# Effect of Glyceollin, a Soybean Phytoalexin, on Feeding by Three Phytophagous Beetles (Coleoptera: Coccinellidae and Chrysomelidae): Dose versus Response

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**ABSTRACT** Laboratory studies showed that the soybean phytoalexin glyceollin, an isoflavonoid previously investigated as an inhibitor of fungal pathogens, is an effective antifeedant for some insect species. Glyceollins extracted from soybean, *Glycine max* Merrill, cotyledons were applied to the surface of common bean leaves, *Phaseolus vulgaris* L., in five concentrations, including physiological concentration, for feeding preference tests. Leaves treated with glyceollin at concentrations below physiological levels were less acceptable to the southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber, and the Mexican bean beetle, *Epilachna varivestis* Mulsant, than untreated leaves. Feeding deterrence was positively correlated with increasing concentrations. The bean leaf beetle, *Cerotoma trifurcata* Forster, was not affected even by very high doses. An ethological concentration ($EC_{50}$) was computed based on the log dose-reduction of acceptance of treated disks. The $EC_{50}$ for the Mexican bean beetle was 6.1 $\mu$g/mg leaf dry weight and 3.5 $\mu$g/mg for the southern corn rootworm. Results indicate that soybean phytoalexins may represent a common defense against microorganisms and insect herbivores.

**KEY WORDS** Insecta, *Epilachna varivestis, Diabrotica undecimpunctata, Cerotoma trifurcata*

RESISTANCE IN SOYBEAN, *Glycine max* Merrill, to leaf-feeding insects and to disease is well documented and varies widely among genotypes (Van Duyn et al. 1971, Ward et al. 1979). The biochemical mechanisms accounting for insect resistance in soybean are multifaceted and probably involve leaf volatiles (Liu et al. 1988), variations in nutrient concentrations, feeding excitants or deterrents, and antibiotics (Kogan 1986). Two distinct modalities of insect resistance in soybean have been identified: constitutive (preformed) (Kogan 1972) and inducible (produced only after the plant is challenged) (Hardin 1979, Hart et al. 1983, Chiang et al. 1986a).

Phenolic compounds, present in soybean, may have a role in deterrence to insect feeding in both categories of resistance (Hardin 1979, Caballero et al. 1986, Chiang et al. 1986a, Fischer et al. 1990). Several simple phenolic acids are present constitutively in higher concentrations in the insect-resistant soybean genotype PI 227687 than in the insect-susceptible cultivar 'Forrest.' These concentrations increase when plants are damaged by insects (Hardin 1979). Some of these compounds are precursors to isoflavonoids (cinnamic and p-coumaric acids), a few of which (e.g., vestitol, hildecarpin, coumestrol, and afrormosin) have been identified as constitutive insect antifeedants in cultivated legumes (Russell et al. 1978, Caballero et al. 1986, Chiang et al. 1986b, Khan et al. 1986, Lwande et al. 1986). Only one inducible isoflavonoid (vestitol) with antibiotic activity has been shown to be an insect antifeedant as well (Russell et al. 1978). But inducible phytoalexins, particularly glyceollins, have been implicated in soybean resistance to the fungal pathogen *Phytophthora megasperma* f. sp. *glycinea* in soybean (Paxton 1983). Kogan & Paxton (1983) have suggested that the soybean phytoalexin response, which can be initiated by several biotic and abiotic stresses or elicitors, may have evolved as a single, energy-conserving chemical defense active against microorganisms and insects.

