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Phytoecdysteroids as antifeedants towards several beetles that include polyphagous and monophagous feeding guilds

Russell A. Jurenka
Iowa State University, rjurenka@iastate.edu

Kathryn Russell
Iowa State University

Matthew E. O'Neal
Iowa State University, oneal@iastate.edu

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Abstract

BACKGROUND

Plants are thought to produce ecdysteroids as a means of protection from insect herbivores. Some insects will not feed on plants containing high amounts of phytoecdysteroids, and this response could be limited to monophagous and oligophagous insects. The aim of this study was to determine whether phytoecdysteroids could inhibit feeding in several species of beetles that range from monophagous to polyphagous.

RESULTS

Here we demonstrate that phytoecdysteroids, including 20-hydroxyecdysone, prevent several beetle species from feeding on preferred host plants, including the polyphagous Japanese beetle *Popillia japonica* (Scarabaeidae). Phytoecdysteroids prevented feeding damage when sprayed onto soybean plants in no-choice and choice assays in a dose-dependent manner. Laboratory assays indicate that other plants could be protected from Japanese beetle herbivory, including linden, wild grape, elm, Virginia creeper and rose leaves. Additional beetle species tested in the family Chrysomelidae included the oligophagous *Cerotoma trifurcata* and *Diabrotica virgifera virgifera* and the monophagous *Trirhabda canadensis*. All species were prevented from feeding when their preferred host plants were treated with phytoecdysteroids.

CONCLUSION

This study demonstrates that beetles, representing polyphagous and monophagous feeding guilds, can be prevented from feeding when phytoecdysteroids are applied to the leaf surface. The phytoecdysteroids could be utilized in pest management towards a variety of beetles, including the more pestiferous polyphagous species, if the compounds are placed on the leaf surface. © 2016 Society of Chemical Industry

Disciplines

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Comments

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Phytoecdysteroids as antifeedants towards several beetles that include polyphagous and monophagous feeding guilds.

Russell Jurenka,* Kathryn Russell, Matthew O'Neal
Department of Entomology, Iowa State University, Ames, Iowa 50011
*Corresponding author: Russell Jurenka, rjurenka@iastate.edu, 515-294-1485

Abstract:
BACKGROUND: Plants are thought to produce ecdysteroids as a means of protection from insect herbivores. Some insects will not feed on plants containing high amounts of phytoecdysteroids and this response could be limited to monophagous and oligophagous insects. The aim of this study was to determine if phytoecdysteroids could inhibit feeding in several species of beetles that range from monophagous to polyphagous.

RESULTS: Here we demonstrate that phytoecdysteroids, including 20-hydroxyecdysone, prevents several beetle species from feeding on preferred host plants, including the polyphagous Japanese beetle, *Papillia japonica* (Scarabaeidae). Phytoecdysteroids prevented feeding damage when sprayed onto soybean plants in no-choice and choice assays in a dose dependent manner. Laboratory assays indicate that other plants could be protected from Japanese beetle herbivory including linden, wild grape, elm, Virginia creeper, and rose leaves. Additional beetle species tested in the family Chrysomelidae included the oligophagous *Cerotoma trifurcata* and *Diabrotica virgifera virgifera*, and the monophagous *Trirhabda canadensis*. All species were prevented from feeding when their preferred host plants were treated with phytoecdysteroids.

CONCLUSION: This study demonstrates that beetles, representing polyphagous and monophagous feeding guilds, can be prevented from feeding when phytoecdysteroids are applied to the leaf surface. The phytoecdysteroids could be utilized in pest management toward a variety of beetles including the more pestiferous polyphagous species if the compounds are placed on the leaf surface.

