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EFFECTIVENESS OF METAL–METAL AND METAL–ORGANIC COMPOUND COMBINATIONS AGAINST *Plutella xylostella*: IMPLICATIONS FOR PLANT ELEMENTAL DEFENSE

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Abstract—Plants that contain elevated foliar metal concentrations can be categorized as accumulators or, if the accumulation is extreme, hyperaccumulators. The defense hypothesis suggests that these plants may be defended against folivore attack, and recent research has indicated that metal concentrations at or below the accumulator range may be defensively effective. This experiment explored the toxicity of four metals hyperaccumulated by plants (Cd, Ni, Pb, and Zn) and asked if combinations of metals, or metals and organic chemicals, might broaden the defensive effectiveness of metals. Metals were used alone and in certain metal + metal (Zn plus Ni, Pb, or Cd) and metal + organic defensive chemical (Ni plus tannic acid, atropine, or nicotine) combinations. Artificial diet amended with these treatments was fed to larvae of the crucifer specialist herbivore *Plutella xylostella*. Combinations of metals and metals + organic chemicals significantly decreased survival and pupation rates, compared to single treatments, for at least some concentrations in every experiment. Effects of combinations were additive rather than synergistic or antagonistic. Because Zn enhanced the toxicity of other metals and Ni enhanced the toxicity of organic defensive chemicals, our findings suggest that the defensive effects of metals are more widespread among plants than previously believed. They also support the hypothesis that herbivore defense may have led to the evolution of metal hyperaccumulation by increasing the preexisting defensive effects of metals at accumulator levels in plants.

Key Words—Accumulation, alkaloids, cadmium, elemental defenses, hyperaccumulation, herbivory, lead, metal, nickel, zinc.

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INTRODUCTION

Metals such as Ni, Zn, Pb, or Cd may reach high concentrations in soils because of geological processes (Reeves et al., 1981; Brooks, 1987) or industrial contamination (Reeves and Brooks, 1983; Baker and Brooks, 1989, 1994). Plants growing under natural conditions vary in tissue metal concentrations, and this variation can be used to classify them into three broad categories: normal, accumulator, and hyperaccumulator plants (Brooks et al., 1977; Baker, 1981; Baker et al., 2000; Reeves and Baker, 2000). These categories, summarized in Table 1, vary depending on the metal. In general, relatively small concentrations of Cd, Co, or Cr are unusual in plant tissues, whereas much larger concentrations of Mn or Zn are considered unusual. Other metals (Cu, Ni, and Pb) are elevated at levels between these extremes (Table 1).

Plants are defended by a variety of mechanisms, including organic chemicals (Gatehouse, 2002). Tannins, alkaloids, and glucosinolates are examples of organic defensive chemicals (Feeny, 1976; Rhoades and Cates, 1976; Schultz, 1988; Clausen et al., 1992; Agrawal, 2000). Certain elements, such as Si, F, Ni, Zn, Se, and Ca, also may have defensive functions for plants (McNaughton and Tarrants, 1983; Twigg and King, 1991; Boyd, 2004) or algae (Hay et al., 1994). Hyperaccumulated elements can defend against herbivores (Pollard and Baker, 1997; Boyd and Moar, 1999; Hanson et al., 2004) by reducing feeding (Pollard and Baker, 1997; Jhee et al., 1999; Hanson et al., 2004) or survival (Boyd and Martens, 1994; Boyd and Moar, 1999; Hanson et al., 2004). Termed "elemental defenses" by Martens and Boyd (1994), these

TABLE 1. NORMAL RANGE, MINIMUM ACCUMULATOR LEVEL, AND MINIMUM HYPERACCUMULATOR LEVEL FOR METALS OFTEN ACCUMULATED BY PLANTS[a]

Metal	Normal range ($\mu g\ g^{-1}$)	Minimum accumulator level ($\mu g\ g^{-1}$)	Minimum hyperaccumulator level ($\mu g\ g^{-1}$)
Cd	0.1–3	20	100
Co	0.03–2	20	1000
Cr	0.2–5	50	1000
Cu	5–25	100	1000
Mn	20–400	2,000	10,000
Ni	1–10	100	1000
Pb	0.1–5	100	1000
Zn	20–400	2,000	10,000

[a]Normal range, minimum accumulator level, and minimum hyperaccumulator level refer to tissue concentrations in field-collected plants and follow Reeves and Baker (2000). All values are expressed as μg metal g^{-1} dry mass.

are based on elements taken from soil and sequestered rather than produced by metabolic pathways. Elemental defenses may be advantageous because they cannot be detoxified or degraded by herbivores (Martens and Boyd, 1994), unlike many organic chemicals (e.g., furanocoumarins, Weimin et al., 2003). They also may defend against specialist herbivores that have circumvented an organic chemical defense (e.g., Jhee et al., 2005).

Hyperaccumulator plants occur in many locations and on many soil types (Baker et al., 2000; Reeves and Baker, 2000; Macnair, 2003). Hyperaccumulators of Ni are typically found on ultramafic soils (Brooks et al., 1977; Reeves and Baker, 2000; Iturralde, 2004). Hyperaccumulators of Zn, Pb, and Cd are often found on Pb/Zn mineralized soils or metal polluted soils near mine sites or smelters (Reeves and Brooks, 1983; Baker et al., 2000). At least 418 hyperaccumulator taxa have been discovered, and most of these (318, or 76%) hyperaccumulate Ni (Reeves and Baker, 2000). Some species can hyperaccumulate multiple metals, such as Zn and Cd (Meerts and van Isacker, 1997; Escarré et al., 2000), Zn and Ni (Reeves and Baker, 1984, 2000), Zn and Pb (Meerts and van Isacker, 1997; Baker et al., 2000), Co and Cd (Reeves and Baker, 2000), or Co and Cu (Reeves and Baker, 2000).

