Oecologia (1997) 112:362–369

David E. Dussourd

Plant exudates trigger leaf-trenching by cabbage loopers, *Trichoplusia ni* (Noctuidae)

Received: 13 January 1997 / Accepted: 6 June 1997

Abstract Cabbage loopers, *Trichoplusia ni*, cut a narrow trench across leaves of plants that release exudate, then feed distal to the trench in an area of reduced exudation. The larvae do not normally trench plant species such as plantain, *Plantago lanceolata*, that lack exudate. To determine what cues elicit trenching, I reared larvae to the final instar on plantain, then applied test solutions to their mouthparts during feeding. Loopers that received latex from *Lactuca serriola* (Asteraceae) or phloem exudate from watermelon, *Citrullus vulgaris* (Cucurbitaceae), often responded by cutting a trench in plantain, even though these larvae had not previously encountered exudate nor previously trenched. Loopers that were allowed to trench and feed on *L. serriola* for 1 day prior to the assay subsequently cut trenches in plantain more frequently and in response to more fluids, including a viscous solution of polyethylene glycol and latex from a non-host, poinsettia (*Euphorbia pulcherrima*). Subsequent bioassays with larvae reared entirely on plantain tested whether bitter cucurbitacins or gelation are essential cues for trenching. Sap from non-bitter cucumber plants (*Cucumis sativus*) caused larvae to trench, showing that cucurbitacins are not required to induce trenching. Loopers also trenched after receiving cucumber sap that did not gel due to the addition of mercaptoethanol. An extract of sap lacking the proteins that cause gelation likewise triggered trenching. Further fractionation revealed that cucumber sap and also butternut squash sap (*Cucurbita moschata*) contain trenching stimulants that are small (molecular weight < 3,000) water-soluble molecules.

Key words *Trichoplusia ni* · Trenching behavior · Plant defense · Phloem sap · Exudate

D.E. Dussourd
Department of Biology,
University of Central Arkansas,
Conway, AR 72035, USA
e-mail: Dussourd@mail.uca.edu; Fax: 501-450-5914

Introduction

In 1980, C.R. Carroll and C.A. Hoffman described a striking adaptation in squash beetles (*Epilachna tredecimnotata*) for overcoming host defenses. The beetles cut a circular trench in cucurbit leaves before feeding within the trench (Carroll and Hoffman 1980). Subsequent research has shown that comparable behaviors occur in diverse insect groups that include various caterpillars, beetles, katydids, and sawflies (Dussourd and Denno 1991; Dussourd 1993 and references cited therein; Becerra 1994; McCloud et al. 1995). These insects cut trenches, sever leaf veins, or girdle petioles before feeding distal to the cuts on the portion of the plant isolated by the cuts.

Although insects could potentially accrue a variety of benefits from these behaviors (Dussourd 1993), two hypotheses have attracted the most interest. Carroll and Hoffman (1980) and Tallamy (1985) proposed that trenching by *Epilachna* beetles functions to prevent the plant from mobilizing bitter cucurbitacins to leaf tissue within the trench. Support for this hypothesis includes (a) evidence that damage to cucurbit leaves induces increases in cucurbitacin titers, (b) behavioral assays showing that *Epilachna borealis* beetles in the laboratory avoid damaged leaves, and (c) feeding trials showing that *E. borealis* has lower survivorship and fecundity when fed damaged foliage (Tallamy 1985; Tallamy and Krischik 1989). Recent observations, however, do not confirm the cucurbitacin hypothesis. Notably, laboratory feeding assays have documented that cucurbitacins actually stimulate feeding by *Epilachna* (Tallamy and McCloud 1991; McCloud et al. 1995). Furthermore, beetles in the field trench nonbitter plants lacking cucurbitacins, not just bitter plants (Dussourd and Denno 1991; Tallamy and McCloud 1991). The beetles often cut multiple trenches in the same plant (even the same leaf), suggesting that tissue outside the trench has not become unpalatable as predicted (Tallamy and McCloud 1991; McCloud et al. 1995).

Alternatively, insects may cut trenches to reduce their exposure to exudates during feeding. Most insects that sever veins or cut trenches occur on plants that, when damaged, emit viscous exudate from latex canals (laticifers), resin ducts, or phloem (Dussourd and Denno 1991). These fluids are stored under pressure within the canals (Buttery and Boatman 1976). The cuts rupture the canals, thus blocking the flow of secretion to the insect's feeding site and draining some secretion from this distal section (Dussourd and Denno 1991). Plant exudates have a twofold effect on insects. The exudates often contain noxious chemicals (Farrell et al. 1991), such as cardiac glycosides in milkweed latex (Seiber et al. 1982; Sady and Seiber 1991) and terpenes in conifer resin (Cates and Alexander 1982; Gershenzon and Croteau 1991). They also often coagulate upon exposure to air, and may even entrap small herbivores or gum up their mouthparts (Rudinsky 1966; Zalucki and Brower 1992; Dussourd 1993, 1995). McCloud et al. (1995) suggest that *Epilachna* beetles cut trenches in cucurbits specifically to reduce their exposure to the physical stickiness of phloem sap. Cucurbits have unusually large pores in their sieve plates (Esau and Cheadle 1959); damage causes immediate exudation of phloem sap that gels rapidly upon exposure to air (Read and Northcote 1983; Alosi et al. 1988).

