Journal of Chemical Ecology, Vol. 12, No. 5, 1986

COEVOLUTIONARY ADAPTATIONS OF ROOTWORM BEETLES (COLEOPTERA: CHRYSOMELIDAE) TO CUCURBITACINS

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(Received June 26, 1985; accepted October 15, 1985)

Abstract—The cucurbitacins are oxygenated tetracyclic triterpenoids produced as secondary plant compounds by nearly all genera of Cucurbitaceae. The very bitter and toxic cucurbitacins are effective semiochemicals acting ecologically as allomones to protect the Cucurbitaceae from attack by a variety of invertebrate and vertebrate herbivores. For the Luperini (Coleoptera: Chrysomelidae: Galerucinae) the cucurbitacins have become kairomones for host selection, affecting the behavior of this large group of 1500 species of Aulacophorina (Old World) and Diabroticina (New World) by arrest and compulsive feeding. When feeding on bitter cucurbits these beetles sequester large amounts of cucurbitacins in their blood and tissues, and these act as allomones to deter predation. Specific detoxification and excretory mechanisms of the Diabroticina enable these beetles to avoid the toxic effects of the cucurbitacins.

Key Words—Rootworm beetle, *Diabrotica* spp., *Aulacophora* spp., Coleoptera, Chrysomelidae, *Cucurbita* spp., *Cucumis* spp., cucurbitacin, allomone, kairomone, Chinese mantis, *Tenodera aridifolia sinensis*, predator, Orthoptera, Mantidae.

INTRODUCTION

Coevolutionary adaptations between terrestrial plants and insect herbivores must have originated in the Carboniferous Period about 300 million years (BP) when land plants were diversifying and insects had begun to evolve from primitive segmented ancestors (Riek, 1970; Taylor, 1982). By the Permian Period 270 million years BP insects had segregated into modern orders, and the first fossil records of insect-damaged leaves are recorded. The first flowering plants oc-

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curred in the Triassic Period about 225 million years BP (Smart and Hughes, 1973).

Over this vast stretch of evolutionary time, many of the estimated 100,000 secondary plant compounds appeared in response to evolutionary pressures for protection of plants from herbivore attack. Semiochemical communication has become the dominant force in coevolutionary relationships between plants and animals, by allomones that benefit the producer, kairomones that benefit the receiver, and synomones where both producer and receiver benefit. Fraenkel (1969) stated the profundity of the case for host plant selection by herbivores: "evidence for the importance of secondary plant substances in host selection is so overwhelming as to need no further proof."

The roles of the cucurbitacins of Cucurbitaceae as allomones and kairomones regulating herbivore attack provide a compelling example of the ecological and behavioral effects of secondary plant compounds (Chambliss and Jones, 1966; Sharma and Hall, 1973, Metcalf et al., 1980). The oxygenated tetracylic triterpenoid cucurbitacins (Cucs) are extremely bitter and toxic substances characteristic of the Cucurbitaceae (Metcalf et al., 1980). An appropriate scenario for the evolutionary and behavioral interactions between Cucurbitaceae and herbivores can be portrayed as follows (DaCosta and Jones, 1971a; Price, 1975; Metcalf, 1979): (1) ancestral Cucurbitaceae with *bi bi* genes for Cuc synthesis are heavily preyed upon by herbivores; (2) mutation in Cucurbitaceae to *Bi bi* forms bitter and toxic Cuc that deters herbivore attack; (3) strong selection pressure spreads *Bi bi* genes throughout evolving Cucurbitaceae species; (4) mutant Cucurbitaceae flourish in absence of herbivore attacks; (5) mutant ancestral Luperini rootworm beetles develop detoxification and excretion pathways to neutralize harmful effects of Cucs; (6) Luperini beetles expand into new ecological niches developing specific receptors for Cuc detection; and (7) Luperini beetles develop high blood and tissue levels of Cuc conjugates for defense against predators.

CUCURBITACINS AS ALLOMONES FOR CUCURBITACEAE

There is no ambiguity about the role of the Cucs as protective semiochemicals of the Cucurbitaceae and allied families against the attacks of herbivores. This is immediately apparent to anyone who has tasted a bitter squash, cucumber, or melon. The Cucs are the bitterest substances yet identified and can be detected by humans at dilutions as great as 1 ppb (Metcalf et al., 1980). The merest trace produces an almost paralytic response in lips and mouth and a persistent aftertaste. Moreover, the Cucs are extremely toxic to mammals with LD_{50} values to mice intraperitoneally of Cuc A, 1.2; Cuc B, 1.1; and Cuc C, 6.8 mg/kg (David and Vallance, 1955); and orally of Cuc I, 5 and Cuc E glycoside, 40 mg/kg (Stroesand et al., 1985).

Mice fed freeze-dried *Cucurbita texana* fruit at 10–20% of the diet died in 3–6 days. When fed this fruit at 1%, 40% died within 10 weeks, and the survivors had severe diarrhea and anemia (Stroesand et al., 1985). Sheep and cattle that consumed bitter fruit of wild *Cucumis leptodermus*, *C. africanus* and *C. myricarpus*, under drought conditions in South Africa were severely poisoned (Watt and Breyer-Brandwyk, 1962). Twenty-two cases of human poisoning from eating *C. pepo* (zucchini) fruits that contained about 1.1 mg Cuc E glycoside per gram occurred in Queensland, Australia, in 1982 (Ferguson et al., 1983a; Herrington, 1983). Symptoms included severe cramps, persistent diarrhea, and collapse occurring within a few hours after eating the zucchini fruits which were apparently mutant reversions to the *Bi* heterozygote (Rhymal et al., 1984).