Hart et al. (1983) demonstrated that soybean cotyledons, when induced to form phytoalexins, became less acceptable to Mexican bean beetle larvae and adults, in dual-choice feeding preference tests. In their studies, however, components such as simple phenolic acids, anthocyanins, or isoflavonoids, that change concentration in the tissue after induction, were not tested individually. Chiang et al. (1986b) reported that consumption of approximately 30% of a trifoliolate leaflet of PI 227687 resulted in increased antifeedants in leaves throughout the plants. They concluded that the chemicals that are responsible are apparently phenylpropanoids (Chiang et al. 1986b). This inducible resistance is correlated with increased L-phenylalanine ammonialyase and L-tyrosine ammoni-

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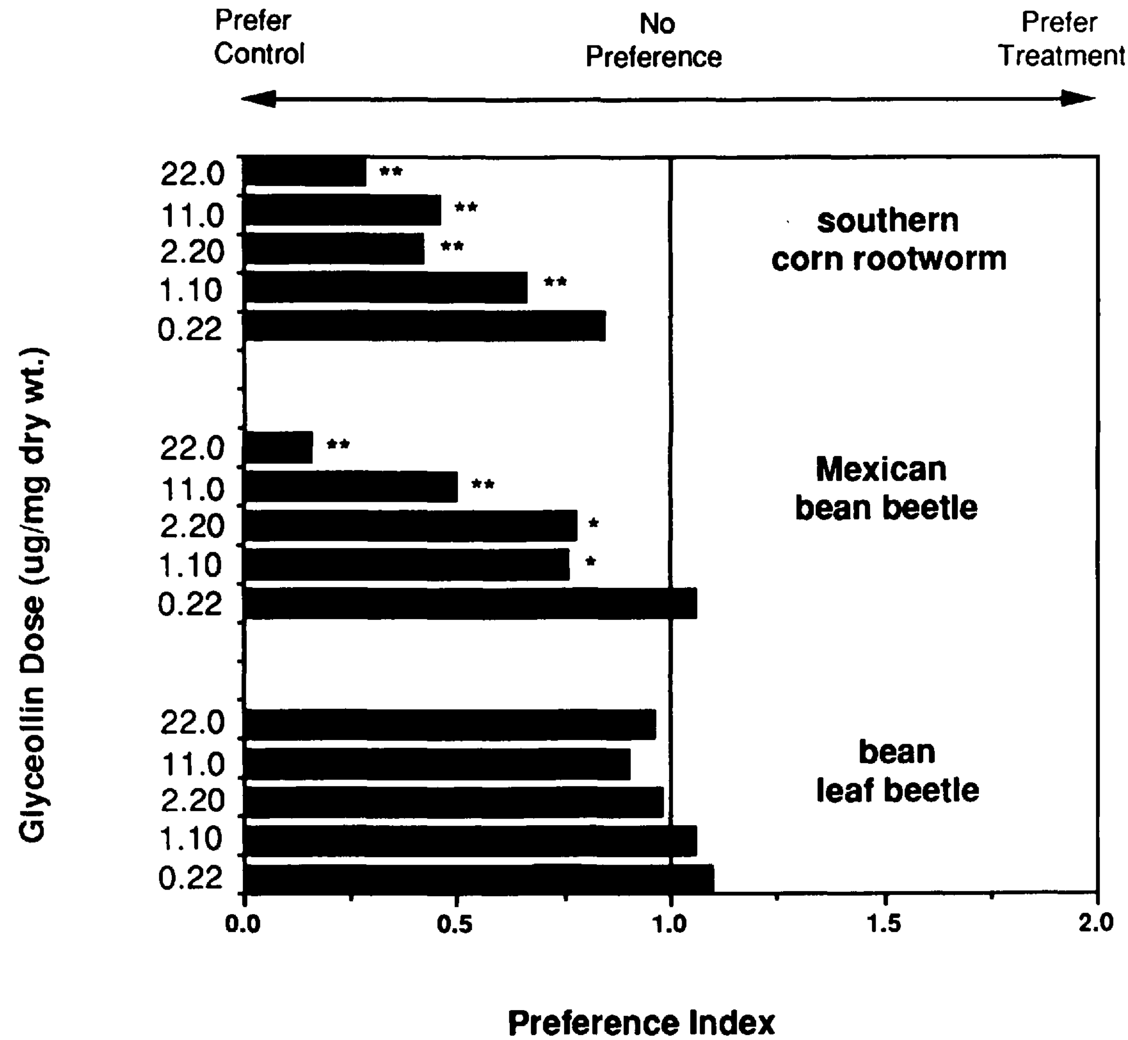