I here describe a novel bioassay with cabbage loopers, *Trichoplusia ni*, that directly implicates plant exudates as the trigger for trenching behavior. Cabbage loopers are generalist folivores that feed on herbaceous plants in over 20 families (Eichlin and Cunningham 1978; Sutherland and Greene 1984). Hosts include plants that emit latex (Asteraceae), resin (Apiaceae), or phloem sap (Cucurbitaceae), as well as many species that do not produce exudates when damaged. Notably, the loopers only cut trenches in plants that release exudate (Dussourd and Denno 1994). On these plants, the loopers nibble back and forth across the leaf before feeding beyond the trench (Fig. 1). On *Lactuca serriola* (Asteraceae), trenching often lasts 1–2 h, although some individuals may require over 6 hours to complete a trench (D.E. Dussourd, unpublished work). Plant species that do not release exudate, such as plantain (*Plantago lanceolata*, Plantaginaceae) are not trenched (Dussourd and Denno 1994). The loopers simply feed directly on the leaf without first cutting leaf veins. This facultative response provides the basis for the bioassay. Loopers can be induced to trench leaves of *P. lanceolata* by applying *L. serriola* exudate to their mouthparts as they begin to feed. This paper compares the effectiveness of different fluids in eliciting trenching, examines the effect of prior trenching experience on larval propensity to trench, tests whether cucurbitacins and gelling proteins are necessary to elicit trenching, and finally describes a partial characterization of trenching stimulants from the phloem sap of cucumber (*Cucumis sativus*) and butternut squash (*Cucurbita moschata*).

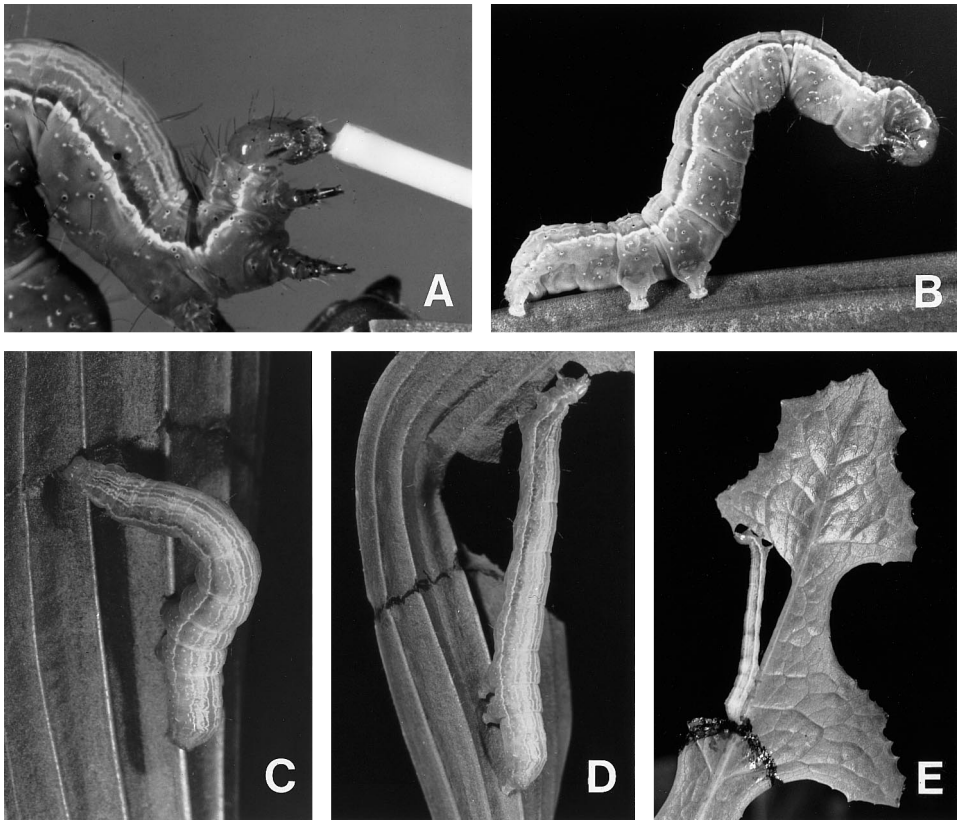

Fig. 1 Cabbage loopers **A** receiving a 1-µl dose of latex from prickly lettuce, *Lactuca serriola*, **B** grooming, **C** cutting a trench in a *Plantago lanceolata* leaf, and **D** feeding beyond the trench. Their response to the latex resembles **E** the normal behavior of cabbage loopers on *L. serriola*. Cabbage loopers normally do not cut trenches in *P. lanceolata*

Materials and methods

Trenching bioassay – general description

Cabbage loopers were reared on potted *Plantago lanceolata* grown in the greenhouse. Upon reaching the mid-final instar, each larva was starved for 1–2 h, then allowed to crawl onto the underside of an excised *P. lanceolata* leaf held in a water pik to maintain turgidity. Flat mature leaves with a maximum width of 1.2–2.1 cm were selected to minimize variation in leaf shape. Wide leaves and leaves with curled edges are more difficult for loopers to trench and thus were avoided. When a larva began feeding, a small drop of fluid was applied with a capillary (Drummond microcap) to the side of the larva's mouth. When the drop was consumed, additional droplets were added until the entire dose was dispensed. Larvae exposed to deterrent solutions often reared back on their prolegs. The remainder of the fluid sample was then applied directly to their mouthparts. In rare cases, loopers walked off the leaf after receiving a test solution (7 out of 382 larvae in all tests). Because these larvae neither fed nor trenched, the tests were repeated with new larvae.

Inexperienced larvae that had not previously encountered exudate nor previously trenched were used in all experiments except for the first study, which examined the effects of prior experience on trenching. The treatments of each experiment were presented to larvae in random order, with the exception again of the first experiment. In all cases, each larva was tested with only a single solution, and a new capillary was used with every larva.

Two minor changes were made in the trenching bioassay over the course of this study. For the later tests (effect of gelling proteins; fractionation of sap; characterization of trenching stimulant), 5-µl Wiretrol micropipettes (Drummond) were used instead of microcaps, because they allow greater precision in delivering fluids. To simulate more accurately the natural outflow of exudate, solutions in some experiments (fractionation of sap; characterization of trenching stimulants) were applied as a series of three to five droplets (per 1.5 µl dose) delivered only to the mouthparts of feeding loopers. Larvae responded to deterrent solutions by wiping and cleaning their mouthparts, and sometimes by moving to new locations to feed. Each subsequent droplet of solution was applied only after the larva resumed feeding. Frequently, with active extracts, only a portion of the dose was applied before a looper began trenching.

