Influence of Cucurbitacins, Sugars, and Fatty Acids on Cucurbit Susceptibility to Spotted Cucumber Beetle[1,2]

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Abstract. Seedlings of 18 plant types from 5 genera of Cucurbitaceae were analyzed for cucurbitacins, total sugars, and fatty acids and these were correlated with spotted cucumber beetle feeding. The species were: *Citrullus colocynthis* L. (Schrad.), *C. lanatus* (Mansf.) *Matsumara: Cucumis anguria* L., *C. dipsaceus* Ehrenb., *C ficifolius* Bouche'*C. longipes* Hook f., *C. melo* L., *C. myricarpus* Naud., *C. prophetarum* L.: *Cucurbita foetidissima* H. B. K., *C. pepo* L.; *Lagenaria siceraria* (Mol.) Stamdl.; *Luffa acutangula* (L.) Roxb., and *L. cylindrica* Roem. Seedling injury ranged from 0 (none) to 3 (severe). Regression analysis indicated that cucurbitacins, total sugars, and the fatty acids (palmitic and linolenic) except linoleic contributed to insect feeding; correlation between concn and feeding was positive. Cucurbitacins, which cucumber beetles were able to locate without feeding, played the major role in seedling susceptibility; next in importance were palmitic acid, linolenic acid, and total sugars. All apparently related to the preference of beetles for specific strains or cultivars. In non-preferred seedlings, preference was induced by topical application of cucurbitacins A, B, C, D, E, and 1 and by the glycosides from *C. foetidissimma* roots. A barrier seemed to obstruct feeding of the beetles on the upper surface of the cotyledons, even when the attractant cucurbitacins were applied.

Differential insect-feeding responses in plant families have been observed and recorded; few researchers, however, have attempted to analyze the factors involved (18). Kennedy and co-workers (15) proposed the theory of dual discrimination which states that host selection is based on the insect's response to two types of stimuli: (a) flavor stimuli from such botanically specific biochemicals as cucurbitacins amd (b) nutrient stimuli apparently by feeding stimulants and deterrents which may or may not constitute required nutrients.

Cotyledons of 18 plant types from 5 genera of Cucurbitaceae were analyzed for certain biochemical constituents (some nutritional and some non-nutritional based on insect response) and the results were correlated with seedling resistance to spotted cucumber beetle. We intended that data provided by our research would link previous extensive studies by Hall and co-workers (10, 17, 27) on identification of distinct crop to crop differences and would also substantiate the key role of cucurbitacins in host resistance or susceptibility reported by Chambliss and Jones (3,4). A new technique of using non-preferred plant material for testing attractance of cucurbitacins is introduced in our study.

Materials and Methods

This study was conducted in Manhattan, Kansas, during 1969 and 1970. Plant materials acquired from 2 Regional Plant Introduction Centers (Experiment, Georgia; Ames, Iowa) and from individual sources, include: 2 species of *Citrullus:* 7 species of *Cucumis;* 2 species of *Cucurbita*; 1 species of *Lagenaria*; and 2 species of *Luffa* (Table 1).

In preliminary experiments during June, 1969. to determine the number of days required for each species to germinate, we found that seedlings germinated more uniformly in the greenhouse than in the field.

Seedlings for the major experiment begun in August 1969 were started in the greenhouse in quartz sand to minimize nutrient x resistance interaction (18). Forty seedlings of each species were grown 10 in a flat. Seeding was scheduled so that different species reached full cotyledon expansion the same day. Flats were then transferred to a screen cage (12 ft x 18 ft) in the field. In both greenhouse and cage, flats were arranged in a randomized complete block design with 4 replications. Field-collected spotted cucumber beetles were released in the cage at a ratio of one beetle per seedling. Beetles were allowed to feed for 44 hr, then cotyledon injury was rated visually on a numerical scale (Fig. 1) (27).