**Fig. 1.** Results of feeding preference tests with glyceollin. Bars represent mean of eight preference tests at each dose with preference indices converted to percent response by the formula: % Response = (1.0 − PI) × 100. *, $P < 0.05$; **, $P < 0.01$ (paired $t$ test).

alyase enzyme activities (Chiang et al. 1986b) indicating that resistance may be due to phenolic-based compounds. Subsequently, Fischer et al. (1990) found that several of the simple phenolic acids are strong feeding deterrents for the Mexican bean beetle. This finding, together with the evidence from Hardin (1979) that phenolic acid levels are elevated after insect feeding takes place, raises the possibility that phenolic acids are inducible resistance factors.

In a series of experiments, we tested the hypothesis that glyceollins may act as feeding deterrents to certain soybean-associated arthropods. We determined the dose-related deterrent effects of glyceollin on feeding behavior of three leaf-eating Coleoptera that consume soybean leaves: the coccinellid Mexican bean beetle, *Epilachna varivestis* Mulsant, and the chrysomelids bean leaf beetle, *Cerotoma trifurcata* (Forster), and southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber.

## Materials and Methods

**Glyceollin Production.** Glyceollins for this investigation were obtained from the cotyledons of 5-d-old 'Williams' soybean seedlings grown in flats of quartz sand in a greenhouse. The seedlings were harvested, and cotyledons were removed in the laboratory. A small area of the abaxial surface was excised with a scalpel, and several drops of an autoclaved extract of cultured cells of *Phytophthora megasperma* f. sp. *glycinea* were applied. The cotyledons were incubated for 48 h and then dried in an oven at 50°C for 12 h.

Glyceollin was extracted for 8 h by covering the cotyledons with ethanol/water (1:1) buffered to pH 7.2 with 0.01 M phosphate buffer. The extracting solution was decanted and the cotyledons rinsed with a second portion of fresh solution. The extracts were combined, and the ethanol was removed in a rotary evaporator (Buchi, Model W, Switzerland) under reduced pressure at 45°C. The crude glyceollins were extracted from the remaining water portion with an equal volume of ethyl acetate. This extract was dried with magnesium sulfate and filtered, and the solvent was evaporated. The crude material was suspended in methylene chloride and loaded onto a Sep-Pak silica chromatography column (Cat. No. 51910, Waters Associates, Milford, Mass.). The glyceollins were removed selectively from the column with 8.0 ml of a solvent mixture (ethyl acetate 70%, hexane 29%, methanol 1%). The eluent was concentrated approximately 20 times and further purified by high performance liquid chromatography (HPLC) using a Waters dual pump (Model M510, Waters Associates) instrument with a preparative octadecylsilane column (1 by 25 cm), 10-$\mu$m particle size (Cat. No. 6231, Alltech Associates, Deerfield, Ill.), and UV detector (254 nm) (Model M481, Waters Associates). The HPLC solvent was 70% methanol in water. The glyceollin fraction (peak corresponding to authentic standard) was collected from the HPLC output and taken to dryness in a rotary evaporator under reduced pressure at 45°C. The identity of the purified glyceollin was corroborated by UV spectroscopy.

**Testing Glyceollins for Behavioral Effects in Dual-Choice Preference Tests.** Common bean, *Phaseolus vulgaris* L., a preferred food plant for the Mexican bean beetle and bean leaf beetle, does not produce glyceollins. *P. vulgaris* plants were grown in potting soil (soil/peat/sand/perlite; 1:1:1:1) under metal halide light and a photoperiod of 14:10 (L:D) in a greenhouse and fertilized once each week. The lateral leaflets of single trifoliolates from 4-wk-old plants were used as treatment or control in preference tests.

