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INFLUENCE OF CNICIN, A SESQUITERPENE LACTONE OF *Centaurea maculosa* (ASTERACEAE), ON SPECIALIST AND GENERALIST INSECT HERBIVORES

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Abstract—The sesquiterpene lactone cnicin was extracted from *Centaurea maculosa* and *Centaurea vallesiaca*. We examined its effects on the ovipositional response and larval development of generalist and specialist insect herbivores associated with *C. maculosa*. For the oviposition trials, three plant species (*C. maculosa*, *Achillea millefolium*, and *Cichorium intybus*), half of which were sprayed with 3% of cnicin, were exposed to the specialist moths *Stenodes straminea*, *Agapeta zoegana*, and *Pterolonche inspersa* in field cages. All three species significantly preferred *C. maculosa* to other plants and *P. inspersa* significantly preferred cnicin-sprayed plants to untreated plants for oviposition. Tested over all species, cnicin significantly increased the number of eggs laid on a given plant. A larval diet test examined the toxicity of cnicin for larvae of the generalist noctuid moth *Spodoptera littoralis*. Cnicin concentrations of 3% and 6% were lethal and 1% and 0.5% seriously inhibited growth and development. The larvae of the *C. maculosa* specialist *Stenodes straminea* survived at 6% cnicin, but none of the pupae hatched. *Agapeta zoegana* was able to survive at 1% and 3% cnicin. Both specialists had difficulties with the artificial diet, but weight increase and survival was not further reduced when cnicin was present compared with on the control diet. In conclusion, cnicin influenced host recognition by the specialist species, and larvae of the generalist did not survive on natural levels of cnicin. Growth and survival of the specialist were not influenced by cnicin but were considerably hampered on artificial diet.

Key Words—*Centaurea maculosa*, sesquiterpene lactone, cnicin, host-plant

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selection, oviposition behavior, antifeedant, attractant, *Spodoptera littoralis*, *Agapeta zoegana*, *Stenodes straminea*, *Pterolonche inspersa*, *Lepidoptera*, *Noctuidae*, *Cochylidae*, *Pterolonchidae*.

INTRODUCTION

Sesquiterpene lactones are secondary compounds characteristic of many Asteraceae. The great majority of the sesquiterpene lactones known by 1977 (Fischer et al., 1979) are from this plant family. Later, Seaman (1982) listed over 1300 sesquiterpene lactones only from Asteraceae, and they occur only infrequently in other families such as Lauraceae, Magnoliaceae, and Apiacea (Kery et al., 1987). Most of these terpenoids have, in addition to other biological activities (Rodriguez et al., 1976), antifeedant properties. They have been found to reduce growth and survival of insects (Jones et al., 1979; Picman et al., 1978; Picman and Picman, 1984) and to be feeding deterrents for herbivore vertebrates and insects (Burnett et al., 1974; Mabry et al., 1977; Nawrot et al., 1983; Harmatha and Nawrot, 1984). The antifeedant properties of sesquiterpene lactones may play a major role in specific plant–animal coevolution (Burnett et al., 1987).

Sesquiterpene lactones have been isolated from many plants, usually from the aerial parts (e.g., Geppert et al., Bloszyk and Drozdz, 1978; Jones et al., 1979). The sesquiterpene lactone cnicin ($C_{20}H_{26}O_7$) was first isolated from *Cnicus benedictus* and is found in many *Centaurea* species (e.g., Nowak et al., 1984), among others in *C. maculosa* (see, e.g., Huneck et al., 1986).

Spotted knapweed, *C. maculosa*, was accidentally introduced to America from Eurasia at the end of the last century and has become one of the most important rangeland weeds in the Northwest of the United States and Canada. Together with *Centaurea diffusa*, it has reduced the grazing potential of many areas by over 80% (Müller-Schärer and Schroeder, 1993). *C. diffusa* extracts were tested for allelopathy to try to explain the rapid spread of this weed (Muir and Majak, 1983). Inhibitory activity was detected in laboratory seed germination tests, but field studies failed to show any allelopathic effects (Muir et al., 1985). Two years later, the phytotoxic compound of *C. maculosa* was recognized to be cnicin, located in glandular trichomes on the epidermal surface (Kelsey and Locken, 1987). Its concentration varies among tissues and by time: in living tissue, Locken and Kelsey (1987) found most cnicin in the small leaves of the stem. They measured 0.5% of dry weight in spring and about 1% in late summer. The highest concentration of 2.8% dry weight was measured in October in dry branches. No cnicin was found in the roots.