Combinations of defensive chemicals can increase plant resistance in two ways. An additive effect of two chemicals can result in a larger combined effect on an herbivore. Compounds may also interact, so that their effects in combination differ from those predicted by adding their individual effects (Nelson and Kursar, 1999). Nelson and Kursar (1999) pointed out that interactions may increase toxicity of compounds in combination (synergism) or may decrease toxicity (antagonism). Most studies of synergism among plant defenses have focused on synergism between organic defenses (e.g., Dyer et al., 2003). Boyd (1998) suggested that multiple elemental defenses, or an elemental defense and an organic defense, may act together to provide greater herbivore resistance than each defense alone. Many Ni hyperaccumulating species belong to families (such as Lauraceae, Rutaceae, Verbenaceae, and Lamiaceae) that possess aromatic substances that may provide resistance to herbivores (Baker et al., 2000; Borhidi, 2001). Many other Ni hyperaccumulators belong to the Brassicaceae, known for production of glucosinolates (Louda and Mole, 1991), or the Rubiacae, known for alkaloids (Borhidi, 2001). Thus, elemental and organic defenses co-occur in plant species, and together they may be more effective than each alone. Pioneering investigations of defensive effects of combinations of elemental and organic chemicals have been conducted for marine algae (e.g., Hay et al., 1994) but, to our knowledge, the possibility that combination effects contribute to the defensive ecology of metal accumulating or hyperaccumulating plants has yet to be formally addressed. Testing these questions is potentially significant for two reasons. First, combination effects may allow elemental defenses to contribute to plant fitness at concentrations

less than expected based on studies of the defensive effects of a metal alone. This could extend the defensive effects of metals to more plant species than otherwise expected. Second, combination effects (either additive effects or synergy) may have contributed to the evolution of metal accumulation and hyperaccumulation by plants (Boyd, 2004). In this scenario, a plant capable of taking up and tolerating elevated levels of a metal could derive a defensive benefit from that ability at a relatively low metal concentration in its tissues. Further stepwise increases in uptake and tolerance abilities could then be selected for in the population, as a result of differential damage from natural enemies, resulting in still greater levels of elemental defenses. By lowering the level of metal at which a defensive benefit first accrues to a plant, combination effects may help explain the evolution of metal accumulation, and later hyperaccumulation, by plants.

Recently, Coleman et al. (2005) used the diamondback moth (DBM), *Plutella xylostella*, to explore the boundaries of defensive effects of eight metals (Cd, Co, Cr, Cu, Mn, Ni, Pb, and Zn) often accumulated by plants (Reeves and Baker, 2000). They found that all metals were toxic to DBM larvae at hyperaccumulator levels. However, all also were toxic to larvae at accumulator concentrations. Five metals (Cd, Mn, Ni, Pb, and Zn) were toxic below accumulator levels, Cd and Pb were toxic near the concentration ranges of normal plants, and Zn was toxic at a concentration within the normal range. Their results indicated that single metals may be effective at concentrations far lower than previously hypothesized.

This study tests the hypothesis that combinations of metals, or combinations of metals and organic defense chemicals, have greater defensive effects than each compound alone. If this is the case, then defensive effects of metals in plants may be more extensive than previously proposed (Coleman et al., 2005). We used DBM as a bioassay herbivore because of its relative ease of colony maintenance and short generation time. We used Zn combined with Cd, Ni, and Pb to represent metals often found in combination in metal accumulator or hyperaccumulator plants. We also used several representative organic defensive compounds to test for combination effects with Ni. Nickel was chosen because it is the metal most often hyperaccumulated by plants (Reeves and Baker, 2000). Tannic acid was used to represent the "digestibility" reducing quantitative defense commonly found in apparent plants (Feeny, 1976; Rhoades and Cates, 1976; Behmer et al., 2002). Nicotine and atropine, both alkaloids, represented qualitative toxins that can defend against generalist herbivores (Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1979; Gómez et al., 2003). The toxic effects of both these alkaloids on herbivores have been well studied (e.g., Muller, 1998; Yildiz, 2004). Our experiments consisted of a series of artificial diet feeding trials using DBM larvae. In each trial, larvae were reared on one of four diet treatments: two treatments consisting of each chemical

added to diet separately, a third diet consisting of both chemicals in combination, and a fourth containing no added chemicals (control). Specifically, we asked the following questions:

1) Does a combination of two metals decrease survival or pupation of DBM larvae compared to each metal alone?
2) Does a combination of Ni and an organic chemical decrease larval survival or pupation compared to Ni alone or to the organic chemical alone?

METHODS AND MATERIALS

Experimental Colony. The diamondback moth, *P. xylostella* (L.) (Lepidoptera: Plutellidae), is an oligophagous herbivore of the Brassicaceae (Talekar and Shelton, 1993). This family contains a large percentage (about 25%) of the known hyperaccumulator species (Reeves and Baker, 2000). Although DBM has not been reported as attacking hyperaccumulators in the wild, surveys of herbivores on hyperaccumulator species are few (for exceptions, see Mesjasz-Przybylowicz and Przybylowicz, 2001; Wall and Boyd, 2002). The laboratory colony (Department of Entomology and Plant Pathology, Auburn University, Auburn, AL, USA) was maintained on an artificial DBM diet (BioServe, Frenchtown, NJ, USA). The diet's exact ingredients are proprietary information, but wheat germ and cabbage leaf powder are two of the main ingredients (Carpenter and Bloem, 2002). Colony maintenance procedures generally followed those of Harvey (2002). Eggs were collected on grooved aluminum foil sheets that had been coated with sterilized collard juice as an oviposition stimulant (Harvey, 2002). A 10% bleach solution was used to soak aluminum foil sheets of DBM eggs for 20 sec. Foil sheets were rinsed with deionized (DI) water for 1 min and allowed to dry. Dried egg sheets were cut into strips containing approximately 300–400 eggs per strip. Each strip was placed into a 250-ml paper cup (Solo, Twin Falls, ID, USA) with about 1 cm of congealed artificial diet covering its bottom. Cups were incubated at 37°C and approximately 30–50% humidity until eggs hatched and the instars first began to feed (~60 hr from egg collection). Larvae were allowed to feed for approximately 10–12 d after hatching. Pupae were placed in screen cages kept at room temperature where eclosed adults could mate and lay eggs on the aluminum foil sheets.