Concurrently, a duplicate planting of seedlings of each species listed in Table 1 was made in the greenhouse. When seedlings had reached the same growth stage as those exposed to cucumber beetles, a 5 g fresh wt composite sample of cotyledons was collected from each flat and immediately frozen for biochemical analysis.

Extraction procedure. A clarified ethanolic extract of 5 g of cotyledons, obtained by a procedure modified from Enslin (6) and Rehm and Enslin (22) was shaken twice with 20 ml of petroleum ether (bp 60° - 80°C); the free fatty acids were separated in the ether layer. The ethanol fraction was shaken twice with 20-ml of chloroform. The chloroform fraction was washed with equal amounts of distilled and deionized water, dried over sodium sulfate, and kept for determination of curcurbitacins. The remaining ethanol extract and the wash

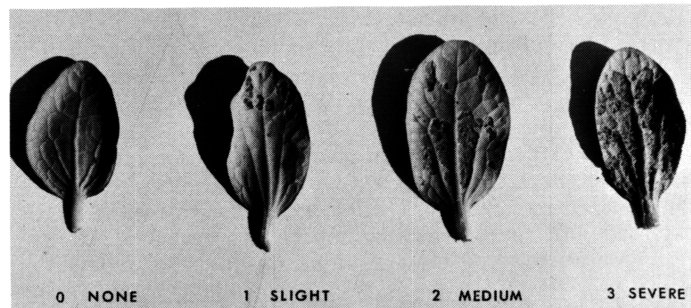

Fig. 1. Rating scale for evaluating cotyledon injury. 0 = none; 1 = slight; 2 = medium; 3 = severe injury. Scale established by Wiseman et al. (27).

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[1]*Diabrotica undecimpunctata howardi* Barber. Coleoptera: Chrysomelidae.

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[4]From dissertation prepared in partial fulfillment of the Ph.D. degree: Role of Cucurbitacin and Some Genetic and Biochemical Aspects of Insect Resistance Cucurbitaceae. 1970. 170 p. Present address: Department of Soil and Plant Science, Alabama A and M University, Normal.

Table 1. Differences in the means for spotted cucumber beetle injury, cucurbitacins, total sugars, and the fatty acids (palmitic, linoleic, and linolenic) for the species studied.[a,b]

Species	P.I. no. & country	Beetle injury[c]	Cucurbitacins mg/g	Total sugar ug/g	Palmitic acid mg/g	Linoleic acid ug/g	Linolenic acid ug/g
CITRULLUS							
C. colocynthis	195927 Ethiopia	2.6 c	0.27 d	3.8 c	4.1 de	5.6 g	27.1 d
C. lanatus	248 774 Africa	2.4 d	0.24 e	4.4 b	4.4 d	5.2 h	21.4 ef
C. lanatus	306367 Angola	1.7 f	0.18 e	3.3 de	2.9 f	9.7 d	24.7 de
CUCUMIS							
C. anguria	196477 Brazil	0.5 ij	0.10 k	5.2 a	5.3 c	3.5 k	41.3 c
C. dipsaceus	193498 Ethiopia	2.4 a	0.22 f	1.6 ghi	2.1 f	5.4 gh	9.9 h
C. ficifolius	233646 Ethiopia	0.4 j	0.10 k	3.0 de	4.4 d	3.9 j	41.0 c
C. longipes	249897 Africa	2.2 e	0.28 f	2.4 f	4.2 de	2.4 l	28.5 d
C. melo	164328 India	1.4 g	0.15 h	0.9 j	2.5 f	2.5 l	25.3 de
C. myriocarpus	282449 S. Africa	2.8 b	0.31 c	1.1 ij	3.7 de	2.6 l	25.0 de
C. prophertarum	193967 Ethiopia	1.8 f	0.16 h	1.6 gh	2.6 f	9.2 e	24.7 de
CUCURBITA							
C. foetidissimma "Wild Gourd"	Kansas U. S. A.	3.0 a	0.49 a	3.4 d	8.7 a	14.0 b	49.7 b
C. pepo ovifera	197671 Mexico	0.5 ij	0.11 k	0.9 j	5.7 c	4.5 i	19.6 f
C. pepo ovifera	232072 S. Africa	0.6 i	0.13 j	1.8 g	3.4 e	4.2 ij	46.2 b
C. pepo cv. Black Zucchini		3.0 a	0.31 c	1.5 ghi	5.2 c	6.2 f	12.7 gh
C. pepo cv. Early Golden Bush Scallop		0.0 l	0.08 l	2.4 f	7.0 b	11.7 c	17.1 fg
LAGENARIA							
L. sicereria	India	1.2 h	0.13 i	2.9 e	4.8 c	9.7 d	24.7 de
LUFFA							
L. acutangula	246931 India	3.0 a	0.42 b	1.3 hij	4.5 d	20.5 a	56.3 a
L. cylindrica	174878 India	0.2 k	0.10 kl	1.2 hij	3.4 e	5.0 h	24.8 de

