CHEMICAL FACTORS INVOLVED IN SELECTION OF HOST PLANT FOR OVIPOSITION BY THE PICKLEWORM MOTH (LEPIDOPTERA: PYRALIDAE)

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ABSTRACT

Studies with caged gravid females of the pickleworm, [*Diaphania nitidalis* (Stoll.)], revealed that leaves of yellow squash (*Curcurbita pepo* L.) contain small (MW <1000), non-volatile and highly polar amphoteric compounds which stimulate oviposition on artificial sites. Several compounds, extracted from paper chromatograms, caused moderate stimulation of oviposition. When these extracts were recombined, the mixture proved highly active. Addition of whole leaf volatiles to the active water-soluble fraction increased oviposition. The whole leaf volatiles mixture could be substituted with volatiles originating from leaf glandular trichomes.

Index Words. Lepidoptera, *Diaphania nitidalis*, oviposition, host plant, squash, *Curcurbita pepo*, trichomes, volatiles.

RESUMEN

Estudios sobre el gusano de los pepinos [*Diaphania nitidalis* (Stoll.)] revelaron que las hojas de la calabaza (*Cucurbita pepo* L.) contienen pequeños (MW<1000) compuestos anfotéricos no volátiles y altamente polares que estimulan la ovoposición en sitios artificiales. Varios compuestos, extraidos a partir de cromatogramas en papel, estimularon moderadamente la ovoposición. Cuando esos extractos fueron recombinados, la mezcla fue muy activa. La adición de volátiles de la hoja completa a la fracción activa hidrosoluble incrementó la ovoposición. La mexcla de volátiles de la hoja completa podría ser sustituida por volátiles originados de los tricomas glandulares de las hojas.

The pickleworm, [*Diaphania nitidalis* (Stoll.)], is a severe pest of cucurbit vegetables in the southeastern United States, mainly because the larvae tunnel into the fruit. Commercially, damage tolerance is low, especially in the pickling industry (Elsey et al. 1989). Infestations in the field vary with cucurbit species as well as with cultivar (Corley 1973, Day et al. 1961, Pulliam 1979, Quisumbing & Lower 1975). Bioassays with cucumber cultivars showed that little antibiosis to larvae exists (Wehner et al. 1985). However, non-preference to oviposition was shown in glabrous mutants of muskmelon and cucumber (Day et al. 1961, Elsey & Wann 1982). Since nearly isogenic lines of glabrous and pubescent cucumbers were evaluated, it appeared that resistance (avoidance) was due to the lack of plant trichomes (Elsey & Wann, 1982). Elsey (1985) showed that resistance in *Cucurbita moschata* was due to oviposition non-preference. A study with whole plants in large field screen cages showed that pickleworm oviposition preference decreased in the order of yellow squash, cucumber and watermelon (Elsey 1981). A similar pattern was observed when ethanol extracts of whole leaves were applied to artificial oviposition sites (Elsey & McFadden 1981).

Because chemical factors were suspected to be involved in oviposition preference by pickleworms, this study was undertaken to isolate these stimulants. Leaves of straightneck yellow squash were used because this yellow squash is a preferred host (Dilbeck & Canerday 1968) and most pickleworm eggs are laid on foliage. Effects of volatile and non-volatile compounds were evaluated and attempts to isolate non-volatile stimulants are described.

MATERIALS AND METHODS

Insect Rearing

Diaphania nitidalis were raised in the laboratory according to methods described by Elsey et al. (1984).

Culture of Plant Material

Seedlings of yellow squash (*Cucurbita pepo* L. var. Early Prolific Straightneck) were planted in the field and raised using standard cultural practices. Healthy leaves, approximately one half ultimate size (10 - 12 cm longest dimension) were collected from the field early in the morning and immediately processed or used for bioassays.

Bioassays

Assays involving oviposition were conducted according to Elsey & McFadden (1981) with some modification to improve quantitative aspects. Pads of building insu-

TABLE 1. OVIPOSITION BIOASSAYS OF FILTRATES OBTAINED BY SEQUENTIAL FILTRATION THROUGH MEMBRANE MOLECULAR FILTERS.