C. maculosa plants in America have practically no natural enemies, although they were introduced over a century ago. On the other hand, many herbivores feed on European plants. According to the plant apparency theory (Feeny, 1976), *C. maculosa* is rather "unapparent" and "unpredictable" and could be expected

to be eaten mostly by generalist insects. The root-feeding insects of *C. maculosa* are, however, mostly specialists, with only 22% of generalists (Müller, 1989). Leaf damage in the field is not very common and only a few species use the leaves as their food niche (Schroeder, 1985). American *C. maculosa* was found to contain generally low cnicin concentrations, compared with European *C. maculosa* (Müller-Schärer, unpublished data).

We examined the effects of cnicin on the ovipositional response and larval development of specialist and generalist herbivores and predicted different responses for specialists and generalists. We asked whether cnicin on the surface of the leaves is an attractant for female specialist moths looking for their host plant to lay their eggs and if they are attracted by other plants if these are coated with cnicin. We next asked if cnicin reduces the growth and fitness of larval specialist and generalist Lepidoptera.

METHODS AND MATERIALS

Cnicin. Cnicin was extracted from *C. maculosa* Lam. and *C. vallesiaca* Jordan, which is taxonomically closely related to *C. maculosa*. The dried small leaves of the stems were ground, extracted twice at room temperature with diethyl ether, and washed with pentane. The separation was performed with column chromatography using chloroform and ethyl acetate. The fractions containing cnicin were found by thin-layer chromatography. It was recrystallized from distilled H_2O (Landau, 1993). NMR showed that the extracted cnicin was over 95% pure (J. A. Robinson, personal communication), compared with a reference (see Huneck et al., 1986). The reference, tested with MS, was 99% pure cnicin (H.-R. Buser, personal communication). Additionally, some samples were analyzed using HPLC as described by Locken and Kelsey (1987). Since the extracted concentrations were very small (0.15–0.2%), we also used reference material isolated by Huneck (see Huneck et al., 1986). Cnicin is white, odorless, more or less crystalline, and has a melting point of 143°C (Merck Index). Compared to other extraction methods (Locken and Kelsey, 1987), the obtained amount of cnicin was very low and the method used is not satisfactory. Namely, diethyl ether is not ideal for extraction of cnicin, and large amounts had to be used for extraction because, according to the Merck Index, cnicin is practically insoluble in ether. The main reason for using this method was, however, its proven evidence of yielding cnicin of suitable quality (see Huneck et al., 1986). Based on the data available from spectroscopic methods (MS, NMR), HPLC, melting point determination, and thin-layer chromatography, a purity of $>97\%$ was ascertained.

Plants. Centaurea maculosa (L.) (Asteraceae) is a biennial or a short-lived perennial and is widely distributed in eastern Europe, where it grows at ruderal

sites. Seeds from *C. maculosa*, *Cichorium intybus* (Asteraceae), and *Achillea millefolium* (Asteraceae) originated from the International Institute for Biological Control in Delémont and were grown in pots (13 cm diameter, 17 cm depth) in a greenhouse.

