Inducible Phytoalexins in Juvenile Soybean Genotypes Predict Soybean Looper Resistance in the Fully Developed Plants[1]

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ABSTRACT

The hypocotyl of different soybean genotypes was tested for its inducible phytoalexin (i.e. glyceollin or coumestrol) accumulation and its inducible soybean looper resistance in response to chemical elicitation. A very highly insect-resistant soybean genotype (PI 227687) produced significantly more phytoalexins than a relatively insect-susceptible one (Davis) in response to the same chemical elicitation. The resultant standardized hypocotyl assay allowed quick categorization of unknown soybean genotypes regarding the level of insect resistance in the fully developed plants. Glyceollin was a better indicator of inducible resistance than coumestrol. Elicitor concentration influenced the amount of glyceollin and coumestrol accumulated. Younger seedlings (4–5 d old) responded stronger to chemical elicitation than did older ones (7–10 d old). The elicited accumulation of glyceollin showed a temporal pattern that peaked at 72 h. Accumulation of coumestrol showed a gradual increase. Elicitation of phytoalexins in juvenile soybean plants by sulfhydryl-binding reagents was found to be useful for the prediction of genotypic differences in the level of insect resistance in the fully developed plants.

The SBL[2] *Pseudoplusia includens* (Walker) is one of the more destructive insect pests of soybean, *Glycine max* (L.) Merr., in the southern United States and Central America (17, 26). Host-plant resistance is a highly useful component of an integrated management system for regulating this insect pest (27). Based on field-cage and greenhouse evaluations with fully developed plants, some soybean genotypes (e.g. PI 227687 and 229358) proved highly resistant and others (e.g. Davis and Centennial) proved more susceptible (19, 22, 23, 27).

Phytoalexins are important contributors to plant resistance to insects, pathogens, and nematodes (5, 6, 8–11, 20, 22, 28, 29). However, the specific roles of phytoalexins in soybean-SBL interactions have not been adequately investigated. In particular, the usefulness of phytoalexins in juvenile plants as a predictor of the SBL resistance level in the fully developed soybeans has not been studied. If juvenile plants could be used reliably to predict a genotype's level of insect resistance in fully developed plants, then important, improved efficiency in soybean breeding and selection may result. Regarding chemical elicitors, some classical sulfhydryl-binding reagents mimic insects in the induction of phytoalexin (e.g. glyceollin) accumulation in fully developed soybean plants (15, 16). Stössel (24, 25) also showed that sulfhydryl-binding reagents could induce glyceollin accumulation in soybean hypocotyls. Glyceollin is one of several isoflavonoids in legumes that have been confirmed as contributors to the plant's antixenosis and antibiosis to insects (5, 6, 20, 22).

In this study, we tested the hypothesis that phytoalexin accumulation in juvenile plants (hypocotyls) of soybean genotypes elicited with sulfhydryl-binding reagents could be used to predict the level of SBL resistance in fully developed plants of those genotypes. Juvenile plants of highly insect-resistant genotypes (e.g. PI 227687) would be expected to accumulate greater quantities of phytoalexins than more susceptible genotypes (e.g. Davis). We also studied the effects of several secondary experimental parameters on such inducible resistance in juvenile soybeans.

MATERIALS AND METHODS

Soybean Seeds

Seeds of *Glycine max* (L.) Merr., cv Davis, PI 227687 and six numerically coded lots were tested. Davis is relatively susceptible to foliar-feeding insects, and PI 227687 is highly resistant (3, 4, 12–14, 19, 20, 22, 23, 26, 27).

Chemical Elicitors

The sulfhydryl-binding reagents, IAA, PMBS, and $AgNO_3$, were obtained from Sigma Chemical Co. (St. Louis, MO). All elicitor solutions were freshly prepared for each experiment.

Chemical Elicitation of Soybean Hypocotyls

Soybean seeds were germinated in a flat of sterilized, moistened vermiculite in a Percival environmental chamber at 27 ± 2°C and 75 ± 5% RH in darkness (13). Five-day-old (or as otherwise described) seedlings were harvested and washed with distilled water to remove the vermiculite and then were arranged horizontally in a row of 15 seedlings on

[2] Abbreviations: SBL, soybean looper(s); PMBS, *p*-chloromercuriphenyl sulfonic acid; PI, plant introduction; HSD, honest significant difference.

an aluminum tray. The roots of each seedling were wrapped in a uniform-sized piece of moistened paper towel (Fort Howard Co., Green Bay, WI).