Filter	Average Oviposition[1,2]
(MWCO)	(Percent of Total)
none	11.5 b
20 K	15.8 bc
5 K	22.1 de
1 K	26.9 e
500	19.9 cd
blank[3]	3.9 a

[1]Average of four independent bioassays.
[2]Means followed by the same letter are not significantly different. Means separation by Fisher's LSD (P = 0.05).
[3]No fraction applied.

lation glasswool (diam 9 cm, thickness 2-3 cm) were placed in petri dishes containing the solutions to be tested. After adsorption was completed, the solvent was evaporated. Subsequently, the pads were suspended from the top of wire mesh cages (120 × 90 × 90 cm), which contained about 100 pairs of 3-day-old moths. The cages were located in a humidified room held at 23 C, and a photoperiod of 16:8 (L:D). Testing material was placed in the cages in the late afternoon (before the start of the scoto period), removed the next morning and the eggs counted.

Common amino acids were tested for stimulation of oviposition by dissolving them in 50% ethanol and applying 50 μg or 200 μg per pad. The following amino acids were used: L-alanine, γ-amino butyric acid, L-arginine, L-asparagine, aspartic acid, citrulline, cysteine, L-glutamic acid, L-glutamine, glycine, histidine, DL-homocysteine, hydroxyproline, isoleucine, leucine, lysine, DL-methionine, phenylalanine, proline, serine, DL-threonine, tryptophan, L-tyrosine and DL-valine. A test consisted of four pads containing one (different) amino acid each, one pad with 0.25 g equivalent of an aqueous polyamide fraction (control, contains no volatiles), and one pad without application (blank).

Volatiles from small whole leaves (6-9 cm longest dimension) or droplets (trichome exudates) were tested by placing them between glass fiber pads. Alternatively, the fresh material was lyophilized for 72 hrs under high vacuum while the volatiles were trapped under liquid nitrogen. The leaves, now free of volatiles, were extracted and tested.

Extractions and Separations

Leaves approximately two-thirds full size [4-7 g fresh weight (FW), 14-18 cm longest dimension of leaf blade] were harvested from greenhouse grown squash plants. The leaves were lyophilized and ground to pass a 0.85 mm mesh screen. The material was subsequently extracted with hexane for approximately 12 hours on a shaker and filtered. The procedure was repeated twice and the residue was extracted with hot 95% ethanol in a homogenizer and filtered. After two repetitions, the combined ethanol extracts were condensed to a syrup, and water was added. The extract was filtered through a series of surface membrane molecular filters under nitrogen pressure in a stirring cell. The filters had a nominal molecular weight cut-off (MWCO) of 20K, 5K,

Lyophilyzed leaves
hexane extraction

Step	Fraction	Mean[1]		SEM
Residue extr with	hexane	3.5	b[2]	0.9
95% EtOH		23.5	a	6.6
condensed to aqueous water added; molecular filtration, MWCO=1000				
Filtrate		30.6	a	5.5
Partition against CH_2Cl_2	CH_2Cl_2	4.9	b	1.9
Aqueous fraction, partition against EtOAc	EtOAc	4.5	b	1.3
Aqueous		33.1	a	2.2

[1]Average percentage of total egg laying corrected for blank (oviposition on non-treated pads), and standard errors of the means.

[2]Means followed by the same letter are not significantly different. Means separation by Fisher's LSD (p = 0.05).

Figure 1. Extraction and partitioning scheme for the isolation of oviposition stimulants. All fractions were assayed simultaneously, and the assay was performed three times. Numbers in parentheses indicate the amount of oviposition as a percent of the total number of eggs deposited. Numbers were corrected for 'background', i.e. eggs deposited on pads that received no extracts.

1K and 500 respectively. Since the 500 MWCO gave rise to losses of activity (Table 1), filtrations of subsequent extracts were limited to a MWCO of 1K. Molecular filtration was followed by partitioning against dichloromethane and ethylacetate respectively. The extraction scheme is presented in Fig. 1.

Chromatography

The aqueous fraction obtained after the last partitioning was subjected to low-pressure column chromatography using polyamide as stationary phase (column dimensions: 25 × 2.6 cm diam). The polyamide was prewashed with one liter each of 0.5% formic acid and water, respectively. An extract aliquot representing up to 100 g leaf fresh weight equivalent (FWE) in 10 ml water was pumped onto the column and eluted stepwise with methanol/acetic acid (995:5), 100% methanol and water respectively. Absorbance was monitored at 280 nm. The fractions were tested for stimulation of oviposition and checked with p-anisidine phthalate to detect reducing sugars (Stahl 1969, p. 857) and with ninhydrin (Stahl 1969, p. 889) or vanillin-potassium hydroxide (Stahl 1969, p. 904) to test for primary or secondary amino functions.

