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DUAL CHEMICAL BARRIERS PROTECT A PLANT AGAINST DIFFERENT LARVAL STAGES OF AN INSECT

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Abstract—The host plants of the native American butterfly, *Pieris napi oleracea*, include most wild mustards. However, garlic mustard, *Alliaria petiolata*, a highly invasive weed that was introduced from Europe, appears to be protected from this insect. Although adults will oviposit on the plant, most larvae of *P. n. oleracea* do not survive on garlic mustard. We used feeding bioassays with different larval stages of the insect to monitor the isolation and identification of two bioactive constituents that could explain the natural resistance of this plant. A novel cyanopropenyl glycoside (**1**), alliarinoside, strongly inhibits feeding by first instars, while a flavone glycoside (**2**), isovitexin-6″-D-β-glucopyranoside, deters later instars from feeding. Interestingly, the first instars are insensitive to **2**, and the late instars are little affected by **1**. Furthermore, differential effects of dietary experience on insect responses suggest that **1** acts through a mechanism of post-ingestive inhibition, whereas **2** involves gustatory deterrence of feeding.

Key Words—Pieris, pieridae, crucifer, feeding deterrent, feeding inhibition, flavonoid, garlic mustard, Alliaria.

INTRODUCTION

Plants are chemically defended from insect herbivores by the presence of repellents, oviposition deterrents, feeding deterrents, and toxins. However, some herbivores inevitably adapt to these defenses and may eventually specialize by utilizing the "defensive compounds" to recognize their host plants (Ehrlich and Raven, 1964; Lindroth, 1988). In the Cruciferae (=Brassicaceae), glucosinolates and their

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hydrolysis products act as a first line of defense against generalist insects, but some of these compounds have become signals for host finding and acceptance for many specialists (Feeny, 1977; Chew, 1988; Chew and Renwick, 1995). Some crucifers, however, produce additional compounds that protect them from specialists. For example, butterflies of the imported cabbageworm, *Pieris rapae* L. are deterred from ovipositing on *Erysimum cheiranthoides* and *Iberis amara* by the presence of cardenolides and cucurbitacin glycosides, respectively (Sachdev-Gupta et al., 1990; Huang et al., 1993). Larvae of this butterfly also are deterred from feeding on these plants by the same and similar compounds (Sachdev-Gupta et al., 1993a,b). The presence of such bioactive chemicals may have contributed to the rapid proliferation of many introduced plants in North America. For example, garlic mustard, *Alliaria petiolata*, a particularly invasive crucifer that was introduced from Europe late in the 19th century, is relatively free of herbivory (Nuzzo, 1993). The native butterflies, *Pieris napi oleracea* and *P. virginiensis*, oviposit on this plant, but their larvae usually do not survive (Bowden, 1971; Chew, 1981). In this study, experiments were conducted to examine the basis for the failure of *P. n. oleracea* larvae to develop on garlic mustard. Two chemicals responsible for inhibiting feeding were identified, and a unique system of selective resistance against different larval stages was discovered.

METHODS AND MATERIALS

Plants. Alliaria petiolata seedlings were obtained from various natural sites in the vicinity of Ithaca, New York, and transplanted into 10 cm cord pots of artificial soil (Cornell Mix A) in an air-conditioned greenhouse that was maintained at *ca* 25°C. Supplementary lighting was provided by 400-W multivapor high intensity discharge lamps.

Chemical Extraction. After 4-6 weeks, foliage from rosette plants was harvested and extracted with boiling ethanol for 5 min. The ethanolic extract was evaporated to dryness under reduced pressure, and lipids were removed by extracting with n-hexane. The defatted residue was dissolved in water and the aqueous solution was partitioned 3 times with 1-butanol. The combined butanol extract and the post-butanol water extract were concentrated under reduced pressure and dissolved in methanol/water mixtures for bioassays.