Different concentrations (0.001–100 mM in deionized distilled water) of the three chemicals were tested for elicitor activity on the hypocotyl of seedlings of Davis or PI 227687. Each hypocotyl was treated with a row of four (5 μL) droplets of elicitor (IAA, PMBS, or AgNO₃) at a given concentration or distilled water as the control, spaced at 0.3-cm intervals. Seedlings of different ages (e.g. 4, 5, 7, or 10 d old) were tested for chemical elicitation. The treated and control seedlings were then incubated in a Percival environmental chamber under the previously described conditions for a given time (48 h or as otherwise described) to allow induction of glyceollin and/or coumestrol. Each treatment was applied to five hypocotyls, and there were at least three replications. At the end of the incubation, the phytotoxic reaction on each hypocotyl was rated on a 0 to 5 scale (0, healthy; 5, completely wilted).

Glyceollin and Coumestrol Determination

Accumulation of glyceollin and coumestrol was measured as an indication of phytoalexin biosynthesis. Glyceollin determination was based on methods adapted from Stössel (24, 25) and Bhattacharyya and Ward (2). A 1.7-cm piece of the treated hypocotyl was excised from the treated or control seedling at the end of the incubation. Five such hypocotyl pieces were put into a glass test tube, mixed with 5 mL of 100% ethanol, and then immersed within the tube for 2 min in boiling water. The ethanol-soluble compounds, including isoflavonoids, were decanted, and the hypocotyl pieces were then extracted two more times with 2 mL of ethanol. The extracted tissues in each tube then were dried and weighed. All ethanol-soluble materials from one tube were combined and reduced to near dryness at 40°C in a rotary evaporator. The residues of the ethanol-soluble materials were extracted three times with 0.5 mL of ethyl acetate, and the solvent was removed to near dryness under nitrogen gas. These ethyl acetate-soluble compounds were then dissolved in 50 μL of ethyl acetate, and all 50 μL were applied to a TLC plate (silica gel, Sigma T-6270, 250 μm thick with 254-nm fluorescent indicator). Each TLC plate was developed in a benzene:methanol (95:8, v/v) solvent system. The resolved glyceollin was detected by cochromatography with authentic samples (obtained from Dr. N.T. Keen, University of California, Riverside, CA, and Dr. J. Ebel, Biologische Institut II der Universität, Freiburg in Briesgau, FRG) by characteristic fluorescence quenching under UV light (254 nm). The TLC bands containing glyceollin were scraped and then extracted with 3 mL of ethanol. The quantity of glyceollin (a mixture of three isomers) was determined from the absorption at 285 nm and the extinction coefficient (1). Coumestrol was determined on the same TLC plates used for the glyceollin analysis. The resolved coumestrol was identified by cochromatography with the authentic chemical (Sigma) and its characteristic bright blue fluorescence under UV light (366 nm). The coumestrol concentration on the TLC plates was determined with a TLC scanner (TLC SCAN II, CAMAG Scientific Inc., Wilmington, NC) under UV light (366 nm) with a CAT3 computing integrator software program. Thus, the quantity of coumestrol could be calculated from a regression equation that related coumestrol concentration to fluorescence.

Glyceollin and coumestrol concentrations were expressed as μg/mg dry weight of tissues.

Insect Dual-Choice Feeding-Preference Assays with Elicited versus Control Hypocotyls

The hypocotyls of 5-d-old seedlings (PI 227687 and Davis) were treated with a row of eight (4 μL) droplets of 1 mM PMBS or distilled water as control, spaced at 0.3-cm intervals. The treated and control seedlings were then incubated in a Percival environmental chamber under the above conditions for 48 h. The chemically elicited and water-control seedlings of each genotype then were washed with distilled water. The treated portion (about 3 cm) of each hypocotyl was excised, rinsed three times with distilled water, and blotted to remove the surface water with clean paper towels. The hypocotyls were weighed individually, and pairs (elicited versus control hypocotyls) of either PI 227687 or Davis were put in a Petri dish (9 cm) with a moistened filter paper. A newly molted fifth-instar SBL (from our laboratory colony maintained in a pinto bean-based artificial diet [21]) was prestarved for 2 h and then introduced into each Petri dish. The dual-choice assay was conducted in an environmental chamber at 25 ± 1°C, 85 ± 5% RH in darkness. Each assay lasted for 5 h. At the end of the assay, the insect was removed, and the weight of each remaining hypocotyl was recorded. The insect-consumed weight of each hypocotyl (elicited versus control) was calculated. Each experiment had 20 replicates and was repeated twice. Differences in feeding on elicited versus control hypocotyls of each soybean genotype were analyzed with a paired t test.

