

M. Brian Traw · Todd E. Dawson

Differential induction of trichomes by three herbivores of black mustard

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Abstract Specificity of plant induction responses may be important to the interactions between mustards and insect herbivores. This study compared the effects of the cabbage white butterfly, *Pieris rapae* (L.), cabbage looper, *Trichoplusia ni* (Hubner), and the mustard flea beetle, *Phyllotreta cruciferae* (Goeze) on induction of leaf trichome density, sinigrin concentration, and nitrogen concentration in black mustard, *Brassica nigra* (L.) Koch. Plants were damaged for 12 h at the four-leaf stage, with effort made to standardize the damage applied. Induction responses were measured on the fifth, seventh, ninth, and 11th leaves counted from the cotyledons. Seventh leaves of plants damaged by *P. rapae* had 76% more trichomes per unit area than controls, whereas equivalent leaves of plants damaged by the other two herbivores exhibited no response. Ninth leaves of plants damaged by *T. ni* had 113% more trichomes per unit area than controls, whereas equivalent leaves of plants damaged by the other two herbivores exhibited no response. Trichome densities of fifth and 11th leaves did not respond to treatments. Leaf sinigrin and nitrogen concentrations were not affected by the damage treatments. Differential plant trichome response to *P. rapae* and *T. ni* may have been due to differences in location of feeding during the damage treatment. Other cues, such as salivary components, may also have differed between the two herbivores. This study is one of the

first to document differential effects of two herbivores from the same guild on induction of morphological resistance.

Keywords Brassicaceae · Glucosinolate · Induced defense · Ontogeny · Phenotypic plasticity

Introduction

Induction of resistance has been widely observed in plants following damage by insect herbivores (Karban and Baldwin 1997; Tollrian and Harvell 1999). Studies have generally addressed only the effects of a single herbivore on induction of resistance in one plant species (Karban and Baldwin 1997). However, herbivores differ in their feeding behaviors, physiology, and mode of attachment to the leaf surface (Root 1973; Strong et al. 1984). Plants are therefore likely to respond differentially to herbivores as has been shown in several studies (Hartley and Lawton 1987; Olson and Roseland 1991; Felton et al. 1994; Stout et al. 1994; Turlings et al. 1998; Agrawal 2000a).

Resistance traits often differ broadly in their efficacy against different herbivores, particularly between specialists and generalists (Feeny 1976). Specialist herbivores, which occur only on one or a few related hosts, often are not affected by secondary compounds of these hosts that are lethal to non-adapted herbivores (Blau et al. 1978). If resistance is costly, plants would be expected to induce secondary compounds following damage by generalists, but not following damage by adapted specialists. In contrast, morphological resistance traits such as leaf toughness or trichome density are often effective against most herbivores (Feeny 1976) and would likely be induced following damage by both generalists and specialists.

Annual plants in the mustard family are particularly suitable for addressing the differential induction of resistance for several reasons. These plants are typically weedy and therefore easy to germinate and grow. The

M.B. Traw (✉) · T.E. Dawson
Cornell University,
Department of Ecology and Evolutionary Biology,
Ithaca, NY 14853, USA
e-mail: btraw@uchicago.edu
Tel.: +1-773-8347690, Fax: +1-773-7029740

Present address:

M.B. Traw, Department of Ecology and Evolution,
University of Chicago, 1101 E. 57th St., Chicago, IL 60637, USA

Present address:

T.E. Dawson, Department of Integrative Biology,
University of California, Berkeley,
3060 Valley Life Sciences Building 3140, Berkeley,
CA 94720–3140, USA

resistance traits have also been well documented (Mauricio and Rausher 1997; Agrawal 1999, 2000b). Last, mustards have been widely studied and much is known about their interactions with generalist and specialist herbivores (Tahvanainen 1972; Root 1973; Feeny 1977; Nielson 1978).