1 Introduction

Phytoecdysteroids are plant produced secondary metabolites that have similar structures to the insect-derived ecdysteroids.[1] The most common is 20-hydroxyecdysone that is the same structure as the true molting hormone of most insects. Plants may produce phytoecdysteroids as a defensive mechanism to prevent feeding by phytophagous insects. In this regard antifeedant properties of some phytoecdysteroids have been demonstrated against a few Lepidoptera larvae [2] and a fungus gnat, *Bradysia impatiens* (Diptera: Sciaridae).[3] Testing of phytoecdysteroids against other Lepidoptera indicate highly polyphagous species are pre-adapted to feeding on phytoecdysteroids with little detrimental effects due to effective detoxification mechanisms.[4] The majority of previous studies have concentrated on Lepidoptera. However, recently phytoecdysteroids were observed to have detrimental effects on two polyphagous arthropods, a whitefly *Bemisia tabaci* (Hemiptera, Aleyrodidae), and a herbivorous mite *Oligonychus perseae* (Arachnida, Tetranychidae).[5] Revealing general patterns of the impact of phytoecdysteriods on herbivores will be helpful if they are to be exploited for pest management.

We hypothesized that within a subset of herbivorous Coleopterans, the impact of phytoecdysteroids would vary by their feeding range. To determine if phytoecdysteroids could be used to control highly polyphagous as well as monophagous species we utilized the Japanese beetle, *Popillia japonica* (Scarabaeidae) and three species of beetles in the family Chrysomelidae with varying degrees of polyphaga. The Japanese beetle is a highly polyphagous herbivore with over 300 known host plants. They were first found in the United States in 1916 and since have spread to most Eastern and mid-Western states.[6] Current control practices utilize synthetic insecticides against the adults and bioinsecticides against the larvae.[7] The oligophagous chrysomelid species tested include the Western corn rootworm, *Diabrotica virgifera virgifera*, and the bean leaf beetle, *Cerotoma trifurcata.* Both are native to the United States and feed on a few native and non-native host plants. The Western corn rootworm feeds primarily on maize and a few other grass species and cucurbits; it is currently a major pest of maize causing an estimated $1 billion dollars of damage yearly.[8,9] The bean leaf beetle feeds primarily on legumes including soybean.[10] The chrysomelid tested as a monophagous species was the goldenrod leaf beetle, *Trirhabda canadensis*, which feeds primarily on a few species of goldenrods (*Salidago*) both as larvae and adults.[11]

To determine if phytoecdysteroids could be used to prevent herbivore feeding, we tested the effect of surface applied phytoecdysteroids on adult beetles. Results indicate that phytoecdysteroids on the surface of leaves will inhibit feeding in representatives of polyphagous, oligophagous, and monophagous beetles.

2 EXPERIMENTAL METHODS

2.1 Insects and chemicals

Japanese beetles, *Popillia japonica*, were collected from wild populations near the Iowa State University (ISU) campus in Ames, Iowa, USA using a commercial Tanglefoot trap (Grand Rapids, Michigan, USA) or were collected by hand from wild grape. Bean leaf beetles, *Cerotoma trifurcata*, and goldenrod leaf beetle, *Trirhabda canadensis*, were collected from wild populations near Ames, Iowa, USA. Newly caught beetles were used in all assays. Western corn rootworm beetles, *Diabrotica virgifera virgifera* were obtained from a non-diapausing laboratory strain maintained at ISU. Adults of both sexes were used in all assays.

20-Hydroxyecdysone was purchased from Sigma-Aldrich (St. Louis, MO, USA) and ecdysone was purchased from SelleckBio (Houston, TX, USA). Phytoecdysteroids were obtained from the plant *Cyanotis vaga* and was purchased as a dry plant extract from Beyond A Century (Greenville, MA, USA). Compounds were dissolved at the indicated concentrations in 100% methanol and either used directly or diluted with water. Field assays using soybeans were treated with the plant extract at the indicated concentrations first dissolved in 100% methanol and then diluted with water to a final concentration of 10% methanol.