Elicitation of Inducible Phytoalexins in Coded Soybean Genotypes with 1 mM PMBS

Six soybean genotypes with previously determined (E.E.H., unpublished data) foliar-feeding insect-resistance levels were supplied by one of us (E.E.H.) to the other three coauthors for experimental use. Each genotype was identified only by a code number (code Nos. 1–6) written on the container bag. Seeds of each coded genotype were germinated as previously described. The seedlings were then treated with a row of four (5 μL) droplets of 1 mM PMBS or distilled water as control; the induced phytoalexins, glyceollin and coumestrol, were quantified as previously described. The induced glyceollin and coumestrol in the six coded genotypes were separately categorized by the HSD multiple range test (P ≤ 0.05), and those genotypic ratings were then compared with the previously determined level of foliar-feeding insect resistance (E.E.H., unpublished data) in fully developed plants of each genotype only after the hypocotyl assays were completed.

RESULTS

Elicitor Activity

The elicitor activity of the chemicals, IAA, PMBS, and AgNO3, was concentration dependent. The range of elicitor

concentrations that induced the larger quantities of phyto-alexins was about 0.5 to 10 mM (Fig. 1). AgNO₃ elicited both glyceollin and coumestrol accumulation in both PI 227687 and Davis soybean hypocotyls as compared with the non-elicited control (P < 0.05, Tukey's HSD multiple range test) (Fig. 2). PMBS induced glyceollin in both genotypes but elicited coumestrol only in PI 227687 (Fig. 2). IAA slightly induced glyceollin, but not coumestrol, accumulation in PI 227687 (Fig. 2) and did not induce the accumulation of either compound in Davis (Fig. 2). Compared to coumestrol, the induced glyceollin was almost 10-fold (one magnitude) greater in response to the same elicitor treatment (Fig. 2).

Temporal Pattern of Elicitation

In response to elicitation by the sulfhydryl-binding re-agents IAA and PMBS (both at 1 mM), glyceollin accumula-tion in Davis soybean hypocotyls significantly increased (P < 0.05, Tukey [HSD] pairwise comparisons) at 24 h, as compared to the quantity at zero time, and peaked (PMBS) or plateaued (IAA) at 72 h. PMBS induced a greater response than IAA (Fig. 3a). Coumestrol accumulated gradually in PMBS-elicited Davis hypocotyls; however, a significant increase (P < 0.05, HSD test) appeared only after 48 h (Fig. 3a).
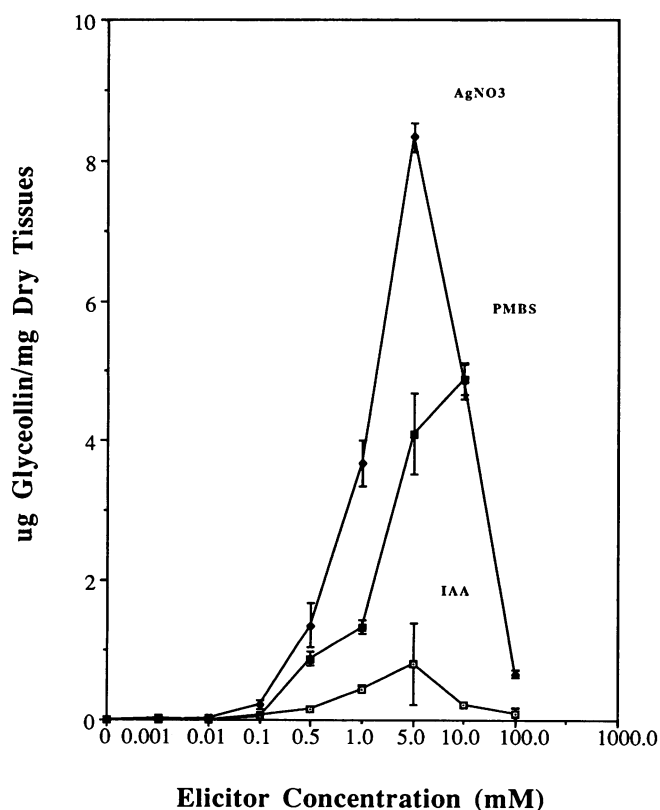

Figure 1. The effects of elicitors (IAA, PMBS, or AgNO₃) and their concentrations on glyceollin biosynthesis in Davis hypocotyls with a 48-h elicitation.

