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A ROLE FOR ISOTHIOCYANATES IN PLANT RESISTANCE AGAINST THE SPECIALIST HERBIVORE *Pieris rapae*

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Abstract—We experimentally reanalyzed the classic interaction between *Pieris rapae*, a specialist lepidopteran herbivore, and isothiocyanates (mustard oils) that are characteristic phytochemicals of the Brassicaceae. Previous investigations have suggested that *P. rapae* is unaffected by isothiocyanates. Using whole plants, root extracts, and a microencapsulated formulation of allyl isothiocyanate, we now show that isothiocyanates reduce herbivore survival and growth, and increase development time, each in a dose-dependent manner. Neither the substrate allyl glucosinolate, nor myrosinase, the enzyme that results in the breakdown of glucosinolates, negatively affected *P. rapae.* Thus, we present strong evidence for a role for isothiocyanates in plant resistance against the specialist herbivore *P. rapae.*

Key Words—*Arabidopsis lyrata, Brassica* spp., Brassicaceae, glucosinolates, herbivory, horseradish, host range evolution, induced plant resistance, microencapsulation, myrosinase, plant–insect interactions.

INTRODUCTION

Organisms specialize on the consumption of particular foods because of trade-offs in fitness when eating alternate resources (Futuyma and Moreno, 1988; Agrawal, 2000a). For herbivorous macrolepidoptera in particular, approximately 25% of species feed on species in one plant family (Schoonhoven et al., 1998). Specialist herbivores restrict their counterdefensive measures to the small range of defensive tactics of the plants on which they specialize, while generalists invest in broad detoxification strategies (Krieger et al., 1971; Schoonhoven et al., 1998). Thus, specialist herbivores have long been predicted to be less affected by a

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given chemical defense in plants compared to generalists (the specialist herbivore paradigm) (Whittaker and Feeny, 1971; Feeny, 1976; Blau et al., 1978; Rhoades, 1979; Van Dam et al., 1993; Dyer, 1995; Giamoustaris and Mithen, 1995; Van Der Meijden, 1996). While the specialist herbivore paradigm must be true to some extent, several studies have challenged this notion by demonstrating strong effects of plant chemicals on specialists (Berenbaum et al., 1989; Adler et al., 1995; Malcolm and Zalucki, 1996; Agrawal, 2000b; Van Dam et al., 2000; Kliebenstein et al., 2002; Traw and Dawson, 2002).

White butterflies in the genus *Pieris* are largely restricted to feeding on plants in the Brassicaceae and have been a prominent focus in the study of the ecology and evolution of host specialization (Verschaffelt, 1910; Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991; Ohsaki and Sato, 1994; Giamoustaris and Mithen, 1995; Renwick, 2001). Although plants in the Brassicaceae contain diverse phytochemicals thought to serve defensively, *Pieris rapae*, a herbivore that widely feeds on plants in the Brassicaceae, is purportedly unaffected by either glucosinolates (and their breakdown products) (Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991) or proteinase inhibitors (Broadway, 1995). In particular, glucosinolates are sulfur- and nitrogen-containing secondary compounds characteristic of the Brassicaceae. Myrosinase is an enzyme stored separately from glucosinolates in plants, and does not react with glucosinolates until tissues are macerated, as occurs when lepidopteran larvae feed on leaves. The resulting hydrolysis products, isothiocyanates or mustard oils, give mustards their characteristic pungent odor and are toxic to a broad range of organisms (Chew, 1988; Louda and Mole, 1991).

The groundbreaking studies on the response of *P. rapae* to plant defense involved feeding larvae diets that were variable in glucosinolate concentration and assumed that hydrolysis to isothiocyanates occurred in the herbivore gut (Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991). These studies confirmed that glucosinolates *per se* were not effective agents of plant resistance against *P. rapae*, and that the compounds serve as both feeding and oviposition stimulants (Slansky and Feeny, 1977; Blau et al., 1978; Chew, 1988; Louda and Mole, 1991; Giamoustaris and Mithen, 1995; Renwick and Lopez, 1999). Although putative plant defenses are known to have manifold effects in communities (Linhart, 1991), stimulatory effects on specialists have reinforced the specialist herbivore paradigm. Nonetheless, the defensive role of the breakdown products of glucosinolates (isothiocyanates) against *P. rapae* has not been rigorously addressed.