Effect of different fluids and prior experience on larval trenching

The trenching bioassay was used to test each of the following solutions with ten cabbage loopers: latex from prickly lettuce, *Lactuca serriola* (Asteraceae); latex from poinsettia, *Euphorbia pulcherrima* (Euphorbiaceae); phloem sap from watermelon, *Citrullus vulgaris* var. Bush Sugar Baby (Cucurbitaceae); a viscous aqueous solution of polyethylene glycol (molecular weight, 15,000–20,000; 0.5 g/ml); and water as a control. Each looper received a 1-µl dose applied with either a 2-µl or 10-µl capillary (Drummond microcaps) depending on solution viscosity. Each 1-µl sample of latex or sap was collected fresh from a potted plant by severing leaf veins or petioles, and collecting 1 µl of exudate directly into the capillary. The number of loopers cutting trenches in response to a test solution versus the aqueous control was compared with 1-tailed Fisher exact tests (Abacus Concepts 1992). The total time a looper spent trenching and the number of times that the looper nibbled across the leaf were also recorded.

To determine how prior exposure to exudate affects larval behavior, some larvae were reared to the early final instar on potted *P. lanceolata*, then were transferred to potted plants of prickly lettuce, *Lactuca serriola*. These larvae were allowed to trench and feed on *L. serriola* for 24 h, then were starved for 1–2 h and tested using the bioassay described above. Henceforth, these loopers will be referred to as experienced loopers, whereas larvae reared entirely on *P. lanceolata* will be labeled inexperienced larvae because they have not previously encountered exudate nor previously trenched. Experienced larvae were tested with prickly lettuce latex, poinsettia latex, polyethylene glycol, and water as described above. Fisher exact tests were used to compare the number of experienced versus inexperienced loopers that cut trenches.

Effect of cucurbitacins on trenching

Varieties of cucumber (*Cucumis sativus*) with and without cucurbitacin have been developed (Andeweg and Bruyn 1959; Robinson et al. 1976). I tested sap from Marketmore 80 cucumber plants, a nonbitter variety lacking cucurbitacins, plus sap from bitter plants of Marketmore 76, which produce cucurbitacins. Sap samples from bitter and nonbitter plants were tested in random order, each with ten cabbage loopers, using the bioassay described previously. Each larva received a single 1-µl dose of sap collected fresh with a 10-µl capillary from the severed vein of a mature leaf. A separate 2-month-old plant was used with each larva. After the test, a small piece of leaf was tasted to confirm that plants were correctly classified as bitter or nonbitter. If larvae cut trenches specifically in response to cucurbitacins, then only loopers exposed to Marketmore 76 sap should cut trenches. Alternatively, if cucurbitacins are absent in cucurbit sap as reported (D.W. Tallamy, unpublished work, cited in McCloud et al. 1995), then no larvae should trench. Finally, if other factors trigger trenching besides or in addition to cucurbitacins, then both bitter and nonbitter sap should elicit trenching.

Another approach for determining the importance of cucurbitacins would be to test these compounds directly. Unfortunately, cucurbitacin in *Cucumis* occurs exclusively as the aglycone (Reum et al. 1957; Guha and Sen 1975), which is not sufficiently soluble in water to test with the trenching bioassay.

Effect of gelling proteins on trenching

Cucurbit sap gels due to the oxidative formation of disulfide bonds between phloem proteins in the exudate (Read and Northcote 1983; Alosi et al. 1988). Gelation can be prevented or reversed by adding a thiol reagent such as mercaptoethanol (Walker and Thaine 1971; Read and Northcote 1983). If the adhesiveness of gelling sap alone elicits trenching, then loopers that experience a sap/mercaptoethanol solution should not be stimulated to trench.

To evaluate the importance of gelation, I tested cabbage loopers with the following three solutions: 2 µl cucumber sap mixed with 0.4 µl water; 2 µl cucumber sap + 0.4 µl 0.3 M mercaptoethanol (producing an overall 0.05 M solution); and a mercaptoethanol control (0.05 M). Each of the three treatments was tested with 15 larvae in a randomized sequence using the trenching assay. Each larva received 1.5 µl of the test solution. Sap was collected fresh from the youngest full-size cucumber leaf by cutting the midvein ~1 cm from the petiole. A separate 2-month-old Marketmore 80 (nonbitter) plant was used with each larva.

Fractionation of sap by molecular weight

To test further the importance of gelation, bulk samples of sap were separated into three fractions by molecular weight: MW > 30,000, MW 3,000–30,000, and MW < 3,000. Sap was collected from four 2-month old Marketmore 80 cucumber plants. Each plant was severed near the base with a clean razor blade. The distal stem was blotted with filter paper to remove contents of ruptured cells, then all sap flowing from the stem was collected with 20-µl Wiretrol capillaries that were discharged into a vial held on ice. When exudation ceased, the stem was severed again ~20 cm from the previous cut and exuding sap was collected in the same fashion, this time from both sides of the cut. Additional cuts were made progressing from the base to the tip until the entire plant had been bled

for sap. The four plants together yielded 2 ml sap. Previous studies have documented that fluid collected with this method is indeed phloem sap (Eschrich et al. 1971; Weber et al. 1974). Exudate was not collected from the base of the plant, which exudes xylem sap (Satoh et al. 1992). To prevent gelation of the phloem sap, mercaptoethanol was added to produce a 0.02 M solution. To remove the gelling proteins, a portion of the sap was placed in Centricon 30 concentrators (Amicon) and spun at 5,000 *g* in a Beckman J2-HS centrifuge for 1 h at 4°C. The retentate containing compounds with a molecular weight greater than 30,000 was dissolved in a solution of 0.02 M TRIS buffer (pH 7.5), 0.02 M mercaptoethanol equal to the initial sap volume. Without mercaptoethanol, this fraction gels, documenting the presence of the phloem protein(s) that causes gelation. In another cucurbit, *Cucurbita maxima,* the protein responsible for gelation has a molecular weight of 96,200 (Read and Northcote 1983). The filtrate was transferred to Centricon 3 concentrators and centrifuged at 6,500 *g* for 2 h at 4°C. Tris buffer (0.02 M, pH 7.5) was added to the retentate fraction, which contains intermediate size molecules (MW 3,000–30,000), to restore the initial volume of the sap. Water was added to the filtrate containing small molecules (MW < 3,000), again to restore the initial volume. A buffer control was also prepared consisting of 0.02 M TRIS buffer (pH 7.5), 0.02 M mercaptoethanol. The three sap fractions and the control were each tested with ten loopers in random order using the trenching bioassay. Each looper received 1.5 µl containing a dose equivalent to 1.5 µl sap.