Allomonal effects of Cucs have also been demonstrated on invertebrate herbivores including red spider mites (Tetranychidae) and the stem borer *Margaronia hyalinate* (DaCosta and Jones, 1971a). The presence of Cucs E and I has been shown to provide feeding deterrents to numerous insect herbivores including the leaf beetles *Phyllotreta nemorum*, *P. undulata*, *P. tetrastigma*, *Phaeodon cochliariae*, and *P. cruciferae* (Nielson et al., 1977). Cuc B applied to bean leaves prevented the feeding of the bean leaf beetle *Ceratoma trifurcata* (Metcalf et al., 1980).

Chemical Identity of Cucurbitacins. Approximately 20 chemically different Cucs have been characterized from plants (Lavie and Glotter, 1971; Guha and Sen, 1975) (Figure 1). Cuc B is the predominant form found in about 91% of all species characterized, followed by Cuc D (69%), Cucs G and H (47%), Cuc E (42%), Cuc I (22%), Cucs J and H (9%), and Cuc A (7%). Cucs C, F, and L were each found in only a single species (2%) (Rehm et al., 1957). It appears that the two primary Cucs are Cuc B and Cuc E and that the other Cucs are formed by enzymatic processes occurring during plant development and matu-

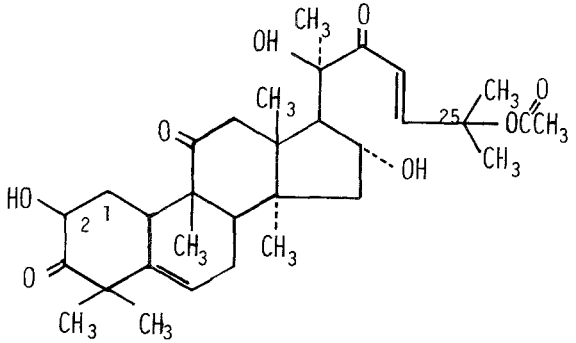

FIG. 1. Structure of cucurbitacin B. Cuc D is C_{25}—OH; Cuc E is C_1=C_2; Cuc I is C_1=C_2, C_{25}—OH; Cuc F is C_2—OH, C_3—OH, C_{25}—OH; Cuc G is C_{24}—OH, C_{25}—OH; Cuc L is C_1=C_2; C_{23}—C_{24}, C_{25}—OH.

ration. Cuc B can be metabolized to Cucs A, C, D, F, G, and H and is characteristic of *Coccinia*, *Cucumis*, *Lagenaria*, and *Trichomeria* (Rehm et al., 1957). Similarily Cuc E can be metabolized to Cucs I, J, K, and L and is characteristic of *Citrullus*. *Cucurbita* contains two groups of species characterized by either Cuc B or Cuc E (Metcalf et al., 1982). Cuc B is converted to Cuc E by Cuc Δ^1 reductase that produces the diosphenol grouping $C_1{=}C_2$, and Cuc Δ^{23} reductase converts the $C_{23}{=}C_{24}$ Cuc B and E series to the dihydro Cucs. Cucs B and E are converted to Cucs D and I by desacetylation by Cuc acetyl esterase (Schwartz et al. 1964, Schabort and Teijema 1968, Lavie and Glotter, 1971).

The Cucs in *Cucumis*, *Lagenaria*, and *Acanthosicyos* are present as free aglycones. However, in most species of *Citrullus* examined, the Cucs are present as glycosides (Rehm et al., 1957). In *Cucurbita* Cucs are present as aglycones in most species but glycosides are found in *C. cylindrata*, *C. foetidissima*, *C. palmata*, and *C. texana* (Metcalf et al., 1982). The presence of glycosides is related to the absence of β-glucosidase (elaterase) which may also be sequestered in intact plant tissues and released by crushing (Enslin et al., 1956).

Although the multiplicity of Cucs and their variable distribution in the Cucurbitaceae presents a complex situation for analysis, certain generalizations are valid: (1) Cucs B and E are the parent substances found most widely and the other Cucs, generally present in much lower quantities, are degradative products. (2) Cuc D is always associated with Cuc B and Cuc I is always associated with Cuc E. (3) Cucs G and H are always associated with Cucs B and D. (4) Cucs J and K are always associated with Cucs E and I. (5) Cuc A is always associated with Cuc B. (6) Cuc C is singular and occurs alone in *Cucumis sativus* (Rehm et al., 1957; Ferguson et al., 1983b). (7) Only Cucs B, C, and E sometimes occur alone.

Distribution of Cucurbitacins in Plants. Cucurbitacins, as the name suggests, are peculiarly associated with the family Cucurbitaceae where they have been characterized in at least 30 genera and more than 100 species (Rehm et al., 1957; Lavie and Glotter, 1971; Pohlmann, 1975). Cucs are also found in a few genera of the related plant families Begoniaceae, Brassicaceae (Cruciferae), and Datisceae, all of the superorder Violoflorae (Thorne, 1981). For example, Cucs have been identified in 16 species of *Iberis* (Brassicaceae) (Lavie and Glotter, 1971; Curtis and Meade, 1971). Cucs have also been identified in a few species of Euphorbiaceae, Scrophulariaceae and Rosaceae (Dryer and Trousdale, 1978).