We were motivated to reinvestigate the role of isothiocyanates as a defense against *P. rapae* because no previous experiments had successfully evaluated the role of isothiocyanates on larval performance. We consistently observe a reduction in the performance of *P. rapae* on plants that have been previously damaged by herbivores, challenging the application of the specialist herbivore paradigm to *P. rapae* (Agrawal, 1998, 2000b; Agrawal et al., 1999b). To demonstrate unambiguously

the effects of the glucosinolate–myrosinase system and their hydrolysis products on *P. rapae*, we took a three-pronged approach employing whole plants, root extracts, and synthetic diets incorporating volatile isothiocyanates in microcapsules. In particular, we first show that phenotypic and genetic manipulations of the glucosinolate–myrosinase system in plants affect *P. rapae*'s growth and development. We then isolate the specific effects of allyl isothiocyanate on *P. rapae* by employing active and deactivated pastes of horseradish root extract and a microencapsulated formulation of isothiocyanate in synthetic diets. Although previous studies have directly attempted to examine the effects of allyl isothiocyanate on herbivores, they have failed because allyl isothiocyanate is volatile; when added to synthetic diets, these isothiocyanates are released and fumigate the larvae (Mcloskey and Isman, 1993). Our final approach involving microencapsulated single molecules of allyl isothiocyanate in β-cyclodextrin surmounts previous difficulties of dosing diets with volatile defense compounds (Usher et al., 1989; Szente et al., 1990).

METHODS AND MATERIALS

Whole Plant Experiments. Induced plant resistance to *P. rapae* was assessed with *Arabidopsis lyrata* and *Brassica oleracea*, two host plants commonly used by *P. rapae* in the field. *A. lyrata* ($N = 52$) were grown in a growth chamber in 500-ml pots. At the 6–10 leaf stage, a newly hatched *P. rapae* larva was introduced to half of the plants. *P. rapae* larvae are sluggish and do not move from plant to plant as long as leaves are not touching. Thus, larvae were not caged or restricted. After 5 days of feeding, less than 20% of the leaf tissue was damaged and the caterpillars were removed. A newly hatched *P. rapae* larva was then added to each plant and weighed to the microgram after 5 days. *Brassica oleracea* var. Marathon ($N = 76$) were grown in a glasshouse. All procedures were the same as above except that damaging *P. rapae* larvae were introduced when the plants had one fully expanded true leaf. Chemical analyses were conducted on 15 replicates of each treatment of *B. oleracea*. The analytical procedure for determining glucosinolates was modified from published protocols, and glucosinolates were identified using predetermined HPLC retention times (Brown and Morra, 1995).

To assess the performance of *P. rapae* on whole plants, we worked with four lines of *Brassica* with genetically determined differences in glucosinolate concentrations. A double haploid line was derived from *B. oleracea* var. Green Duke, and three additional lines were produced from its progeny with three wild *Brassica* species (*B. drepanensis, B. villosa*, and *B. atlantica*) (Faulkner et al., 1998). Methods and glucosinolate inheritance are reported in Faulkner et al. (1998). Seeds from these crosses were grown in a glasshouse as described earlier. At the four-leaf stage, a single, newly hatched *P. rapae* larva was introduced to each

plant. The plants were not caged, and caterpillars were not restricted. When the caterpillars entered the wandering phase, approximately 4 days before pupation, we confined the larvae to deli cups and provided them with cut foliage from the plants on which they were feeding.