Insects. P. n. oleracea larvae for bioassays were from a colony started with insects that were collected by Dr. F. S. Chew (Tufts University) in Vermont and maintained in the laboratory at *ca*. 25°C. The colony was renewed or supplemented with field-collected butterflies on an annual basis. Oviposition occurred on cabbage plants in greenhouse cages, and the larvae were reared on cabbage (*Brassica oleracea* L var Golden Acre). Naïve neonates (no experience with a plant) were

obtained by allowing butterflies to oviposit on a strip of Parafilm that was wrapped around a beaker immediately below a cabbage leaf, on which butterflies made tarsal contact (Webb and Shelton, 1988).

Bioassays. Choice bioassays for feeding deterrent activity were conducted with early 4th instar larvae, using 1.5 cm diameter cabbage leaf discs as test substrate. Each side of test discs was treated with 20 μl of solution to provide a total of 0.1 gram leaf equivalents (gle) per disc, whereas pure solvent was applied to control discs. The tests were conducted in waxed paper ice cream cups (250 ml), each containing a paraffin wax layer at the bottom, which was covered with moist filter paper. Two treated and two control discs were arranged alternately in each cup and supported on insect pins. Four larvae were placed in the center of each cup, which was covered with a perforated plastic lid. After a period of 6 hr in the dark, the remaining area of each disc was measured with a LiCor-3100 area meter. The area consumed was obtained by subtracting the remaining area of each eaten disc from the average measurement of all discs that showed no signs of feeding. Feeding inhibition was expressed as a feeding deterrent index (FDI): $\mathrm{FDI} = 100 \times (\mathrm{C} - \mathrm{T})/(\mathrm{C} + \mathrm{T})$, where C is the area of control discs consumed and T is the area of treated discs consumed (Dimock et al., 1991). An FDI value of zero means that there is no preference, a positive value means that there is a preference for the control, and a negative value means a preference for the treatment. Subsequent bioassays with 2nd, 3rd, and 5th instars were conducted in the same manner. The time period for these assays was adjusted to allow for different consumption rates of the different instars. In each case, assays were terminated when approximately 50% of the control discs had been consumed.

For neonate assays, one treated and one control disc were pinned at the same height near the center of each cup so that they overlapped and larvae could readily move from one disc to the other. Five larvae were introduced to the overlapping area and the assays were continued for 24 hr, when measurable feeding had usually occurred. To determine the effects of diet on larval responses to the active compounds, eggs were allowed to hatch on a wheat germ diet (Bell et al., 1979) in styrofoam cups (250 ml), which were kept in an incubator at 27°C until the larvae at the different stages were used for assays.

Chemical Isolation. Semi-preparative HPLC was performed by using a C_{18} Bondex 10 (Phenomenex) column, 300 × 7.8 mm, at a flow rate of 2 ml/min. Subsequent analytical separation and isolation of pure compounds was accomplished by using a Luna C_{18} column, 250 × 4.6 mm (Phenomenex) at a flow rate of 1 ml/min. For both HPLC systems, a gradient of water and acetonitrile was used: CH_3CN 0% at time 0, 15% at 5 min, 30% at 25 min, and 10% at 30 min. The HPLC effluent was monitored by a diode array detector at 254 nm and at 218 nm.

RESULTS AND DISCUSSION

Previous studies have shown that water-soluble fractions from ethanolic extracts of *A. petiolata* foliage contain both oviposition stimulants and deterrents for *P. n. oleracea* butterflies, and the deterrent compounds could be extracted into butanol. Despite the presence of these unidentified deterrents, acceptance for oviposition could be attributed to the overwhelming effect of allylglucosinolate that is present as a stimulant (Huang et al., 1995). In this study, we tested for the presence of feeding inhibitors in similar extracts of rosette leaves of *A. petiolata*, before and after partitioning with 1-butanol. In choice bioassays with 4th instar larvae, we found that butanol extracts have strong antifeedant activity. Cabbage leaf discs treated with 0.1 gram leaf equivalents (gle) of the butanol extract were consumed by larvae significantly less than control discs that were treated with solvent alone (FDI $= 35.74 \pm 4.94$, $N = 10$). Reverse phase HPLC analysis of the butanol fraction using a water-acetonitrile gradient gave a chromatogram which showed 4 significant peaks (Figure 1). The UV-spectra corresponding to all but the first of these peaks were typical of flavonoids. Four major fractions (A–D), each of which included material associated with one major peak, were collected for bioassays. Feeding by 4th instar larvae of *P. n. oleracea* was deterred only by the fraction B (Figure 2a).