Metal + Metal Combination Experiments. Artificial diet was amended with metals to examine the effect of single, combination, and control treatments on DBM larvae. Each combination experiment compared DBM survival on diet containing each metal alone, diet containing both metals, and a control of unamended diet. Three combination experiments were conducted, using Zn

paired with Cd, Ni, or Pb. Metals were obtained as chloride salts from Sigma (St. Louis, MO, USA). Metal salts were dissolved in DI water to form stock solutions of 0.02 M Ni, 0.2 M Zn, 2 mM Pb, or 0.4 mM Cd. For each combination experiment, 100 ml of diet were amended with stock solutions to yield the concentrations of metal listed in Table 2. Experimental concentrations of metals were selected based on preliminary results that determined approximate lethal concentrations. Approximately 2–3 ml of diet were distributed into each 30-ml plastic rearing cup to give 12 replicates of each treatment within a combination experiment. A separate cup of 30 ml of diet from each treatment was saved for later metal concentration analysis.

DBM eggs were collected from a single cage containing adults that were 2–4 d posteclosion. Eggs were collected and sterilized in a 10% bleach solution and rinsed with DI water for 1 min. After egg sheets were dried, they were cut into strips containing approximately 80–100 eggs. Strips of eggs were arbitrarily distributed into diet cups by adding a strip to each cup in the first replicate (i.e.,

TABLE 2. METAL AND CHEMICAL CONCENTRATIONS IN ARTIFICIAL DIET[a]

Experiment	Chemical	Units	Unamended diet	Amended diets			
Zn + Ni	Zn	μM Zn g^{-1}	0	12	18	24	140
		μg Zn g^{-1}	22[b]	760	1200	1500	8900
	Ni	μM Ni g^{-1}	0	1.2	1.9	2.9	3.7
		μg Ni g^{-1}	3.4[b]	73	110	170	220
Zn + Pb	Zn	μM Zn g^{-1}	0	11	19	25	150
		μg Zn g^{-1}	21[b]	740	1200	1600	10,000
	Pb	μM Pb g^{-1}	0	4.6	83	120	170
		μg Pb g^{-1}	0.088[b]	9.5	17	26	36
Zn + Cd	Zn	μM Zn g^{-1}	0	12	18	23	150
		μg Zn g^{-1}	22[b]	750	1200	1500	10,000
	Cd	μM Cd g^{-1}	0	19	58.1	118	189
		μg Cd g^{-1}	0.063[b]	2.1	6.5	13	21
Ni + Tannic acid	Ni	μM Ni g^{-1}	0	1.3	1.9	2.9	4.0
		μg Ni g^{-1}	4.3[b]	77	110	170	230
	Tannic acid	mg ml^{-1}	0	0.5	1	1.5	2
Ni + Atropine	Ni	μM Ni g^{-1}	0	1.3	1.9	2.6	3.4
		μg Ni g^{-1}	3.6[b]	74	110	150	200
	Atropine	mg ml^{-1}	0	0.04	0.05	0.08	0.1
Ni + Nicotine	Ni	μM Ni g^{-1}	0	1.3	1.8	2.7	3.8
		μg Ni g^{-1}	3.4[b]	77	110	160	220
	Nicotine	mg ml^{-1}	0	0.02	0.03	0.035	0.04

[a]Molar concentrations are amounts added by dilution of stock solutions during diet preparation. Data for metals in μg g^{-1} are from dry mass elemental analyses of diet samples.
[b]Amounts of these elements were present in unamended (control) diets.

metal 1, metal 2, combination, control), then a strip to each cup in the second replicate, etc., until the cups in all 12 replicates had received an egg sheet.

Diet cups were placed into an incubator at 37°C and approximately 30–50% humidity. Egg sheets were removed from cups after larvae hatched and had begun to feed (approximately 60 hr after the sheets were collected). When egg sheets were removed, we counted the number of first instars in each cup. The number of live larvae was counted every 2–3 d thereafter. Once pupation began, numbers of live pupae also were counted, and each pupa was recorded as a surviving individual. Counting for all cups within a combination experiment ended when adults began to eclose from the control treatment (approximately 14–17 d after eggs were collected).

Nickel + Organic Chemical Experiments. Artificial diet was amended with combinations of Ni and an organic defense chemical. Each combination experiment compared DBM survival on diet containing each chemical alone, diet containing both chemicals, and a control of unamended diet. Nickel was paired with tannic acid, atropine, or nicotine. Organic chemicals were obtained from Sigma. To create a stock solution of tannic acid, we dissolved 400 mg of powdered tannic acid in 4 ml of ethanol and then diluted with DI water to form a 5-mg ml^{-1} concentration stock solution. Atropine stock solutions were formed by dissolving 200 mg of atropine, in powdered form, in 4 ml of methanol and diluting with DI water to create a 1-mg ml^{-1} solution. The stock solution of nicotine was made by dissolving 50 mg of powdered nicotine into 1 ml of ethanol and diluting with water to create a 0.25-mg ml^{-1} solution. For each combination experiment, 100 ml of diet were amended with the Ni and organic chemical concentrations listed in Table 2. Experimental concentrations of metals and organic chemicals were selected based on preliminary results that determined approximate lethal concentrations to DBM. Diet was distributed into 30-ml plastic cups as with the metal–metal experiments, and a separate cup of 30 ml of diet from each treatment was saved for later metal concentration analysis. Addition of eggs, incubation, and counting of larvae and pupae followed the procedures of the metal–metal combination experiments described.

Elemental Analysis. Plant metal concentrations in the literature on accumulation and hyperaccumulation are expressed in μg g^{-1} dry mass (e.g., Brooks, 1987; Reeves and Baker, 2000). We performed diet analyses to provide comparable data on metal concentration in each treatment expressed as μg g^{-1} dry mass of diet. The 30-ml sample of diet retained from each metal concentration was dried at 60°C for 5 d and ground to a fine powder. Four 0.5-g dry mass subsamples from each metal concentration were wet digested using 10 ml of acid mix (700 ml concentrated HNO_3 + 300 ml concentrated $HClO_4$) within 250-ml glass digestion tubes for 24 hr. The next day, tubes were heated on a block digester within a perchloric acid fume hood at 190°C until digestion was complete. Once the tubes cooled, 2.5 ml of 1 M HCl were added

to each tube, and contents were transferred to 25-ml volumetric flasks. Contents of the volumetric flasks were brought to 25 ml by adding DI water and transferred to 100-ml plastic storage bottles (Nalgene, Rochester, NY, USA). Metal concentrations were determined using an inductively coupled argon plasma (ICP-AE) spectrophotometer (SPECTRO CIROS CCD, Cleves, Germany).