Root Extract Experiment. To examine the specific effects of allyl isothiocyanate on *P. rapae*, we employed diets that vary in their allyl isothiocyanate levels. We used Japanese Horseradish Powder (JFC International, Inc., San Francisco, CA), which contains only horseradish, mustard seed, cornstarch, and coloring. Allyl isothiocyanate is the primary hydrolysis product released by damage to horseradish (Yu et al., 2001). The powder was obtained by freeze-drying plant tissues, to remove water without letting the reaction between glucosinolates and myrosinase take place. When this powder is wetted, the reaction takes place in a few minutes, as evidenced by the pungent odor and burning taste of horseradish. Our hypothesis was that higher doses of active horseradish extracts would result in larger negative effects on the herbivore. We prepared three types of insect diet: (1) active horseradish paste (1:1 mixture of dry powder and tap water based on mass), (2) deactivated horseradish paste (dry powder wetted to release volatile isothiocyanates, then dried and ground using a mortar and pestle); and subsequently rewetted at the same (1:1) powder-to-water ratio, and (3) 10% active horseradish paste (with a 1:9 ratio of active to deactivated powder). The deactivated paste had the same appearance and texture as the active paste, but none of the spicy, vaporous emanations indicative of isothiocyanate production. Sixty, newly hatched *P. rapae* larvae were initially introduced singly to 30-ml plastic cups with approximately 0.5 g of paste ($N = 20$ on active paste and 40 on deactivated paste). The horseradish paste was refreshed each day of the experiment. After 3 days, the surviving caterpillars on the deactivated paste were split into two equal groups, one maintained on deactivated paste and one on 10% active paste.

Synthetic Diet Experiments. We prepared synthetic diets suitable for *P. rapae* (Webb and Shelton, 1988) and assessed the consequences of dosing these diets with microencapsulated allyl isothiocyanate, allyl glucosinolate, or myrosinase on *P. rapae*'s growth and development. Natural host plants of *P. rapae* range in the concentrations of allyl isothiocyanate up to 1.7 μmol/g fresh mass (Crucifer Genetics Cooperative, University of Wisconsin – Madison). Our synthetic diets had 0, 0.282, 0.565, 1.129, and 1.693 μmol/g fresh mass of allyl isothiocyanate (Sigma Chemical Co.) microencapsulated in β-cyclodextrin ($N = 18$ of each concentration). β-Cyclodextrin is a cycloamylose molecule containing 7 glucose units obtained by the action of *Bacillus macerans* on starch. The cyclodextrin cavity is slightly hydrophobic and large enough to envelop allyl isothiocyanate in a 1:1 ratio. An additional sham control was prepared with an equal amount of empty β-cyclodextrin as in the highest allyl isothiocyanate treatment ($N = 18$). The microencapsulated allyl isothiocyanate was mixed with prepared diet (when cooled $< 50^{\circ}$C) made with agar that does not gel until 28°C. The diet was prepared in a

single large batch (to avoid differences between batches) and then split into equal groups to which the microcapsules were added and thoroughly mixed. Larvae were individually reared in 30-ml plastic cups from freshly hatched eggs.

Experiments with the substrate (allyl glucosinolate) and enzyme (myrosinase) that ultimately result in the production of allyl isothiocyanate were conducted as above, employing synthetic diets and singly reared larvae. Diets containing allyl glucosinolate (Sigma Chemical Co.) were prepared at six incremental concentrations ranging from 0 to 30 μmol/g fresh mass, $N = 15$. The myrosinase experiment compared control diets to diets containing 0.156 mg/g myrosinase (Sigma Chemical Co.); myrosinase was added when the diet cooled below 45°C because temperatures above 60°C denature myrosinase. The amount we added would produce 4.8 μmol glucose per minute when reacting with allyl glucosinolate at pH 6 at 25°C (Bones et al., 1994).

Statistical Analyses. For analyses of continuous data, we employed *t* tests where there were two treatment groups, and ANOVA where there were more treatment groups (or additional variables included in the model such as sex of the caterpillar). Where our treatments involved increasing doses of compounds, we assessed the effect of dose on caterpillars by employing response-curve analyses using polynomial contrasts following ANOVA (Dawkins, 1981, 1983). Response-curve analyses are superior to discrete ANOVAs followed by contrasts because the hypothesis tested is a prediction about an ordered set of treatments. However, response-curve analyses do not assume that the predictor variable is continuous as in regression (Dawkins, 1981, 1983). The polynomial contrast procedure in Systat version 9 (SPSS, Inc., Chicago, IL) was employed. In all response-curve analyses, results represent the first-order polynomial (linear) analysis; no higher order polynomial analyses were significant. *G* tests were employed to test for differences in mortality of the caterpillars.