2.2 Laboratory experiments

Laboratory assays using Japanese beetles were conducted using 1 liter glass jars with screen lids. Leaves were removed from various preferred host plants that were growing on the ISU campus and cut leaves were treated within an hour of removal from the plant by dipping the leaf in solutions of methanol, allowed to dry at 22 ± 2 ºC, and placed in the jars with the cut end in a vial of water. Methanol was used to dissolve phytoecdysteroids because it evaporated quickly and it caused an even spreading of the compounds on leaf surfaces. No visible phytotoxicity was seen in any of the leaves after the methanol treatment. In no-choice assays one treated leaf was placed in the jar and then ten adult beetles were added, and allowed to feed for 20 hrs at 22 ± 2ºC and 14:10 L:D. Leaves were removed and visualized for feeding damage. Leaves were scanned and images used to determine percentage of area eaten using Adobe Photoshop CS5 (Adobe Systems, Mountain View, CA, USA). Choice assays were conducted using five leaves treated with 10, 5, 1, 0.1, and 0 g/l of phytoecdysteroid per 1 liter jar along with 25 beetles, unless indicated otherwise. To determine the concentration per leaf area required to inhibit feeding a specific concentration of phytoecdysteroid was applied based on the area of the leaf. The compounds were applied to leaves in specific volumes using a hand-held micropipette. Leaf area was determined before application using Adobe Photoshop CS5 on a scanned leaf. Phytoecdysteroid was applied using methanol to both sides of the leaf to produce a final concentration ranging from 0.1 to 400 $\mu g/cm^2$. After drying, the leaves were used in a no-choice assay as described above.

Choice laboratory assays using bean leaf beetles were conducted in the same manner as just described for the Japanese beetles. Soybean leaves with the cut end in a vial of water were dipped in various concentrations of phytoecdysteroids in methanol, allowed to dry and placed in a 1 liter glass jar with 10 beetles. After feeding for 20 hrs the percentage of leaf area eaten was determined. Choice assays using Western corn rootworms were conducted in a similar manner except the leaves were removed from a 2-3 week old maize plant grown in a greenhouse. The leaves were cut to an area of approximately 1 cm^2 and placed on the surface of 2% agar in a round 9 cm diameter petri dish to prevent the leaves from drying. The phytoecdysteroids in methanol were pipetted onto the surface of the leaves, allowed to dry and then placed in a cage (15 cm cube) containing 20 adult beetles. After feeding for 20 hrs the percentage of leaf area eaten was determined.

2.3 Field experiments

No-choice field assays were conducted using soybeans, *Glycine max* (IA3027), planted at the Curtiss Research farms located near Ames, Iowa, USA. Four-week old plants were thinned to 60 cm between plants and cages were placed around a single plant with the open end buried in the soil. The next day plants were treated in a randomized complete block design with phytoecdysteroids at 0, 0.1, 1, 5, and 10 g/l in 10% methanol in water with 4 replications per treatment. The compounds were applied with a hand-held plastic spray bottle by spraying each trifoliate on both sides three times, saturating the leaf surface. The plants were allowed to air-dry before 20 Japanese beetles were added to each cage. The amount of defoliation was estimated as a percentage at the end of 2 weeks by visualization of leaves. The number of beetles alive in the cages was determined as was plant height after two weeks. In a separate experiment choice assays were conducted where two plants were in one cage and treated in three ways: 1. Both plants treated with phytoecdysteroids at 10 g/l using 10% methanol in water. 2. One plant treated with phytoecdysteroids at 10 g/l using 10% methanol in water and one plant treated with 10% methanol in water control. 3. Both plants treated with 10% methanol in water.

2.4 Statistical analysis

All laboratory experiments were conducted per a randomized design, using ANOVA followed by Fisher LSD to determine if treatments affected defoliation (Systat Software Inc., San Jose, CA). Within the field experiment, we calculated means for defoliation and the number of beetles on each plant on a per-cage basis. After confirming these data were normally distributed, they were analyzed as a two-factor ANOVA, with application of the phytoecdysteriod (treated vs untreated) and choice (both plants treated or

untreated, or one of each) as the two factors. This allowed us to test whether the phtyoecdysteriod prevented defoliation and if this occurred only when beetles had a choice between treated and untreated plants.