Insects. The generalist *Spodoptera littoralis* (Boisduval) (Lep.: Noctuidae), the Egyptian cotton leafworm, is widely distributed in Africa, the Middle East, and the circum-Mediterranean region. The larvae feed on about 40 plant families (Navon, 1985). The larvae used in the experiments were reared at the Sandoz Agrobiological Station (Witterswil, Switzerland). The oligophagous moth *Agapeta zoegana* (L.) (Lep.: Cochylidae) is widely distributed in Europe and is associated with the roots of *C. maculosa* (Müller et al., 1988). *Pterolonche inspersa* (Stgr.) (Lep.: Pterolonchidae), is also closely associated with *C. maculosa* (Dunn et al., 1989). Both species were introduced into North America for the biological control of *C. maculosa* (Müller-Schärer and Schroeder, 1993). *Stenodes straminea* (Haw.) (Lep.: Cochylidae) feeds on *Artemisia*, *Scabiosa*, and *Centaurea* species and therefore have a wider host range than the other described specialists (Müller, 1983). All three species lay their eggs on rosette leaves, and the hatching larvae mine in the leaves before they tunnel into the roots (Müller et al., 1988). The larvae of *A. zoegana* and *P. inspersa* were collected in roots of *C. maculosa* in Hungary, 60 km east of Budapest. *Stenodes straminea* larvae were collected in Lalden (Wallis, Switzerland), where they live in the bases of the rosette leaves and the root collar of *Centaurea vallesiaca*. For the larval diet test, the hatching *A. zoegana* larvae were transferred to *C. maculosa* rosettes in pots and reared in a greenhouse.

Oviposition Test. The experiment was conducted in field cages (1 × 1 × 1 m). Four specimens of three plant species were placed in each cage and were presented at ground level by burying their pots. The plants *Cichorium intybus* and *Achillea millefolium* were used in addition to *C. maculosa* (control). *A. zoegana* had laid on average 7.6 (five replicates) and 0 (three replicates) eggs per female per test, respectively, on these plants in previous oviposition tests (Müller et al., 1988). The plants were cut to the same size and randomly assigned to two groups. The dry weight of each species was estimated, and the amount of cnicin, which would give 3% of the dry weight, was calculated. Using a Biomat spray, half the plants were carefully coated with cnicin, which had been dissolved in a methanol–water solution (2:1). Twelve pots were randomly placed within each of the three cages (blocks). The newly hatched moths were introduced into the cages, where they lived for about nine days. They were offered a honey solution and a bunch of flowers as food. A few days later and at the end of the experiment, i.e., after six to nine days, the eggs were counted. The results were tested with repeated-measures ANOVA with plant species and cnicin as a between- and time as a within-subject factor. This allows testing, averaged over time, for differences among plant species and effect of cnicin.

Since there were two measurements recorded on each individual plant, using a repeated-measures analysis of variance design is recommended (Sokal and Rohlf, 1981).

Larval Diet Tests. Small tubes with artificial diet (Sandoz Standard) were used for all experiments. For the different diet concentrations, cnicin was dissolved in acetone and added to the liquid diet. For the two specialists, *A. zoegana* and *S. straminea*, 3% of powdered *C. maculosa* root was added. Larvae were taken from *Centaurea* roots and immediately transferred into the tubes. The treatments were always randomly assigned within blocks. *Spodoptera littoralis* were weighed every second day. Because *A. zoegana* and *S. straminea* mine within their webs, they could not be weighed during the experiment.

The larvae of *S. littoralis* were put into small tubes containing 3 ml of artificial diet, enough to reach pupation. In the first test, 0% (control), 3%, and 6% cnicin were tested on second-instar larvae. They were kept at 25°C, 65% relative humidity, 16-hr photoperiod. Later, third-instar larvae were used to test cnicin concentrations of 0%, 0.5%, and 1%. They were kept at 22°C, 85% relative humidity, 15-hr photoperiod. The tubes for *S. straminea* (tested at the fourth to sixth instar) contained 2 ml diet plus 0% (control) and 6% cnicin. They were kept in the dark at 20°C, 60% relative humidity. *A. zoegana* (fifth and sixth instar) were tested with cnicin concentrations of 0%, 1%, and 3% in 2 ml of diet. They were kept in the dark at 22°C, 85% relative humidity 15-hr photoperiod. The results were tested with repeated-measures ANOVA. Block effects were not significant and are therefore omitted everywhere (Sokal and Rohlf, 1981, p. 350). Means are given with ± 1 SE.

RESULTS

Oviposition Test. For *Stenodes straminea*, a total of 917 eggs were found in the three cages. Although eggs were laid in all treatments, 96% of the eggs were found on *C. maculosa* (Figure 1a), being responsible for the significant effect of the plant species (Table 1). The fact that many larvae hatched indicates that cnicin was not toxic as a substrate.