Characterization of trenching stimulant

In the preceding experiment, only the filtrate fraction containing small molecules (MW < 3,000) elicited trenching. To characterize the trenching stimulant further, the filtrate was extracted with a solvent series. Sap was collected from four Marketmore 80 cucumber plants and fractionated with Centricon 30 and Centricon 3 concentrators, as previously described. The filtrate was rinsed with hexane followed by ethyl acetate, then dried under vacuum. The residue was rinsed with methanol, and the insoluble remnants were taken up in a volume of water equal to the initial volume of the filtrate. The hexane, ethyl acetate, and methanol extracts were dried, then water was added to each to restore the initial filtrate volume. These three solvent extracts and the aqueous extract were each tested with ten loopers in the trenching bioassay. Each looper received 1.5 µl containing an amount of material equivalent to ~3 µl sap (hereafter sap equivalents).

Only the aqueous extract elicited trenching in the previous experiment. To determine if the active chemical in this fraction was proteinaceous, the aqueous extract was digested with proteinase K. Proteinase K is a nonspecific proteinase (Kraus and Femfert 1976; Butler et al. 1991) that is often used to digest proteins in cell fractions (Berger and Kimmel 1987; Sambrook et al. 1989). Sap was collected from an additional five cucumber plants and fractionated using filtration and solvent extraction as described previously. Following extraction with methanol, the filtrate residue was taken up in 0.01 M TRIS (pH 7.8) instead of water. Proteinase K (50 µl/ml) was added to a portion of this fraction, which was then incubated at 37°C for 1 h. A second portion was incubated without proteinase K. A control consisting of TRIS buffer (0.01 M) + proteinase K (50 µl/ml) was also incubated. Ten loopers were tested with each of the three fractions using the trenching bioassay. Each looper received 2 µl containing ~3 µl sap equivalents.

To determine if the presence of a small water-soluble trenching stimulant was unique to cucumber sap, I repeated the above fractionation steps using sap from butternut squash (*Cucurbita moschata*). Sap was collected from 1-month-old plants of Waltham butternut. Butternut fruits were just beginning to form. As previously described, the stems were sequentially cut into sections beginning at the base. The stem was a poor source of exudate, so sap was also collected from flower stalks and young fruits, which emitted sap in greater quantity. A total of 5.8 ml was collected from 30 plants. To prevent gelation, mercaptoethanol was again added to produce a 0.02 M solution. The chilled sample was then centrifuged in Centricon 30 concentrators at 5,000 *g* and 4°C for 3 h. The retentate was placed in 0.02 M TRIS buffer (pH 7.5), 0.02 M mercaptoethanol equal to the original sap volume. A white precipitate in the retentate (also in the original sap/mercaptoethanol solution) did not dissolve in the buffer solution and could not be tested adequately with the bioassay. The filtrate was transferred to Centricon 3 units and spun for 4.5 h at 6,500 *g* and 4°C. To restore the original sap volume, TRIS buffer (0.02 M, pH 7.5) was added to the retentate, and water was added to the filtrate. The three sap fractions were tested in the trenching bioassay, together with raw sap + mercaptoethanol (0.02 M) and a buffer control (0.02 M TRIS, pH 7.5 + 0.02 M mercaptoethanol). Each of the five treatments was tested with ten cabbage loopers. Each looper received a dose of 1.5 µl containing the equivalent of 1.5 µl sap.

A sample of the filtrate from the Centricon 3 concentrators was next subjected to solvent extraction. As previously with cucumber sap, the filtrate sample was sequentially extracted with hexane, ethyl acetate, methanol and water. The hexane fraction lacked detectable weight and was not tested in the bioassay. Water was added to the other dried fractions to restore the initial sap volume. The three fractions and a portion of the filtrate were tested with ten loopers. Each looper received 1.5 µl corresponding to 1.5 µl sap equivalents.

Results

Effect of different fluids and prior experience

Cabbage loopers reared exclusively on *Plantago lanceolata* often cut a trench after receiving a 1-µl drop of prickly lettuce latex or watermelon sap (Table 1A). Even though these larvae had not previously encountered exudate nor previously trenched, the single exposure to exudate sufficed to elicit the behavior. Water alone, polyethylene glycol, and poinsettia latex did not stimulate trenching (Table 1A). The lack of trenching in response to poinsettia latex is surprising because poinsettia is listed as a host plant for *T. ni* (Eichlin and Cunningham 1978). However, initial observations suggest that poinsettia is not an acceptable host, at least for the cabbage loopers tested here. Two hundred newly emerged larvae placed on green leaves of a potted poinsettia failed to feed, as did five final-instar larvae sleeved individually on leaves for 2.5 days (D.E. Dussourd, unpublished work). Only exudate from the two suitable hostplants, prickly lettuce and watermelon, triggered trenching.