3 RESULTS

3.1 Laboratory experiments

Results of laboratory assays based on dipping leaves into a solution with phytoecdysteroids indicate that feeding by Japanese beetles could be inhibited in both choice and no-choice assays. In the choice assay 5 and 10 g/l concentrations were significantly effective in inhibiting beetle feeding ($F = 19.23$; df = 4, 20; $P = 0.001$). At the highest concentration, this inhibition resulted in less than 2% defoliation, compared to 60% defoliation on control leaves (Fig 1). To determine the approximate concentration required to inhibit feeding per leaf area, specific amounts were applied directly to leaves of known area (Table 1). The 100 µg/cm^2 concentration was the lowest concentration tested that almost completely inhibited feeding and could be comparable to dipping leaves in a 5 g/l solution. The most common phytoecdysteroid, 20-hydroxyedysone, was tested as a pure compound and was found to inhibit feeding at 10 µg/cm^2 leaf area. Ecdysone also inhibited feeding at similar concentrations.

To determine if phytoecdysteroids can inhibit Japanese beetle feeding on various plants, leaves were collected and tested in a no-choice assay by dipping in methanol solutions at the indicated concentrations. All plant leaves tested were protected from feeding by Japanese beetles at the higher concentrations (Table 2). These concentrations were similar to those found in the choice assay. Rose leaves were the least protected at the 5 and 10 g/l concentrations.

The effect of phytoecdysteroids applied to the leaf surface of a preferred host plant was also determined using three species of chrysomelid adult beetles in choice assays. Bean leaf beetles, *Cerotoma trifurcata*, were tested using soybean leaves. Western corn rootworms, *Diabrotica virgifera virgifera*, were tested using maize, *Zea mays*, leaves. Goldenrod leaf beetles, *Trirhabda canadensis*, were tested using leaves from goldenrod, *Solidago canadensis*. As shown in Table 3, phytoecdysteroids at the 5 and 10 g/l concentration were very effective in preventing feeding by adults of all three species of beetles. Last instar goldenrod leaf beetle larvae were also tested at the highest dose of 10 g/l and preferred to feed on the untreated control.

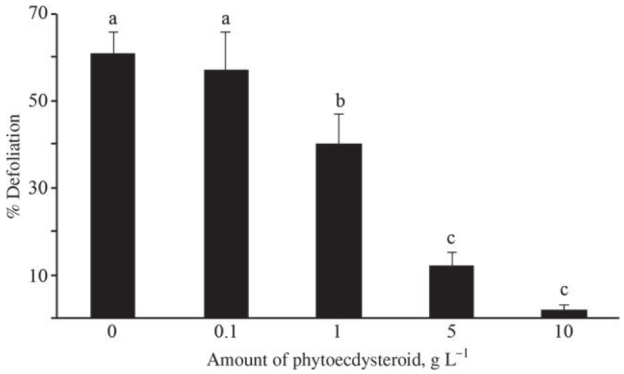

Figure 1. Effect of various concentrations of phytoecdysteroid on defolia- tion of linden, (*Tilia tomentosa*) leaves by Japanese beetles in a choice assay. Mean ± SEM, $n = 5$. Letters at the top of each bar indicate significant differ- ences between treatments $P < 0.05$ (ANOVA followed by Fisher's LSD test).

Table 1. Ecdysteroids reduced feeding by Japanese beetles of elm (*Ulmus americana*) leaves in a no-choice assay. Defoliation varied by concentration was achieved by application of indicated compounds to leaves of known area. Values represent mean percentage defoliation ± SEM[a]

Concentration (µg cm^{-2})	Phytoecdysteroid[b] $n = 5$	20-OHecdysone[c] $n = 3$	Ecdysone[d] $n = 3$
0.1	56 ± 7 a	50 ± 6 a	74 ± 15 a
1	36 ± 4 b	34 ± 10 a	47 ± 13 b
10	33 ± 8 b	4 ± 2 b	15 ± 6 c
100	14 ± 6 c	3 ± 2 b	6 ± 2 c
200	4 ± 3 c		
400	4 ± 1 c		

[a] Lower-case letters indicate significant differences between treatments within a column $P < 0.05$ (ANOVA followed by Fisher's LSD test). [b] $F = 7.06$; df = 4, 20; $P = 0.001$.
[c] $F = 15.83$; df = 3, 8; $P = 0.001$.
[d] $F = 9.01$; df = 3, 8; $P = 0.006$.