Purified glyceollins were dissolved in reagent grade acetone and applied to the leaf surface using an aerosol chromatography sprayer and a motorized turntable as described by Fischer et al. (1990). Leaves were treated immediately after excision. Five concentrations of glyceollin were tested; 0.22, 1.1, 2.2, 11.0, and 22.0 $\mu$g/mg dry weight of leaf tissue.

Treated leaf tissue was subjected to feeding preference tests performed in arenas constructed from glass Petri dishes (18.0 cm diameter) filled to a depth of 1 cm with hardened plaster of paris, saturated with tap water, and covered with filter paper. For each glyceollin concentration, three treated disks and three acetone-sprayed control disks were arranged alternately in a circular pattern in the arena.

Mexican bean beetles and southern corn rootworms were obtained from laboratory colonies at the Illinois Natural History Survey, Champaign, Ill. Mexican bean beetle adults and larvae were reared on common bean leaves. Southern corn rootworm larvae were reared on corn roots. Adults were fed a dry, pollen based diet and both colonies were maintained in a greenhouse. Bean leaf beetles were collected from alfalfa plants during the month of August and maintained on greenhouse-grown common bean leaves until testing.

**Table 1. Results of paired *t* test for areas eaten of treated and control disks in dual-choice preference tests**

| Glyceollin concentration, $\mu$g/mg dry wt | Mean PI | *t* | df | *P* |
|---|---|---|---|---|
| Southern corn rootworm | | | | |
| 0.22 | 0.84 | 2.114 | 7 | 0.0724 |
| 1.10 | 0.66 | 5.053 | 7 | 0.0015 |
| 2.20 | 0.42 | 4.453 | 7 | 0.0030 |
| 11.0 | 0.46 | 6.703 | 7 | 0.0003 |
| 22.0 | 0.28 | 5.736 | 7 | 0.0007 |
| Mexican bean beetle | | | | |
| 0.22 | 1.08 | 0.511 | 7 | 0.6250 |
| 1.10 | 0.84 | 2.538 | 7 | 0.0388 |
| 2.20 | 0.76 | 3.964 | 7 | 0.0107 |
| 11.0 | 0.44 | 3.818 | 7 | 0.0066 |
| 22.0 | 0.17 | 15.371 | 7 | 0.0001 |
| Bean leaf beetle | | | | |
| 0.22 | 1.10 | 0.940 | 7 | 0.3786 |
| 1.10 | 1.06 | 1.192 | 7 | 0.2723 |
| 2.20 | 0.98 | 0.342 | 7 | 0.7430 |
| 11.0 | 0.90 | 0.557 | 7 | 0.5951 |
| 22.0 | 0.96 | 0.823 | 7 | 0.4375 |

For each test, four adult beetles of one species were placed in the arena and allowed to eat until approximately half of the leaf material was consumed (usually within 6–8 h). Test arenas were kept in the dark in a rearing chamber at 27 ± 2°C and relative humidity near saturation within the arenas. Beetles were starved for approximately 12 h before testing and used in no more than three tests on consecutive days. At the end of each test, all disks were removed and measured with a leaf area meter (Model 3000, LI-COR, Lincoln, Nebr.) to determine leaf area eaten from each disk. The test was replicated eight times (in the same day) for each concentration and the same five dosage levels were tested for each species.

A preference index (PI) was calculated for each paired comparison according to the formula: PI = 2T/(T+C); T = area eaten from the treated disks and C = area eaten from the untreated control disks. Values in this preference test range from 0.0 to 2.0 with 0.0 indicating absolute preference for the control disks, 1.0 indicating no preference, and 2.0 indicating absolute preference for the treated disks (Fig. 1). The PI values for each test were used to compute the concentration of glyceollin that produced a behavioral response resulting in a 50% reduction in acceptance of the bean leaf disks (ethological concentration or $EC_{50}$).