[a]Quantities expressed are on fresh wt basis.

[b]Numbers in each column followed by the same letter are not significantly different from each other at 5% level.

[c]Injury rating: 0 = none; 1 = slight; 2 = moderate; and 3 = severe.

were pooled for analysis of total sugars.

Quantitative determination of cucurbitacins was achieved by ultraviolet (uv) spectroscopy (on a Cary Recording Spectrophotometer at 230 mμ in spectrograde methanol). A standard curve was obtained from 10 readings for each of 5, 10, 20, and 40 μg/ml concentration of pure cucurbitacin B (provided by P. R. Enslin, Pretoria, South Africa). Total sugars were determined by using Walborg's (26) aniline/acetic, orthophosphoric acid procedure.

Gas-liquid chromatography was employed for quantitative estimation of palmitic (16:0), linoleic(18:2), and linolenic (18:3) acids. A methylation procedure was developed, using BF_3-methanol reagent (Suppelco, Inc.) Petroleum ether extract equivalent to 1/2 g (fresh wt) of plant tissue was transferred to a glass vial with a polyethlene stopper. The solvent was evaporated under N and 1/2 ml of benzene and 2 ml of BF_3-methanol reagent were added. Different temp and time combinations were attempted. Methylation by heating the extract with BF_3-methanol in such vials yielded low quantities or esters; methylation at room temp did not and was satisfactory. Fifteen hr was found to be the optimum reaction time (Table 2). Distilled water (2 ml) was added to stop methylation. Methyl esters were separated in 5 ml of hexane

Table 2. Methylation of 3 fatty acids[a] in the petroleum-ether fractions of 'Early Golden Bush Scallop' seedling extracts at different time lengths at room temp.

Time	Palmitic	Linoleic	Linolenic
1 hour	3.21	5.61	11.62
3 hours	3.92	6.61	12.36
7 hours	4.70	8.46	14.27
9 hours	5.59	9.64	15.26
15 hours	6.50	10.48	16.57
24 hours	6.42	10.43	16.6

[a]Calculated on peak-area basis; ug/g fresh wt reported.

and dried over sodium sulfate. The solvent was driven out and esters were reconstituted with exactly 0.2 ml of hexane and injected in a Barber-Coleman gas chromatograph (series 5000). Operating conditions were: Column: 9' diethleneglycol succinate (7.5%) on chromosorb G.A.W. DMCS, 70-80 mesh, U-shaped, glass, 9' long 1/4" diam, temp 190°C, isostatic, carrier gas (N) flow 80 ml/min. Detector and injector: flame ionization detector, hydrogen 80 ml/min, detector 230°C; injector 222°C.