The current study compares effects of three herbivores on induction of resistance traits in black mustard, *Brassica nigra* (L.) Koch. This introduced annual grows abundantly near Ithaca, N.Y. (Feeny and Rosenberry 1982) from May to September (Hicks and Tahvanainen 1974). Herbivores were chosen to represent a range of feeding styles and degree of association with the host plant in nature. The mustard flea beetle, *Phyllotreta cruciferae* (Goeze), and cabbage white butterfly, *Pieris rapae* (L.), are both primary herbivores in local populations of *B. nigra* (Traw, personal observation). However, these two herbivores differ in style of feeding. *Phyllotreta cruciferae* adults make pits on the leaf surface (Kinoshita et al. 1979; Wylie 1979), whereas *Pieris rapae* larvae chew along the leaf margin (Root 1973). Both herbivores are specialists on mustards. The cabbage looper, *Trichoplusia ni* (Hübner) feeds in a similar manner to *Pieris rapae*. However, it is a generalist herbivore with a diet that includes plants in many families (Tietz 1972) and occurs only occasionally on *B. nigra* (B. Traw, personal observation). These three herbivores are hereafter referred to as *Phyllotreta*, *Pieris*, and *Trichoplusia*, respectively.

Resistance traits of *B. nigra* include non-glandular trichomes and glucosinolates. Trichomes provide physical protection against herbivores in many crops (Levin 1973) and mustard species (Ågren and Schemske 1993; Palaniswamy and Bodnaryk 1994; Mauricio and Rausher 1997). Sinigrin, also known as allylglucosinolate, accounts for more than 95% of the total glucosinolate concentration in *B. nigra* leaves (Feeny and Rosenberry 1982). Sinigrin is toxic to generalist herbivores (Blau et al. 1978), including *Trichoplusia* (Wadleigh and Yu 1988; Shields and Mitchell 1995; Stowe 1998), but actually stimulates feeding by *Pieris* and *Phyllotreta* (Hicks 1974; Feeny 1977; Blau et al. 1978; Chew 1988; Siemens and Mitchell-Olds 1996). Both trichomes and glucosinolates have been shown to increase in mustards following damage by herbivores (Agrawal 1998).

Leaf nitrogen concentration is highly positively correlated with both photosynthetic rate (Evans 1989) and herbivore performance (Slansky and Feeny 1977; Mattson 1980). Little is known about how leaf nitrogen concentrations respond following herbivory to plants (Karban and Baldwin 1997). Several studies have found no induction of leaf nitrogen concentration (Gowda 1997; Wold and Marquis 1997). However, nitrogen concentration in some plants might increase following damage if they compensate for the loss of leaf material by increasing photosynthetic rates.

Specifically, the current study addressed three questions. First, do leaf trichome density, sinigrin concentration, or nitrogen concentration change in leaves produced following damage? Second, do plants differ in their

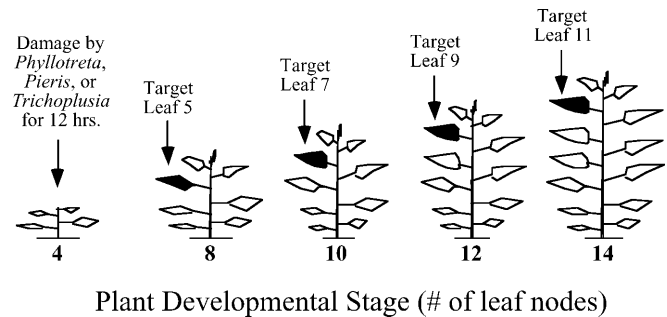


Fig. 1 Relationship between leaf sampling and plant development. Cotyledons are not included

responses to the three herbivores? Third, what is the time course of plant induction response?