Additional bioassays with newly hatched larvae indicated that the total butanol extract of *A. petiolata* was even more inhibitory to neonates than to the 4th instars (mean FDI 54.6 vs 35.7, $N = 10$) . However, the fraction B, which was most inhibitory to 4th instars, was relatively inactive against neonates. Instead,

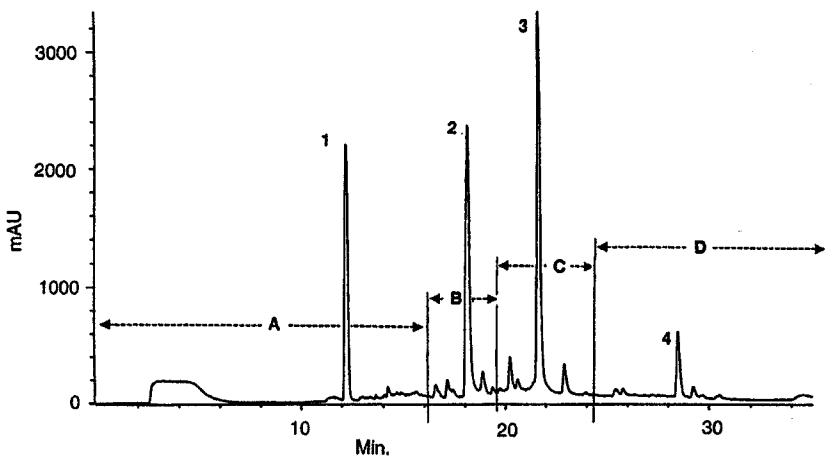

FIG. 1. High pressure liquid chromatogram of the 1-butanol fraction obtained from aqueous extract of *Alliaria petiolata*, with UV monitoring at 218 nm.

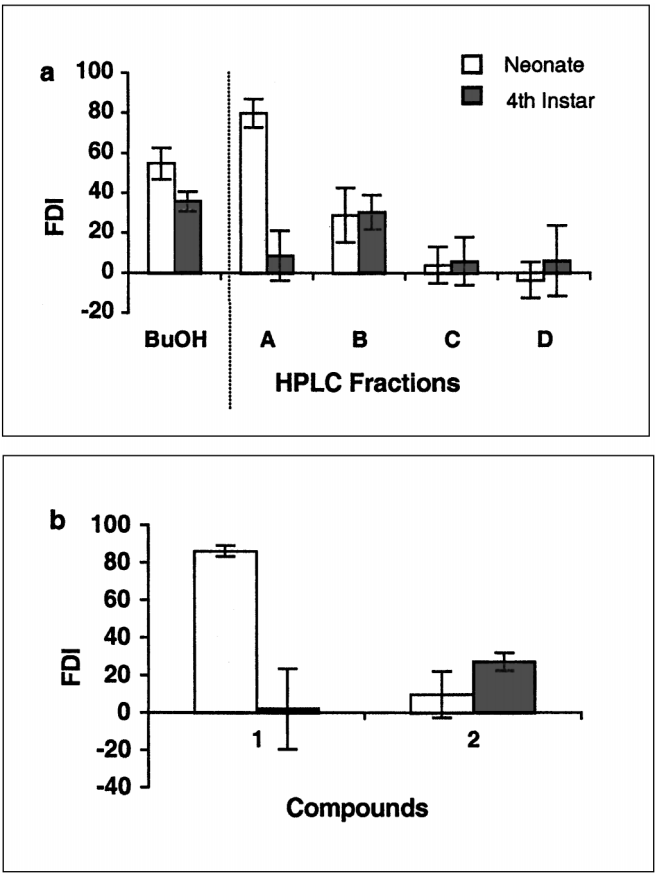

FIG. 2. Feeding inhibition (Feeding Deterrent Index, mean ± SE, $N = 10$) of neonate and 4th instar *P. napi oleracea* by **a**: total butanol extract (BuOH) and individual HPLC fractions, and **b**: compounds **1** and **2** isolated from these fractions by HPLC, at concentrations of 0.1 gram leaf equivalents/disc.