Fatty acids were characterized by the equivalent chain length procedure (11), which required plotting carbon numbers of known saturated fatty acids against retention time (Fig. 2). Dr. W. E. Klopfenstein, Department of Biochemistry, synthesized and provided the haneicosanoic acid (21:0) used as an internal standard. That fatty acid does not occur in plants. The peak area (height x width at 1/2 height) was calculated for the three fatty acids. Typical separation of three fatty acids is shown in Fig. 3.

Host susceptibility to the spotted cucumber beetle was artifically induced in seedlings of non-preferred 'Early Golden Bush Scallop' (EGBS). Solutions containing 0.05% (low) and 0.1% (high) pure cucurbitacins (supplied by Dr. P. R. Enslin, South Africa and Dr. David Lavie, Israel) were topically applied to the upper and lower sides of the fully expanded EGBS cotyledons. In addition, glycosides from *C. foetidissimma* root extracted according to the methods described (6) were also applied. Five seedlings replicated 4 times for each treatment (Table 5) were placed in a 12' x 18' cage and the spotted cucumber beetles were released at the rate of 4 beetles per seedling. The experiment was terminated 34 hr after the release of the beetles and insect injury was rated (Fig. 1.).

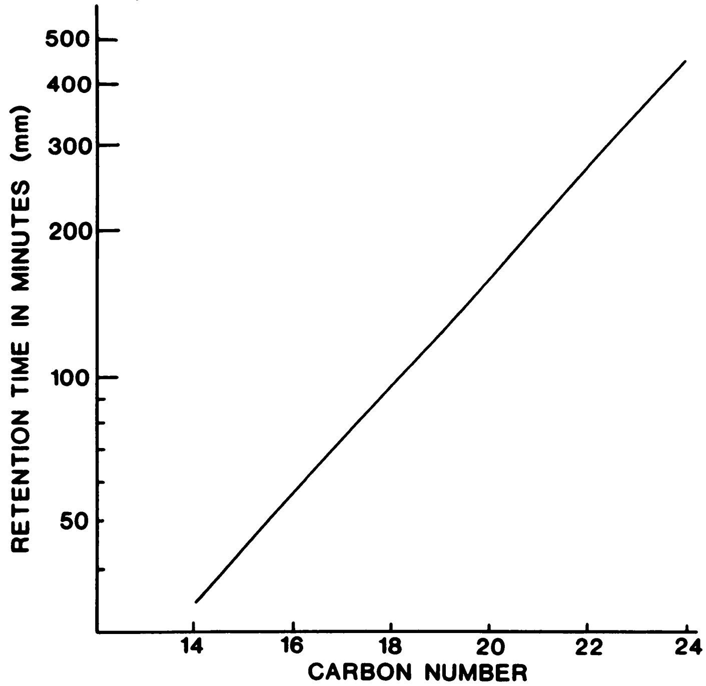

Fig. 2. Equivalent chain-length graph for diethyl glycol succinate column, showing relationship of retention time and carbon numbers of saturated straight-chain esters of fatty acids.