For *Agapeta zoegana*, of a total of 82 eggs, only three were not laid on *C. maculosa*. The factors plant species, but also time and their interaction were significant (Table 2), as more eggs were laid in the second half of the experiment. Although the cnicin-sprayed plants were preferred (Figure 1b), this factor was not statistically significant (Table 2).

For *Pterolonche inspersa*, the factors plant species, cnicin, and their interaction were highly significant. The females clearly preferred the cnicin-sprayed *C. maculosa* plants (Table 3 and Figure 1c).

When tested with Fisher's Combining Test (Sokal and Rohlf, 1981), the

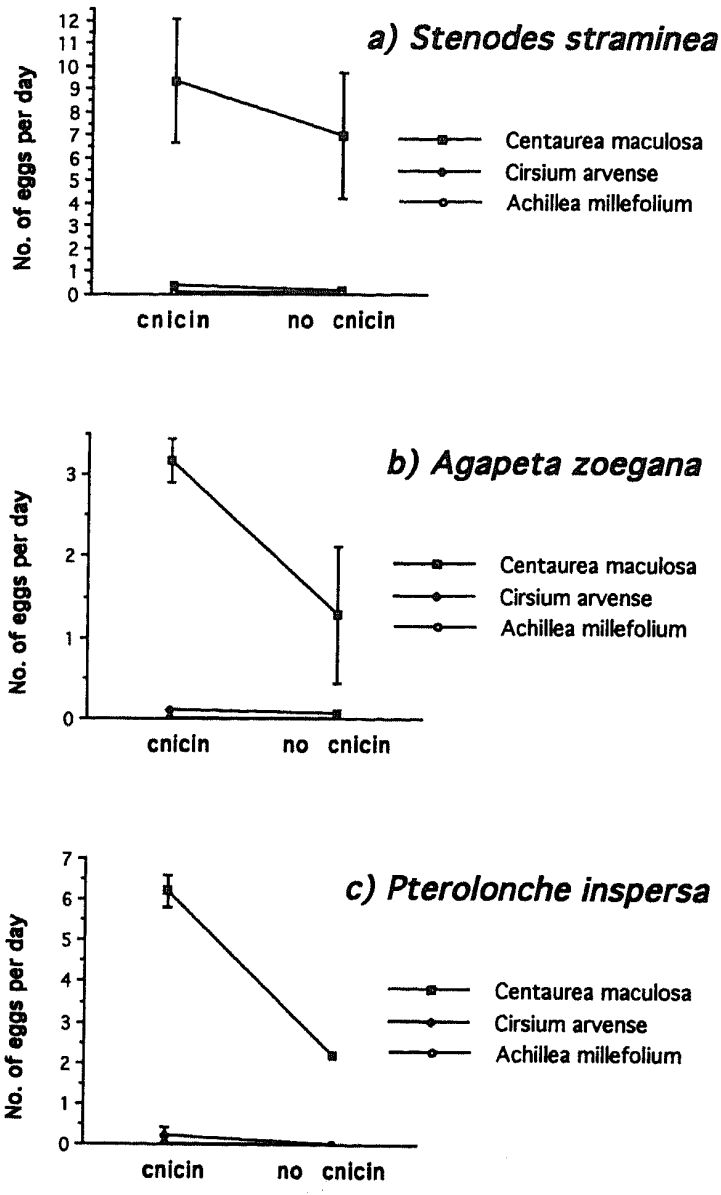

FIG. 1. The numbers of eggs (±SE) laid on the different plant species with and without cnicin.

specialists overall laid significantly more eggs on the cnicin-sprayed plants ($\chi^2_{df=6} = 24.94$, $P < 0.001$) (Figure 1).