Cuc synthesis in *Cucumis*, *Citrullus*, *Cucurbita*, and *Lagenaria* is initiated by a single dominant gene *Bi*. Nonbitter fruit may develop from bitter seedlings in the presence of a modifier gene *Su Bi* (Robinson et al., 1976). It is probable that a few major genes control the chemical nature of the Cuc formed and Cuc B is dominant over Cuc E (Ferguson et al., 1983b).

Cucs are found in all parts of the plant in the Cucurbitaceae: roots, stems, leaves, fruit, and occasionally in the seeds.

Roots. The concentration in the roots increases with age and can reach very high levels in perennial plants, e.g., 1.4% in *Citrullus naudinianus*, 1.1% in *Acanthosicyos horrida*, and 0.9% in *Colocynthis ecirrhosa* (Rehm, 1960). In 18 species of *Cucurbita*, Cucs B–D were detected in the roots of seven, up to 0.43% in *C. ecuadorensis*, and Cucs E–I were identified in six up to 0.38% in *C. palmata* (Metcalf et al., 1982).

Leaves. Although Rehm (1960) indicates that the leaves of Cucurbitaceae seldom contain Cucs even when the roots have very high concentrations, this generalization has many exceptions. Young rapidly growing leaves of *Colocynthis* (*Citrullus*) *vulgaris* and *C. ecirrhosa* have only about 0.01% Cucs, but this reaches 0.1–0.3% by the end of the vegetative season. Of 18 species of *Cucurbita* examined, Cucs B–D were found in seven species up to 0.059% in *C. lundelliana*, and Cucs E–I in six species up to 0.1% in *C. okeechobeensis* (Metcalf et al., 1982).

Fruits. The fruits of a variety of Cucurbitaceae contain high amounts of Cucs. Rehm et al., (1957) found Cuc concentrations of >0.1% in the fruits of *Citrullus colocynthis* and *C. ecirrhosis* (Cuc E), *Cucumis angolensis* and *C. longyies* (Cuc D), *C. myriocarpus* (Cuc A), and *C. sativus* (Cuc C). Of 18 species of *Cucurbita* examined, the fruits of seven contained Cucs B–D up to 0.31% in *C. andreana*, and the fruits of five contained Cucs E–I up to 0.23% in *C. foetidissima* (Metcalf et al., 1982).

Seeds. Cucs are not commonly found in the seeds of Cucurbitaceae. Rehm et al. (1957) reported that three of 45 species had bitter seeds, presumably from Cucs present in the surrounding tissue.

CUCURBITACINS AS KAIROMONES FOR ROOTWORM BEETLES

The ancestral association between the leaf beetles of the tribe Luperini (Coleoptera: Chrysomelidae: Galerucinae) and the plants of the family Cucurbitaceae seems to have been effected through the presence of the cucurbitacins acting secondarily as kairomone cues for host selection by the beetles. The scope of this association is presently worldwide. The Luperini is comprised of two very similar but geographically isolated subtribes, the Diabroticina of the New World, containing about 993 species all evidently radiating from tropical America, and the Aulacophorina of the Old World, containing about 535 species apparently radiating from tropical Australasia (Maulik, 1936; Wilcox, 1972). The Aulacophorina are presently all restricted to Asia except *Aulacophora foveicollis* whose range extends to Europe and *A. africanus* extending into Africa. Maulik (1936) cites the remarkable similarity between these two

large groups of phytophagous beetles: "In the Old World, Aulacophora represents Diabrotica . . . In larval, pupal and adult structures, in breeding habits and in food plants, there is a remarkable resemblance between the two genera." The present distribution of the two subtribes of the Luperini is shown in Metcalf (1985).

At least 50 species of Luperini, representing more than 80% of the available host records, have been recorded as feeding on Cucurbitaceae (Wilcox, 1972; Takizawa, 1978; Metcalf, 1985). The common names of notable pest species attest to the significance of this relationship:

Diabroticina (New World): *Diabrotica balteata*, the banded cucumber beetle; *D.* (*Paranapiacaba*) *connexa*, the saddled cucumber beetle; *D. picticornis*, the painted cucumber beetle; *D. speciosa*, the cucurbit beetle; *D. u. howardi*, the spotted cucumber beetle; and *A. vittatum*, the striped cucumber beetle.

Aulacophorina (Old World): *Aulacophora abdominalis*, plain pumpkin beetle; *A. femoralis*, cucurbit leaf beetle; *A. foveicollis*, red pumpkin beetle; and *A. hilaris*, pumpkin beetle.

