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INDUCIBLE VERSUS CONSTITUTIVE PI 227687 SOYBEAN RESISTANCE TO MEXICAN BEAN BEETLE, *Epilachna varivestis*

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Abstract—Contrary to constitutive resistance, inducible resistance to Mexican bean beetle (MBB) (*Epilachna varivestis*) herbivory in PI 227687 soybean leaves was positively correlated with total phenolic content and temporally unique, increased L-phenylalanine ammonia-lyase (PAL) and L-tyrosine ammonia-lyase (TAL) activities. Initial expression of the induced resistance was localized at or near the site of herbivory. Systemic parameters of the induced resistance also were observed. Inducible MBB resistance in PI 227687 soybeans apparently involves increased phenylpropanoid metabolism.

Key Words—Plant resistance, constitutive, inducible, soybean, Mexican bean beetle, *Epilachna varivestis*, Coleoptera, Coccinellidae, PI 227687, phenylpropanoid metabolism, PAL, TAL, total phenols.

INTRODUCTION

Plant resistance to insect pests involves at least two (i.e., constitutive and inducible) categories of parameters (Kogan and Paxton, 1983; Chiang and Norris, 1985). Constitutive parameters are expressed by the plant independent of environmental stresses. Such chemical parameters which are effective against Mexican bean beetle (MBB), *Epilachna varivestis* Mulsant, feeding have been studied partially in *Glycine max* (L.) Merr. PI 227687 soybeans by Chiang et

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al. (1986). In this work, L-phenylalanine ammonia-lyase (PAL) and L-tyrosine ammonia-lyase (TAL) activities in given-age leaves of nonstressed PI 227687 plants showed characteristic temporal patterns which were distinct from those of such leaves on nonstressed, insect-susceptible "Davis" soybeans. Activities of these enzymes have previously been positively correlated with the biosynthesis of phytoalexins and other flavonoids which include antifeedants for insects (Ebel et al., 1984).

Inducible versus constitutive (i.e., stress-dependent versus stress-independent) chemical parameters of PI 227687 resistance to MBB feeding have now been compared. This study contributes new understanding toward an ultimate holistic comprehension of PI 227687 soybean resistance to *E. varivestis* and other herbivores.

METHODS AND MATERIALS

Rearing of E. varivestis. Egg masses of the Mexican bean beetle (MBB) were removed from snapbean (*Phaseolus vulgaris* L.) plants on disks cut from leaves with a No. 8 cork borer. Masses on disks were sterilized for 10 min in Clorox® bleach–distilled H_2O (1:50), and then rinsed for 10 min in distilled H_2O. Egg masses on leaf disks were then placed on a dry filter paper in the bottom of a Petri dish which contained a water-wetted filter paper attached to the inside of the lid. Such eggs were held for three to four days at 25°C for hatching. Larvae were returned to vigorous snapbean plants growing within cages in 16-hr photoperiod, 27°C, and 65% relative humidity in a greenhouse. Feeding larvae and adults were provided daily with vigorous snapbean plants.

Growing of Soybean Plants. Relatively insect-resistant *G. max* PI 227687 plants were grown and studied. Seeds were germinated in moistened vermiculite in flats under the same environmental conditions as were used for the experimentation. Such conditions were: (1) 15-hr photophase, using Metalarc® high-intensity (1000-W), full-spectrum metal halide lighting, 27°C, and 65% relative humidity in a greenhouse or (2) 14-hr photophase with 12 hr of full light intensity, i.e., 300–500 $\mu E/m^2/sec$; day temperature, 27 ± 1°C, and night temperature, 20 ± 1°C; and relative humidity, 65 ± 5%, in the University of Wisconsin Biotron.

Germinated plants were transplanted at the first-leaf stage into individual plastic pots. In greenhouse experiments, plants were watered two times per day in a sterilized potting mixture (i.e., compost–soil–sand–vermiculite, 5:5:2:1). Plants in biotron studies were potted in a support medium and were provided with 30–40 ml of one-half-strength Hogland's nutrient solution (Hammer et al., 1978) four times per day (i.e., every 6 hr). All experiments involved plants between the V2 and V3 stages of development (Fehr and Caviness, 1977).