Effect of Seedling Age

Age influenced the response of Davis seedlings to chemical elicitation. Induced glyceollin or coumestrol concentrations were significantly higher (P < 0.05, Tukey [HSD] pairwise comparisons) in elicited 4- to 5-d-old versus 7- to 10-d-old seedlings. The regression of induced glyceollin concentration on seedling age was $y = 2.5518 - 0.27051x$, $r^2 = 0.802$ (Fig. 3b); the regression of induced coumestrol concentration was $y = 0.3456 - 0.03738x$, $r^2 = 0.833$. The glyceollin and coumestrol concentrations in control (nonelicited) seedlings of different ages remained quite stable at low levels (Fig. 3b).

Effect of Soybean Genotype

PI 227687 showed a greater (P < 0.05, HSD multiple range test) glyceollin and coumestrol inducibility than Davis in response to chemical elicitation (Fig. 2). Induced glyceollin differed (P < 0.01, t test) between the two soybean genotypes at all tested concentrations of PMBS or AgNO₃ and at 0.5 and 1 mM IAA (Fig. 2, left). The highly phytotoxic 5 mM AgNO₃ was the only treatment in which the glyceollin con-centration in Davis exceeded that in PI 227687. High concen-tration (5 mM) of IAA inhibited PI 227687 glyceollin accu-mulation as compared to the two lower (0.5 and 1 mM) amounts (Fig. 2, left). Induced coumestrol differed between the two genotypes only at 1 mM PMBS and 0.5 and 1 mM AgNO₃ (P < 0.05 or 0.01) (Fig. 2, right).

In the nonelicited controls, PI 227687 hypocotyls contained slightly more glyceollin (P < 0.01, t test), but less coumestrol (P < 0.05, t test), than those of Davis (Fig. 2).

Phytotoxic Reactions

The phytotoxicity in the hypocotyls depended on the chemical and its concentration (Table I). AgNO₃ caused the most severe phytotoxic symptoms, and PMBS caused the least. Higher concentrations caused the more severe phyto-toxic symptoms. Phytotoxic reactions of the two genotypes (Davis and PI 227687) to the same elicitor were quite similar except for 1 mM IAA (rating 3.0 versus 3.9) (Table I). How-ever, phytotoxic reaction showed a poor correlation with glyceollin or coumestrol accumulation.

Dual-Choice Preference Assay

SBL larvae fed less (P < 0.01, paired t test) on elicited versus control hypocotyls of PI 227687 (17.29 versus 28.54 mg). The insect feeding on elicited (26.94 mg) versus control (35.17 mg) hypocotyls of Davis was not significantly (P > 0.05, paired t test) different (Fig. 4).