The role of cucurbitacins in host selection by Luperini is exemplified by two recent studies relating Cuc content of *Cucurbita* cotyledons to damage by rootworm beetles, rated on a five-point scale. For *A. foveicollis* attacking *Cucurbita moschata* cultivars there was a substantial correlation between Cuc content and feeding damage, $r = 0.62$, $N = 32$, SD = 0.14[2], and it was concluded that "low cucurbitacin content appeared to impart resistance" (Pal et al., 1978). In a similar study of *D. u. howardi* and *A. vittatum* attacking *Cucurbita pepo* cultivars, the correlation between beetle damage and Cuc content was, $r = 0.77$, $N = 12$, SD = 0.20, and it was concluded that there was a "strong positive correlation between seedling cucurbitacin content and Diabroticina beetle attacks" (Ferguson et al., 1983b). Similar correlations were found between the average numbers of Diabroticina beetles feeding on crumpled leaves or sliced fruits of various *Cucurbita* spp. and their total Cuc content (Metcalf et al., 1982): with *D. u. howardi*, leaves $r = 0.74$, $N = 16$, SD = 0.20; fruits $r = 0.70$, $N = 11$, SD = 0.24. With *D. v. virgifera*, the correlations were leaves $r = 0.64$, $N = 16$, SD = 0.24; and fruits $r = 0.58$, $N = 11$, SD = 0.27.

The systematic relationships between Aulacophorina and Diabroticina and their common responses to Cucs indicate a common ancestral relationship with a Cucurbitaceae progenitor during a geologic period no later than the Miocene ca. 30 million years BP when continental land bridges were still present (Metcalf, 1979). This argument is supported by the presence of fossil *Diabrotica* spp. in Florisant shales of Colorado, ca. 25–30 million years BP (Wickham, 1914). Isozyme studies of the Diabroticina have produced evidence of the divergence of *Acalymma* from *Diabrotica* at about 2.7 genetic distances or approximately 45 million years BP (unpublished data).

[2] r = correlation coefficient, N = number of observations.

TABLE 1. LIMIT OF RESPONSE (LR) OF DIABROTICINA BEETLES TO PURE CUCURBITACINS ON SILICA GEL PLATES

Species	Cuc LR (μg)							
	B	D	E	F	G	I	L	E-gly
D. balteata	0.01			10	3	5		0.1
D. l. barberi	0.1		0.3					5
D. cristata	0.1	1.0	0.3	>10	3		>1.0	50
D. u. howardi	0.001	0.03	0.01	1.0	3.0	0.1	0.01	0.05
D. u. undecimpunctata	0.003		0.03					
D. v. virgifera	0.01	0.1	0.3	0.1	3	0.3	1.0	0.03
A. vittatum	0.3		10					50

Sensory Receptors of Diabroticina Beetles for Cucurbitacins. The high degree of sensitivity to the Cucs and the specificity of the behavioral responses of arrest and compulsive feeding induced by proximity to Cucs demonstrate that specific receptor organs are present in the beetles. Both sexes of *D. u. howardi*, *D. v. virgifera*, and *A. vittatum* responded to pure Cucs with identical arrest and feeding behavior. Amputation of the antennae of *D.u. howardi* beetles had little effect on the feeding response to Cucs but amputation of the maxillary palpi abolished arrest and feeding (Metcalf et al., 1980). Scanning electron microscopy of the maxillary palpi of the several species of Diabroticina studied (Table 1) has demonstrated *sensilla basiconica* arranged around the tip of the maxillary palpus that seem likely to contain the Cuc receptor. Detailed study of the comparative morphology in the several species is in progress (J.R. Larsen, in preparation).

Diabroticina Beetle Sensitivity to Cucurbitacins. Field observations of the mass attacks of various Diabroticina beetles on cotyledons, leaves, and fruits of Cucurbitaceae suggest that the Cucs present are effective at low concentrations in producing arrest and compulsive feeding of adult beetles.

Qualitatively, all the species of Diabroticina investigated (see Table 1) will feed to some degree on pure crystalline Cucs A, B, C, D, E, F, G, I, and L placed on filter paper or silica gel thin-layer chromatography (TLC) plates (Chambliss and Jones, 1966; Metcalf et al., 1980). The various beetle species produced identical spectra of feeding patterns on TLC plates developed from chloroform extracts of *Cucurbita andreana* (Cucs B–D), *C. okeechobeensis* (Cucs E–I), or *C. texana* (Cuc E glycoside) (Metcalf et al., 1982). These "beetle prints" (Figure 1) are valuable in characterizing the distribution of Cucs in the Cucurbitaceae. When the TLC plates developed from plant extracts were presented simultaneously, Cuc B was always fed upon before Cuc E and Cuc D before Cuc I, indicating the greater beetle sensitivity to the Cuc B–D compounds (Table 1). The beetle bioassay used in conjunction with TLC to separate

the individual Cucs is both qualitative and semiquantitative as the areas eaten from the TLC plates are proportional to the amounts of Cucs present.

Quantitatively, there are substantial differences in the threshold amount of the various chemically purified Cucs that produces a detectable feeding response and different species of beetles show different responses to the individual Cucs (Table 1). This quantity is the limit of response (LR) and is measured on silica gel TLC plates exposed to about 100 Diabroticina beetles for four days. The LR represents the relative degree of complementarity of the various Cucs to the maxillary palpi receptors of the Diabroticina (Metcalf et al., 1980). *D. u. howardi* and *D. u. undecimpunctata* beetles consistently responded to 1–3 ng of Cuc B under these standard conditions as shown in Table 1. This beetle bioassay is therefore about 250× more sensitive than HPLC (Ferguson et al., 1983b) and 1000× more sensitive than UV spectrophotometry (Metcalf et al., 1982).