RESULTS AND DISCUSSION

P. rapae larvae grew 32 and 12% more slowly when feeding on previously damaged plants of *Arabidopsis lyrata* and *Brassica oleracea* than when feeding on the respective undamaged plants (Figure 1, *A. lyrata*, $t = 3.21$, df $= 50$, $P = 0.002$; *B. oleracea*, $t = 2.09$, df $= 44$, $P = 0.042$). This increased resistance in *B. oleracea* was correlated with a 176% and a 94% increase in isothiocyanate-producing indolyl ($t = 5.55$, df $= 28$, $P < 0.001$) and allyl ($t = 2.55$, df $= 28$, $P = 0.017$) glucosinolates, respectively (Figure 1). These results challenge the specialist herbivore paradigm because *P. rapae* is negatively affected by induced responses of plants, and this effect is correlated with induction of compounds previously believed to be ineffective against *P. rapae.* Other recent studies have reported negative effects of induced plant responses on performance of *P. rapae* (Agrawal et al., 1999a; Agrawal, 2000b; Traw and Dawson, 2002).

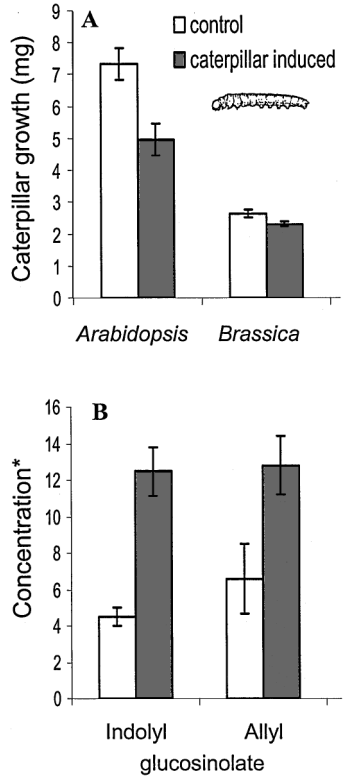

FIG. 1. (A) Induced resistance of *Arabidopsis lyrata* and *Brassica oleracea* to *Pieris rapae*. Low growth of caterpillars on *B. oleracea* was correlated with significant increases in (B) isothiocyanate-producing indolyl and allyl glucosinolate (sinigrin). *Units for indolyl glucosinolate are μmol/g dry mass while units for allyl glucosinolate are 1×10^{-2} μmol/g dry mass. Shown are means $\pm$ SE.

Our experiments with four plant lines with a range of isothiocyanate-producing glucosinolates from 7 to 154 μmol/g dry mass (Faulkner et al., 1998) also showed negative effects on *P. rapae*. Glucosinolate concentration in the *Brassica* lines was negatively correlated with *P. rapae* pupal mass, a strong indicator of butterfly fitness (Figure 2, response-curve ANOVA, $F_{1,44} = 17.354$, $P < 0.001$, sex: $F_{1,44} = 8.059$, $P = 0.007$). Other recent evidence, using lines of *B. rapa* artificially selected for high and low concentrations of glucosinolates, also supported a role for these secondary compounds in defense against *P. rapae* (Stowe, 1998). Neither approach of using induced plant responses nor bred lines of plants, however, unambiguously addresses the role of isothiocyanates in plant resistance.

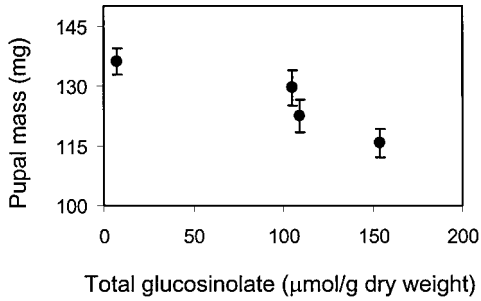

FIG. 2. Pupal mass of *Pieris rapae* as affected by four lines of *Brassica* that varied in their glucosinolate content. From left to right, the varieties are *B. oleracea* var. Green Duke (GD), GD X *B. drepanensis*, GD X *B. villosa*, and, GD X *B. atlantica*) (Faulkner et al., 1998). Shown are least squares means ± SE.