Mexican Bean Beetle Feeding Assays. The substrate utilized in feeding bioassays was an 18-mm-diameter leaf disk. Each disk was positioned with its abaxial side up. Assay insects were given a choice between two PI 227687 disks, each from a plant which had received a specific "stress" treatment, presented in an opposed arrangement in a Petri disk arena adapted from Norris and Baker (1967).

Only adult female *E. varivestis* beetles that were 2 weeks old and had been starved 24 hr, but were water-satiated, were used in bioassays. Two MBB females were released in each Petri dish arena. Assays were run for 22 hr in complete darkness at 24 ± 1°C and 60 ± 5% relative humidity.

Evaluation of beetle feeding in the bioassays involved measuring area (cm^2) of leaf disk eaten using a model LI-3100 Area Meter (LICOR, Inc., Lincoln, NE).

Estimation of Phenols. The used analytical method was based on techniques of Price and Butler (1977). Single fresh leaves, just removed from the plant, were weighed and then homogenized in 5 ml 60% methanol in a 10-ml ground-glass hand homogenizer. Homogenate plus 1 ml 60% methanol rinse was filtered through Whatman No. 1 paper in a glass funnel using vacuum. The approximate 6 ml of filtrate were added to 50 ml double-distilled (dd) H_2O in a 250-ml flask. Three milliliters of each of two reagents (i.e., A and B) were next added to the flask. Reagent A was 0.1 M $FeCl_3$ in 0.1 N HCl and reagent B was 0.008 M $K_3Fe(CN)_6$. The mixture was allowed to react for 10 min for color development. After 10 min, the optical density of samples was determined with a B&L Spectronic 20 at 720 nm. A blank of identical composition except for omission of the homogenate was analyzed and its reading at 720 nm was subtracted from all other sample readings.

Results were expressed as catechin equivalents (CEs), using a standard curve prepared daily from fresh solutions of commercial D-catechin. A catechin equivalent is the milligrams of catechin/100 mg of soybean leaf tissue that would be required to give the observed absorbance.

Assays for L-*Phenylalanine Ammonia-Lyase (PAL) and* L-*Tyrosine Ammonia-Lyase (TAL).* Extraction and assay of PAL from soybean tissue employed the method of Zucker (1965) as modified by Ciepiela (1984). A 1.5-g aliquot of dried acetone extractables from PI 227687 soybean leaves was homogenized in 10 ml of 0.1 M borate buffer, pH 8.8, using a glass homogenizer. The homogenate was centrifuged at 14,000 *g* for 15 min at 4°C. An aliquot (0.7 ml) of the resultant supernatant was mixed directly with 1 ml 0.03 M L-phenylalanine, 1 ml 0.1 M borate buffer, pH 8.8, and enough dd H_2O to bring the total volume to 3 ml. Incubation was 60 min in a 37°C water bath, and enzyme activity in such aliquot mixtures was then determined spectrophotometrically as the increase in absorbance at 290 nm. The enzymatic reaction in samples was killed by addition of 1 ml 1 N HCl. Specific enzyme activity was

expressed as units per milligram protein in the enzyme extract. One unit of enzyme activity equals the amount of PAL required to produce 1 μmol of *t*-cinnamic acid in 1 hr under specific conditions. Soluble protein content of samples was determined using the reagent and methods of Bradford (1976).

Extraction and assay of TAL were conducted similarly to the methods used for PAL, except that the substrate, L-tyrosine, is not dissolved completely in 0.1 M borate buffer, pH 8.8, so the reaction mixture must be continuously shaken at 37°C. At the completion of incubation, 1 ml 1 N HCl was added to kill the reaction and to clear the otherwise cloudy solution so required spectrophotometry could be readily performed. Activity of TAL was measured as the change in absorption at 333 nm.