Table 1 also shows that there are major differences in the LR values for the various Diabroticina beetles to the individual Cucs. These differences have evolutionary and behavioral significance. The conclusions that can be drawn from the data in Table 1 are: (1) Cuc B was consistently detected in the lowest amount and is probably the parent Cuc to which Diabroticina receptors are attuned, as it was found in 91% of the species characterized. (2) Cuc B was consistently detected at levels of about 0.1 that of Cuc E. (3) The acetoxy Cucs B and E were detected at levels about 0.1 those of the corresponding desacetoxy Cucs D and I, respectively. (4) Saturation of the desacetoxy Cucs at $C_{23}=C_{24}$ double bond (Cuc L) had little effect on level of detection. (5) Sensitivity to the 2-OH, 3-C=O Cuc D was greater than to the 2-OH, 3-OH Cuc F. (6) The *D. undecimpunctata* subgroup (*D. balteata*, *D.u.u.*, *D.u.h.*) is more sensitive to Cucs than the *D. virgifera* subgroup (*D.l. barberi*, *D. cristata*, and *D.v. virgifera*). (7) *A. vittatum* is substantially less sensitive to Cucs than the *Diabrotica* spp.

The range of variations in maxillary receptor sensitivity to the various Cucs (Table 1) is relatively consistent for all the species examined. It appears that Cuc B has maximum complementarity to the Cuc receptor. Receptor depolarization most probably results from allosteric changes in the conformation of the receptor protein resulting from interactions of the free paired electrons (dipoles) associated with the several oxygen atoms of the Cuc molecules (Metcalf et al., 1980). The structural change from Cuc B to Cuc E by introduction of a single C=C bond at C_1-C_2 in ring A (Figure 1) seems trivial, yet this change produces a 10-fold decrease in receptor affinity (Table 1). The introduction of the C=C bond into ring A changes the orientation of the three contiguous O atoms (C_3=O, C_2—OH, and C_{11}=O) from a staggered configuration on the cyclohexyl moiety of Cuc B to a planar configuration in Cuc E. From observations of molecular models, this change seems ample to decrease receptor affinity and depolarization. Cucs D and I exhibit about a 10-fold decrease in receptor affinity

as compared to the C_{25} acetoxy derivatives B and E (Figure 1). This suggests that the acetoxy C=O dipole must also be involved in complete binding to the receptor.

CUCURBITACINS AS ALLOMONES FOR DIABROTICINA BEETLES

The incredibly bitter taste of Cucs and their toxic effects on vertebrates after ingestion suggest a further role in the behavioral ecology of the Diabroticina beetles, i.e., as protective allomones against predators. Thus the ingestion of Cucs by Diabroticina species feeding on bitter Cucurbitaceae host plants may provide protection against birds and predaceous vertebrates, as first suggested by Howe et al. (1976).

Storage and Sequestration of Cucs. Diabroticina beetles can concentrate and sequester relatively large amounts of Cucs in free and derivatized form. A single elytron of *D. undecimpunctata* or *D. balteata* fed on bitter *Cucurbita andreana* × *C. maxima* cultivar (Rhodes et al., 1980), containing 1–3 mg Cuc B–D per gram fresh weight, had an extremely bitter taste and the numbing effect on lips and tongue was perceptible 2 hr later (personal observation).

Groups of *D. balteata* adults were fed on bitter *C. andreana* × *C. maxima* fruit for various periods of time and the Cuc content was measured in the hemolymph by UV spectrophotometry after isolation by TLC. The presence of a Cuc conjugate (R_f 0.4 in chloroform–methanol 1 : 1) was readily detected in 1 μl or less of insect hemolymph following TLC, after one and two days of feeding of the adult beetles on bitter squash (Ferguson et al., 1985). The concentration of the Cuc conjugate rapidly increased in the hemolymph of *D. balteata* and reached an apparent plateau level after 30–40 days when blood levels were about 30 μg/μl (3%). Similar blood measurements on *D. u. howardi* adults collected from bitter squash plants indicated hemolymph levels of Cucs of 20–26 μg/μl. This Cuc content is equivalent to about 15 mg Cucs per gram of body weight. No Cucs were detectable in *D. balteata* adults reared on pollen food (Ferguson et al., 1985).

Adverse Effect of Cucurbitacin Sequestration upon Predators. The Chinese mantis *Tenodera aridifolia sinensis* is an omnivorous insect predator and has been shown to acquire learned aversion to cardiac glycosides sequestered in the milkweed bug, *Oncopeltus fasciatus* (Berenbaum and Miliczky, 1984). Paired experiments where mantids were offered first Diabroticina beetles reared on pollen and then beetles reared on bitter squash fruit showed that 72% of *D. balteata*, 46% of *D. u. howardi*, and 24% of *D. v. virgifera* fed on bitter fruits were rejected. The results were highly significant ($P < 0.001$–$P < 0.05$) (Ferguson and Metcalf, 1985). No mantid ever rejected a beetle fed on pollen, but the rejection reaction of the Cuc-containing beetles typically consisted of the

mantid violently flinging away the beetle after a single bite on an elytron. Approximately 70% of the rejected beetles survived such encounters. The predator was obviously disturbed by the Cucs and underwent a period of excessive grooming, unsteadiness, and/or regurgitation. In some instances the mantis would fall from its perch and, after holding the bitter prey away from its body, would taste it one or two more times prior to discarding it.