the fraction A that showed no activity against 4th instars was most inhibitory to neonates (Figure 2a). The major compound in each active fraction (**1** from A and **2** from B) was isolated by HPLC and tested for activity against both 1st and 4th instars. Compound **1** inhibited feeding by neonates, whereas compound **2** was active against the 4th instars (Figure 2b).

To determine the stage of development at which larval sensitivity changes from one compound to the other, we measured the responses of larvae to each compound at each stage in their development. Batches of larvae were reared from egg to 5th instar on cabbage or on wheat germ diet, and groups of larvae were

removed for assays at each stage. Previous work with *Pieris rapae* showed that larval responses to feeding deterrents can depend on dietary experience, and specific constituents of wheat germ are responsible for suppressing development of sensitivity to deterrents Huang and Renwick, 1995, 1997). Our experiments with *P. n. oleracea* now show that the larval response of this species to compound **1** is unaffected by diet, but sensitivity to the feeding inhibitor decreases steadily to zero by the time larvae molt to the 4th instar (Figure 3a). In contrast, the larval response to compound **2** is clearly dependent on diet. Larvae reared on wheat germ diet

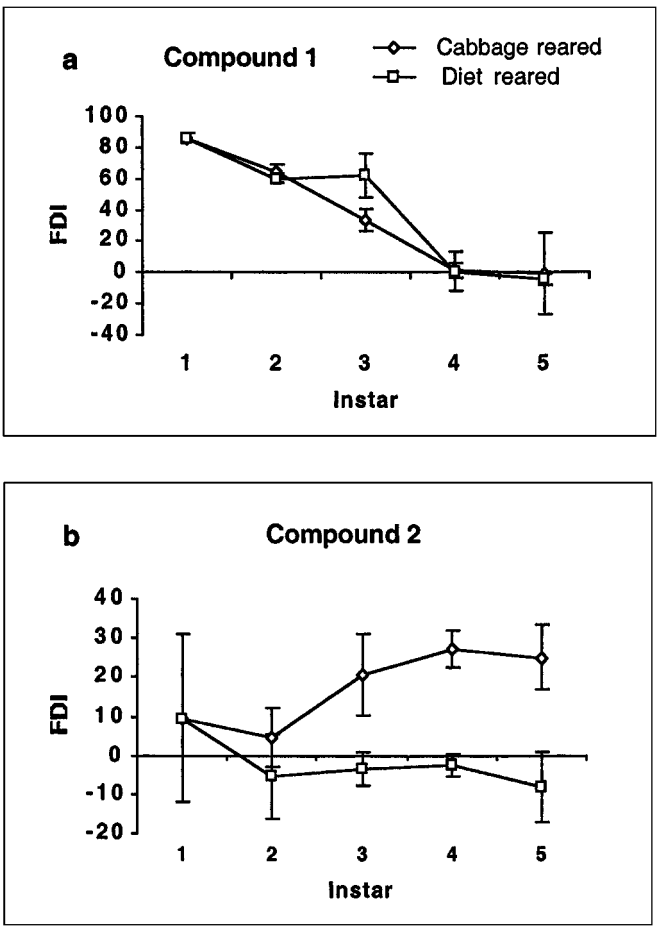

FIG. 3. Feeding inhibition by compounds **1** and **2** on different instars of cabbage-reared and wheat germ-reared larvae of *P. napi oleracea* (Feeding Deterrent Index, mean ± SE, $N = 10$).

remain insensitive to compound **2** throughout their development, whereas larvae feeding on cabbage develop sensitivity to reach a maximum during the last two instars (Figure 3b).