RESULTS

Comparative Antiherbivory to Mexican Bean Beetle. Experimentally inflicted MBB herbivory on the middle leaflet of the second leaf (i.e., first trifoliolate leaf) of PI 227687 soybeans resulted in a significant ($P < 0.01$ or 0.05) reduction in subsequent MBB feeding on other leaflets of this second leaf at both 12 and 24 hr after the stress (i.e., MBB herbivory) treatment (Table 1). Systemic MBB antiherbivory effects of the experimentally inflicted MBB herbivory on PI 227687 second leaves were observed on the first (i.e., unifoliate) leaves ($P < 0.05$) at 48 hr after the stress treatment (Table 1). Other antiherbivory comparisons of first or second leaves between MBB herbivory-stressed

TABLE 1. COMPARATIVE MEXICAN BEAN BEETLE (MBB) FEEDING ON LEAF DISKS FROM LEAVES (OR LEAFLETS) ON PI 227687 PLANTS EXPERIENCING PRIOR MBB HERBIVORY OR NO HERBIVORY IN STANDARDIZED BIOASSAY

Hours after treatment	Leaf No.	Mean ± SD cm^2 eaten per leaf disk[a]	
		Herbivory stressed	No herbivory
12	2[b]	0.30 ± 0.1[c]	0.53 ± 0.2
24	2[b]	0.22 ± 0.04[d]	0.32 ± 0.02
48	1	0.21 ± 0.03[e]	0.33 ± 0.01

[a] Results from all other bioassays of leaves 1–3 at various times after treatment were not significantly different, $P < 0.10$, *t* test, $N = 4$.
[b] Significantly different feeding on "other" leaflets, *t* test, $N = 4$.
[c] Significantly different, $P < 0.01$.
[d] Significantly different, $P < 0.05$.
[e] Significantly different feeding, $P < 0.05$, *t* test, $N = 4$.

versus -unstressed PI 227687 at 12, 24, 48, or 72 hr gave no significant difference ($P < 0.10$) attributable to treatment.

Total Phenolic Content of Leaves. At 4 or 8 hr after MBB herbivory, no leaves of the stressed PI 227687 soybeans showed a significantly ($P < 0.05$) greater total phenolic content than corresponding leaves on nonstressed plants. However, by 12 hr after this stress treatment, the herbivory-wounded (i.e., fed on) middle leaflet of the first trifoliolate leaf contained a significantly ($P < 0.05$) larger amount of total phenolics (Table 2). At 24 hr after the stress treatment, this wounded (middle) leaflet of the first trifoliolate leaf still contained a significantly larger ($P < 0.05$) amount than was in such leaflets from unwounded PI 227687 plants (Table 2). By 48 hr after the stress treatment, the greater average CEs ($P < 0.05$) were found in the nonwounded (other) leaflets of the wounded second trifoliolate leaf and in the unwounded first (unifoliate) leaf. By 72 hr after the MBB herbivory, none of the leaves (i.e., 1-3) on the wounded (stressed) plants had significantly ($P < 0.05$) more total phenols than did corresponding leaflets or leaves from nonstressed PI 227687 soybean plants.

L-Phenylalanine Ammonia-Lyase (PAL) and L-Tyrosine Ammonia-Lyase (TAL) Activities. MBB herbivory on the middle leaflet [Leaf 2(M), Figures 2 and 4] of the first trifoliolate leaf of PI 227687 soybeans significantly ($P < 0.05$ or 0.01) increased levels (i.e., units per milligram protein) of both PAL and TAL in plants (Figures 1-4). The temporal pattern of activity for each enzyme also differed distinctly from that in the nonstressed plants. Major in-

TABLE 2. COMPARATIVE TOTAL PHENOL CONTENT IN PI 227687 SOYBEAN LEAVES AT INDICATED INTERVAL AFTER MBB HERBIVORY (TREATMENT) ON MIDDLE LEAFLET OF FIRST TRIFOLIOLATE LEAF OR IN LEAVES OF NONSTRESSED (CONTROL) PLANTS