Loopers that cut a trench exhibited a characteristic sequence of behaviors. Upon contacting latex or sap, the larvae typically wiped their mouthparts on the leaf, then reared back on their prolegs. As the remaining exudate was added to their mouthparts, the loopers often wiped each drop of exudate off on the leaf, although some loopers consumed the entire dose. Many of the loopers moved their mandibles together and apart repeatedly as though chewing, then often lowered their head to the legs to groom legs and mouthparts, often for several minutes and sometimes for over half an hour (Fig. 1). Before beginning to trench, the loopers first positioned themselves on the plantain leaf. They often walked to the leaf tip, then returned to the middle. After turning toward the tip again and stretching with their head to

Table 1A–C Percentage of cabbage loopers, *Trichoplusia ni*, that cut trenches in *Plantago lanceolata* leaves after a 1-μl drop of fluid was applied to their mouthparts ($n = 10$ larvae/solution). The caterpillars were reared to the final instar on *P. lanceolata* and had either no previous experience with exudates and trenching (inexperienced loopers), or had fed for 1 day on the latex-bearing *Lactuca serriola* just prior to the test (experienced loopers). The loopers responded to some solutions by nibbling back and forth across the leaf before beginning to feed. The total time spent trenching and the number of passes across the leaf is recorded. *Asterisks* indicate treatments in experiments **A** and **B** that differ significantly from the water control ($P < 0.05$, Fisher exact test comparing number of loopers trenching) (*MW* molecular weight)

	Percent *trenching*	Trenching time *Mean ± SD (min)*	No. of passes *Mean ± SD*
A Inexperienced loopers tested with exudates and other fluids			
Prickly lettuce latex	40*	5.8 ± 2.6	4.8 ± 1.3
Poinsettia latex	0		
Watermelon sap	90*	8.2 ± 5.9	4.0 ± 1.9
Polyethylene glycol (MW 15,000–20,000; 0.5 g/ml)	0		
Water control	0		
B Experienced loopers tested with exudates and other fluids			
Prickly lettuce latex	100*	9.9 ± 3.2 ($N = 9$)	6.3 ± 1.7 ($N = 9$)
Poinsettia latex	70*	8.4 ± 3.8	6.3 ± 3.6
Polyethylene glycol (MW 15,000–20,000; 0.5 g/ml)	20	7.5	5.5
Water control	0		
C Inexperienced loopers tested with cucumber sap			
Sap from nonbitter plants	70	7.9 ± 3.4	5.0 ± 2.2
Sap from bitter plants	60	6.5 ± 2.2	4.8 ± 1.2

each leaf margin, they began nibbling a shallow trench across the underside of the leaf. Upon reaching the far margin, they nibbled back along the trench. After making multiple passes across the leaf, requiring several minutes (Table 1A), the loopers started to feed at the edge of the trench and proceeded towards the leaf tip (Fig. 1). Their behavior was identical to the trenching of cabbage loopers on prickly lettuce, except that loopers on these plants often trench for a longer duration (D.E. Dussourd, unpublished work). Loopers on plants with exudate position themselves so the trench transects a narrow portion of the leaf, then always feed distal to the trench on the section of the leaf with reduced exudation (Dussourd and Denno 1994).

Experienced cabbage loopers, which were allowed to trench and feed on prickly lettuce for 24 h, cut trenches in plantain not only in response to prickly lettuce latex, but also to poinsettia latex and polyethylene glycol (Table 1B). Watermelon sap was not tested with the experienced larvae. Only the water control failed to elicit trenching; the water drops were readily consumed, typically with no interruption of feeding. The experienced loopers trenched more frequently than inexperienced loopers in response to both prickly lettuce latex ($P = 0.01$) and poinsettia latex ($P = 0.002$, Fisher exact tests). Two of the experienced loopers even trenched a plantain leaf before solution was applied to their mouthparts. These two larvae were replaced and were not included in the analysis. In contrast, in tests with over 800 inexperienced cabbage loopers (this paper; D.E. Dussourd, unpublished work), the larvae have never cut trenches in plantain, except after exudate was artificially administered to their mouthparts.

Effect of cucurbitacins and gelling proteins

Cabbage loopers cut trenches in response to sap from both bitter and nonbitter cucumber plants (Table 1C). There was no significant difference in number of loopers trenching ($P > 0.9$ Fisher exact test), trenching time ($P > 0.6$ Mann-Whitney *U*-test), or number of passes the larvae made across the leaf ($P > 0.8$ Mann-Whitney *U*-test). The cucumber sap gelled rapidly and adhered readily to larval mouthparts. Several larvae had to bite through thin strands of congealing sap that temporarily glued them to the capillary or even to the leaf as they attempted to wipe their mouthparts. Upon receiving a drop of sap, the typical larva wiped its mouthparts vigorously on the leaf, chewed on the remaining sap, groomed, then positioned itself on the leaf for trenching. Most larvae began to trench before receiving the entire microliter of sap.

Adding mercaptoethanol to cucumber sap eliminated gelation, but not trenching (Table 2A). Indeed, more larvae cut trenches in response to the sap/mercaptoethanol solution than to sap/water, although this difference was not significant ($P = 0.16$, Fisher exact test). Mixing water with the sap did not prevent gelation; indeed, 7 of the 15 larvae tested with the sap/water solution were temporarily glued to the capillary by congealing sap. In another cucurbit, *Cucurbita moschata*, dilution actually causes gelation to progress more rapidly (Alosi et al. 1988). The reduced trenching with the sap/water solution suggests that rapid gelation decreases trenching in this assay, not the converse. The mercaptoethanol control had little effect on larval feeding and did not elicit trenching.