Larval Diet Tests. For *Spodoptera littoralis*, the effect of cnicin, time, and their interaction had highly significant influences on the survival and weight of the larvae (Table 4 and 5). The concentrations of 3% and 6% were lethal; after five days all second-instar larvae had died, whereas the larvae in the control diet grew quickly (Figure 2). In the test with 0.5% and 1% cnicin, none of the larvae died during the first 30 days, but the larvae in the two cnicin diets showed reduced growth (Figure 3). The developmental rate was also reduced by cnicin:

TABLE 1. ANOVA FOR OVIPOSITION OF *Stenodes straminea*

	df	SS	*F* value	*P* value
Plant (A)	2	20700.028	17.138	<0.001
Cnicin (B)	1	268.347	0.444	0.510
A × B	2	409.694	0.339	0.715
Error	30	18117.417		
Time (C)	1	183.681	1.133	0.296
A × C	2	412.028	1.271	0.295
B × C	1	0.681	0.004	0.949
A × B × C	2	5.028	0.016	0.985
Error	30	4863.083		

TABLE 2. ANOVA FOR OVIPOSITION OF *Agapeta zoegana*

	df	SS	*F* value	*P* value
Plant (A)	2	26.661	24.284	0.001
Cnicin (B)	1	2.535	4.618	0.075
A × B	2	4.598	4.188	0.073
Error	6	3.294		
Time (C)	1	1.042	21.277	0.004
A × C	2	1.286	13.138	0.006
B × C	1	0.002	0.034	0.860
A × B × C	2	0.006	0.066	0.937
Error	6	0.294		

TABLE 3. ANOVA FOR OVIPOSITION OF *Pterolonche inspersa*

	df	SS	*F* value	*P* value
Plant (A)	2	92.631	3100.058	<0.001
Cnicin (B)	1	12.756	853.806	<0.001
A × B	2	21.388	715.781	0.001
Error	6	0.090		
Time (C)	1	0.196	0.042	0.845
A × C	2	0.100	0.011	0.990
B × C	1	0.511	0.109	0.753
A × B × C	2	0.397	0.042	0.959
Error	6	28.272		

TABLE 4. ANOVA FOR ARCSIN-TRANSFORMED SURVIVAL RATE OF *Spodoptera littoralis* LARVAE WITH 3% AND 6% CNICIN AFTER 1, 2, AND 4 DAYS

	df	SS	*F* value	*P* value
Cnicin (A)	2	7.608	50.455	<0.001
Error	15	1.131		
Time (B)	2	7.608	61.667	<0.001
A × B	4	5.757	23.333	<0.001
Error	30	1.851		

TABLE 5. ANOVA FOR *Spodoptera littoralis* LARVAL WEIGHT WITH 0.5% AND 1% CNICIN DURING FIRST TEN DAYS

	df	SS	*F* value	*P* value
Cnicin (A)	2	6314438.8	384.0	<0.001
Error	33	271337.1		
Time (B)	7	5118781.8	441.0	<0.001
A × B	14	5609378.8	241.6	<0.001
Error	231	383066		

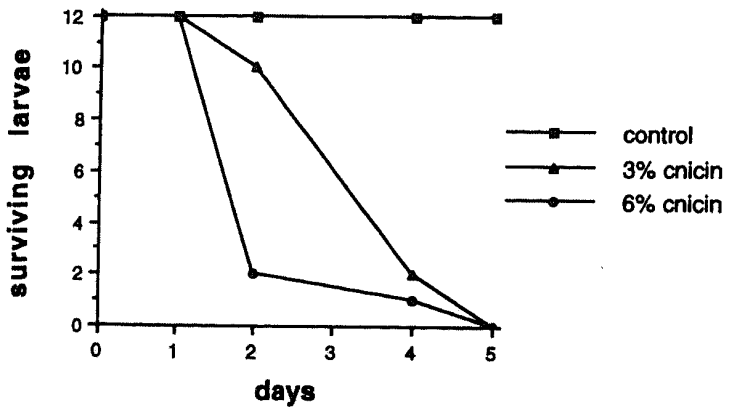

FIG. 2. *Spodoptera littoralis*: survival rate of second-instar larvae.

after 10 days, the larvae on the control diet started to pupate. Table 6 shows different larval stages on the 10th, 21th, 31th, and 38th day. At day 38, the experiment was stopped because the diet was very old. The pupae with cnicin were often malformed; pupation was incomplete for seven of 11 larvae in the 0.5% diet, and for four of seven larvae in the 1% diet; they were also very