Hours after treatment	Leaf No.	CE[a] (Mean ± SD per leaf)	
		Herbivory stressed	No herbivory
4	2[b]	0.58 ± 0.03[NS]	0.63 ± 0.05
8	2[b]	0.52 ± 0.04[NS]	0.54 ± 0.02
12	2[b]	0.83 ± 0.07**	0.58 ± 0.02
24	2[b]	1.82 ± 0.09**	0.63 ± 0.03
48	2[c]	1.76 ± 0.05**	0.88 ± 0.05
48	1	1.33 ± 0.09**	0.49 ± 0.03

[a] Results from all other assays of leaves 1-3 at various times after herbivory treatment were not significantly (NS) different from nonwounded controls, $P < 0.10$, t test, $N = 8$, ** significantly different at $P < 0.01$. A catechin equivalent (CE) is milligrams of catechin/100 mg of soybean leaf tissue that would be required to give the observed absorbance.
[b] Middle (wounded) leaflet on the first trifoliolate (second true) leaf.
[c] Other lateral (nonwounded) leaflet on the first trifoliolate leaf.

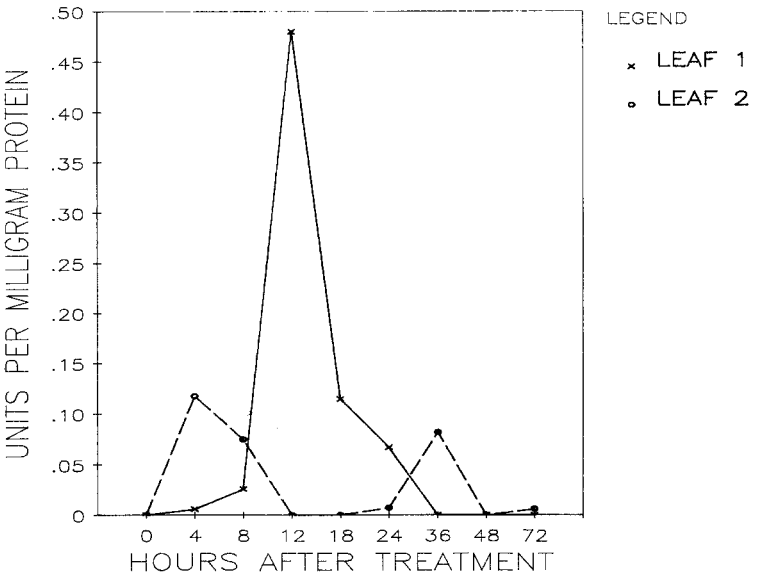

FIG. 1. L-Phenylalanine ammonia-lyase (PAL) activity in the indicated leaf of healthy (nonstressed) PI 227687 soybean plants, at the given time after treatment, growing in a biotron room.

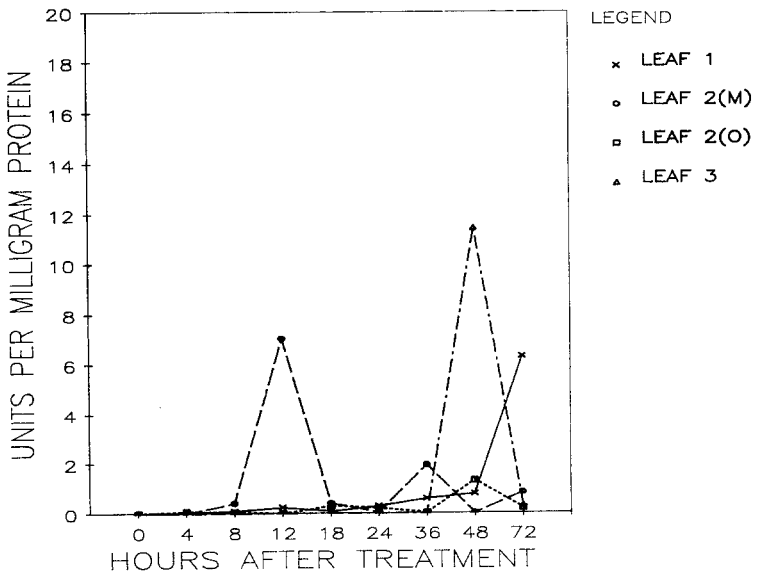

FIG. 2. L-Phenylalanine ammonia-lyase (PAL) activity in the indicated leaf, or leaflet, of experimentally stressed PI 227687 soybean plants, at the given time after the herbivory (stress) treatment, growing in a biotron room. LEAF 2(M) refers to the middle leaflet of the first trifoliolate leaf; and LEAF 2(O), to the outside (lateral) leaflet(s) of such a leaf.