Elicitation of Inducible Phytoalexins in Coded Soybean Genotypes

Based on the standardized elicitation of both glyceollin and coumestrol in seedlings of the six numerically coded soybean genotypes, three levels of hypocotyl phytoalexin accumula-tion and insect resistance were discerned (Table II). As com-pared to the controls, elicitation of the six soybean genotypes (code Nos. 1–6) yielded a significantly increased (P < 0.01,
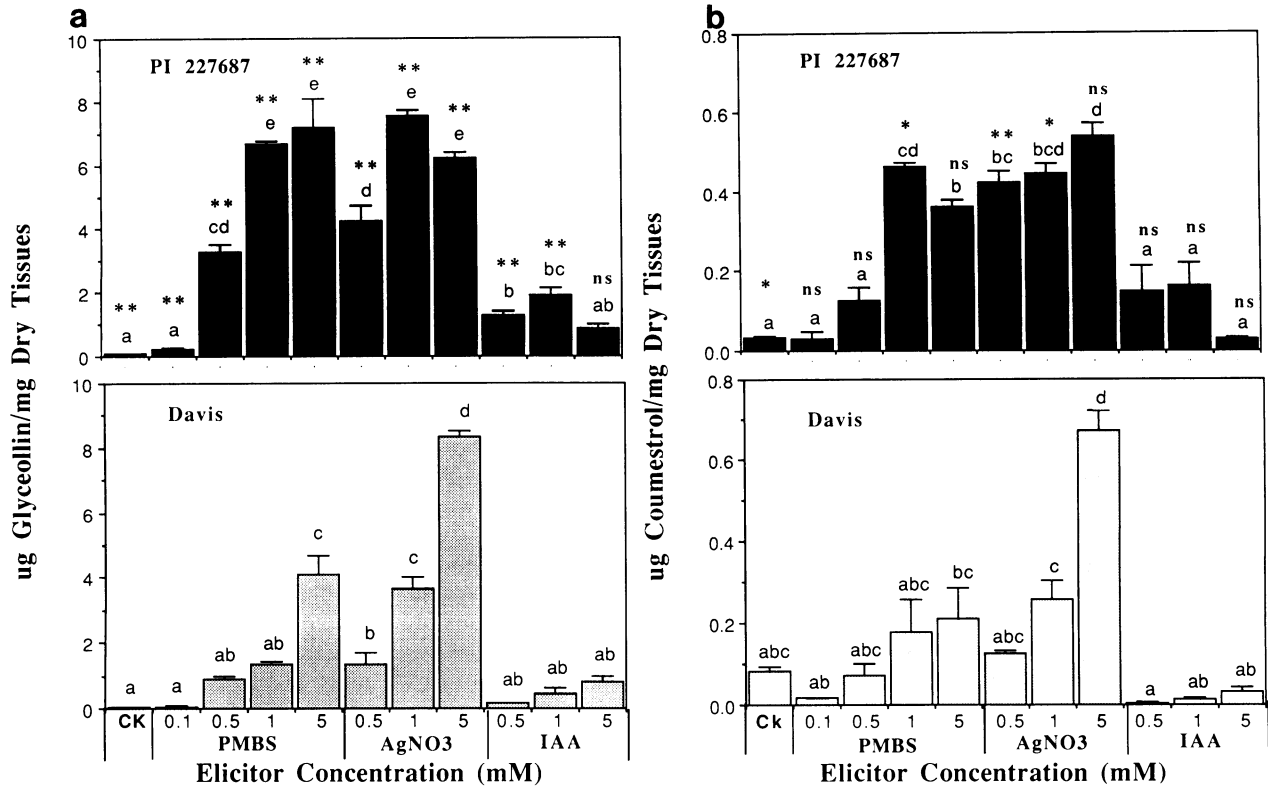
Figure 2. Comparison of phytoalexin (glyceollin [left] and coumestrol [right]) inducibility between two soybean genotypes (PI 227687 versus Davis) in response to a 48-h chemical elicitation. The effects of chemical elicitation on glyceollin (left) and coumestrol (right) inducibility in hypocotyls were analyzed. Results are means ± sE. CK is the nonchemical elicited control. For a given genotype, the bars not having the same letter on the top differ at P < 0.05 (Tukey [HSD] multiple range test). * and ** indicate that the mean values for the two genotypes differ at P < 0.05 and 0.01 (*t* test), respectively; NS, P > 0.05.

t test) glyceollin accumulation in all codes but a significantly increased (P < 0.01, *t* test) coumestrol accumulation only occurred in code Nos. 5 and 6 (Table II).

DISCUSSION

This study provided evidence that the amounts of glyceollin and coumestrol induced in juvenile plants of soybean genotypes by elicitation with sulfhydryl-binding reagents can be used to predict a level of foliar-feeding insect resistance in the fully developed plants. The amounts of glyceollin and coumestrol in elicited versus control hypocotyls allowed classification of resistance to foliar-feeding insects as relatively susceptible (includes Hartwig's "S" and "IM"), highly resistant (Hartwig's "HR"), and very highly resistant (Hartwig's "VHR"). The demonstrated effectiveness of this hypocotyl assay may allow plant breeders to reduce significantly the time required for evaluation of genotypes.