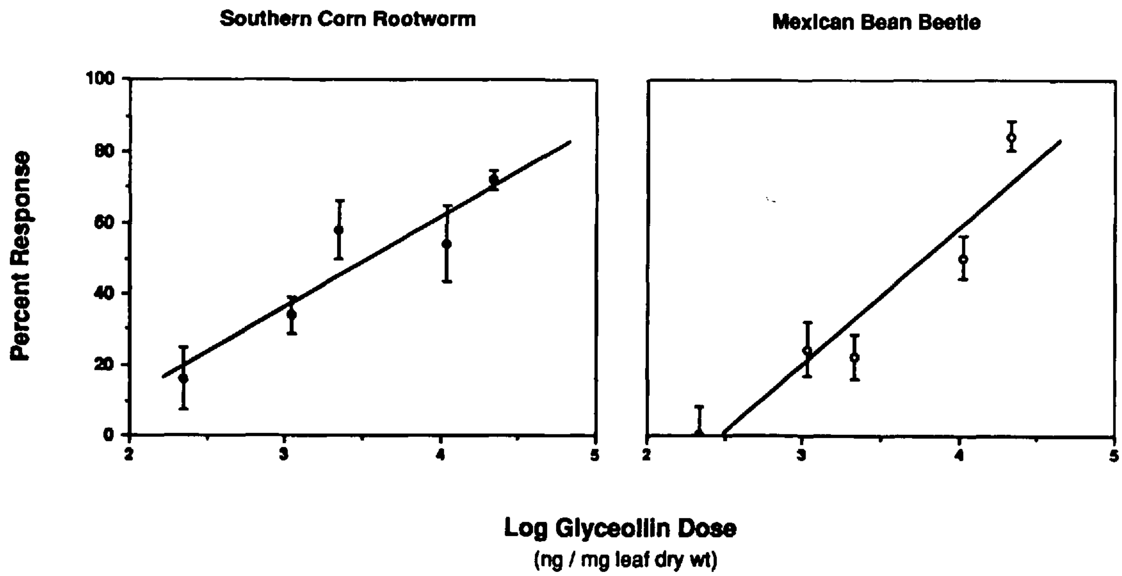


Fig. 2. Response by Mexican bean beetle and southern corn rootworm to increasing glyceollin concentration on leaf disks in dual choice feeding preference tests. For the Mexican bean beetle $n = 5$, slope = 38.23 ± 7.34 SE, $r = 0.948$, $EC_{50}$ = 6.1 μg/mg leaf (dry wt), 95% CL = 14.86 – 61.60. For the southern corn rootworm $n = 5$, slope = 25.47 + 5.97 SE, $r = 0.926$, $EC_{50}$ = 3.5 μg/mg leaf (dry wt), 95% CL = 6.48 – 44.46.

## Results and Discussion

The range of glyceollin concentrations tested in these experiments was centered around the normal physiological concentration of these compounds in induced soybean leaves. Pathogen infection (*Phytophthora megasperma* f. sp. *glycinea*), as well as abiotic elicitors, such as silver nitrate, cause accumulation of 1–5 μg glyceollin/mg dry wt in soybean tissue (Stossel 1982, Classen & Ward 1985, Long et al. 1985). The highest glyceollin concentration we used was approximately four times the maximum found in induced soybean tissue, and the lowest concentration was five times lower than the lowest commonly achieved by induction with the pathogens.

Our results confirmed that glyceollin has a deterrent effect on feeding by two of the phytophagous Coleoptera (Table 1). The combined results of 120 preference tests (40 per species) for these three insects are given in Fig. 1. Mexican bean beetles and southern corn rootworms were deterred from feeding by the application of glyceollin at concentrations normally induced by pathogens and abiotic elicitors. Bean leaf beetles did not appear to be deterred from feeding by these levels of glyceollin.