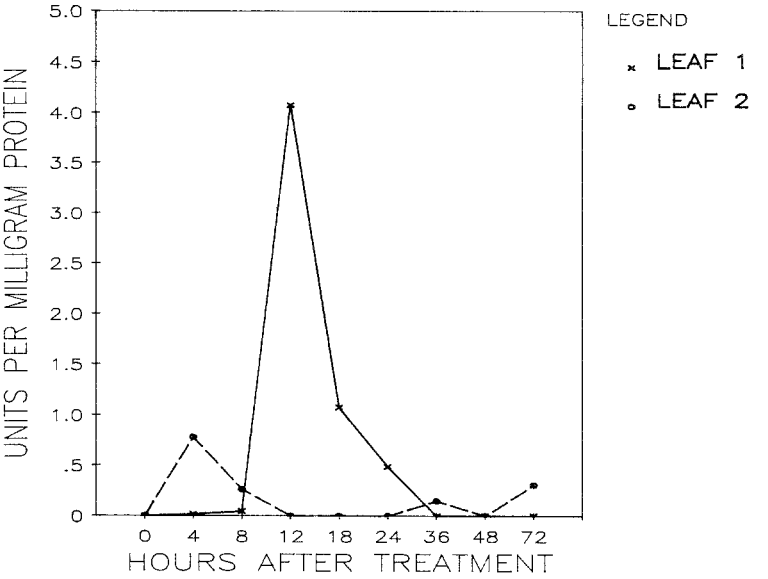

FIG. 3. L-Tyrosine ammonia-lyase (TAL) activity in the indicated leaf of healthy (non-stressed) PI 227687 soybean plants, at the given time after treatment, growing in a biotron room.

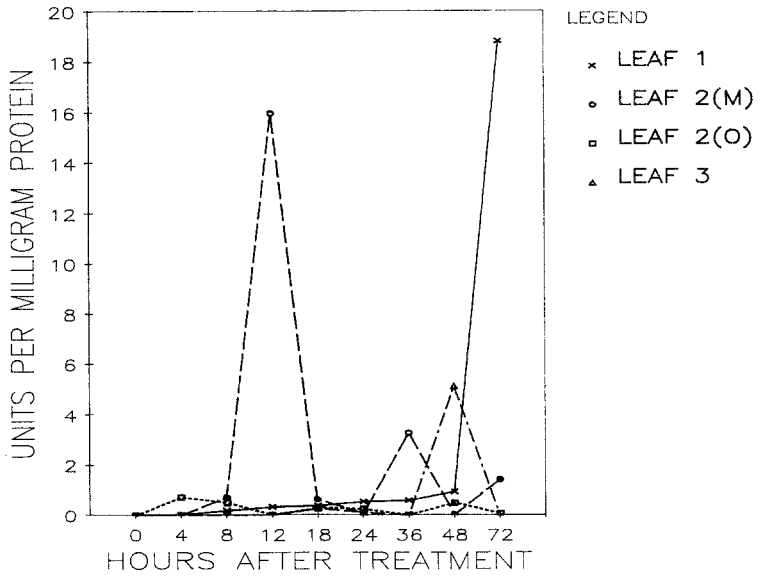

FIG. 4. L-Tyrosine ammonia-lyase (TAL) activity in the indicated leaf, or leaflet, of experimentally stressed PI 227687 soybean plants, at the given time after the herbivory (stress) treatment, growing in a biotron room. LEAF 2(M) and LEAF 2(O) have the same meanings as in Figure 2.

duced activity ($P < 0.01$) for each enzyme was limited to the "attacked" middle leaflet [Leaf 2(M), Figures 2 and 4] until 48 hr after the herbivory treatment. In each case, induced activity ($P < 0.01$) next appeared in leaf 3 (i.e., the youngest leaf present) (Figures 2 and 4). Activity of both enzymes increased ($P < 0.01$) markedly in the unifoliate leaf (i.e., the oldest leaf present) by 72 hr after treatment.