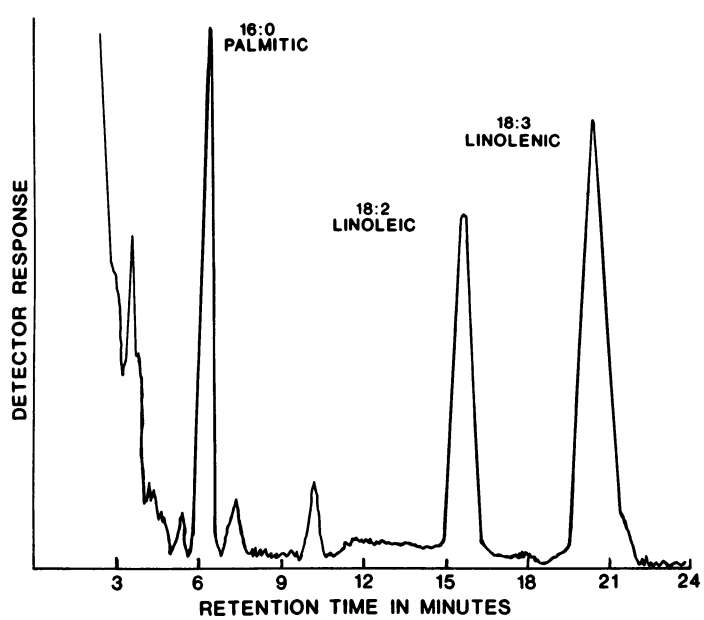

Fig. 3. Gas chromatogram showing typical separation of palmitic, linoleic, and linolenic acids on a 9' diethyl glycol succinate column.

Results

Means for insect injury, cucurbitacins, total sugars, palmitic acid, linoleic acid, and linolenic acid for various species are given in Table 1. Equality of means was tested by Duncan's New Multiple Range Test (5). Two-way analysis of variance showed that differences were highly significant among species (treatments) but nonsignificant among blocks. Distinct differences in insect injury were observed, e.g. *L. cylindrica* seedlings were injured only slightly; those of *L. acutangula* were destroyed (Fig. 4).

To compare unique associations between two variables, a simple correlation analysis was run. As shown in Table 3, cucurbitacins were positively correlated with palmitic, linoleic, and linolenic acids. Cucurbitacins and total sugars showed a very low level of correlation. Total sugars were positively related to palmitic and linolenic, but negatively to linoleic acid. All three fatty acids correlated positively with each other, indicating that their content in the species considered was either low or high. The highest correlation existed between cucurbitacins and insect injury. But that relationship was more clearly shown in a multiple regression analysis.

The coefficients of determination for independent variables are shown in Table 4. Cucurbitacins, total sugars, and palmitic and linolenic acids contributed significantly to cotyledon injury (dependent variable); linoleic acid showed nonsignificant correlation. Hence, the nonsignificant variable was deleted and the 4 independent variable regression equation was used to predict spotted cucumber beetle injury to the cotyledons of a species:

Cucumber beetle injury = 9.86 + 10.169 cucurbitacins (mg/g) - 0.213 x palmitic acid (μg/g) - 0.024 x linolenic acid (μg/g) + 0.1190 x total sugars (mg/g).

Results shown in Table 4, indicated that the greatest contribution to R^2 was made by cucurbitacins (78.7%); palmitic and linolenic acid contributed approximately 10% and 6%, respecively, toward spotted cucumber-beetle injury. Total sugars, the lowest of significant factors, contributed less than 2%.

Treatment means for artificially induced beetle injury are reported in Table 5. Analysis of variance and orthogonal comparisons showed that highly significant differences existed between the solvent- and cucurbitacin-treated seedlings; and between low (0.05%) and high (0.1%) concns of cucurbitacins.

Discussion

Cucurbitacins were more important in cotyledon susceptibility to spotted cucumber beetles than four other biochemical substances. In our study, species or varieties with more than 0.2 mg/g fresh wt of cucurbitacins were susceptible to beetle injury and received an injury rating of 2 or 3. We propose that in areas where the cucumber beetle is a serious problem, cucurbitaceous crops should be checked for cucurbitacin level to predict susceptibility to cucumber beetle.

Rehm and Enslin reported variations in cucurbitacin concn (20, 21). We also observed intraspecific variations in cucurbitacin content and thus in cucumber beetle injury. 'Black Zucchini' was high in cucurbitacin and susceptible whereas, 'Early Golden Bush Scallop' (both *C. pepo*) was low and resistant to *C. pepo.* Within genera, similar differences were evident in *C. foetidissimma* and *C. pepo ovifera* and between *C. dipsaceus* and *C. anguria.*