Statistical Analysis. Percent survival of DBM larvae for all treatments was calculated based on the final number of survivors (larvae plus pupae) on d 16 for all treatments (d 15 for the Ni + tannic acid experiment). Because our experiment was not a complete factorial design, we compared percent survival among metal + metal experiment treatments and Ni + organic chemical experiment treatments by using pairwise orthogonal contrasts after one-way analysis of variance (ANOVA) with JMP IN 5.1 (SAS Institute, 2005). Prior to analysis, percent survival data were arcsine square root-transformed to better fit the assumptions of ANOVA (Zar, 1996). The orthogonal contrasts allowed us to answer two questions for each combination experiment:

1) Did chemicals singly or in combination decrease survival relative to the control?
2) Did the combination of chemicals result in decreased survival compared to each chemical singly?

Differences in survival were considered significant at $\alpha \leq 0.05$.

We further examined the data to subdivide combination effects into additive or interactive (synergistic or antagonistic) effects. We converted DBM survival data into mortality data and, by using the technique of Salama et al. (1984), calculated an expected mortality for each combination treatment using the mean mortality from each chemical in a combination. These expected mortalities were compared to the actual combination mortality by using a chi-square test at $\alpha \leq 0.05$ (Salama et al., 1984). Significant deviation from expected values would indicate either synergy (if actual mortality were greater than expected) or antagonism (if actual mortality were lesser than expected).

To detect sublethal effects of treatments on DBM larvae, we compared pupation rates between treatments at the time pupae were first observed in the control treatment for each experiment. Thus, this measure included both mortality effects and decreased development time due to treatments. Pupae counts used for each experiment were taken from the data at 14 d for Zn + Ni, 10 d for Zn + Pb, 10 d for Zn + Cd, 9 d for Ni + tannic acid, 12 d for Ni + atropine, and 11 d for Ni + nicotine. We calculated % pupation by dividing the number of pupae in each cup by the maximum number of larvae that had been counted for that cup. Percent pupation values were arcsine square root-transformed to better fit the assumptions of ANOVA (Zar, 1996) and analyzed by one-way ANOVA to determine if treatments significantly affected pupation,

and pairwise orthogonal contrasts were used to compare means using JMP IN 5.1 (SAS Institute, 2005).

As with survival data, we further examined pupation data in cases where combinations had greater effects than single compounds to determine if this was caused by an additive or by an interactive (synergistic or antagonistic) effect. We calculated an expected percentage of larvae that did not pupate for each combination treatment by using the mean "failure to pupate" value from each chemical in a combination. As above, comparison of actual and expected values showed if synergy or antagonism had occurred.

RESULTS

Data analysis revealed statistically significant treatment effects for all experiments (ANOVA, $\alpha < 0.05$ in all cases). Here, we focus on the results from the pairwise orthogonal contrasts for each experiment. We use these to determine the concentrations of chemicals at which a combination effect was observed, defining a combination effect as when the survival or pupation rate for the combination treatment differed from that of both chemical treatments alone. We then ask if each combination effect is an additive or an interactive (synergy or antagonism) effect.

Metal + Metal Combination Experiments. For Zn + Ni, combination effects on survival were found at the two highest concentrations used: 1500 μg Zn g^{-1} + 170 μg Ni g^{-1} (Figure 1C) and 8900 μg Zn g^{-1} + 220 μg Ni g^{-1} (Figure 1D). Both these combination effects were additive. A combination effect for pupation (sublethal effect) occurred for the 1200 μg Zn g^{-1} + 110 μg Ni g^{-1} treatment, for which a combination effect was not detected from survival data. This combination effect was additive. Greater concentrations of Zn + Ni did not show combination effects for pupation because the Zn treatment produced almost no pupation in those cases.

For Zn + Pb, combination effects on survival were detected at 1200 μg Zn g^{-1} + 17 μg Pb g^{-1} and 10,000 μg Zn g^{-1} + 36 μg Pb g^{-1} (Figure 2B and D). These effects were additive. For pupation data, combination effects were observed for all but the lowest concentrations examined (Figure 2E–H). These effects also were additive.

Combination effects on larval survival for Zn + Cd treatments were detected for all concentrations except 1200 μg Zn g^{-1} + 6.5 μg Cd g^{-1} (Figure 3B). The effects were additive in all three cases (Figure 3A, C, and D). Pupation data revealed combination effects in all four trials (Figure 3E–H). These effects were striking, as pupation was zero for the combination treatments of all four trials, whereas at least some larvae pupated in all single metal treatments. The effects for the pupation data were additive in all cases.