DISCUSSION

Stress (MBB herbivory) -induced higher levels of PI 227687 resistance to subsequent MBB feeding occurred first locally at or near the site of the initial stress (i.e., herbivory), which was the middle leaflet of the first trifoliolate leaf. This increased antiherbivory was positively correlated with an elevated level of total phenolics and temporally and quantitatively altered patterns of active PAL and TAL enzymes in such tissues. Such altered phenolic and enzyme levels occurred within 12 hr after the MBB herbivory. Systemic effects from the herbivory were evidenced as significant increases in PAL and TAL activities in leaf 3 by 48 hr after treatment. Increased antiherbivory activity and altered levels of total phenolics and active PAL and TAL enzymes in leaf 1 by 48 hr after the initial treatment provided further evidence of systemic effects.

Temporal aspects of the observed induced greater MBB resistance, including increases in the total phenolics and PAL and TAL activities, are compatible with the reported timetables of similar events associated with microorganismal induction of phytoalexins in soybeans (Sequeira, 1983; Darvill and Albersheim, 1984; Ebel et al., 1984).

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REFERENCES

BRADFORD, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

CHIANG, H.S., and NORRIS, D.M. 1985. Expression and stability of soybean resistance to agromyzid beanflies. *Insect Sci. Appl.* 6:265–270.

CHIANG, H.S., NORRIS, D.M., CIEPIELA, A., OOSTERWYK, A., SHAPIRO, P., and JACKSON, M. 1986. Comparative constitutive resistance in soybean lines to Mexican bean beetle. *Entomol. Exp. App.* 42:19–26.

CIEPIELA, A. 1984. Wpływ zerowania mszycy zbozowej (*Sitobion avenae* F.) na akumulacje i przemiany białek i aminokwasow w wybranych gatunkachj i odmianach zboz. Unpublished doctoral thesis. Szkoła Głowna Gospodarstwa Wiejskiego Akademia Rolnicza [SGGW-AR].

DARVILL, A.G., and ALBERSHEIM, P. 1984. Phytoalexins and their elicitors—a defense against microbial infection in plants. *Annu. Rev. Plant Physiol.* 35:243–275.

EBEL, J., SCHMIDT, W.E., and LOYAL, R. 1984. Phytoalexin synthesis in soybean cells: Elicitor induction of phenylalanine ammonia-lyase and chalcone synthase mRNAs and correlation with phytoalexin accumulation. *Arch. Biochem. Biophys.* 232:240–248.

FEHR, W.R., and CAVINESS, C.E. 1977. Stages of soybean development. Special Report 80. Coop. Ext. Serv. Agric. Home Econ. Exp. Stn. Iowa State University, Ames.

HAMMER, P.A., TIBBITTS, T.W., LAUGHANS, R.W. and MCFARLANE, J.C. 1978. Base-line growth studies of "Grand Rapids" lettuce in controlled environments. *J. Am. Soc. Hortic. Sci.* 103:649–655.

KOGAN, M., and PAXTON, J. 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.

NORRIS, D.M. and BAKER, J.E. 1967. Feeding responses of the beetle *Scolytus* to chemical stimuli in the bark of *Ulmus*. *J. Insect Physiol.* 13:955–962.

PRICE, M.L., and BUTLER, L.G. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. *J. Agric. Food Chem.* 25:1268–1273.

SEQUEIRA, L. 1983. Mechanisms of induced resistance in plants. *Annu. Rev. Microbiol.* 37:51–79.

ZUCKER, M. 1965. Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.* 40:779–785.