Studies show that young seedlings of most species contain primary cucurbitacins B or E in their cotyledons (22). In some cases, however, a small amount of cucurbitacin D with B, and I with E may also be formed particularly if the growth of the seedling is advanced (20, 22). With the help of thin layer chromatography we determined that the species we studied contained only cucurbitacins B or E at the stage when cotyledons were harvested. In order to use *uv* spectroscopy as a routine technique for large samples, 230 mμ wave length was selected to determine cucurbitacin B or E in the cotyledons. In a recent study it was reported that both cucurbitacins B or E follow Beer's law at 230 mμ (16). The measurement of plant extracts containing several cucurbitacins at one wavelength in the maximum absorption regions, 229-234 mμ, (except for cucurbitacins J, K and L, which have absorption maxima around 270 mμ) may result in over- or under-estimation of some cucurbitacins. Nevertheless, *uv* spectroscopy is probably the most sensitive technique for routine analysis of a large number of samples containing cucurbitacins.

Fig. 4. Cucumber beetle feeding on *Luffa cylindrica* (left) and *L. acutanqula* (right).

Table 3. Simple correlation coefficients for seedlings of several species analyzed for 6 variables.

	1 Cucurbitacin	2 Total sugar	3 Palmitic acid	4 Linoleic acid	5 Linolenic acid
1. Cucurbitacin					
2. Total sugar	0.01				
3. Palmitic acid	0.32**	0.31**			
4. Linoleic acid	0.50**	-0.01	0.37**		
5. Linolenic acid	0.34**	0.22*	0.28*	0.40**	
6. Insect injury	0.88**	0.01	0.01	0.28*	0.19

* and ** indicate significance at the 5% and 1% levels, respectively.
Error degrees of freedom = 72.

Table 4. Multiple regression analysis showing contribution of independent variables to R^2 and their significance.[a]

Independent variables	Contribution to R^2	df	F
Cucurbitacin	0.787	16	59.14**
Palmitic acid	0.098	15	12.73**
Linolenic acid	0.059	14	15.11**
Total sugar	0.017	13	5.84*
Linoleic acid	0.002	12	0.63

[a]Cucumber beetle injury was the dependent variable.
* and ** indicate significance at 5% and 1% levels, respectively.

Topical application of high and low concns of cucurbitacins resulted in an injury rating averaging above 2 on a 0 – 3 rating scale. Cucurbitacins D and I which lack the acetyl group in the side chain showed attractance to spotted cucumber beetle at slightly lower levels than cucurbitacins B and E. As shown, the use of non-preferred plant material as EGBS can be suitable in testing biochemicals (e. g., cucurbitacins) for their possible role in host preference.

It was intriguing to observe that even when cucurbitacins were applied to both sides of cotyledons spotted cucumber beetles fed only on the lower sides. This may imply that there is a natural barrier which overrides the strong attraction of cucurbitacins and therefore obstructs or repels the beetles from feeding on the upper sides of the cotyledons. Further work in this and in the area of topical application to induce host susceptibility should provide a greater insight into insect plant relationships.

The regression equation reported above is based on our work with cotyledons containing mainly primary cucurbitacins. In cases where a mixture of cucurbitacins occur, qualitative differences in relative attactance of cucurbitacins as reported by Chambliss and Jones (4) and above should be considered while formulating such a relationship.

The contribution of palmitic, linoleic, and linolenic acid and total sugar composition of cotyledons to host susceptibility can be explained thus: (a) Some or all are a nutritional necessity to the cucumber beetle. (b) Some, although essential in the strict sense, may act as "feeding stimulants." (c) The green plant is a complex biochemical system, and the relationship here observed between each of the 4 biochemicals and injury could be due partly to factors not studied in this experiment. Besides being a chemical stimulus, a plant also provides the physical aspect of diet and microenvironment to the pest. What was measured here as insect injury could have been partly the result of those factors.