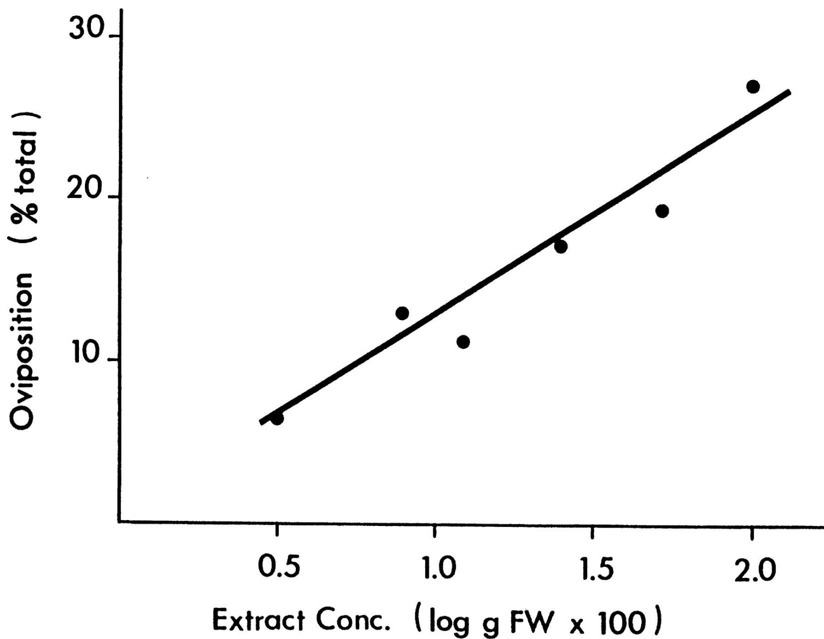


Figure 2. Dose-response curve of oviposition (% of total number eggs produced per bioassay) versus the logarithm of the amount of extract used. All concentrations were represented once in a bioassay and the entire assay was performed four times. Regression analysis: $y=1.18 + 11.86x$, $r^2=0.91$, $P<0.01$.

The active fraction from the polyamide column (water fraction) was condensed and used for paper chromatography on Whatman 3 MM sheets (46 × 57 cm). Development was in the descending mode using tert-butanol: acetic acid:water-3:1:1. After drying, the sheets were sprayed on the sides with ninhydrin and heated at 110°C. The area corresponding with the colored bands, as well as the areas in between, were extracted with 50% ethanol and bioassayed.

The active fraction from the polyamide column was also run through a Dowex 50 × 8 cation exchange column (18 cm × 1 cm diam; sample 10 g FWE). The sample was adjusted to pH 2.5 with concentrated formic acid and eluted with 8N formic acid, water, and triethylamine (in 20% aqueous acetone, pH=10). After the column had been standing for three days, more activity could be eluted with triethylamine [Table 3, $Et_3N(2)$]. The fractions were checked with ninhydrin and tested for activity.

Hydrolysis was performed on the active polyamide fraction. The reaction was conducted in a sealed vial at 110°C for 24 hrs, in 2N HC1. The products were condensed to dryness, redissolved and bioassayed. Seven such tests were performed.

Sources of Chemicals

L-glutamine, L-asparagine, L-glutamic acid, L-arginine, proline, L-tyrosine and tryptophane were obtained from Aldrich Chemical Co., Milwaukee, WI. L-alanine, aspartic acid, γ-amino butyric acid, citrulline, cysteine, glycine, histidine, DL-homocysteine, isoleucine, leucine, lysine, DL-methionine, phenylalanine, hydroxy-L-proline,

serine, DL-threonine, DL-valine and ninhydrin were obtained from Sigma Chemical Co., St. Louis, MO. p-Anisidine phthalate was obtained from Pfaltz and Bauer, Inc. Waterbury, CT. Polyamide was obtained from Universal Scientific Inc. Atlanta, GA. Hexane, dichloromethane and ethylacetate (HPLC grade) were obtained from Burdick and Jackson, Muskegon, MI. Tertiary butanol was obtained from J. T. Baker Inc., Philipsburg, NJ.

Statistical Analyses

Statistical analyses, consisting of one-way ANOVA, students t-test, and regression techniques, were performed where appropriate on a personal computer with the SOLO statistical system (BMDP Statistical Software, Inc., Los Angeles, CA). Means separations were by Fisher's LSD (P=0.05).