Table 2A–D Percentage of cabbage loopers that cut trenches in *P. lanceolata* leaves after 1.5 µl of test solution was daubed on their mouthparts (2 µl in **D**). The caterpillars were reared to the final instar on *P. lanceolata* and had no previous experience with exudates or trenching. Ten larvae (experiments **B**, **C**, **D**) or 15 larvae (experiment **A**) were tested with each solution. *Asterisks* indicate treatments in experiments **A**, **B**, and **D** that differ significantly from the controls ($P < 0.05$, Fisher exact test comparing number of loopers trenching)

		Percent *trenching*
A	Nonbitter cucumber sap + water	27*
	Nonbitter sap + mercaptoethanol (0.05 M)	53*
	Mercaptoethanol control (0.05 M)	0
B	Nonbitter cucumber stem sap, mercaptoethanol (0.02 M)	100*
	MW > 30,000 fraction in 0.02 M TRIS (pH 7.5), 0.02 M mercaptoethanol	0
	MW 3,000–30,000 fraction in 0.02 M TRIS (pH 7.5)	0
	Filtrate (MW < 3,000) + water	60*
	0.02 M TRIS (pH 7.5), 0.02 M mercaptoethanol control	0
C	Filtrate (MW < 3,000)	70
	Hexane extract in water	0
	Ethyl acetate extract in water	0
	Methanol extract in water	0
	Aqueous extract of residue	70
D	Aqueous extract in 0.01 M TRIS (pH 7.8)	60*
	Aqueous extract in 0.01 M TRIS (pH 7.8) + proteinase K (50 µg/ml)	50*
	0.01 M TRIS (pH 7.8) + proteinase K (50 µg/ml) control	0

Table 3A,B Percentage of cabbage loopers that cut trenches in *P. lanceolata* leaves after 1.5 µl of test solution was daubed on their mouthparts. The caterpillars were reared to the final instar on *P. lanceolata* and had no previous experience with exudates or trenching. Ten larvae were tested with each solution. *Asterisks* indicate treatments in experiment A that differ significantly from the control ($P < 0.05$, Fisher exact test comparing number of loopers trenching)

		Percent *trenching*
A	Butternut squash sap, mercaptoethanol (0.02 M)	100*
	MW > 30,000 fraction in 0.02 M TRIS (pH 7.5), 0.02 M mercaptoethanol	10
	MW 3,000–30,000 fraction in 0.02 M TRIS (pH 7.5)	0
	Filtrate (MW < 3,000) + water	80*
	0.02 M TRIS (pH 7.5), 0.02 M mercaptoethanol control	0
B	Filtrate (MW < 3,000) from butternut squash sap	80
	Ethyl acetate extract in water	0
	Methanol extract in water	20
	Aqueous extract of residue	80

When sap constituents were fractionated according to size, only the filtrate containing small molecules (MW < 3,000) elicited trenching (Table 2B). The large molecule fractions, which contained the gelling proteins, were completely inactive. Due to the addition of mercaptoethanol, none of the solutions in this test gelled. Nevertheless, larvae still cut trenches in response to the filtrate and also the sap/mercaptoethanol mixture. This mixture was not diluted with an aqueous solution as in the previous experiment, which may explain the higher percentage of larvae trenching (100% vs. 53% in Table 2A).

Characterization of trenching stimulant

When the small molecule fraction (MW < 3,000) was extracted with solvents, only the aqueous fraction elicited trenching (Table 2C). The same number of larvae trenched with the aqueous fraction as the unaltered filtrate, indicating that the initial extractions with hexane, ethyl acetate, and methanol removed little or no activity. Subjecting the aqueous fraction to proteinase K had little effect on larval trenching (Table 2D). Evidently, the active compound in the aqueous fraction is not proteinaceous.

As with cucumber, sap from butternut squash proved to be a potent stimulant of trenching, even when mixed with mercaptoethanol (Table 3A). The filtrate fraction (MW < 3,000) from the Centricon concentrators again was most active. The aqueous extract of this fraction elicited the most trenching. It was significantly more active than the methanol extract ($P = 0.01$, Fisher exact test), which also triggered trenching (Table 3B). Thus, both cucumber sap and butternut squash sap contain water-soluble trenching stimulants with molecular weights less than ~3,000.

Discussion

Exudates of prickly lettuce, watermelon, and cucumber suffice to elicit trenching behavior in cabbage loopers. No prior experience with these host plants nor previous attempts at trenching are required. Even final-instar larvae show the entire behavioral repertoire after a single

exposure to latex or sap. Their behavior is similar to normal trenching on plants with exudate except that the trench is completed in less time. However, trenching can be prolonged simply by placing an additional drop of exudate on a larva's mouthparts when it starts to feed (D.E. Dussourd, unpublished work). Presumably, on plants with secretory canals, a looper repeatedly encounters exudate during trenching. The larva continues trenching until all canals crossing the trench have been severed and distal pressures lowered, thus reducing larval exposure to trenching stimulants.

All exudates tested in this study congealed upon exposure to air. Their viscosity and adhesiveness can undoubtedly be detected; Lepidoptera larvae have both mechanoreceptors and chemoreceptors on their maxillae and other mouthparts (Hanson 1983; Grimes and Neunzig 1986; Frazier 1992). Larvae often responded to exudates by masticating the congealing solutions and by grooming at length. A viscous solution of polyethylene glycol elicited trenching by experienced loopers suggesting that viscosity or stickiness does elicit trenching. However, inexperienced loopers cut trenches twice as frequently when the sap did not gel (Table 2A). Apparently, other cues are more significant than gelation. Rapid hardening of sap may actually prevent larvae from detecting chemical factors in the sap that elicit trenching.

The presence or absence of cucurbitacins in cucumber plants had no detectable effect on larval response to sap. Whether cucurbitacins trigger trenching is still unresolved, however, because cucurbitacins may not occur in cucumber sap, even when the plants are bitter. This study documents that sap fractions lacking both cucurbitacins and gelling proteins still stimulate trenching. Another factor must be present. This factor (or factors) in cucumber sap has a molecular weight $< 3{,}000$, is highly polar (soluble in water, but not methanol), and is unlikely to be a protein. Butternut squash sap also contains a small (MW $< 3{,}000$) polar molecule(s) that elicits trenching. It is not known whether the trenching stimulants are merely proximate cues or the ultimate reason why loopers trench. However, the recent discovery that such potent neurotoxins as nicotine, decamethonium, and tubocurarine elicit trenching by cabbage loopers (D.E. Dussourd and D.F. Wiemer, unpublished work) suggests that trenching stimulants may have negative effects on larvae. Thus, the trenches may function partly or primarily to reduce larval exposure to noxious stimulants. A complete characterization of natural trenching stimulants would not only clarify the function of trenching, but also potentially provide new opportunities in pest management.