An $EC_{50}$ was calculated as a measure of the behavioral effect of glyceollin on Mexican bean beetles and southern corn rootworms (see Fig. 2). For our purposes $EC_{50}$ is defined as the concentration resulting in a preference index (PI) of 0.5 (50% reduction in acceptance of treated leaf disks). Log of dosage was plotted against percent response to a treatment to obtain the $EC_{50}$ (percent response = 1.0 − PI × 100). The $EC_{50}$ is 3.5 μg/mg for the southern corn rootworm and 6.1 μg/mg for the Mexican bean beetle. It was not possible to calculate an $EC_{50}$ for the bean leaf beetle because the highest doses used (above physiological levels) never reached the 50% deterrency level. None of the insects was significantly deterred by the lowest dose, 0.22 μg/mg, which is about one-tenth of the normal concentration in induced tissue. Even though the $EC_{50}$ was above the minimal physiological levels for Mexican bean beetle and southern corn rootworm, all doses above the minimum dose significantly deterred feeding by these species; thus, the threshold of response is well below physiological concentrations.

The southern corn rootworm is considered a polyphagous species and has numerous recorded hosts across many taxa, including legumes (soybean and peanut, *Arachis hypogoea* L.). However, adults, though present in soybean fields and observed feeding on leaves, consume only small amounts and are not considered pests of soybean. Agricultural practice in Illinois assures that there is constant contact with soybean providing the opportunity for the southern corn rootworm to use the plant as a regular host. Large populations of the southern corn rootworm develop on corn and squash, and both crops are inevitably planted close to soybean. Our results suggest that glyceollin may have a role in the mechanisms that limit the acceptance of soybean leaves by the corn rootworm.

The Mexican bean beetle is able to survive on soybean and even become a pest in several soybeangrowing regions of the United States (Kogan & Turnipseed 1987). Soybean, however, is a marginal host for the Mexican bean beetle (Kogan 1972), and many varieties of soybean are unsuitable for supporting its development undoubtedly because of chemical defenses. The Mexican bean beetle is closely adapted to *Phaseolus* spp. in its native central Mexico and is restricted to this narrow host range (Thomas 1924, Gordon 1975). Thus there is a likelihood that an inducible soybean defensive chemistry is active against the Mexican bean beetle.

The bean leaf beetle, a species native to the United States, is better adapted to soybean and feeding is much less deterred by resistant soybean varieties than is the Mexican bean beetle, suggesting a tolerance for resistance factors in soybean. The native hosts of the bean leaf beetle are believed to be members of the genus *Desmodium* and several other legume species. Thus, the bean leaf beetle is more polyphagous within the Leguminoseae than the Mexican bean beetle. Therefore, the bean leaf beetle may have been preadapted to soybean defenses, whereas the Mexican bean beetle was not.

It is clear that the phytoalexin glyceollin, heretofore known as an inducible pathogen resistance factor, can deter feeding on soybean by some insects.

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## References Cited

**Caballero, P., C. M. Smith, F. R. Fronczec & N. H. Fischer. 1986.** Isoflavones from an insect-resistant variety of soybean and the molecular structure of afrormosin. J. Nat. Prod. 49(6):1126–1129.

**Chiang, H. S., D. M. Norris, A. Ciepiela, P. Shapiro & A. Oosterwyk. 1986a.** Inducible versus constitutive PI 227687 soybean resistance to Mexican bean beetle, *Epilachna varivestis*. J. Chem. Ecol. 13(4): 741–749.

**Chiang, H. S., D. M. Norris, A. Ciepiela, A. Oosterwyk, P. Shapiro & M. Jackson. 1986b.** Comparative constitutive resistance in soybean lines to Mexican bean beetle. Entomol. Exp. Appl. 42(1): 19–26.