Although the Chinese mantis consistently rejected *D. balteata*, *D. u. howardi*, and *D. virgifera* adult beetles fed on bitter squash in favor of those fed on pollen, the striped cucumber beetle, *A. vittatum*, was an exception, in that there was no significant difference in rejection rate between beetles fed pollen and those fed bitter squash (Ferguson and Metcalf, 1985). However, larvae of the *A. vittatum* beetles were reared on the roots of *Cucurbita maxima* (blue hubbard squash). The roots of this species contain relatively large amounts of Cucs, and taste and beetle bioassays showed the presence of large amounts of Cucs in both the pollen-fed and bitter-squash-fed adults. Thus, the larvae feeding on the bitter roots sequestered Cucs that were transferred to the adult beetles during metamorphosis. The larval sequestration of Cucs was effective and persistent, and *D. u. howardi* adult beetles fed as larvae on bitter *Cucurbita* and then placed as adults on a pollen diet for three months were still rejected by the Chinese mantid. Bioassay by TLC and beetle feeding demonstrated that the hemolymph of these beetles free from exposure to Cucs for six weeks still contained quantities of Cucs comparable to those fed continuously on bitter fruit (Ferguson and Metcalf, 1985).

The feeding deterrent effect of pure Cuc B was evaluated by topical application in acetone of 14 μg to the elytra of pollen fed beetles devoid of Cucs. Such treated beetles were consistently rejected by the Chinese mantid (Ferguson and Metcalf, 1985).

The protective effect of Cucs against predation of Diabroticina species appears to extend to larvae and to eggs. The larvae of several species reared on roots of bitter squash had hemolymph that was distinctly bitter. Females of *D. u. howardi*, *D. balteata*, and *A. vittatum* laid eggs that contained substantial quantities of Cucs as detected by beetle bioassay (Ferguson et al., 1985) (Figure 2). These "bitter" eggs could be effective in discouraging ant egg predators such as by *Solenopsis geminata* and *Pheidole* spp. (Risch, 1981).

Metabolism of Cucurbitacins by Diabroticina. The ability of Diabroticina beetles to grow, develop, and reproduce on host bitter *Cucurbita* containing as much as 0.32% fresh weight of Cucs (Metcalf et al., 1982) demonstrates the evolutionary development of effective metabolic mechanisms for disposition of these toxic triterpenoids. For example, groups of 25 *D. u. howardi* or *D. v. virgifera* adults completely consumed 1 mg of Cuc B in 72 hr without any perceptible ill effect. These insects weighed about 20 and 10 mg, respectively, so that the oral LD_{50} values for Cuc B are $>>2000$ mg/kg (Metcalf et al., 1980).