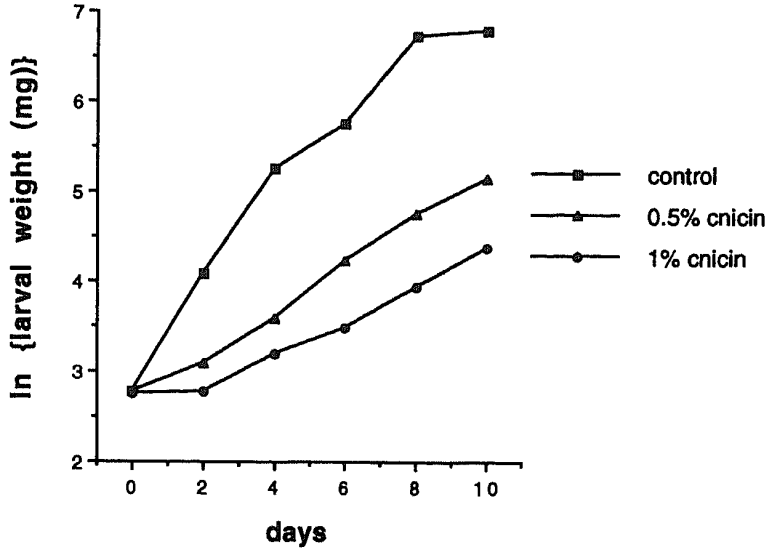

FIG. 3. Weight increase of *Spodoptera littoralis* larvae.

small or otherwise different from normal pupae. Pupal weight, which is generally correlated with fecundity of the adults, was lower with cnicin (Table 6).

For *Stenodes straminea*, rearing on artificial diet was difficult. Some larvae walked around the tubes for days without making a web. Within four days, they all lost about 65% of their weight. After that, they were left in their diet until they pupated or died. The results are given in Table 6. Although some larvae pupated at 6% cnicin, none of these emerged as adults. Pupal weight was also reduced ($df = 6$, $t = 6.5$, $P < 0.001$). Due to limited availability of cnicin, tests with lower concentrations could unfortunately not be made.

For *Agapeta zoegana*, after 43 days, the diet was very old and the experiment was stopped. None of the larvae, which were all in the fifth and sixth instar, when the experiment was started, pupated. Of the 27 larvae, 19 died. The reason for this was not cnicin, because they died in the control diet as well (Table 6). Cnicin influenced neither survival nor larval weight ($F_{5,7} = 2.1$, $P = 0.22$).

DISCUSSION

The widespread distribution of sesquiterpene lactones within the family Asteraceae implies that antifeedant properties of these compounds may have been an important factor in the evolution (Burnett et. al., 1987). The result of our larval diet tests showed that for generalist larvae of *S. littoralis* cnicin was noxious. In high concentrations (3% and 6%) the larvae died after four to five days, probably as a direct consequence of cnicin and not because of starvation. In a pilot experiment, starving third-instar larvae died only after seven to eight

TABLE 6. SUMMARY OF RESULTS OF LARVAL DIET TEST

	Cnicin treatment	N	Stage after 10 days	Stage after 21 days	Stage after 31 days	Stage after 38 days	Pupal weight (mg)
Spodoptera littoralis	Control	12	12 × 6[a]	12 × P	12 × A		332 ± 16.8
	0.5%	12	12 × 5	12 × 6	9 × P; 3 × 6	1 × A; 4 × P; 7 × dead	241 ± 41.9
	1%	12	11 × 4; 1 × 5	5 × 5; 7 × 6	1 × P; 10 × 6; 1 × 5	4 × P; 3 × 6; 1 × 5; 4 × dead	243 ± 41.9
			Days till pupation	Larvae pupated	Pupal weight (mg)	% pupae emerged	
Stenodes straminea	Control	10	18.6	5	19.4 ± 1.4	100	
	6%	10	28.6	3	6.9 ± 0.7	0	
			Larvae survived	Larval weight (mg) after 43 days			
Agapeta zoegana	Control	9	2	26			
	1%	9	2	11.2			
	3%	9	4	12.4			