The observed amounts of glyceollin and coumestrol in juvenile plants of PI 171451 did not account for its Hartwig rating (i.e. field resistant) regarding SBL feeding based on field-cage studies (E.E.H., unpublished data) but did agree with its Hartwig rating (i.e. laboratory susceptible) based on excised-leaf feeding conducted in the laboratory. These differing results from field-cage and laboratory studies suggest that different chemicals (e.g. volatiles versus nonvolatiles

[18]) dominate the insect behavior in field-cage versus laboratory assays, respectively. Our results indicate that PI 171451 lacks the phytoalexin-based (e.g. glyceollin) resistance to insect feeding, and such a lack could explain why PI 171451 is susceptible in the Hartwig laboratory assays.

Another example of how our findings from the hypocotyl assays may contribute to an improved understanding of the chemical bases of soybean genotypic differences in insect (i.e. stress) resistance involves our results for D75–10169 and PI 229358. D75–10169 (7) was developed as an improved agronomic type having PI 229358 as its known source of resistance to foliar-feeding insects. Feeding studies in field cages in which plants were exposed to a heavy population of SBL suggested to Hartwig that D75–10169 had a slightly higher level of resistance than PI 229358. In our hypocotyl assays, D75–10169 also showed a greater phytoalexin (e.g. glyceollin and coumestrol) inducibility than did PI 229358. The greater phytoalexin accumulation in D75–10169 could explain Hartwig's observed higher level of resistance to SBL feeding compared to PI 229358. Our results indicate that D75–10169 apparently not only inherited the insect resistance of PI 229358 but also obtained additional resistance through the crossing process.

The levels of induced glyceollin and coumestrol in a given soybean hypocotyl depended on seedling age, the chemical
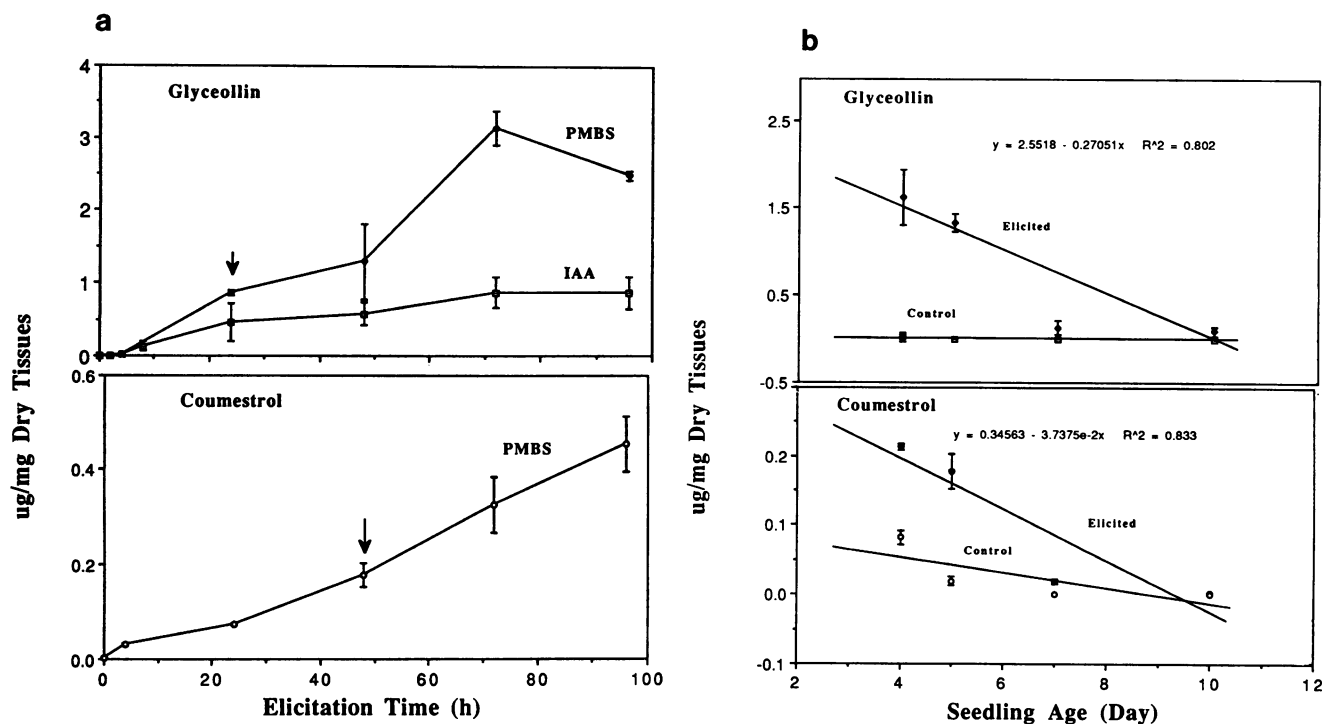
Figure 3. a, Temporal patterns of glyceollin and coumestrol biosynthesis in Davis soybean hypocotyls after elicitor (1 mM IAA or PMBS) treatment. Arrow indicates a significant increase (P < 0.05, HSD test) of glyceollin or coumestrol at that time as compared to the quantity at time (0). b, The effects of Davis seedling age (d) on glyceollin and coumestrol biosynthesis with a 48-h elicitation.

