni-based defences also can be influenced by the stage of leaf development. Ni concentrations were higher in the older foliage of both *Psychotria* and *Ficus* in this study. Greater Ni concentrations in older leaves indicate that Ni is an immobile element that accumulates in tissues over time. Since Ni levels were lowest in the young, palatable leaves for both species, it appears that young leaves of *Psychotria* are less defended by Ni. However, the mean Ni level in young *Psychotria* leaves observed in this study (13 700 $\mu g\ g^{-1}$) is likely to be acutely toxic to generalist folivores. For example, Martens & Boyd (1994) demonstrated that a leaf Ni concentration of 3000 $\mu g\ g^{-1}$ was toxic to a generalist folivore (*Pieris rapae* L.) feeding on the Ni hyperaccumulator, *Thlaspi montanum* var. *montanum* P. Holmgr. (Martens & Boyd, 1994). In fact, further experimentation with artificial diet studies showed that Ni concentrations of only 1000 $\mu g\ g^{-1}$ were fatal to *Pieris rapae* larvae (Martens & Boyd, 1994). Prior studies of *Psychotria douarrei* have shown that leaf Ni levels vary greatly: 13 400 (Lee *et al.*, 1977), 14 900–27 700 (Boyd *et al.*, 1999), 19 900 (Kelly *et al.*, 1975), and 47 000 $\mu g\ g^{-1}$ (Jaffré & Schmid,

Table 2 Effects of age and simulated herbivore damage treatment on foliar elemental composition of the Ni hyperaccumulator shrub, *Psychotria douarrei*

	Young foliage			Old foliage			*P*-values		
	None	Moderate	Severe	None	Moderate	Severe	Age	Damage	A x D
Metals									
Ni	13900 (± 1100)	13500 (± 930)	13800 (± 1000)	20000 (± 1100)	18700 (± 1200)	19700 (± 870)	<0.0001	0.729	0.898
Zn	59.2 (±7.1)	105 (± 29.3)	70.2 (± 6.4)	99.2 (± 6.1)	92.7 (± 12.5)	95.9 (± 6.1)	0.118	0.323	0.152
Mn	45.9 (± 4.3)	47.6 (± 4.1)	44.2 (± 3.5)	36.5 (± 1.7)	46.8 (± 8.7)	32.2 (± 1.9)	0.056	0.153	0.460
Cr	5.5 (± 0.7)	5.0 (± 0.6)	5.2 (± 0.5)	16.5 (± 1.6)	21.8 (± 3.7)	16.0 (± 2.3)	<0.0001	0.307	0.229
Co	13.8 (± 0.9)	13.5 (± 0.8)	15.0 (±1.1)	20.0 (± 1.1)	19.1 (± 1.4)	20.9 (± 0.9)	<0.0001	0.278	0.981
Pb	31.1 (± 2.1)	29.5 (± 2.2)	30.3 (± 2.2)	44.8 (± 2.3)	57.1 (± 16.0)	45.1 (± 2.1)	0.002	0.667	0.552
Nutrients									
N (%)	1.7 (± 0.04)	1.7 (± 0.06)	1.6 (± 0.05)	1.6 (± 0.03)	1.6 (± 0.04)	1.5 (± 0.11)	0.051	0.462	0.792
P	790 (± 30)	860 (± 100)	770 (± 30)	600 (± 20)	640 (± 60)	560 (± 10)	<0.0001	0.247	0.939
K	15900 (± 970)	16200 (± 1200)	17400 (± 880)	6300 (± 620)	6500 (± 980)	6800 (± 820)	<0.0001	0.524	0.846
Ca	7100 (± 560)	6900 (± 560)	7100 (± 500)	8100 (± 500)	8000 (± 700)	8100 (± 500)	0.026	0.949	0.993
Mg	5900 (± 260)	6200 (± 370)	5700 (± 280)	6500 (± 250)	6700 (± 590)	6100 (± 280)	0.095	0.329	0.929

Elemental concentrations are expressed as µg g^{-1} unless otherwise noted. Data are means ± SE; $n = 20$.

Table 3 Effects of age and simulated herbivore damage treatment on foliar elemental composition of the nonhyperaccumulator shrub, *Ficus webbiana*