Results and Discussion

Preliminary experiments showed that increasing amounts of crude 95% ethanol extracts of fresh squash leaves applied to glasswool pads increased egg-laying. At approximately 1 g FWE, i.e., the amount obtained from 1 g fresh leaf material, no further increase was obtained, and at higher dosages very erratic dose-response data were obtained. A linear dose-response ($r^2 = 0.91$) was obtained when the percent of the total number of eggs laid was plotted against the logarithm of the extract concentration (Fig. 2).

The partial isolation scheme (Fig. 1) shows that the oviposition stimulants remained in the aqueous fractions. Oviposition increased considerably when fractions of low polarity were removed (Fig. 1) or when molecular filtration was performed (Table 1). Although this may suggest the presence of inhibitors, the fractions with low polarity showed activity above 'background', i.e., when applied to glasswool pads, they received more eggs than non-treated pads. In addition to the molecular filtration down to a MWCO=1000, a filter with a MWCO=500 was used; in this case a significant amount of activity was lost. When the pH of the final aqueous fraction (Fig. 1) was adjusted to either 10 or 2.5, no activity could be extracted by ethyl acetate, suggesting that oviposition stimulants were highly polar molecules.

The final active aqueous fraction obtained through partitioning (Fig. 1) was subjected to low pressure column chromatography, using polyamide as the stationary phase, and the fractions were tested for activity (Fig. 3). The first fraction (aqueous) contained virtually all of the activity. The first eluate from the polyamide column may contain salts, organic acids, sugars (Winter & Hermann 1986) and some water soluble

TABLE 2. EFFECT OF HYDROLYSIS OF THE AQUEOUS POLYAMIDE (ACTIVE) FRACTION[1] ON OVIPOSITION BY THE PICKLEWORM MOTH.

Treatment	Oviposition (% of Total)							Mean	SEM
Non-hydrolyzed	50.3	25.1	37.2	35.6	49.0	49.2	51.3	42.5 a[2]	3.8
Hydrolyzed	35.4	61.8	46.5	46.8	37.1	34.8	40.2	43.2 a	3.6
Blank (no application)	14.3	13.2	16.4	17.6	13.9	16.0	8.5	14.3 b	1.1
Total oviposition	796	1595	807	1585	2132	762	995		

[1]0.25 g FWE applied to oviposition sites.
[2]Means followed by the same letter are not significantly different. Means separation by Fisher's LSD (P = 0.05).

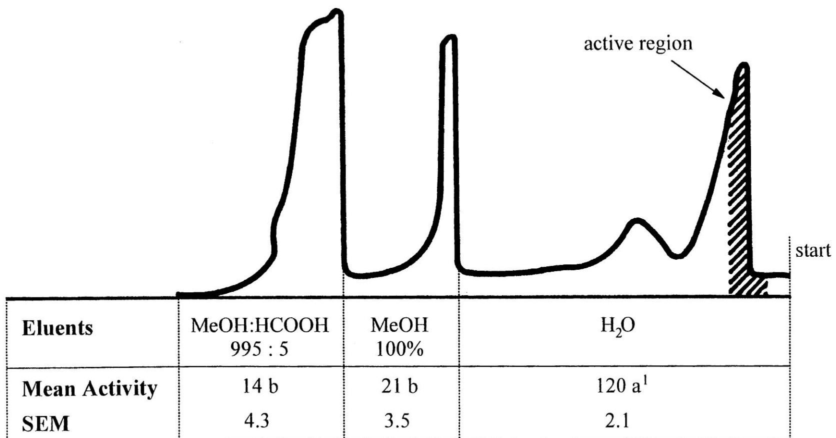
TABLE 3. CATION EXCHANGE CHROMATOGRAPHY OF ACTIVE AQUEOUS FRACTION FROM POLYAMIDE CHROMATOGRAPHY.