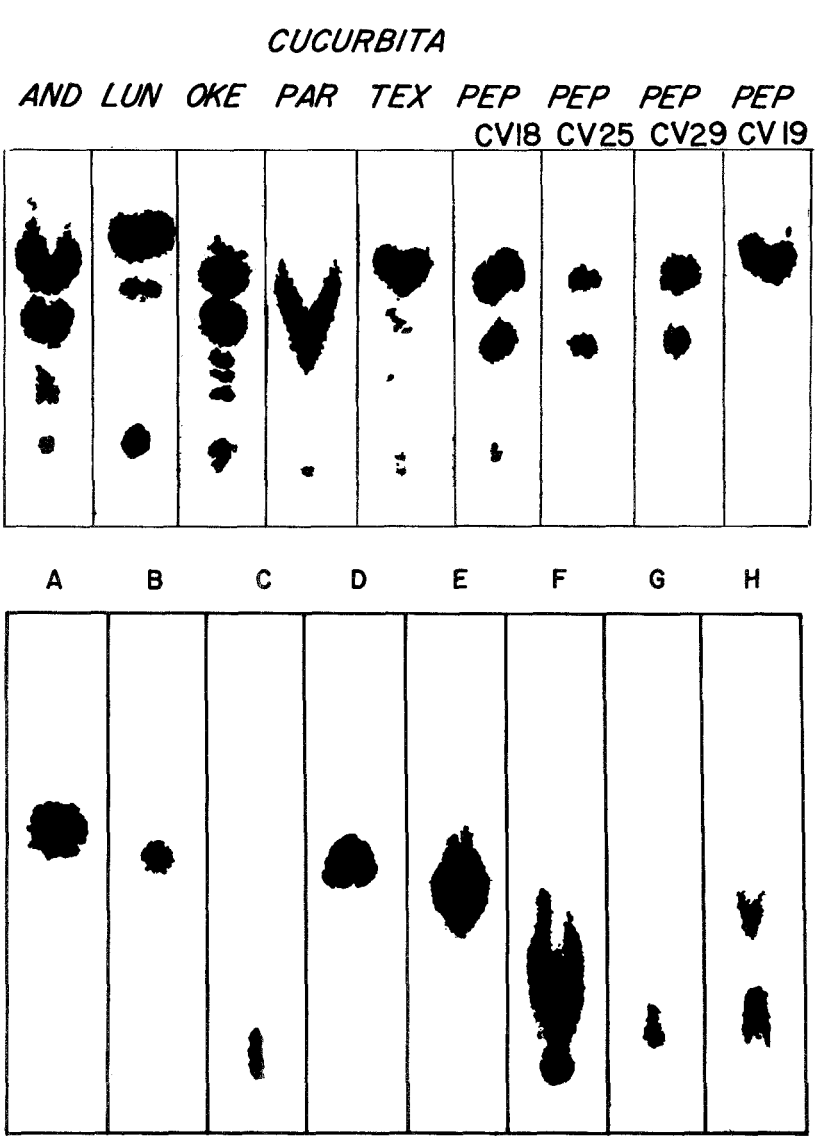

FIG. 2. Profiles of thin-layer chromatograms showing areas eaten from silica gel plates, "beetle prints," by feeding of adult *Diabrotica u. howardi*. Top: chloroform extracts of cotyledons of *Cucurbita* spp. and cultivars. AND = *andreana*, LUN = *lundelliana*, OKE = *okeechobeensis*, PAR = *palmeri*, TEX = *texana*, PEP = *pepo* cv. Ambassadore, Diplomat, Greenbay, and Black, respectively. (Ferguson et al., 1983b). Bottom: A, B = Cuc B and D standards, C = 0.75 μl of hemolymph from field collected *D. u. howardi*, D. = same hemolymph treated with pectinase, E = chloroform extract of excreta from *D. u. howardi*, F = chlorofrom extract of the body of *D. u. howardi* with no exposure to Cucs for six weeks, G = chloroform extract of 200 eggs from *D. u. howardi* adults fed on Cucs, and H = extract of 400 eggs of *A. vittatum* exposed to Cucs only as larvae (Ferguson et al., 1985).

[^{14}C]Cuc B was synthesized from DL-[2-^{14}C]mevalonate in seedlings of *C. maxima* and purified by preparative TLC to specific activity of 2.5 μCi/m mol. Approximately 1 mg of the [^{14}C]Cuc B was fed to groups of 20 one- to two-week-old Diabroticina beetles over a period of 48 hr. The disposition of ^{14}C-labeled products was examined in excreta, hemolympyh, gut, and body remainder after extraction and TLC. The total amounts of ^{14}C label excreted ranged from 67.2% for *A. vittatum* to 94.6% for *D. balteata*. Cuc B was identified in the excreta ranging from 1.7% in *D. u. howardi* to 30.0% in *D. v. virgifera*, but only traces of Cuc D were found, suggesting that deacetylation is not a major step in the formation of excretory products. Most of the ^{14}C was excreted in all five species of Diabroticina as three polar metabolites, R_f 0.0, 0.15, and 0.23 in TLC with chloroform–methanol 95:5. These polar metabolites comprised from 46.8% of the total excreted ^{14}C in *D. v. virgifera* to 91.0% in *D. balteata*. The major excretory metabolite in all five species, R_f 0.0, was a Cuc conjugate that comprised from 24.7% of the total excreted ^{14}C in *A. vittatum* to 43.8% in *D. cristata* (Ferguson et al., 1985). A small proportion of a Cuc-conjugate was permanently sequestered in the hemolymph, from 0.98 to 2.76% of the total ^{14}C. The remainder of the ^{14}C was retained, largely as conjugates, in the body and gut. In the five species examined, there were markedly different ratios of unmetabolized Cucs/polar metabolites, probably related to the present-day dietary habits of the beetles.

When *D. u. howardi* were fed ^{14}C Cucs B–D followed by two weeks on an artificial pollen diet, 93.9% of the total ^{14}C was excreted, but the hemolymph retained 1.12% of the total ^{14}C and 1058 eggs collected contained 0.21% (Ferguson et al., 1985). The characteristic polar metabolite of the hemolymph in all five species of beetle fed Cuc B had R_f 0.40 upon TLC in chloroform–methanol 1:1. This metabolite was further purified by HPLC (Ferguson et al., 1985), a fraction with retention time (RT) of 4.26 min eliciting strong beetle feeding on TLC plates (Figure 2). This Cuc conjugate was incubated overnight with pectinase to free the cucurbitacin which was extracted with chloroform. The cucurbitacin had an R_f 0.46 upon TLC with chloroform–methanol 95:5, and a RT on HPLC of 5.60 min, identical to that of Cuc D. Mass spectrometry by electron impact produced a fragmentation pattern identical to Cuc D (Audier and Das 1966). When beetles were fed *Cucurbita okeechobeensis* fruit containing Cuc E, or *C. texana* fruit containing Cuc E glycoside (Metcalf et al., 1982), hemolymph conjugates were formed that released Cuc I (R_f 0.52, blue-violet color with $FeCl_3$) after treatment with pectinase. Pure Cucs B and E, when incubated with pectinase, were not deacetylated to Cucs D and I as demonstrated by TLC. Thus, the deacetylation to produce the hemolymph conjugates was occurring in vivo in the beetles (Ferguson et al., 1985).