3.2 Field experiments

Phytoecdysteroid reduced defoliation of soybean leaves by Japanese beetles in a no-choice, field setting ($F = 37.49$; df = 4, 20; $P = 0.001$). The percent defoliation that occurred after 14 days of Japanese beetle feeding on soybean plants was significantly less in the plants treated with 10 and 5 g/l of phytoecdysteroid (Fig. 2). Plant height was not significantly different between treatments (data not shown). The number of beetles remaining active in the cage after 14 days also varied by phytoecdysteroid concentration applied to leaves ($F = 11.17$; df = 4, 15; $P = 0.0002$). The plants treated with 10 and 5 g/l phytoecdysteroids had significantly fewer beetles than the other treatments (Fig. 3). The beetles that could not be found in the cages after 14 days most likely burrowed into the soil.

Table 2. Application of phytoecdysteroid reduced defoliation of leaves from various plants in a no-choice assay by Japanese beetles in a concentration-dependent manner. Leaves were dipped in a methanolic solution of phytoecdysteroid. Values represent mean percentage defoliation ± SEM ($n = 5$)[a]

Concentration (g L^{-1})	Linden[b]	Elm[c]	Virginia creeper[d]	Rose[e]	Wild grape[f]
0	76 ± 7a	48 ± 10 a	42 ± 3a	37 ± 4a	75 ± 6a
0.1	49 ± 8b	30 ± 6b	26 ± 5b	43 ± 4a	74 ± 8a
1	40 ± 9b	27 ± 5b	18 ± 4b	20 ± 4b	33 ± 6b
2.5	21 ± 5c				
5	7 ± 3 cd	6 ± 2c	5 ± 3c	16 ± 2b	4 ± 1c
10	2 ± 1d	2 ± 1c	3 ± 1c	12 ± 3b	2 ± 1c

[a] Linden, *Tilia tomentosa*; Elm, *Ulmus americana*; Virginia creeper, *Parthenocissus quinquefolia*; Rose, *Rosa* 'knock out'; wild grape, *Vitis riparia*. Lower-case letters indicate significant differences between treatments within a column $P < 0.05$ (ANOVA followed by Fisher's LSD test).
[b] $F = 24.42$; df = 5, 24; P = 0.0001.
[c] $F = 10.81$; df = 4, 20; P = 0.0001.
[d] $F = 21.11$; df = 4, 20; P = 0.0001.
[e] $F = 14.93$; df = 4, 20 P = 0.0001.
[f] $F = 41.93$; df = 4, 20; P = 0.0001.

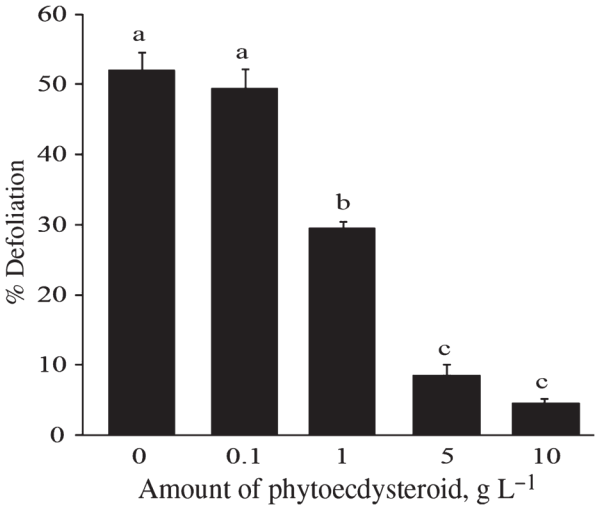

Figure 2. Effect of various concentrations of phytoecdysteroid on defolia- tion of soybean (*Glycine max*) leaves by Japanese beetles in a caged field assay. Mean ± SEM, $n = 4$. Letters at the top of each bar indicate significant differences between treatments $P < 0.05$ (ANOVA followed by Fisher's LSD test).