Eluent	Vol. (ml)	Ninhydrin Reaction	Mean Oviposition (% of Total)[1]	SEM
HCOOH (8N)	106	negative	28.52 b[3]	2.30
H_2O	86	trace	13.54 a	1.68
Et_3N (1)[2]	84	posit. (purple)	25.09 b	2.42
Et_3N (2)	92	posit. (purple)	26.21 b	1.78
Blank			6.63 a	0.43

[1]Six experiments were performed (n=6). A total of 700, 1345, 1320, 1198, 1610, and 1819 eggs were layed per experiment.
[2]Et_3N = triethylamine.
[3]Means followed by the same letter are not significantly different (Fisher's LSD, P = 0.05).

phenolic glycosides (Thieme 1964). Activity was also found in the very first part of the aqueous eluate, before any absorption at 280 nm was observed, excluding phenolic compounds. Para-anisidine phthalate gave no reaction, indicating that no reducing sugars were present (Stahl 1969). Vanillin-potassium hydroxide or ninhydrin gave positive reactions, indicating the presence of primary or secondary amino groups

Polyamide Column Chromatography
monitored at 280 nm



Eluents	MeOH:HCOOH 995 : 5	MeOH 100%	H_2O
Mean Activity	14 b	21 b	120 a[1]
SEM	4.3	3.5	2.1

Figure 3. Polyamide column chromatography of active aqueous extract (see Fig. 1), monitored at 280 nm. Activity is expressed as percent of the activity in the pre-column matrix (active solution applied to column; activity = 100%). Numbers were corrected for the blank (no application). Four independent assays were performed and data were averaged.

TABLE 4. PAPER CHROMATOGRAPHY[1] OF ACTIVE FRACTION (AQUEOUS) OF POLYAMIDE COLUMN CHROMATOGRAPHY.

		Oviposition (% Control)[3]	
Band	hRf[2]	Mean	SEM
1	0-11.1	10.7 de[4]	3.9
2	11.1-19.5	10.1 de	3.7
3	19.5-26.5	6.4 e	4.2
4	26.5-34.7	48.6 a	32.9
5	34.7-40.5	17.6 cde	2.3
6	40.5-47.9	33.9 b	11.0
7	47.9-50.8	25.3 bc	4.4
8	50.8-57.7	22.0 bcd	6.4
9	57.7-63.8	11.6 de	3.1
10	63.8-68.3	24.6 bc	12.5
11	68.3-76.2	14.9 cde	1.6
12	76.2-100	52.8 a	18.3
Recombined[5]		255	65.4
Blank[6]		15	2.6

[1]Solvent system: tert-BuOH:HOAc:H_2O-3:1:1. 4.0 g FWE of the aqueous fraction of the polyamide chromatography applied to paper sheets. Control: 0.25 g FWE of the polyamide fraction.

[2]All bands stained with ninhydrin, except number 12. hRf: relative traveling distance of compound, 0-compound did not move, 100-compound moved with solvent front.

[3]Data obtained from 4 chromatograms. Oviposition for the 12 bands and the recombined sample was expressed as follows:

$$\frac{\text{sample - blank (no applic.)} \times 100\%}{\text{control - blank}}$$

[4]Means followed by the same letter are not significantly different. Means separation by Fisher's LSD (P = 0.05).

[5]0.5 g FWE of each chromatographic band was recombined.

[6]No application of sample.

(Stahl 1969). Hydrolysis of the active aqueous fraction from the polyamide column did not change the activity significantly (Table 2). These results indicate that no active small peptides were present, or, if present, did not play a significant role.

The active fraction from the polyamide column was further separated on a cation exchange column. Activity was present in all fractions except the aqueous (Table 3), indicating acidic and basic compounds were involved in stimulation of oviposition.

Numerous paper chromatograms (PC) were developed from the active polyamide fraction. The developed papers were sprayed on the side with ninhydrin, and the entire chromatograms stained (hues of blue, purple and pink), except the front area. Twelve bands were cut, eluted and tested. Also tested were all the extracted regions recombined. An example of the PC and bioassay data, presented in Table 4, show that various regions were active, and that the recombined sample was very active. Since PC and parallel bioassays showed that active regions, with the exception of one, could be stained with ninhydrin, primary or secondary amino functions, were involved. Furthermore, ion-exchange data showed that acidic and basic compounds were involved. These compounds could not be extracted from an aqueous solution at high or low pH, indicating amphoteric characteristics of the active compounds. These properties strongly indicate amino acids. For this purpose common amino acids were tested. The

TABLE 5. INFLUENCE OF COMMON AUTHENTIC AMINO ACIDS[1] ON OVIPOSITION BY THE PICKLEWORM MOTH.