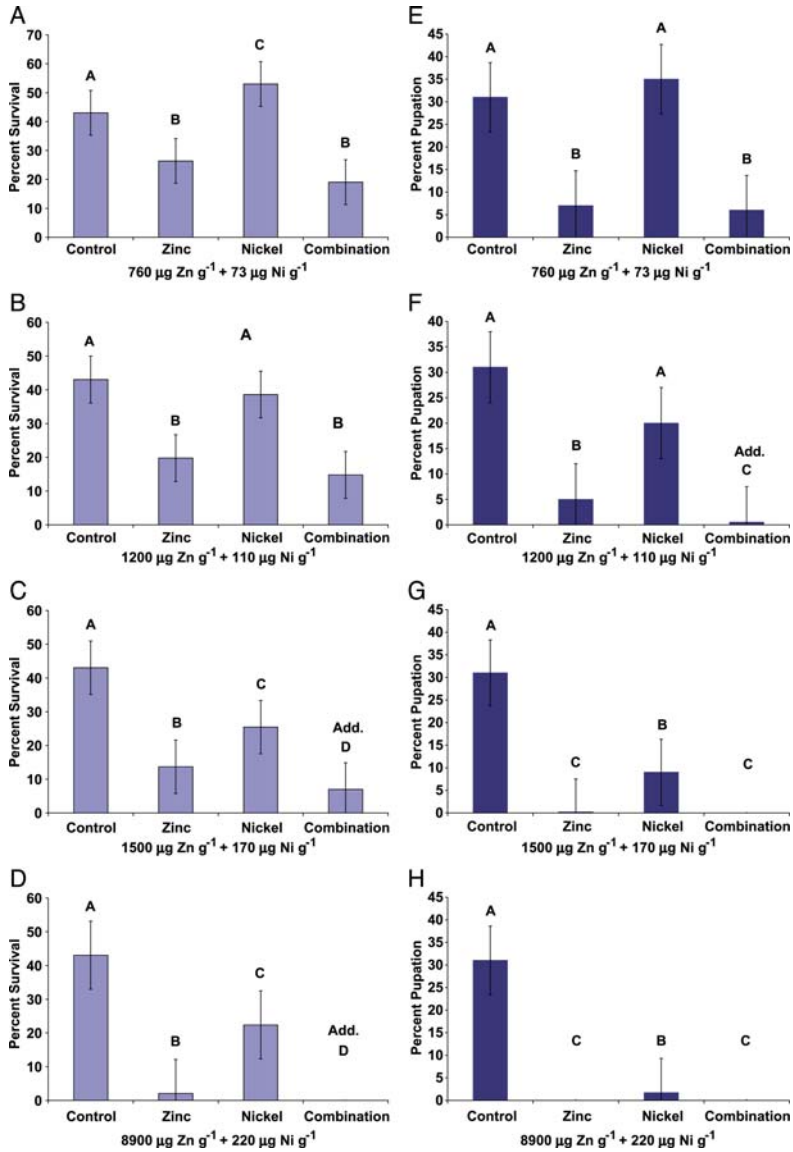

FIG. 1. Effects of Zn and Ni on diamondback moth (DBM) survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation "Add." denotes those experiments for which a significant additive combination effect was detected.

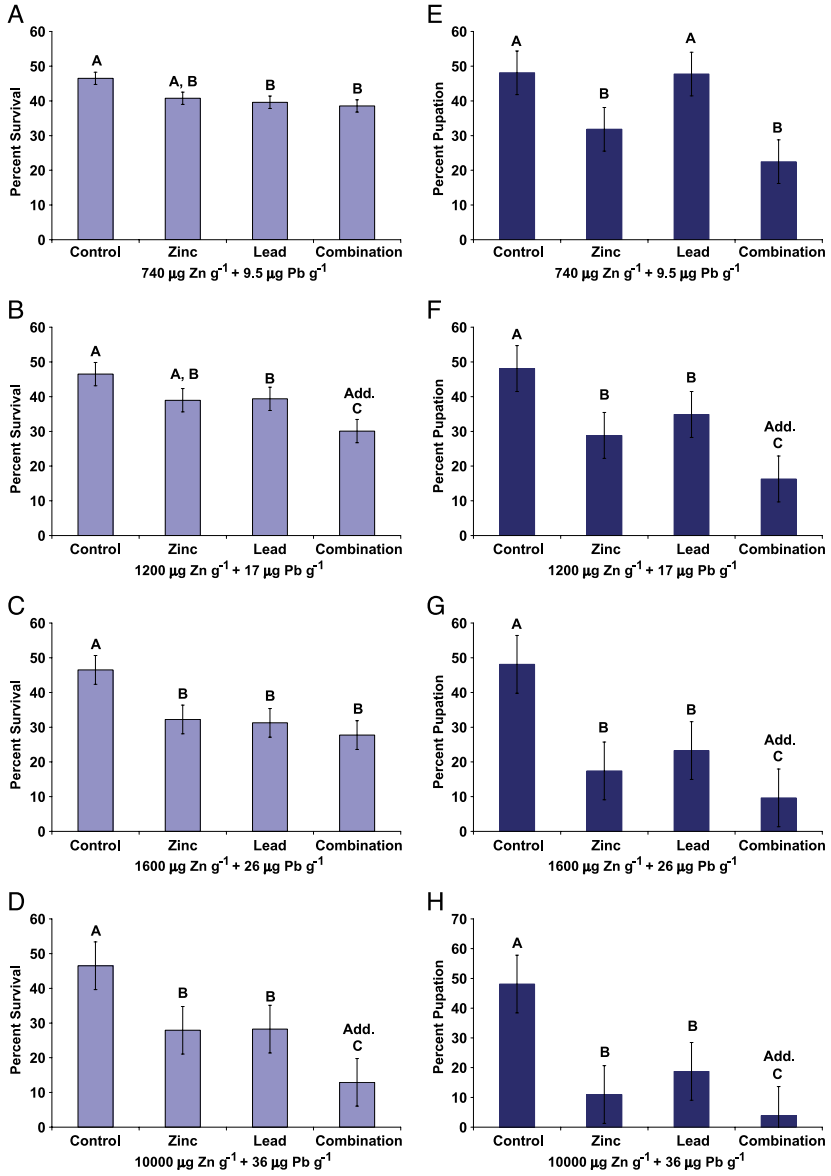

FIG. 2. Effects of Zn and Pb on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation "Add." denotes those experiments for which a significant additive combination effect was detected.