Trenching stimulants for cabbage loopers may also have significant effects on other cucurbit herbivores, including *Epilachna*. The discovery that cucurbitacins do not reduce *Epilachna* fitness and actually stimulate feeding suggests that some other unknown compound in cucurbit leaves is responsible for the poor performance of beetles on damaged leaves (Tallamy and McCloud 1991). It remains to be seen if trenching stimulants for cabbage loopers cause the low fitness of squash beetles on damaged foliage, and whether these sap constituents trigger trenching by the beetles. Considerable evidence suggests that phloem sap causes *Epilachna* to trench. McCloud et al. (1995) note that *E. borealis* adults cut trenches less frequently in severed leaves allowed to wilt than in freshly cut leaves that emit greater volumes of sap. Also, *Epilachna* species found on cucurbit species that emit copious amounts of sticky sap cut trenches, whereas beetles on cucurbits that emit comparatively little sap do not (McCloud et al. 1995). The potential importance of sap stickiness, however, is supported only by the observation that dipping beetle heads in sap causes prolonged grooming and inhibits further feeding, often for hours (Tallamy and McCloud 1991). While this observation clearly indicates the potential hazards of gelling sap for herbivorous insects, the application of sap was artificial. Final-instar *E. borealis* larvae cutting trenches in zucchini (*Cucurbita pepo*) often step into and place their mouthparts in drops of exudate. However, the exudate appears to have little effect; typically, larvae only stop to groom after reaching the leaf edge, and then for a few minutes at most (D.E. Dussourd, unpublished work).

Cabbage loopers not only cut trenches in cucurbits, but also in Asteraceae bearing latex canals (tribe Cichorieae) and in Apiaceae, which have resin canals (Dussourd and Denno 1994). The trenching bioassay could be used to direct the isolation of trenching stimulants from any of these host plants. Another generalist moth, *Erinnyis ello*, feeds on numerous latex-bearing plants in the tropics (Winder 1976), and has been observed to cut trenches or constrict petioles in members of the Euphorbiaceae, Caricaceae, and Moraceae (Dillon et al. 1983; D.E. Dussourd, unpublished work). It also feeds on plants that do not release exudate (Winder 1976). Likewise, *Epilachna admirabilis* beetles cut trenches in some members of the Cucurbitaceae, but not others (McCloud et al. 1995). A bioassay similar to the *T. ni* trenching assay could be developed using *Erinnyis ello* or *Epilachna admirabilis* to identify trenching stimulants from their host plants. Even insects that feed exclusively on plants with exudates could potentially be used to identify biologically significant chemicals in exudate. Late-instar larvae of the monarch (*Danaus plexippus*), for example, cut a furrow in the midrib or petiole of a milkweed leaf before feeding on the distal section (Dussourd and Eisner 1987; Zalucki and Brower 1992). When a drop of fresh latex is placed next to the mouthparts of a feeding larva, it often stops to chew again on its prior midrib cut, as though its initial effort had been inadequate (Dussourd 1990). Whether latex extracts would likewise elicit additional vein cutting is not known.

In summary, the bioassay described herein provides evidence that plant exudates elicit trenching behavior in cabbage loopers. The assay offers the potential for guiding the isolation of plant chemicals of significance to insect herbivores – chemicals of sufficient importance to have selected for behavioral counteradaptations in multiple insect lineages.

Acknowledgements Thanks to D.F. Wiemer, S.W. Runge, and W.M. Moran for helpful advice and use of equipment, to W.L. Roelofs and K. Poole (Geneva Experiment Station, Cornell University) for repeatedly supplying *T. ni* eggs, to J.J. McAllister, N.R. Robertson, N.J. Sanders, and A.B. Goble for assistance in the lab and greenhouse, to the Department of Entomology, University of Maryland at College Park and R.F. Denno for providing a hospitable setting for the initial stages of this work, and to M.D. Bowers, P.V. Hamilton, K.C. Larson, D.W. Tallamy, D.F. Wiemer, and an anonymous reviewer for helpful comments on the manuscript. I am also grateful for financial support from the Cooperative Research Service, USDA (Competitive Research Grant 93-37302-9507) and the University of Central Arkansas Research Council.

References

Abacus Concepts (1992) StatView. Abacus Concepts, Berkeley

Alosi MC, Melroy DL, Park RB (1988) The regulation of gelation of phloem exudate from *Cucurbita* fruit by dilution, glutathione, and glutathione reductase. Plant Physiol 86:1089–1094

Andeweg JM, DeBruyn JW (1959) Breeding of non-bitter cucumbers. Euphytica 8:13–20

Becerra JX (1994) Squirt-gun defense in *Bursera* and the chrysomelid counterploy. Ecology 75:1991–1996

Berger SL, Kimmel AR (1987) Guide to molecular cloning techniques. Academic Press, New York

Butler GH, Kotani H, Kong L, Frick M, Evancho S, Stanbridge EJ, McGarrity GJ (1991) Identification and characterization of proteinase K-resistant proteins in members of the class Mollicutes. Infect Immun 59:1037–1042

Buttery BR, Boatman SG (1976) Water deficits and flow of latex. In: Kozlowski TT (ed) Water deficits and plant growth, vol 4. Academic Press, New York, pp 233–289

Carroll CR, Hoffman CA (1980) Chemical feeding deterrent mobilized in response to insect herbivory and counteradaptation by *Epilachna tredecimnotata*. Science 209:414–416

Cates RG, Alexander H (1982) Host resistance and susceptibility. In: Mitton JB, Sturgeon KB (eds) Bark beetles in North American conifers. University of Texas Press, Austin, pp 212–263