Amino Acid	Oviposition[2]	
	50 μg	200 μg
L-alanine	4.5	-2.5
γ-amino butyric acid	1.7	1.9
L-arginine	-0.3	-3.2
L-asparagine	4.8	2.3
Aspartic acid	1.1	4.4
Citrulline	4.6	1.8
Cysteine	3.7	4.0
L-glutamic acid	3.3	2.4
L-glutamine	2.0	1.3
Glycine	4.5	3.4
Histidine	2.4	4.1
DL-homocysteine	5.8	2.3
Hydroxy-L-proline	-0.4	1.7
Isoleucine	0.3	-1.4
Leucine	0.4	5.2
Lysine	5.2	3.5
DL-methionine	1.7	2.1
Phenylalanine	0.4	4.9
Proline	0.8	3.4
Serine	3.9	4.6
Threonine	-0.8	3.9
Tryptophane	8.3	3.0
L-tyrosine	2.6	1.0
DL-valine	3.5	-1.6

[1]Amino acids were tested in random order, three per test, at amounts of 50 μg and 200 μg per oviposition site.
[2]Oviposition was expressed as follows:

$$\frac{\text{sample - blank (no application)} \times 100\%}{\text{control - blank}}$$

where the extract used was 0.25 g FW squash leaf equivalent, obtained by extraction with 95% ethanol. Total eggs layed per experiment ranged from 1341 to 3778.

highest response was 8.3% of control (Table 5), all other responses were considerably lower, sometimes slightly negative. These responses were far below those obtained by extracts of the PC bands (Table 4), where six bands caused increases in excess of 20% of the control and the highest response was 52.8%. It therefore seems likely that some of the non-protein amino acids, which are ubiquitous in the cucurbit family (Noe & Fowden 1960, Fang et al. 1961, Dunnill & Fowden 1965) are responsible for stimulation of oviposition. Since these particular non-protein amino acids seem to be limited to the cucurbits, a particular composition with respect to identity and relative concentrations could explain pickleworm host plant specificity.

TABLE 6. INFLUENCE OF 'WHOLE LEAF' VOLATILES[1] ON OVIPOSITION ON ARTIFICIAL SITES TREATED WITH SQUASH LEAF EXTRACTS.[2]

Treatment	Oviposition (% Total)					Mean	SEM
Extr. without volatiles	38.4	37.9	45.4	42.8	34.4	39.8 a[3]	1.9
Extr. with volatiles	59.3	54.7	53.6	46.6	49.8	52.8 b	2.2
Blank (no application)	2.4	7.4	1.0	10.6	15.8	7.4 c	2.7
Total oviposition	4687	686	3415	1985	2210		

[1]A small whole leaf sandwiched between glass fiber pads; bottom of leaf facing treated side.
[2]Extracts free of volatiles; 0.25 FWE of aqueous polyamide fraction.
[3]Means followed by the same letter are not significantly different. Means separation by Fisher's LSD (P = 0.05).

TABLE 7. INFLUENCE OF LEAF GLANDULAR TRICHOME EXUDATES[1] ON OVIPOSITION ON ARTIFICIAL SITES TREATED WITH SQUASH LEAF EXTRACTS.[2]

Treatment	Oviposition (% Total)					Mean	SEM
Ext. without volatiles	40.8	40.9	45.7	42.9	43.1	42.7 a[3]	0.89
Ext. without volatiles, With trichome droplets	56.4	56.0	52.3	54.8	54.3	54.8 b	0.72
Blank (no application)	2.8	3.1	2.0	2.3	2.7	2.6 c	0.20
Total oviposition	5231	6840	9283	7230	4663		

[1]Approximately 200 droplets (trichome exudates) applied between two layers of glass fiber pads.
[2]Extracts free of volatiles; 0.25 g FWE of aqueous polyamide fraction.
[3]Means followed by the same letter are not significantly different. Means separation by Fisher's LSD (P = 0.05).

Volatiles originating from whole leaves increased oviposition significantly (Table 6). A similar increase was obtained when 'whole leaf' volatiles were substituted by volatiles originating from droplets produced by leaf trichome glands (Table 7). Subsequent research (Peterson, et al. 1994) showed that these volatiles primarily cause increased attraction rather than stimulation of oviposition. Small, highly polar and non-volatile compounds serve as oviposition stimulants. Most, if not all of these compounds carry a charge, are amphoteric, and most react positively for primary or secondary amino functions. The active compounds were stable and hydrolysis did not decrease activity.

ENDNOTE

Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Dep. Agric. and does not imply its approval to the exclusion of other products that also may be suitable.

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