Table 3. Various concentrations of phytoecdysteroid affected feeding by three species of chrysomelids on preferred host plants in a choice assay. Values represent mean percentage defoliation ± SEM[a]

Concentration (g L^{-1})	BLB[b] $n = 3$	WCRW[c] $n = 5$	GLB adult[d] $n = 3$	GLB larvae[e] $n = 7$
0	31 ± 6a	65 ± 6a	61 ± 7a	58 ± 9 a
1	27 ± 3a	36 ± 7b	60 ± 10 a	
5	1 ± 1b	7 ± 5c	8 ± 1b	
10	1 ± 1b	1 ± 0.3 c	2 ± 2b	12 ± 3b

[a] BLB = bean leaf beetle feeding on soybean leaves; WCRW = western corn rootworm feeding on maize leaves; GLB = goldenrod leaf beetle feeding on common goldenrod.
Lower-case letters indicate significant differences between treatments within a column $P < 0.05$ (ANOVA followed by Fisher's LSD test).
[b] $F = 21.89$; df = 3, 8; $P = 0.0003$.
[c] $F = 25.69$; df = 3, 8; $P = 0.0002$.
[d] $F = 23.23$; df = 1, 21; $P = 0.0004$.
[e] $F = 37.67$; df = 3, 16; $P = 0.0001$.

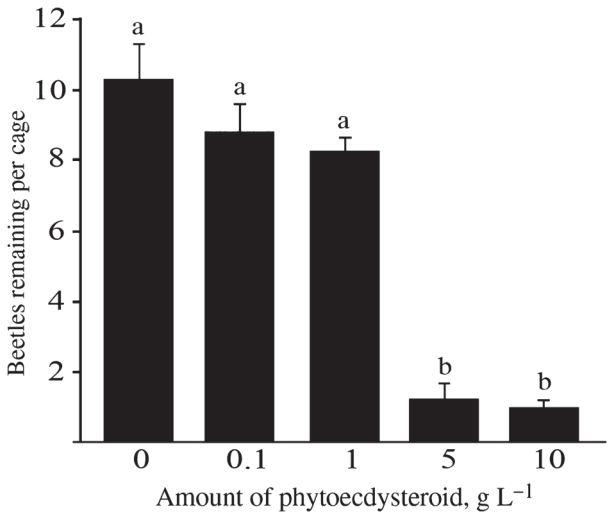

Figure 3. Number of beetles remaining in cages of soybean plants, *Glycine max*, treated with the indicated concentration of phytoecdysteroid after 14 days. Each cage contained 20 beetles at the start of the experiment. Mean ± SEM, $n = 4$. Letters at the top of each bar indicate significant differences between treatments $P < 0.05$ (ANOVA followed by Fisher's LSD test)

Japanese feeding and activity on soybean leaves was consistent in both choice and no-choice settings (Table 4). Regardless of whether a choice could be made by Japanese beetles between treated or untreated plants, all treated plants had fewer Japanese beetles feeding on the plant and fewer damaged leaves compared to untreated plants. In the cages where a choice could be made between treated and untreated plants more beetles were observed feeding on the untreated plants and these plants had a higher incidence of damaged leaves. Although feeding was not completely prevented on treated plants in a no-choice setting, it was reduced by nearly half compared to untreated plants.

Table 4. Japanese beetle feeding on field-grown soybean plants caged in choice or no-choice settings is reduced when leaves are treated with a phytoecdysteroid, as measured by the number of leaves damaged and the number of beetles actively feeding. Values are mean ± SEM ($n = 4$)

	Number of leaves damaged[a]		Number of beetles feeding[b]	
	no-choice	choice	no-choice	choice
Treated	7.4 ± 2.3	7.8 ± 2.7	0.5 ± 0.4	0.3 ± 0.2
Untreated	15.8 ± 3.8	18.3 ± 3.8	2.5 ± 1.3	4.3 ± 2.2

Statistical differences were found between treated and untreated plants (ANOVA).
[a] $F = 9.75$; df = 1, 23; $P = 0.0054$.
[b] $F = 7.19$; df = 1, 23; $P = 0.0143$.
No statistical differences were found between no-choice and choice plants (ANOVA).
[a] $F = 0.23$; df = 1, 23; $P = 0.6396$.
[b] $F = 0.45$; df = 1, 23; $P = 0.5103$.