elicitor, its concentration, and the elicitation time. Plant age proved to be a major factor when evaluating inducible resistance in juvenile plants. Our results regarding the desirable plant age support those of Stössel (25) who found that younger (4–5 d old) soybean seedlings accumulated more glyceollin than did 7-d-old or older plants exposed to the same elicitor.

The efficiency of chemicals as elicitors differs significantly even among the tested sulfhydryl-binding reagents, IAA, PMBS, and AgNO₃, which are confirmed inducers of phytoalexins, especially glyceollin, in soybean (15, 16, 25). The order of efficiency of these chemicals as elicitors of glyceollin and coumestrol in Davis hypocotyls was $AgNO_3$ > PMBS >> IAA, but in PI 227687 hypocotyls the order was $AgNO_3$ ≥ PMBS >> IAA. In the present study, PMBS showed both relatively low phytotoxicity and highly efficient induction of glyceollin and coumestrol accumulations as compared to the low elicitation by IAA or the high phytotoxicity of $AgNO_3$.

Table I. Phytotoxic Reaction[a] by Davis and PI 227687 Soybean Seedlings 48 h after Treatment with the Indicated Elicitor at the Given Concentration

Concentration	IAA		PMBS		AgNO₃	
	Davis	PI 227687[b]	Davis	PI 227687	Davis	PI 227687
mM						
0.001	0		0.13		0	
0.01	1.25		1.0		2.0	
0.1	2.0		1.75	1.55	2.75	
0.5	2.6	2.1	2.53	2.35	3.0	3.25
1.0	3.0	3.9	2.93	3.05	4.0	3.95
5.0	4.5	4.75	3.1	3.27	5.0	5.0
10.0	5.0		3.55		5.0	
100.0	5.0				5.0	

[a] Scale of phytotoxic reactions is 0 to 5: 0, healthy; 1, very slight spot just on the treated area, no color change; 2, small spot with a slight color change; 3, brown spot with a slight indentation in the hypocotyl surface; 4, severe brown spot with a cavity half-way through the hypocotyl; 5, complete wilt. The values are means of 15 replicates. [b] PI 227687 was only tested with a selected range of elicitor concentrations.
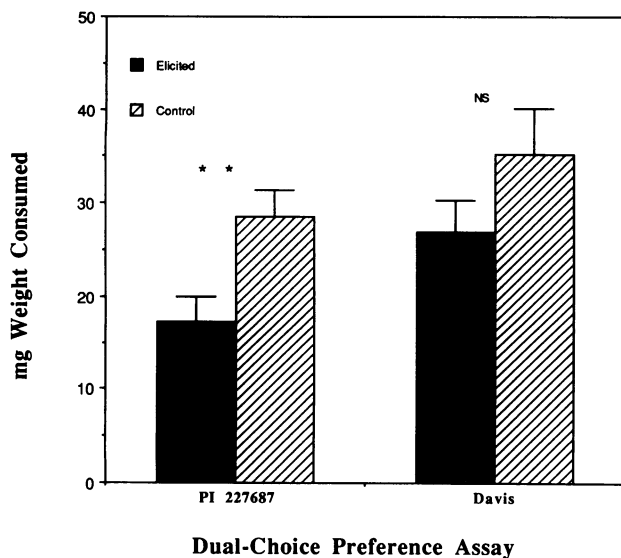
Dual-Choice Preference Assay

Figure 4. Mean weights (± SE; n = 40) of hypocotyls consumed by fifth-instar SBL in a dual-choice assay (elicited versus control). ** indicates that the values differ at P < 0.01 (paired t test); NS, P > 0.05.