	Young foliage			Old foliage			*P*-values		
	None	Moderate	Severe	None	Moderate	Severe	Age	Damage	A x D
Metals									
Ni	43.9 (± 12.6)	47.1 (± 14.7)	47.5 (± 13.1)	139 (± 30.0)	129 (± 31.3)	124 (± 27.3)	<0.0001	0.967	0.914
Zn	27.1 (± 3.0)	36.3 (± 7.8)	30.7 (± 5.3)	53.4 (± 17.3)	34.3 (± 7.7)	29.8 (± 3.6)	0.316	0.577	0.234
Mn	38.7 (± 3.7)	35.1 (± 3.2)	38.8 (± 3.1)	53.4 (± 4.5)	50.6 (± 4.1)	51.6 (± 4.2)	<0.0001	0.682	0.938
Cr	6.4 (± 1.2)	4.9 (± 1.3)	5.9 (± 1.5)	9.3 (± 1.0)	9.2 (± 1.4)	6.6 (± 1.3)	0.011	0.473	0.379
Co	1.1 (± 0.2)	0.9 (± 0.3)	1.0 (± 0.3)	1.6 (± 0.3)	1.9 (± 0.5)	0.9 (± 0.3)	0.079	0.313	0.233
Pb	5.9 (± 1.0)	4.5 (± 1.0)	4.8 (± 1.3)	7.8 (± 1.1)	8.9 (± 1.6)	5.4 (± 1.4)	0.026	0.321	0.312
Nutrients									
N (%)	1.3 (± 0.03)	1.3 (± 0.03)	1.2 (± 0.03)	1.0 (± 0.06)	1.0 (± 0.04)	1.1 (± 0.05)	<0.0001	0.749	0.011
P	720 (± 30)	770 (± 50)	640 (± 30)	610 (± 30)	560 (± 20)	560 (± 20)	<0.0001	0.109	0.171
K	13900 (± 590)	14000 (± 750)	12800 (±650)	9200 (± 790)	8300 (± 520)	9300 (± 510)	<0.0001	0.676	0.258
Ca	14200 (± 770)	13500 (± 1010)	14900 (± 710)	16400 (± 830)	16800 (± 990)	16000 (± 580)	0.002	0.939	0.441
Mg	5200 (± 340)	5000 (± 240)	5500 (± 290)	6500 (± 250)	6400 (± 250)	7100 (± 260)	<0.0001	0.068	0.848

Elemental concentrations are expressed as µg g^{-1} unless otherwise noted. Data are means ± SE; $n = 20$.

1974) have all been reported. Since these values range from 13 to 47 times greater than the amount of Ni necessary to be acutely toxic to an unadapted herbivore (Martens & Boyd, 1994), defence is a likely function of Ni in *Psychotria* leaves at all developmental stages.

Plant defences are not always static; in fact, many defences are induced by herbivory. Although tannins are generally not considered to be inducible (but see Baldwin & Schultz, 1983; Stock *et al.*, 1993), little is known about the inducibility of metal-based defences (Davis & Boyd, 2000). In the current study, neither tannin nor Ni concentrations were increased by simulated herbivore damage, implying noninducibility. There were, however, significant reductions for some measures of organic defence compounds. Total carbon content was less in severely damaged leaves than in moderately damaged leaves for both species (with the exception of mature *Ficus* leaves), indicating that carbon export increased with increased severity of damage. Lower levels of total phenolics and condensed tannins in severely damaged leaves of *Ficus*, and condensed tannins in severely damaged, young *Psychotria* leaves, could be due to mobilization of carbon to other plant organs or failure to reinvest carbon to replace compounds lost from the normal turnover costs of cellular metabolism. It should be noted that the measure of ecological activity of tannins, or protein precipitation ability, was not affected by damage for either species. These patterns in response to damage for both tannins and Ni accumulation indicate that neither defence is induced by simulated herbivory in either *Psychotria* or in *Ficus*. This evidence supports both the widely accepted concept that tannins are constitutive defences (Feeny, 1970) and the more recent idea that Ni hyperaccumulation is also a constitutive trait (Boyd & Martens, 1998b).

Metal hyperaccumulators may have better metal uptake abilities than nonhyperaccumulator plants. Obviously the Ni-hyperaccumulator *Psychotria* should contain more Ni than the nonhyperaccumulator *Ficus*, however, the difference in Ni levels between the two species is staggering; mean Ni content for *Psychotria* leaves was 16 400 $\mu g\ g^{-1}$ d. wt vs 90 $\mu g\ g^{-1}$ d. wt for *Ficus* leaves. In addition, Zn, Cr, Co, and Pb concentrations were 2.5x, 1.5x, 13.0x, and 6.5x higher in *Psychotria* leaves (Table 1). Furthermore, elevated levels of Zn, Cr, Pb, and Co suggest that *Psychotria* has a more efficient metal uptake system (or a less efficient system of exclusion) than *Ficus*. The 'inadvertent uptake' hypothesis, reviewed by Boyd & Martens (1992) and Boyd & Martens (1998), suggests that the evolution of metal hyperaccumulation resulted from a preadapted, enhanced nutrient uptake system, and may not have originated solely in response to herbivore selection pressures. Our data provide support for this hypothesis.

Few studies have attempted to describe multiple defence mechanisms within a species (Pennings, 1996). Unfortunately, chemical (and physical) defences may have subtle interactive effects that can be overlooked by conventional studies focusing on single defensive characteristics. Synergism among defences should increase their effectiveness for overall herbivore protection. It is possible, however, that chemical defences may have antagonistic effects. Goldstein & Spencer (1985) showed that tannins inhibit cyanogenesis in *Carica papaya* L. Similarly, the possibility exists for an antagonism between tannins and Ni. Pizarro *et al.* (1994)) showed that herbal infusions that are high in tannins may inhibit gastrointestinal absorption of Fe, Zn and Cu. A similar interaction may occur between Ni and tannins within the gut of herbivores, thereby limiting the toxicity of Ni. However, preliminary artificial diet studies have shown that Ni and tannic acid produce a synergistic, negative effect on the growth and mortality of *Spodoptera exigua* Hübner larvae (data not shown). Undoubtedly, complete comprehension of the evolution of plant defences of hyperaccumulators cannot occur without consideration of all possible defences within the 'green arsenal.' As our understanding of chemical defences increases, it becomes more apparent that chemical defences work in concert rather than in absence of each other.

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