Dillon PM, Lowrie S, McKey, D (1983) Disarming the "evil woman": petiole constriction by a sphingid larva circumvents mechanical defenses of its host plant, *Cnidoscolus urens* (Euphorbiaceae). Biotropica 15:112–116

Dussourd DE (1990) The vein drain; or, how insects outsmart plants. Nat Hist 90(2):44–49

Dussourd DE (1993) Foraging with finesse: caterpillar adaptations for circumventing plant defenses. In: Stamp NE, Casey T (eds) Ecological and evolutionary constraints on caterpillars. Chapman and Hall, New York, pp 92–131

Dussourd DE (1995) Entrapment of aphids and whiteflies in lettuce latex. Ann Entomol Soc Am 88:163–172

Dussourd DE, Denno RF (1991) Deactivation of plant defense: correspondence between insect behavior and secretory canal architecture. Ecology 72:1383–1396

Dussourd DE, Denno RF (1994) Host range of generalist caterpillars: trenching permits feeding on plants with secretory canals. Ecology 75:69–78

Dussourd DE, Eisner T (1987) Vein-cutting behavior: insect counterploy to the latex defense of plants. Science 237:898–901

Eichlin TD, Cunningham HB (1978) The Plusiinae (Lepidoptera: Noctuidae) of America north of Mexico, emphasizing genitalic and larval morphology. US Dep Agric Tech Bull 1567:1–122

Esau K, Cheadle VI (1959) Size of pores and their contents in sieve elements of dicotyledons. Proc Natl Acad Sci USA 45:156–162

Eschrich W, Evert RF, Heyser W (1971) Proteins of the sieve-tube exudate of *Cucurbita maxima*. Planta 100:208–221

Farrell BD, Dussourd DE, Mitter C (1991) Escalation of plant defense: do latex and resin canals spur plant diversification? Am Nat 138:891–900

Frazier JL (1992) How animals perceive secondary compounds. In: Rosenthal GA, Berenbaum MR (eds) Herbivores: Their interactions with secondary plant compounds. Academic Press, New York, pp 89–134

Gershenzon J, Croteau R (1991) Terpenoids. In: Rosenthal GA, Berenbaum MR (eds) Herbivores: their interactions with secondary plant compounds. Academic Press, New York, pp 165–219

Grimes LR, Neunzig HH (1986) Morphological survey of the maxillae in last-stage larvae of the suborder Ditrysia (Lepidoptera): mesal lobes (Laciniogaleae). Ann Entomol Soc Am 79:510–526

Guha J, Sen SP (1975) The cucurbitacins – a review. Plant Biochem J 2:12–28

Hanson FE (1983) The behavioral and neurophysiological basis of food plant selection by lepidopterous larvae. In: Ahmad S (ed) Herbivorous insects: host-seeking behavior and mechanisms. Academic Press, New York, pp 3–23

Kraus E, Femfert U (1976) Proteinase K from the mold *Tritirachium album* Limber: specificity and mode of action. Hoppe-Seyler's Z Physiol Chem 357:937–947

McCloud ES, Tallamy DW, Halaweish FT (1995) Squash beetle trenching behavior: Avoidance of cucurbitacin induction or mucilaginous plant sap? Ecol Entomol 20:51–59

Read SM, Northcote DH (1983) Chemical and immunological similarities between the phloem proteins of three genera of the Cucurbitaceae. Planta 158:119–127

Reum S, Enslin PR, Meeuse ADJ, Wessels JH (1957) Bitter principles of the Cucurbitaceae. VII. The distribution of bitter principles in this plant family. J Sci Food Agric 8:679–691

Robinson RW, Munger HM, Whitaker TW, Bohn GW (1976) Genes of the Cucurbitaceae. HortSci 11:554–568

Rudinsky JA (1966) Host selection and invasion by the Douglas-fir beetle, *Dendroctonus pseudotsugae* Hopkins, in coastal Douglas-fir forests. Can Entomol 98:98–111

Sady MB, Seiber JN (1991) Field test for screening milkweed latex for cardenolides. J Nat Prod 54:1105–1107

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Plainview

Satoh S, Iizuka C, Kikuchi A, Nakamura N, Fujii T (1992) Proteins and carbohydrates in xylem sap from squash root. Plant Cell Physiol 33:841–847

Seiber JN, Nelson CJ, Lee SM (1982) Cardenolides in the latex and leaves of seven *Asclepias* species and *Calotropis procera*. Phytochem 21:2343–2348

Sutherland DWS, Greene GL (1984) Cultivated and wild host plants. In: Lingren PD Green GL (eds) Suppression and management of cabbage looper populations. US Dep Agric Tech Bull 1684:1–13

Tallamy DW (1985) Squash beetle feeding behavior: an adaptation against induced cucurbit defenses. Ecology 66:1574–1579

Tallamy DW, Krischik VA (1989) Variation and function of cucurbitacins in *Cucurbita*: an examination of current hypotheses. Am Nat 133:766–786

Tallamy DW, McCloud ES (1991) Squash beetles, cucumber beetles and inducible cucurbit responses. In: Tallamy DW, Raupp MJ (eds) Phytochemical induction by herbivores. Wiley, New York, pp 155–181

Walker TS, Thaine R (1971) Proteins and fine structural components in exudate from sieve tubes in *Cucurbita pepo* stems. Ann Bot 35:773–790

Weber C, Franke WW, Kartenbeck J (1974) Structure and biochemistry of phloem-proteins isolated from *Cucurbita maxima*. Exp Cell Res 87:79–106

Winder JA (1976) Ecology and control of *Erinnyis ello* and *E. alope*, important insect pests in the New World. Pest Artic News Summ 22:449–466

Zalucki MP, Brower LP (1992) Survival of first instar larvae of *Danaus plexippus* (Lepidoptera: Danainae) in relation to cardiac glycoside and latex content of *Asclepias humistrata* (Asclepiadaceae). Chemoecology 3:81–93