Materials and methods

Experimental design

The experiment was conducted in a glasshouse at Cornell University from 7 June to 2 August 1998. Seeds were collected in 1996 from a large population at the Southwest Park, a landfill just outside of Ithaca, N.Y. Progeny of a single maternal family were used in order to reduce phenotypic variation in resistance among replicates. Seeds were sown in 6.5-cm-diameter by 25-cm-deep Deepots (Stuewe and Sons, Corvallis, Ore.) containing a 1:1 mixture of topsoil and fritted clay. Seedlings were thinned to one per pot on the 13th day following germination. Temperature was maintained at 25°C (day) and 20°C (night). Natural lighting was supplemented by halogen bulbs to extend day length (14 h). Plants were fertilized on days 41 and 46 with 50 ml of 20:20:20 Peter's NPK fertilizer at a concentration of 1.3 g/l. Plants were randomly assigned to receive either no herbivores, two third instar *Trichoplusia*, two fifth instar *Pieris*, or 40 adult *Phyllotreta*. A small trial suggested that these proportions would provide equivalent amounts of damage. *Phyllotreta* were obtained via sweep netting of the *B. nigra* population at the locality where the seeds were collected. Only adult beetles were used in the study. Voucher specimens of the beetles were identified by a coleopterist and deposited in the United States National Museum collection. *Pieris* and *Trichoplusia* were obtained from laboratory populations maintained at the Boyce Thompson Institute, Ithaca, N.Y. After 21 days of growth, all seedlings, including controls, were enclosed in mesh bags that were secured to the pot by a rubber band. Herbivores were placed on the soil surface, allowed to forage freely on plants for 12 h, and then were removed along with the mesh bags. Leaves were traced before and after damage to document feeding by *Trichoplusia* and *Pieris*. Tracings were scanned into a graphics program (Image 1.22 for Macintosh) along with a scale bar for estimation of herbivory and total area. Leaf damage by *Phyllotreta* was estimated through counts of the number of pits per leaf, multiplied by an average pit area of 0.0145 cm² (SE=±0.0003, n=129) determined from detailed tracings of five leaves.

Within each treatment, plants were randomly assigned to be harvested upon reaching a growth stage of 8, 10, 12, or 14 leaves (Fig. 1). Plants were harvested by developmental stages, rather than by chronological age, to control for ontogenetic variation in trichome density on leaves within a plant. There were 11 replicate plants in each treatment harvested at each developmental stage, for a total of 176 plants. One plant exposed to *Pieris* larvae died and was removed from the study. A second plant was scheduled to receive *Pieris*, but received *Trichoplusia* larvae instead and therefore was analyzed in the latter treatment. Plants were distributed in a completely randomized design on the greenhouse benches and randomized again once every 2 weeks.

Measurement of leaves

Resistance was measured on only one leaf per plant (Fig. 1). The fourth leaf down from the apex was chosen because it was the youngest fully-expanded leaf. During the first harvest, leaf length was not measured. For subsequent harvests, leaf length was measured and used to estimate leaf area, based on a regression equation ($\text{area}=4.93 \times \text{length}-26.16$, $r^2=0.78$, $n=39$) calculated from a subset of leaves for which both length (cm) and area were measured. A standard hole punch was used to remove a disk at 1 cm from both sides of the midrib (total area=0.58 cm²). Trichomes were counted on the top of each leaf disk under a dissecting microscope. Total counts were divided by total disk area to determine leaf trichome density. Following hole punching, the leaf blade and petiole were frozen in liquid nitrogen and stored at -80°C for later measurement of sinigrin concentration.

Leaf sinigrin content was determined on a high performance liquid chromatograph (HPLC) following open-column desulfation (Hugentobler and Renwick 1995). Approximately 0.5 g of frozen leaf material was crumbled into a test tube, weighed, and boiled in 5 ml water for 4 min. Liquid extract was stored at 4°C and particulate matter was discarded. Open columns consisted of a 14 cm Pasteur pipette packed with 0.1 g DEAE Sephadex A-25 (Sigma) dissolved in a buffer solution of 0.23 ml glacial acetic acid, 0.32 ml pyridine, and 19.5 ml water. Column were conditioned with 6.0 ml of the same buffer solution, followed by 12 ml water. Columns were loaded with 1 ml of extract and flushed with 8 ml of water, followed by 0.002 g of sulfatase enzyme (Sigma S-9626 type H-1) dissolved in 2 ml water. After 15 h, 4 ml water was added and the resulting eluate collected and stored at 4°C. Eluate was forced through a nylon filter (Fisher Scientific 09-913-7) and 0.5 ml injected onto a Waters 600 HPLC (Millipore, Milford, Mass.) using a 4.6 mm×25 cm Beckman Ultrasphere ODS 5µ column (Varian). Gradient elution started with 100% water for 2 min, followed by a linear change to 20% acetonitrile at 5 min, 30% acetonitrile at 15 min, and 100% acetonitrile at 20 min. Absorbance of the desulfated sinigrin was measured at 225 nm using a Waters 991 photodiode array detector (Millipore). Peak area was converted to sinigrin concentration using a standard curve ($y=1602703793x-73403$; $r^2=0.99$; $n=5$) derived from a desulfated solution of purified sinigrin (Acros Organic Chemicals 10295-1) of known concentration. Frozen tissue left over from glucosinolate analysis (≈0.3 g) was dried at 60°C for 14 days, then homogenized. Leaf nitrogen concentration was calculated on a 3 mg subsample using a Carlo Erba C:N autoanalyzer (Milan, Italy).