Consumption of Bitter Cucurbita and Diabroticina Longevity. Paired experiments were conducted where 50 newly emerged beetles were fed ad libitum on uniformly sized pieces of either bitter *C. andreana* × *C. maxima* F_1 hybrid

squash fruit containing 1–2 mg/g of Cuc B–D or on *Cucurbita* cultivars, *C. pepo* or *C. moschata*, devoid of Cucs (Ferguson et al., 1985). The total Cuc consumption was estimated over the lifetime of the beetles as 1–2 mg per beetle for *D. balteata*, 1.7–3.4 mg for *D. virgifera*, and 0.8–1.6 mg for *A. vittatum*. Beetle mortality was recorded daily, and the weekly mortality pattern and mean longevities for each species were determined (Ferguson et al., 1985). The lifespans of the adult beetles are remarkably long, up to 250 days. However, exclusive feeding on Cuc-containing fruit significantly decreased the mean longevity of *D. virgifera* from 126 days to 59 days ($P < 0.001$) and of *D. balteata* from 129 days to 70 days ($P < 0.001$). With *A. vittatum*, there was no significant difference in the longevity between the beetles fed on sweet fruits, mean 136 days, and those fed on bitter fruit, mean 122 days.

There were significant differences in the accumulated mortalities of male versus female beetles: the longevity of male *D. virgifera* was reduced 147% by feeding on bitter fruit as compared with only 39% for the female. With *A. vittatum*, feeding on bitter fruit decreased the male longevity by 27% ($P = 0.018$) but had no effect on the female longevity (Ferguson et al. 1985).

These effects on longevity can be interpreted in terms of the stress produced over the insects' lifetimes for energy requirements for metabolism, excretion, and tissue storage of the Cucs. These costs appear to be greatest in *D. virgifera*, whose normal life-style as a Poaceae feeder does not expose it to Cucs. The stress factor is less for *D. balteata* that commonly feeds on Cucurbitaceae and was barely perceptible for *A. vittatum* that is monophagous on Cucurbitaceae.

Incorporation of Cuc D into a pollen diet at 0.5 mg/g increased male food consumption substantially over that of females: *D. balteata* male 36%, female 3 %; *D. howardi* male 25%, female 1%; *D. virgifera* male 15%, female 9%; and *A. vittatum* male 24%, female 7% (Ferguson, 1985). This suggests that the male beetles in the longevity experiments may have consumed more Cucs, thus reducing their survival rate.

COEVOLUTIONARY IMPLICATIONS OF DIABROTICINA AND CUCURBITACEAE ASSOCIATION

The original coevolutionary strategy of the Cucurbitaceae to synthesize the intensely bitter and toxic Cucs as allomones to restrict herbivory has been very successful, as major herbivores are relatively few. This evolutionary strategy has produced three major variations in the structures of these Cuc allomones, (1) C_1-C_2 unsaturation, (2) acetylation at C_{23}, and (3) glucosylation.

A comparison of Cuc content of *Cucurbita* spp. with phyllogeny as constructed from genetic compatibilities and numerical taxonomy (Rhodes et al., 1968) suggests that the primitive form of Cucs was *B–D* and that Cucs *E–I* and the glycosides appeared as secondary modifications. These two modifications

each evolved at least twice independently in *Cucurbita*, i.e., $C_1 = C_2$ (E, I) in *C. martinezii* and *C. palmata* subgroups, and formation of glycosides in *C. texana*, *C. palmata*, and *C. cylindrata* (Metcalf et al., 1980, 1982).

All species of Diabroticina examined have specific Cuc receptors on the maxillary palpi that are structurally attuned to Cuc B. The presence of trace quantities of Cuc B elicits arrest and compulsive feeding in these Diabroticina species whether they are monophagous on Cucurbitaceae as *A. vittatum*, oligophagous on Cucurbitaceae as *D. balteata*, polyphagous as *D. u. howardi*, oligophagous on Poaceae as *D. l. barberi* and *D. v. virgifera*, or monophagous on *Andropogon* as *D. cristata*.

Stimulation of the Cuc receptors in Diabroticina beetles produces behavioral responses of arrest and compulsive feeding that are so fundamental as to override even the male response to the female sex pheromones, e.g., in *D. u. howardi* (Hummel and Andersen, 1982).

All species of Diabroticina examined have well-developed detoxification and storage mechanisms to partially metabolize, conjugate, and concentrate Cucs. Thus they appear to be immune to the general repellent and acutely toxic effects of Cucs. (Ferguson et al., 1985).

Major natural enemies of the Diabroticina appear to be few, and judged from the incredibly bitter taste of beetles fed on bitter *Cucurbita* and their repellent effect to a mantid predator (Ferguson et al., 1985), the coevolutionary strategy of the Diabroticina to conjugate Cucs and concentrate these bitter products in the hemolymph provides a successful survival mechanism.

Acknowledgments—The research reviewed was supported in part by Competitive Grants 5901-0410-8-0067-0 and 59-2171-1-1-6590 from the USDA, SEA Grants Office. Any opinions, findings, and conclusions or recommendations are those of the authors and do not necessarily reflect the view of USDA.

It is a pleasure to acknowledge the invaluable contributions of my coworkers: A.M. Rhodes, R.A. Metcalf, E.R. Metcalf, J.E. Ferguson, D.C. Fischer, J.F. Andersen, R.L. Lampman, Po-Yung Lu, Shen-yang Chang, and Wayne Howe. Samples of pure cucurbitacins were kindly supplied by D. Lavie, and P.R. Enslin.

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