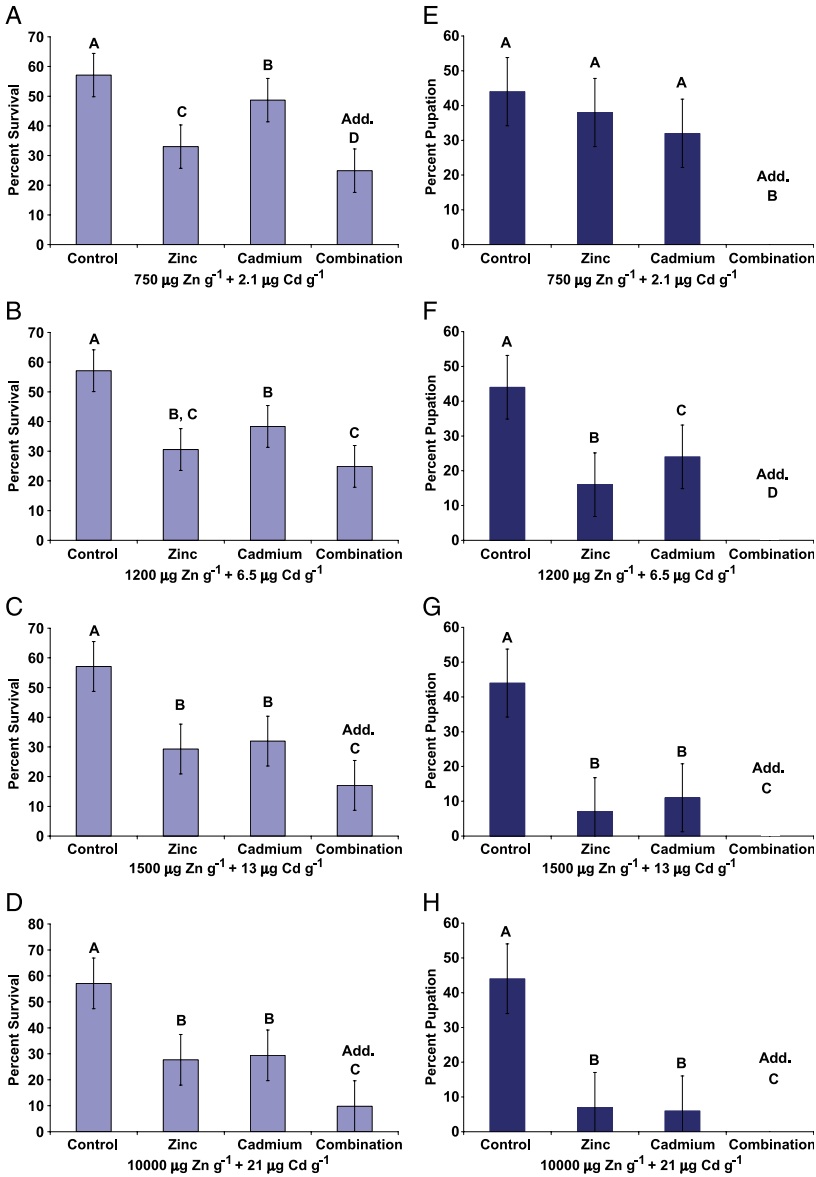

FIG. 3. Effects of Zn and Cd on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation "Add." denotes those experiments for which a significant additive combination effect was detected.

Nickel + Organic Chemical Combination Experiments. Combination effects for Ni + tannic acid were detected for survival data for all but the lowest concentrations used (77 μg Ni g^{-1} + 0.5 mg tannic acid ml^{-1}; Figure 4A). All were additive. Pupation data showed effects for all concentrations (Figure 4E–H), and all were additive.

For Ni + atropine, combination effects for survival data were detected for the two greatest concentrations used (150 μg Ni g^{-1} + 0.08 mg atropine ml^{-1} and 200 μg Ni g^{-1} + 0.1 mg atropine ml^{-1}; Figure 5C and D). Both the combination effects were additive. Pupation data showed effects for all trials (Figure 5E–H), and all were additive.

Combination effects on DBM survival in the Ni + nicotine experiment were detected for the two greatest concentrations (160 μg Ni g^{-1} + 0.035 mg nicotine ml^{-1} and 220 μg Ni g^{-1} + 0.04 mg nicotine ml^{-1}; Figure 6C and D). These were additive. For pupation data, effects were detected for all trials except that using the least concentrations (77 μg Ni g^{-1} + 0.02 mg nicotine ml^{-1}; Figure 6E), and these effects were additive.

DISCUSSION

Our experiments provide a first test against an herbivore of the effects of metals in combination with other metals and with organic compounds. Defining a combination effect as a significantly greater impact in combination than for either chemical alone, we detected a combination effect for each pair of metals and for each Ni + organic chemical pairing (Figures 1–6). Furthermore, combination effects were found for both survival and pupation data. Results for the pupation data extended combination effects to still lower concentrations than were detected by the survival data for all experiments except Ni + tannic acid (in which all treatments showed significant combination effects; Figure 4) and Zn + Pb (Figure 2). Thus, we show that combination effects can magnify the protective effects of metals, and that metals may provide protection against herbivores at lower concentrations than previously believed. We note that combination effects were detected only at concentrations for which at least one of the chemicals alone had a significant negative effect on DBM relative to the control treatment (Figures 1–6). This indicated that the toxicity of one chemical made DBM more susceptible to the negative effects of the second chemical. Further tests will be needed to explore other metal + metal and metal + organic chemical combinations, but our results suggest that combination effects are common for the metals accumulated by plants.

All the combination effects we detected were additive. Boyd (1998) speculated that synergy between metals and organic defenses might magnify the effectiveness of each type of defense. We found no cases of synergy. We also