Statistical analysis

Leaf trichome density, sinigrin concentration, and nitrogen concentration were each tested by a two-way ANOVA containing herbivore treatment and leaf position as main effects. Within each ANOVA, Fisher pairwise contrasts were made between means of each herbivore treatment and control for each leaf position (Snedecor and Cochran 1989). An adjusted *P* value of 0.017 was used to provide an overall Bonferroni confidence of $\alpha < 0.05$ at each leaf position (Rice 1989).

Results

Pieris and *Trichoplusia* both removed 25% of total canopy area (Table 1). In contrast, *Phyllotreta* consumed 17% of total canopy area, which was a significantly lower amount by one-way ANOVA ($F_{2,130}=4.76$, $P=0.010$, Table 1). *Trichoplusia* removed substantially more area from cotyledons, but less area from leaf positions one and two than did *Pieris* as shown by a significant interaction term by two-way ANOVA ($F_{5,422}=9.47$, $P < 0.0001$, Fig. 2).

Table 1 Mean values (\pm SE) of herbivory area (cm²) and percent of total leaf area consumed by 40 adult *Phyllotreta cruciferae*, two fourth instar *Pieris rapae*, or two third instar *Trichoplusia ni*

Treatment	<i>n</i>	Herbivory area	Percent herbivory
<i>Phyllotreta</i>	44	3.74±0.82	16.7±0.56
<i>Pieris</i>	43	4.81±0.34	25.0±2.00
<i>Trichoplusia</i>	45	4.64±0.22	25.0±2.32

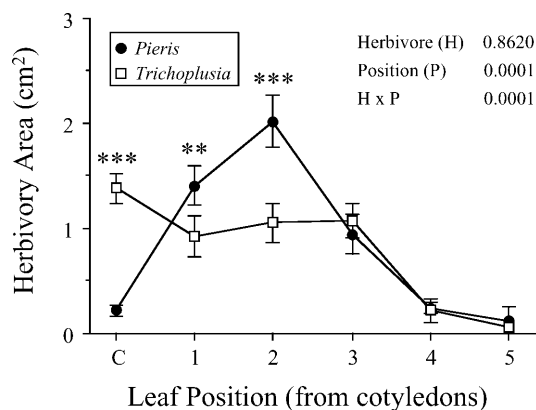


Fig. 2 Differences in the feeding locations of *Pieris rapae* and *Trichoplusia ni* during the damage application. Each mean represents 10–12 replicates. Error bars represent 1 SE; C cotyledons. Overall *P* values for two-way ANOVA are shown. Significant differences between treatment means are indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Table 2 Effect of herbivory treatment and leaf position on leaf trichome density, sinigrin concentration, nitrogen content, and area. *P* values in bold were significant following Bonferroni correction

Source	<i>df</i>	MS	<i>F</i>	<i>P</i>
Trichome density (<i>n</i> /cm ²)				
Herbivore	3	869	0.2	0.9070
Leaf	3	85,280	18.1	0.0001
Herbivore×Leaf	9	14,152	3.0	0.0025
Residual	159	4,713		
Sinigrin (% wet mass)				
Herbivore	3	3.65	0.42	0.7373
Leaf	3	14.79	1.71	0.1672
Herbivore×Leaf	9	3.92	0.45	0.9040
Residual	159	8.65		
Nitrogen (% dry mass)				
Herbivore	3	0.17	0.29	0.8305
Leaf	3	5.12	9.05	0.0001
Herbivore×Leaf	9	0.31	0.55	0.8377
Residual	121	0.57		