Increasing the concentration of a given elicitor increased the phytotoxic reaction but did not necessarily induce a higher glyceollin or coumestrol accumulation. Glyceollin or coumestrol accumulation did, however, increase with the given elicitor concentration up to a certain level (e.g. 5 mM). Above such a level, glyceollin or coumestrol accumulation was inhibited, but phytotoxicity, including cell and tissue death, was increased. For instance, glyceollin accumulation in Davis hypocotyls increased as IAA or $AgNO_3$ concentration was increased from 0 to 5 mM but then dramatically decreased as the elicitor concentration was increased from 5 to 100 mM (Fig. 1). At lower elicitor concentrations that may cause lower phytotoxic reactions, plants may maintain active biosynthesis or metabolism. At higher elicitor concentrations cell or tissue death may advance so rapidly that net glyceollin synthesis is reduced. Lower elicitor concentrations that cause lower phytotoxicity (i.e. PMBS ≤ 5 mM; $AgNO_3$ or IAA ≤ 1 mM), thus, are preferred in such chemical elicitation.

The glyceollin accumulation showed a characteristic temporal pattern for each elicitor. Our observed "time-to-peak" glyceollin accumulation differed from that observed by Stössel (24) (72 versus 48 h, respectively). This difference probably was due to different soybean genotypes, treatment methods, and/or elicitors. In our study, a significant increase in the amount of glyceollin or coumestrol (P < 0.05, HSD test) compared to that at zero time appeared in soybean hypocotyls only after 24 or 48 h, respectively; thus, a stand-

Table II. Effects of 1 mM PMBS Elicitation on Soybean Genotypic Glyceollin and Coumestrol Content in Hypocotyls at 48 h

Code No.	Mean ± SE (Control)[a]		Hartwig Description of Resistance[c]	Name of Genotypes[c]
	Glyceollin[b]	Coumestrol[b]		
	μg/mg of dry tissue			
1	0.532 ± 0.010* (0.043 ± 0.005)	0.020 ± 0.003* (0.007 ± 0.001)	S	Centennial
2	0.608 ± 0.016* (0.032 ± 0.001)	0.015 ± 0.002* (0.011 ± 0.002)	IM	Tracy-M
3	0.424 ± 0.013* (0.033 ± 0.002)	0.015 ± 0.003* (0.010 ± 0.001)	FR + LS	PI 171451
4	1.283 ± 0.082† (0.049 ± 0.006)	0.062 ± 0.011† (0.095 ± 0.005)	HR	PI 229358
5	2.610 ± 0.028‡ (0.030 ± 0.005)	0.209 ± 0.004‡ (0.057 ± 0.004)	HR-VHR	D75-10169
6	2.718 ± 0.137‡ (0.081 ± 0.007)	0.183 ± 0.005‡ (0.033 ± 0.003)	VHR	PI 227687

[a] Mean value from three replicates with SE. Values in the same column not followed by the same symbol differ at P < 0.05, HSD test. The mean value and SE for nonelicited control are in parentheses.   [b] As compared to nonelicited controls, all six of the treated coded genotypes (code Nos. 1–6) showed a significant (P < 0.01, t test) increase in glyceollin, but only codes 5 and 6 had a significant (P < 0.01, t test) increase in coumestrol.   [c] An independent description of the level of insect resistance in fully developed plants of the genotype based on field-cage or greenhouse evaluation by Dr. E.E. Hartwig, personal communication, 1991: S, susceptible; IM, intermediate; FR + LS, resistant in the field-cage, but susceptible in laboratory feeding assays; HR, highly resistant; HR-VHR, highly resistant to very highly resistant; VHR, very highly resistant. The name identification of each soybean genotype and Dr. Hartwig's description of its resistance level were received from him only after our hypocotyl analysis was completed.

ardized measurement for inducible glyceollin and coumestrol might be feasible at 48 h.

The results of the dual-choice feeding-preference assays demonstrate that the inducible resistance was significantly different between soybean genotypes. Such differences apparently were correlated with the accumulation of inducible phytoalexins.

The use of a sulfhydryl-binding-reagent elicitor on juvenile plants to predict quickly the level of fully developed soybean plant resistance to insects and other environmental stresses seems feasible. The usefulness of the technique may be enhanced by further refinements that improve the ability to distinguish smaller differences in resistance.

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