Compound **2** has been identified previously as a flavone glycoside, isovitexin 6"-*O*-β-D-glucopyranoside (Figure 4) (Haribal and Renwick, 1998). Compound **1** was collected by preparative HPLC of butanol extracts to provide mg quantities of material for spectral analysis, resulting in its identification as 4-β-D-pyranoglucosyloxyl-2(Z)-propene-1-nitrile (Haribal et al, 2001) (Figure 4).

Behavioral observations during bioassays provided clues about the mechanisms involved in the rejection of *A. petiolata* as a food plant. When neonate larvae were presented with a cabbage disc that was treated with a butanol extract of *A. petiolata* foliage or with isolated compound **1**, limited nibbling at the surface was observed, without any visible damage. However, some ingestion was evident from the green color visible in the larval gut, which is translucent at this stage. Similarly, when neonates were placed on the surface of a rosette leaf of *A. petiolata*, about one-quarter to one-third of the gut became green within a period of 4 hr, after which the larvae became motionless and made no further attempt to feed. In contrast, those 4th instars that rejected *A. petiolata* rosettes or cabbage discs treated with compound **2** made no attempt to feed or even to bite the plant tissue. (Note: the FDIs reflect combined data from larvae that fed to some extent and those that rejected treated discs outright). This observation would indicate that no ingestion

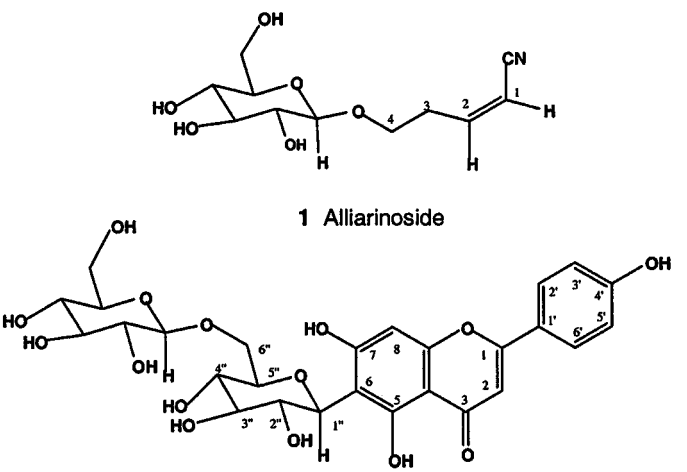

FIG. 4. Structures of compounds **1** and **2** identified as feeding inhibitors from *Alliaria petiolata.*

occurred before rejection by these individuals, whereas extensive palpation with the mouthparts would suggest that taste was involved in their rejection behavior (unpublished data).

Two distinct mechanisms appear to be involved in the resistance of garlic mustard to these pierid caterpillars. Compound **1** inhibits feeding by early instars through an apparent post-ingestive feedback mechanism (Frazier, 1991), whereas compound **2** acts as a direct feeding deterrent that is perceived by taste receptors on the mouthparts of late instars. To our knowledge, this is the first report of such selective barriers in a plant against different larval stages of an insect herbivore. The phenomenon of differential larval responses appears to be a result of simultaneous development of post-ingestive tolerance and taste sensitivity as the larvae feed and develop on their host plants.

Exploitation of garlic mustard as a potential host plant by *P. n. oleracea* is apparently thwarted by the presence of these two unrelated chemicals. Compound **1** effectively blocks feeding by first instars, so that any development of larvae from hatching eggs is unlikely. In addition, the potential migration of late instars from neighboring host plants is likely to fail as a result of the deterrent activity of compound **2**. Although it is clear that the response of such larvae to the deterrent will depend on their previous diet, experience on a preferred host such as cabbage is likely to result in relatively high sensitivity.

Subsequent studies have resulted in the identification of additional flavonoids in foliage of *A. petiolata*, but these do not appear to play a significant role in protecting the plant from *P. napi oleracea*. However, seasonal and population variation in content of the active constituents could potentially affect the susceptibility of garlic mustard to herbivory. (Haribal and Renwick, 2001).

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