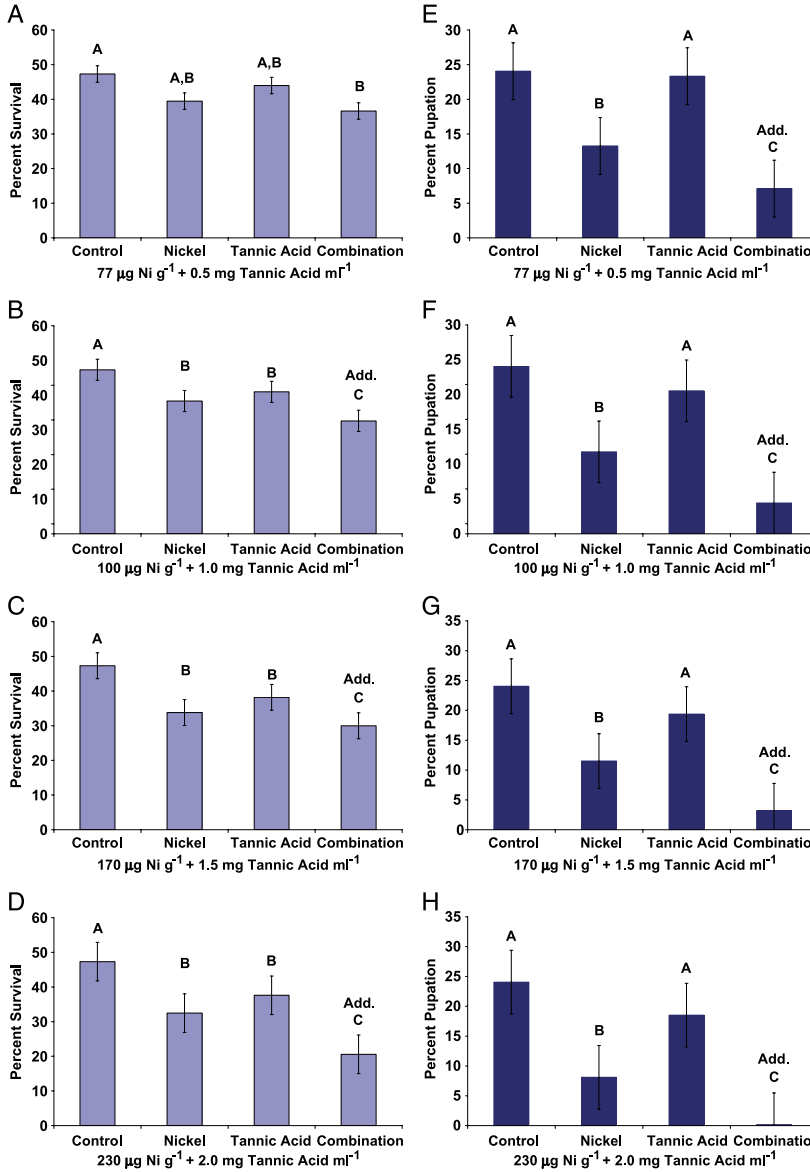

FIG. 4. Effects of Ni and tannic acid on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation "Add." denotes those experiments for which a significant additive combination effect was detected.

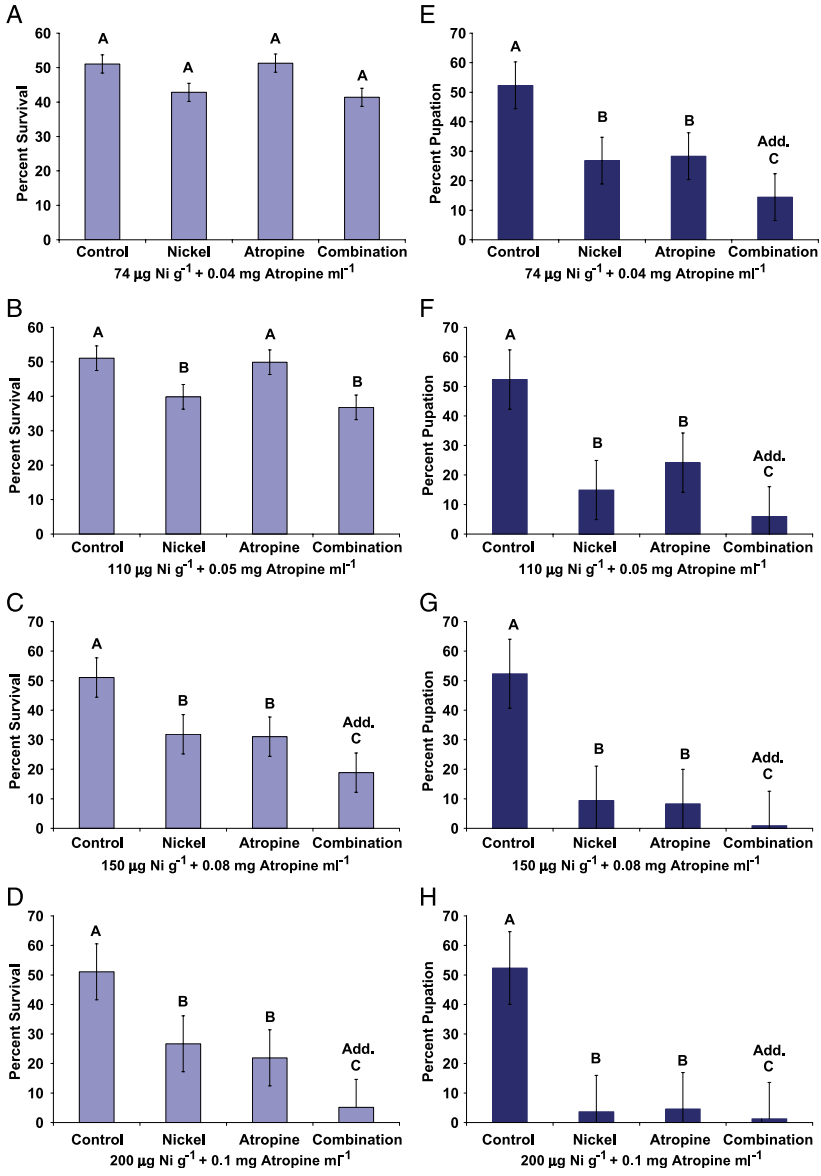

FIG. 5. Effects of Ni and atropine on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation "Add." denotes those experiments for which a significant additive combination effect was detected.