4 DISCUSSION AND CONCLUSIONS

Most plants have the capability of producing phytoecdysteroids.[1] The function of phytoecdysteroids in plants is hypothesized to be the deterrence of phytophagous insects by either acting like an antifeedant or affecting development when ingested.[1, 12] Phytoecdysteroids can produce developmental effects if ingested in some species of insects. In others relatively high levels of ecdysteroids can be ingested with apparently little harm to the insect. It is hypothesized that the more adapted species are polyphagous and have adapted ways of detoxifying the ingested phytoecdysteroids.[4] Larvae of several lepidopterans apparently can sense phytoecdysteroids and will not consume food containing phytoecdysteroids.[2] Apparently phytoecdysteroids can be detected by gustatory sensilla in Lepidoptera larvae[13, 14] as well as adult females searching for oviposition sites.[15]

Our studies indicate that phytoecdysteroids can deter feeding by the highly polyphagous Japanese beetle. We demonstrate that phytoecdysteroids present on the surface of the leaf can prevent Japanese beetle feeding at concentrations of about 100 $\mu g/cm^2$. The pure compounds, 20-hydroxyecdysone and ecdysone, also inhibited beetle feeding at similar concentrations. These results indicate that the hydroxyl group on C20 is not critical for activity. In other studies, a variety of phytoecdysteroids have been tested for insecticidal activity with variable results. For example, makisterone A was found to have the highest toxicity when mixed with diet fed to the Indian meal moth, *Plodia interpunctella*.[16]

Although it has been proposed that polyphagous herbivores are less sensitive to antifeedants including phytoecdysteroids[1], our results demonstrate that phytoecdysteroids can inhibit feeding by adult beetles that are considered both polyphagous and monophagous. The Japanese beetle is highly polyphagous while *T. canadensis* is monophagous. The western corn rootworm and bean leaf beetle are considered to be oligophagous. Recently however, phytoecdysteroids have been shown to negatively affect the growth of several polyphagous herbivores including the Indian meal moth, *P. interpunctella* [16], the sweetpotato whitefly, *B. tabaci*, and the persea mite, *O. perseae*.[5] We have tested only 4 species of beetles from two families. Although it is unlikely that phytoecdysteroids will deter feeding in all species of beetles, our results suggest that feeding breadth will not predict which species will be deterred.

The antifeedant mode of action of the phytoecdysteroids is most likely as a deterrent in that the beetles do not appear to physically eat leaves before making a choice not to eat. At the highest concentrations of phytoecdysteroids the leaves remained intact so the beetles are sensing phytoecdysteroids on the surface. Most likely the phytoecdysteroids are being detected by maxillary and labial palps. Further research is required to determine where on the beetles the sensilla are located that detect phytoecdysteroids. In several moth larvae the taste sensilla detecting phytoecdysteroids were found on the galea of the maxilla.[13, 14] Female adult European corn borers, *Ostrinia nubilalis*, have sensilla on the tarsi that can detect phytoecdysteroids.[15]

In our study we treated the surface of plants with phytoecdysteroids. It is not known exactly where the phytoecdysteroids are found naturally in plants but are most likely found in the vacuole of plant cells.[17] Presumably little is found on hydrophobic leaf surfaces because most phytoecdysteroids are relatively hydrophilic.

The phytoecdysteroids used in this study were obtained from a commercial source that used *Cyanotis arachnoidea* and *Cyanotis vaga* as source plants. These plants are known to contain high concentrations of primarily 20-hydroxyecdysone with smaller amounts of 20-hydroxyecdysone 3-acetate and even lower amounts of a variety of other phytoecdysteroids.[18, 19] The total amount of phytoecdysteroids can reach about 2% by dry weight of total plant mass. This is a relatively high level that has been exploited to obtain relatively pure phytoecdysteroids in large quantities. Our lab and field experiments demonstrate that these plant extracts could have potential in pest management to prevent feeding by herbivorous insects if sprayed onto the surface of leaves.

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