To test more directly for the effects of isothiocyanates on *P. rapae,* we fed larvae diets of active and deactivated horseradish root extracts. After 3 days, mortality was 100% (20 out of 20) on the active paste and 12.5% (5 out of 40) on the deactivated paste (G test, $G = 13.78$, $P < 0.001$). On the 4th day, the surviving caterpillars on the deactivated paste were randomly split into two equal groups, one maintained on deactivated paste and one on 10% active paste. After three more days, no additional mortality occurred, but caterpillars on 10% active paste grew 45% slower than caterpillars on deactivated paste (Figure 3, $t = 3.03$, df $= 33$, $P = 0.005$). Although this approach directly implicates isothiocyanates in plant resistance to *P. rapae*, it has the drawback of volatile loss (and possible fumigation) as in previous studies (Mccloskey and Isman, 1993).

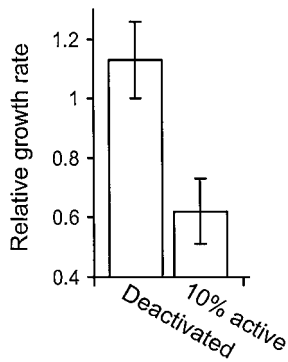

FIG. 3. The relative growth rate of *Pieris rapae* larvae growing on pastes of deactivated and 10% active horseradish root extract. Shown are means ± SE.

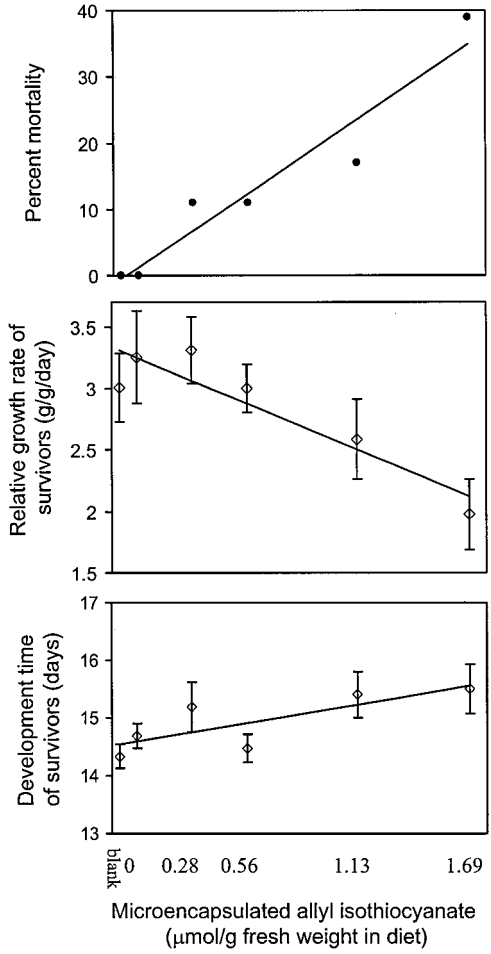

FIG. 4. Effects of microencapsulated allyl isothiocyanate on performance of *Pieris rapae* larvae. (A) percent mortality, (B) relative growth rate, and (C) development time. Shown are means ± SE. The left most data points above "blank" represent larvae on diet with empty β-cyclodextrin microcapsules.

Our final approach to examine the effects of isothiocyanates on *P. rapae* was to compare performance of *P. rapae* on synthetic diets containing microencapsulated allyl isothiocyanate. Microencapsulation prevented the volatilization of the isothiocyanate. Mortality of larvae was dependent on treatment, with no mortality on control diets, but strongly increasing mortality thereafter (Figure 4A, G test, $G = 17.419$, $P < 0.001$). For the survivors, the dose of allyl isothiocyanate negatively

affected the relative growth rate of larvae (Figure 4B, response-curve ANOVA, $F_{1,96} = 10.885$, $P = 0.001$), positively affected development time (Figure 4C, $F_{1,83} = 6.863$, $P = 0.010$), but did not affect pupal mass ($F_{1,89} = 0.079$, $P = 0.779$, sex: $F_{1,89} = 5.720$, $P = 0.019$). These data unequivocally point to isothiocyanates as effective agents of resistance to *P. rapae* via effects on mortality, growth, and development.