**Classen, D. & E. W. B. Ward. 1985.** Temperature-induced susceptibility of soybeans to *Phytophthora megasperma* f. sp. *glycinea:* Production and activity of elicitors of glyceollin. Physiol. Plant. Path. 26(2): 289–296.

**Fischer, D., M. Kogan & J. Paxton. 1990.** Role of free phenolic acids in soybean resistance against the Mexican bean beetle (*Epilachna varivestis*): Behavioral evidence. J. Entomol. Sci. 25(2): 230–238.

**Gordon, R. D. 1975.** A revision of the Epilachninae of the western hemisphere (Coleoptera: Coccinelledae) Technical Bulletin 1493. Agricultural Research Service, USDA, Washington, D.C.

**Hardin, J. M. 1979.** Phenolic acids of soybeans resistant and nonresistant to leaf feeding larvae. Masters thesis, University of Arkansas, Fayetteville.

**Hart, S. V., M. Kogan & J. Paxton. 1983.** Effect of soybean phytoalexins on the herbivorous insects Mexican bean beetle and soybean looper. J. Chem. Ecol. 9(6): 657–672.

**Khan, Z. R., D. M. Norris, H. S. Chiang, N. E. Weiss & A. S. Oosterwyk. 1986.** Light-induced susceptibility in soybean to cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). Environ. Entomol. 15(4): 803–808.

**Kogan, M. 1972.** Feeding and nutrition of insects associated with soybeans. 2. Soybean resistance and host preferences of the Mexican bean beetle, *Epilachna varivestis*. Ann. Entomol. Soc. Am. 65(3): 675–683.

**1986.** Natural chemicals in plant resistance to insects. Iowa State J. Res. 60(4): 501–527.

**Kogan, M. & J. Paxton. 1983.** Natural inducers of plant resistance to insects, pp. 153–171. *In* P. A. Hedin [ed.], Plant resistance to insects. American Chemical Society, Washington, D.C.

**Kogan, M. & S. G. Turnipseed. 1987.** Ecology and management of soybean arthropods. Annu. Rev. Entomol. 32: 507–538.

**Liu, S. H., D. N. Norris & E. Marti. 1988.** Behavioral response of female adult *Trichoplusia ni* to volatiles from soybean versus a preferred host, lima bean. Entomol. Exp. Appl. 49: 99–104.

**Long, M., P. Barton-Willis, B. J. Staskawicz, D. Dahlbeck & N. T. Keen. 1985.** Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to *Pseudomonas syringae* pv. glycinea. Phytopathology 75(2): 235–239.

**Lwande, W., M. D. Bentley & A. Hassanali. 1986.** The structure of hildecarpin, an insect antifeedant 6a-hydroxypterocarpan from the roots of *Tephrosia hildebrandtii* Vatke. Insect Sci. Applic. 7(4): 501–503.

**Paxton, J. D. 1983.** Phytophthora root and stem rot of soybean, pp. 19–27. *In* J. A. Gallow [ed.], Biochemical plant pathology, Wiley, London.

**Russell, G. B., O. R. W. Sutherland, R. F. N. Hutchins & P. E. Christmas. 1978.** Vestitol: a phytoalexin with insect feeding-deterrent activity. J. Chem. Ecol. 4(5): 571–579.

**Stossel, P. 1982.** Glyceollin production in soybean. Phytopatol. Z. 105(2): 109–119.

**Thomas, F. L. 1924.** Life history and control of the Mexican bean beetle. Alabama Agricultural Station Bulletin 221. Auburn.

**Van Duyn, J. W., S. G. Turnipseed & J. C. Maxwell. 1971.** Resistance in soybeans to Mexican bean beetle. I. Source of resistance. Crop Sci. 11(4): 572–573.

**Ward, E. W. B., G. Lazorrovits, C. H. Unwin & R. I. Buzzel. 1979.** Hypocotyl reactions and glyceollin in soybeans inoculated with zoospores of *Phytophthora megasperma* var. *sojae*. Phytopathol. 69(9): 951–955.

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