[a] 12 × 6 = 12 larvae in the sixth instar; P = pupae; A = adult moth.

days. In addition, starving larvae lost 12% and 6% of their weight in the first and second days, respectively. During the same period, the larvae reared on 3% and 6% cnicin had already lost 35% after the first day and 13% and 26% after the second days, respectively. This strongly supports the hypothesis of a noxious effect of cnicin. Cnicin concentrations of 0.5% and 1% retarded the growth and larval developmental rate of *S. littoralis*. Almost half of the insects died either as larvae (sixth instar) or as pupae, and only one moth emerged. The experiment had to be stopped after 38 days because the diet was quite old and some larvae and pupae were diseased. All control larvae had emerged by day 31. As even 0.5% retarded and inhibited development, cnicin is probably a strong insect feeding deterrent. Harmatha and Nawrot (1984) found feeding by insects was deterred by a 1% solution. Mabry and Gill (1979), however, used only 0.125%, 0.25%, and 0.5% of glaucolide-A (a sequiterpene lactone from the genus *Vernonia*) for their larval diet tests and found reduction in growth for some species. Cnicin itself was only harmful to *Spodoptera littoralis* larvae when it was ingested. In a supplementary test, second-instar larvae were sprayed with a solution of cnicin. Even relatively high concentrations were not harmful (Gobeli, personal communication).

The specialist larvae of *Stenodes straminea* were able to develop and some to pupate, when reared in the diet containing 6% cnicin, but no emergence took place. Since none of the larvae in the cnicin diet metamorphosed, we can say that the tolerable concentration for complete development is under 6%. In nature, the larvae encounter rosette leaves, which contain about 0.3% to 1% cnicin (Locken and Kelsey, 1987).

Cnicin concentrations of 1% and 3% did not affect the survival rate of the other specialist *A. zoegana*, but overall survival was only 30%, and none of the larvae pupated. The temperature of 22°C might have been too high for these root specialists, or the consistency of the diet may not have been appropriate. In general, it very difficult to rear food specialists on artificial diets and more experiments with these species are needed. So far, *A. zoegana* have only been reared in *C. maculosa* roots (e.g., Müller and Steinger, 1990).

Preliminary results of a feeding test with adult beetles *Larinus obtusus* (Col. Curculionidae) (a *C. maculosa* specialist) showed that they, too, significantly preferred cnicin-sprayed leaves of the nonhost plant *Cirsium arvense* (Asteraceae) over control leaves sprayed only with methanol–water solution (same method as oviposition test) (Landau, 1993). This confirms the role of cnicin as a key substance for *C. maculosa* specialists.

The experiments made clear that specialists can tolerate a higher concentration of cnicin than generalists. Some *S. straminea* pupated with 6% cnicin, whereas 3% caused immediate death of *S. littoralis*. On the other hand, the specialists, too, had difficulties with high concentrations of cnicin and a tolerable level was not found, because of the difficulties of rearing the larvae in artificial

diet. It will probably be around the concentrations found in the plants. Mabry et al. (1977) tested specialists and generalists of *Vernonia* species with natural concentrations of a sesquiterpene lactone. Here, too, larval development was retarded for the generalists, but not for the specialists.

The impact of cnicin on the ovipositional behavior of *C. maculosa* specialists was clear; they preferred more cnicin on the plants. Cnicin may consequently not be a deterrent for them, but an attractant. It is thus not clear how they can distinguish different cnicin concentrations. Since not all eggs were laid on cnicin-treated plants, other factors such as leaf surface, plant and leaf form, odor, and taste may be important for host-plant selection. These could be eliminated by working with artificial leaves.

Discrimination of cnicin-sprayed plants was least pronounced in *S. straminea* (Tables 1–3, Figure 1), consistent with *S. straminea* not being specialized enough as a biological control agent to be introduced to America (Müller, 1983). This suggests that cnicin may be more important with increasing food specialization of insect herbivores.

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