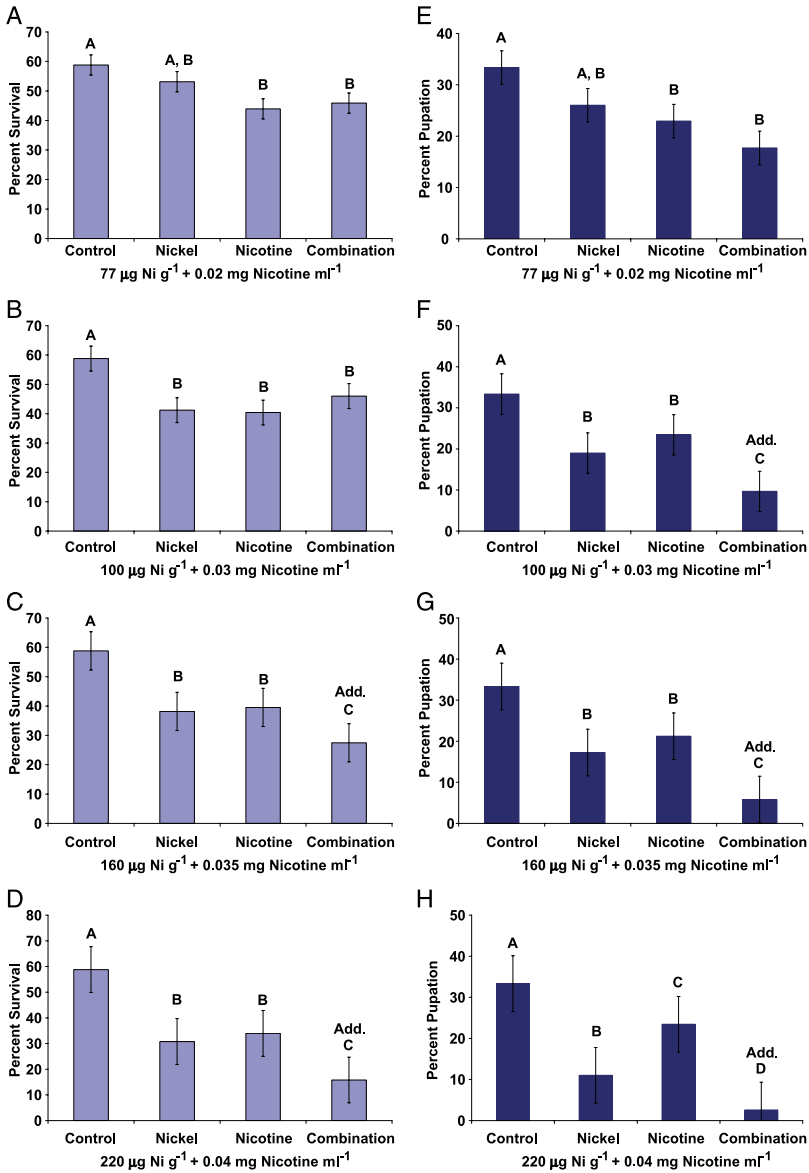

FIG. 6. Effects of Ni and nicotine on DBM survival (A–D) and pupation (E–H). Bars on means denote SE. For each experiment, means with the same letters are not significantly different at $\alpha \leq 0.05$ (orthogonal contrasts of transformed data). Note that means and SE values in graphs are calculated for untransformed data. The notation "Add." denotes those experiments for which a significant additive combination effect was detected.

found no cases of antagonism, which would decrease the effectiveness of combinations of defensive chemicals. Our ability to detect interactive effects was limited because of the relatively high mortality rates of DBM larvae in our experimental trials (Hay, 1996; Pennings, 1996). This mortality rate was not unusual for a DBM colony (Shelton and Collins, 2000), but further explorations should be made for synergy/antagonism between metals, and between metals and organic chemicals, using another experimental system.

The demonstration of combination effects between metals and organic compounds (Figures 4–6) suggests that plants may be able to reduce production of organic defenses by sequestering metals. Martens and Boyd (1994) suggested that a trade-off between elemental and organic defenses allows hyperaccumulators to invest less carbon in the construction of organic defenses and yet remain defended. Tolrà et al. (2001), studying the Zn hyperaccumulator *Thlaspi caerulescens* J. & C. Presl. (Brassicaceae), demonstrated that hyperaccumulating plants possessed significantly lower concentrations of glucosinolates than low-Zn plants. Our study shows that additive effects between Ni and organic compounds enable Ni combined with a low concentration of organic compounds to provide the same defensive effect as higher levels of those organic compounds alone. Thus, plant uptake of metals to relatively low but defensively effective concentrations can allow reduced organic chemical production. Because organic chemical production must have a physiological cost (Agrawal, 2005), then the cost reduction may be significant.

Accumulation or hyperaccumulation of multiple metals may have selective value because plant enemies differ in susceptibility to different elemental defenses. For example, Boyd and Shaw (2004) showed that a plant pathogenic bacterium (*Xanthomonas campestris*) was particularly sensitive to Cu but not to Co. Therefore, an accumulator of both Cu and Co might be protected against this pathogen by its Cu concentration but not its Co concentration. However, Coleman et al. (2005) showed that low levels of Co (40 $\mu g\ g^{-1}$) are defensively effective against DBM, so that Co would protect a Co/Cu accumulator against DBM. Similar considerations regarding multiple natural enemies may explain contrasting results of tests of the defense hypothesis for single metals. For example, Zn hyperaccumulation in *T. caerulescens* is effective against some pathogenic fungi and some herbivores (lepidopteran larvae and grasshoppers; Pollard and Baker, 1997; Jhee et al., 1999) but ineffective against other herbivores (snails; Huitson and Macnair, 2003; Noret et al., 2005).

The evolution of hyperaccumulation is likely complex and may have occurred multiple times for each hyperaccumulated element (Borhidi, 2001) and under different selection pressures. However, Boyd (1998, 2004) suggested that defensive effects of elemental accumulation at levels below the hyperaccumulation threshold might have led to the evolution of hyperaccumulation. Coleman et al. (2005) showed that low concentrations (accumulator concentrations or less)

of most metals increase the mortality of DBM larvae. Our current work shows that relatively low concentrations of metals can be effective defenses because metal + metal and metal + organic chemical combinations increase the effectiveness of these metals. This suggests that low concentrations of metals in plants, even lower than those documented by Coleman et al. (2005), can contribute to plant defense. If these lower concentrations result in increased plant fitness, then plant traits responsible for relatively low levels of metal uptake and sequestration will be favored in plant populations faced with natural enemies. Metal accumulation ability may spread in a population and be enhanced in a stepwise process driven by increased defensive benefits of increased metal accumulation. These defensive effects, therefore, provide a likely pathway for the evolution of accumulation and hyperaccumulation of metals by plant populations.

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