Plants responded to damage by increasing the density of trichomes on subsequent leaves, but the response depended greatly upon the herbivore and leaf position, as shown by the significant interaction between herbivory treatment and leaf position in the two-way ANOVA ($F_{9,159}=3.0$, $P=0.0025$, Table 2). Plants damaged by *Pieris* exhibited the fastest induction, which was observed on the seventh leaf, measured on average 14 days following

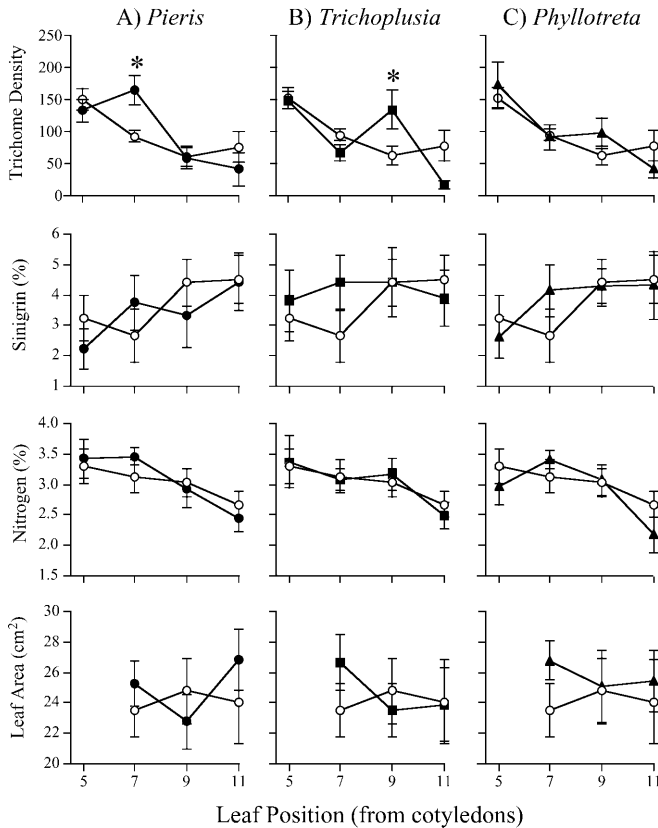


Fig. 3 Effects of three herbivores on trichome density (n/cm^2), sinigrin concentration ($\mu\text{mol}/\text{mg}$ wet mass), nitrogen concentration (% dry mass), and area (cm^2) of newly produced leaves: **A** *Pieris*, **B** *Trichoplusia*, **C** *Phyllotreta*. Each herbivory treatment (solid symbols) is graphed against the control (open circles). Each mean represents 10–12 replicates, with the exception of nitrogen means which represent 6–11 replicates. Error bars indicate 1 SE. Contrasts with P values less than cutoff of $P < 0.017$ are denoted with an asterisk

the damage. The seventh leaf of plants damaged by *Pieris* exhibited an increase of 71.6 trichomes per cm^2 or 76% more than the equivalent leaf of control plants, which was significant by pairwise contrast ($P = 0.0155$, Figs. 3A, 4). Plants damaged by *Trichoplusia* and *Phyllotreta* exhibited no response at the seventh leaf relative to control plants (Fig. 3B, C).

The ninth leaf of plants damaged by *Trichoplusia* increased trichome density by 71.2 trichomes per cm^2 or 113% relative to the same leaf position of control plants, which was significant by pairwise contrast ($P = 0.0140$; Fig. 3B). Plants damaged by *Pieris* and *Phyllotreta* showed no response at the ninth leaf relative to control plants (Fig. 3B, C). On the 11th leaf, there was no significant difference in trichome density between damaged plants and control plants.