Except for cucurbitacins, all of the compounds studied have been shown to play a nutritional role for some insect (9, 12, 13). Albritton (1) has given data on sugar utilization for 8 species of phytophagous insects. In one study, European corn borer *Pyrausta nubilallis* (Hubn.) larvae, highly sensitive to differences in sugar concn, selected the highest concn offered (2). Although most insects probably synthesize all the required fatty acids, a few species are known to depend on other sources for certain unsaturated fatty acids (13). Rate of growth in *Agria affinis* has been promoted by palmitic acid (14). Omitting fat from the diet of *Pectinophora gossypiella* caused some of the insects to die early in the pupal stage. Adding 80 mg of linoleic acid per 100 g of diet permitted normal development (25). In a study on sugarbeet webworm, sterile females did not contain fatty acids with more than one double bond (19).

We could not conclude that total sugar and fatty acids were a dietary necessity for spotted cucumber beetle, because little has been done on dietary requirement of this insect. Fraenkel has suggested (8), "A peculiar feature of history of this subject is the fact that of more than 1 million described insect species, only 20 or 30 have so far been used with any success in nutritional investigations. The nutrition of other types of insects is largely an unexplored field. These include especially important groups of leaf eaters, etc."

Table 5. Treatment means for artificially induced spotted cucumber beetle injury by topical application of pure cucurbitacins to cotyledons of "Early Golden Bush Scallop" (EGBS).

Treatments	Mean insect injury rating[1]
EGBS	0.40
EGBS + solvent[2]	0.50
EGBS + 0.05% A cucurbitacin	2.35
EGBS + 0.10% A	2.90
EGBS + 0.05% B	2.40
EGBS + 0.10% B	2.95
EGBS + 0.05% C	2.10
EGBS + 0.10% C	2.95
EGBS + 0.05% D	2.30
EGBS + 0.10% D	2.85
EGBS + 0.05% E	2.50
EGBS + 0.10% E	3.00
EGBS + 0.05% I	2.25
EGBS + 0.10% I	2.80
EGBS + 0.05% glycosides	2.70
EGBS + 0.10% glycosides	3.00

[1]Insect injury rating scale: 0 = none, 1 = slight, 2 = medium, and 3 = severe insect injury. Mean score is from 4 replications with 5 seedlings each.

[2]Solvent, 70% ethanol.

Fraenkel (7) has proposed that host specificity and resistance are based entirely on 'odd' or 'secondary biochemicals' in the plant tissue. It is generally known, however, that simple carbohydrates (sugars), amino acids, fatty acids, organic acids, and various other metabolic products occur in plants in the free and dynamic form. Some of these nutrients in plants act as gustatory indicators of a suitable food substrate to an insect. Thus, there is little reason that we should not class such nutrients (free metabolites) as token stimulants (24). The role of free forms of metabolic products, or 'sapid' nutrients as feeding stimulants is also supported by Thorsteinson (23). This may explain the minor role played by palmitic and linolenic acids and total sugars in host preference of spotted cucumber beetle.

This study revealed that cucurbitacins, but not the other nutrients discussed here play the major role in host susceptibility to spotted cucumber beetle. Where feeding was observed, beetle population was relatively heavy, cotyledons of species containing a very low concn of cucurbitacins were completely devoid of feeding marks. That suggested either that cucurbitacin had a characteristic odor, which the insect recognized, or that physical contact with the plant was required. But no feeding seemed necessary to locate this compound precisely. Generally insects will not feed on resistant seedlings until the susceptible cotyledons have been consumed. In our study 12 resistant seedlings in the cage were exposed to hundreds of mature cucumber beetles. Very few visited the seedlings, and then fed minimally; others slowly starved. However, when the same number of susceptible seedlings high in cucurbitacins were introduced, the beetles recognized them immediately and the cotyledons were entirely consumed in a day.

Selecting cucurbitaceous plants low in cucurbitacins, and thus achieving resistance by non-preference can reduce the serious damage caused by the insect to seedlings of such commercial crops as watermelon, cucumber, gourd, squash, and many staple–diet vegetables and fruits in tropical as well as in temperate regions throughout the world.

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