Some studies have suggested that specialist herbivores may prevent the defensive enzymatic reactions from occurring upon plant damage, thereby disarming the plant's defense (Engler et al., 2000; Ratzka et al., 2002). For example, a recent study has identified enzymes released by a *Brassica* specialist (*Plutella xylostella*) that prevent isothiocyanates from being formed following ingestion. Sulfatase activity of the enzyme produced by *P. xylostella* competes with myrosinase for attachment to glucosinolates, resulting in very little exposure to isothiocyanates in the herbivore gut. Two lines of evidence suggest that *P. rapae* is not preventing the glucosinolate–myrosinase reaction. First, Ratzka et al. (2002) report that *P. rapae* does not produce glucosinolate sulfatases like *P. xylostella*. Second, the frass of *P. rapae* feeding on *Brassica* contains allyl isothiocyanate, indicating that hydrolysis of glucosinolates does indeed occur during digestion (Agelopoulos et al., 1995). Thus, *P. rapae* does not "disarm the mustard oil bomb" (Ratzka et al., 2002).

There was no negative relationship between phytochemicals and *P. rapae* performance when only the substrate, allyl glucosinolate, was added to synthetic diet (effect on mortality: $G = 4.763$, $P = 0.445$; time to pupation: $F_{1,13} = 0.478$, $P = 0.502$; pupal mass: $F_{1,13} = 0.589$, $P = 0.457$). Similarly, when we added myrosinase to synthetic diets at realistic concentrations found in fresh *Brassica* leaves, *P. rapae* was not affected in growth rate ($t = 0.826$, df $= 38$, $P = 0.414$), development time ($t = 0.056$, df $= 37$, $P = 0.955$), or pupal mass ($t = 0.246$, df $= 37$, $P = 0.870$) (Table 1). Thus, as has been suspected for decades, neither glucosinolate nor myrosinase that together result in the production of isothiocyanate has an independent negative impact on *P. rapae.*

Even if specialists are, to some extent, able to cope with their hosts' phytochemicals, we suggest that a coevolutionary interaction where specialist herbivores reduce plant fitness must impose natural selection for more effective plant defenses against specialists (Berenbaum and Zangerl, 1998; Thompson and Cunningham, 2002). Both glucosinolate and myrosinase are strongly inducible

TABLE 1. EFFECTS OF DIETS CONTAINING 0.156 MG/G MYROSINASE ON THE MEANS ± 1 SE GROWTH AND DEVELOPMENT OF *Pieris rapae*

	Mass day 8 (mg)	Development time (days)	Pupal mass (mg)
Control diet	29.9 ± 5.1	14.3 ± 0.4	167.4 ± 5.4
Myrosinase diet	25.7 ± 3.8	14.1 ± 0.4	164.2 ± 5.4

following herbivory by specialists (Bodnaryk, 1992; Siemens and Mitchell-Olds, 1998; Agrawal et al., 1999b; Bartlet et al., 1999). Some studies find that specialist herbivores of the Brassicaceae feed less on previously damaged plants than controls (Bodnaryk, 1992; Agrawal, 1998, 2000b; Agrawal et al., 1999a; Bartlet et al., 1999). Thus, although many of these same studies find that glucosinolates are attractive to adults of the specialists (e.g., Giamoustaris and Mithen, 1995), differential effects on attraction of adults versus larval performance may explain the paradoxical induction of these compounds that are seemingly beneficial to herbivores. Similar results have been reported for specialist Buckeye butterflies (*Junonia coenia*) on *Plantago lanceolata*, where iridoid glycosides are attractive to adults (Bowers, 1991), but negatively affect larvae (Adler et al., 1995). Ultimately, the net effects of the putative defense compounds will determine the outcome of the plant–herbivore interaction. Indeed, correlations between the myrosinase-glucosinolate system and damage by specialist herbivores in the laboratory and field are often negative (Louda and Mole, 1991; Siemens and Mitchell-Olds, 1998; Stowe, 1998; Li et al., 2000).

Our experiments with microencapsulated isothiocyanates allowed for a realistic evaluation of the effects of these compounds against *P. rapae*. Our results resurrect a long-standing debate on plant–herbivore chemical coevolution and point to multiple positive and negative ecological roles for secondary plant metabolites. While certain aspects of the glucosinolate–myrosinase system may be essential for recognition, oviposition, and feeding by specialist herbivores, these same chemicals reduce larval performance of *P. rapae* and are likely to serve as a plant defense. More generally, differential effects of phytochemicals on different life stages of herbivores may resolve the paradox of why plants appear to be producing compounds seemingly beneficial to specialist herbivores (Malcolm and Zalucki, 1996; Agrawal, 2001).

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