Leaf area estimates for the seventh, ninth, and 11th leaves were not significantly different between damaged and control plants (Fig. 3). Neither leaf sinigrin nor nitrogen concentration differed between leaves of damaged and control plants (Table 2; Fig. 3). Leaves produced at higher positions along the growing shoot had significantly

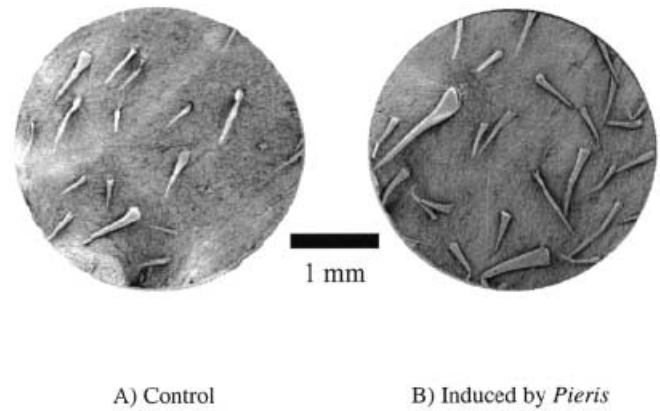


Fig. 4 Scanning electron micrographs of the top surface of the seventh leaf from a control plant (A) and a plant damaged by *Pieris* larvae (B)

lower trichome densities ($F_{3,159} = 18.1$, $P < 0.0001$, Table 2) and nitrogen concentrations ($F_{3,159} = 9.05$, $P < 0.0001$, Table 2), but no significant change in sinigrin concentration ($F_{3,159} = 1.71$, $P = 0.1672$, Table 2).

Discussion

This study tested whether leaf trichome density, sinigrin concentration, or nitrogen concentration were induced on newly produced leaves of *Brassica nigra* and whether plants differed in their responses to three herbivores.

Leaf trichome densities did change following damage, whereas leaf sinigrin and nitrogen concentration did not (Table 2). Trichome induction has been previously observed for three other mustards (Agrawal 1998, 1999, 2000b), other annuals (Pullin and Gilbert 1989; Mutikainen and Walls 1995), cacti (Myers and Bazely 1991), and trees (Baur et al. 1991; Young and Okello 1998). An increase in trichome density could reflect either increases in number of trichomes per leaf or a decrease in leaf area (Karban and Baldwin 1997; Roy et al. 1999). In the current study, leaf area was not significantly affected by herbivory treatments and therefore higher densities were due to more trichomes being produced per leaf. In contrast, Agrawal (1999) found that wild radish, *Raphanus sativus*, did not increase trichome density of leaves produced following damage, but did increase leaf area and therefore total number of trichomes per leaf.

Absence of induction of leaf sinigrin production was a surprise, since another study had shown that mechanical damage alone increased sinigrin concentration of *B. nigra* by 59% relative to control plants (Koritsas et al. 1991). Although Koritsas et al. (1991) analyzed whole plant sinigrin concentrations, their timing of damage and collection of plant material were quite similar to the current study. Other studies have found that aliphatic glucosinolates, such as sinigrin, are less prone to induction than are indole glucosinolates following application of jasmonic acid (Bodnaryk 1992, 1994). Agrawal (2000b) found no induction of leaf sinigrin production in *Lepidium*

virginicum following damage by *Pieris rapae*. In the current study of *B. nigra*, it is possible that some tissues did increase sinigrin concentration, but were not sampled. Subsequently, a large-scale study of 188 maternal families from the same population suggests that the average seventh leaf increases sinigrin concentration by 19% over leaves of control plants (Traw and Dawson 2002). However, 30% of maternal families exhibited no change or declines in sinigrin concentration following damage, suggesting that there is extensive variation in response among maternal families.

Leaf nitrogen concentration did not differ between new leaves on damaged plants and controls. Nitrogen is often limiting for herbivore growth (Slansky and Feeny 1977; Mattson 1980) and there has been some speculation that decreased leaf nitrogen concentration following herbivore damage might be an induced resistance trait (Karban and Baldwin 1997). Few measurements of systemic changes in leaf nitrogen concentration have been reported for plants experimentally damaged by herbivores. Wold and Marquis (1997) found that artificially damaged oak trees did not exhibit a systematic change in leaf nitrogen concentration relative to control plants. Gowda (1997) found no change in leaf nitrogen concentration of *Acacia* trees following artificial damage. It remains to be seen whether these studies represent a general pattern. Plants such as *Nicotiana attenuata* (Baldwin et al. 1998) would likely have a systemic increase in leaf nitrogen concentration following damage because nicotine, a nitrogen-rich compound, is translocated to leaves from roots following damage.

Each of the three herbivores in this experiment had a different effect on the induction of trichomes. Damage by *Pieris* caused a faster induction response than damage by *Trichoplusia*, even though both caterpillars consumed statistically identical amounts of plant material (Table 1) and are similar-sized "strip feeders" (Root 1973). In contrast, *Phyllotreta* had no significant effect on leaf trichomes at any leaf position. The finding of a differential impact of three herbivores on induction is likely the first for a morphological resistance trait. Previous work has revealed differential effects of herbivores on the chemical induction responses of both corn (Turlings et al. 1998) and tomato (Stout et al. 1994, 1998).

Lack of an effect of *Phyllotreta* on trichome induction may simply reflect the lower amount of damage applied by this herbivore (Table 1). However, a subsequent experiment suggests that even when the amount of damage is equal, plants respond less to damage by *Phyllotreta* than to damage by *Pieris* (Traw, unpublished data). Because the feeding by *Phyllotreta* creates pits that are distributed across the leaf surface (Traw, personal observation), the damage may stimulate less of a response than concentrated damage by leaf chewers such as *Pieris* and *Trichoplusia* (Mauricio et al. 1993).

The more interesting finding of the current study was that damage by *Pieris* and *Trichoplusia* larvae caused qualitatively different timings of plant trichome induction even though the amount of damage was similar (Table 1).

Damage by *Pieris* increased trichome density of the seventh leaf whereas damage by *Trichoplusia* increased trichome density of the ninth leaf (Fig. 3). Possible differences between the herbivores might include salivary concentrations of β -glucosidase, a protein shown to induce volatile production by wounded *Brassica oleracea* (Mattiacci et al. 1994, 1995), or volicitin, a chemical implicated in differential corn responses to different herbivores (Turlings et al. 1998). It is also possible that differences in timing of trichome induction existed because herbivores fed at different positions during the application of damage (Fig. 2). *Trichoplusia* larvae consumed more of the cotyledons while *Pieris* larvae consumed more of first and second true leaves. While these differences in feeding location may have contributed to the quantitative differences in the observed trichome induction, it seems unlikely that the foraging pattern explains the fact that plants induced trichomes at the seventh and ninth leaves of *Pieris* and *Trichoplusia* damaged plants, respectively.

There was no evidence that host breadth of the herbivore influenced the degree to which it induced resistance. Of the three herbivores, *Trichoplusia* has the broadest diet (Tietz 1972), yet caused an intermediate induction response. It had less impact than *Pieris*, but more impact than *Phyllotreta* (Fig. 3). Agrawal (2000a) also found no general pattern between diet breadth and induction of wild radish plants damaged by two specialists and two generalists.

Whether trichome induction benefits plants by reducing subsequent herbivory remains to be answered for *B. nigra*. There is evidence from wild radish that early damage by *Pieris* does decrease the performance of *Pieris* larvae reared later on those plants (Agrawal 2000a). For *Trichoplusia*, on the other hand, Agrawal (2000a) found that early herbivory did not influence subsequent performance of conspecifics on those plants. Given that *Pieris* and *Trichoplusia* larvae take only 3 weeks on average to complete development (Traw, personal observation), it may be that the induction response does not have an effect on current larvae. Because both *Pieris* and *Trichoplusia* are multivoltine, the benefits of induction may therefore be in preventing feeding or oviposition by subsequent generations.

In summary, the experiment demonstrated increased trichome density, but not sinigrin concentration, in leaves of *B. nigra* following damage by three herbivores. Plants responded more quickly to damage by *Pieris* than to damage by *Trichoplusia*, even though the amount of damage was similar. This is one of the first demonstrations that two herbivores of the same guild can differ in their effects on plant resistance.

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