The Role of Phytoalexins in the Resistance of Soybean Leaves to Bacteria: Effect of Glyphosate on Glyceollin Accumulation

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ABSTRACT

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The herbicide glyphosate (*N*-phosphonomethyl glycine) inhibited glyceollin accumulation in hypersensitive soybean leaves inoculated with an incompatible race of *Pseudomonas syringae* pv. *glycinea*. Aminooxyacetic acid, aminooxyphenylpropionic acid, and benzyloxycarbonyl aminooxyphenylpropionic acid all reported to inhibit phenylpropanoid biosynthesis in other plants, were not effective inhibitors of glyceollin production in soybean leaves. Glyophosate has been shown to inhibit the conversion of shikimate to chorismate in other plants and this mechanism of action may explain the inhibition of glyceollin accumulation in soybean. Significantly, glyphosate did not block hypersensitive host cell necrosis in leaves inoculated with incompatible bacteria. Incompatible bacterial-cell populations in resistant leaves treated with glyphosate were twofold higher than in untreated leaves, but compatible-cell populations were eightfold higher than incompatible-cell populations in glyphosate-treated leaves. Thus, inhibition of glyceollin accumulation by glyphosate only partially prevented the expression of resistance to bacteria. Pretreatment of plants with phenylalanine and tyrosine, however, restored glyceollin accumulation in glyphosate-treated plants and resulted in complete resistance expression to incompatible bacteria. The results suggest that glyceollin accumulation is a component, but not the only mechanism, of resistance to bacteria in hypersensitive soybean leaves.

Phytoalexin accumulation can be inhibited by treating inoculated plants with transcription or translation inhibitors, and these have been used to assess the role of phytoalexins in the resistance of plants to incompatible pathogens (8,17,25,26). In soybean leaves, the translation inhibitor blasticidin S blocked glyceollin accumulation and hypersensitive host cell death and caused normally incompatible races of *Pseudomonas syringae* pv. *glycinea* to become compatible in resistant soybean cultivars (17). Evidence obtained from the use of transcription or translation inhibitors is inconclusive, however, because such compounds may be expected to block all metabolic events requiring de novo protein synthesis. To more critically test the possible association of phytoalexin production with the resistance of soybean leaves to bacteria, we examined several compounds reported to selectively inhibit phenylpropanoid biosynthesis but not primary plant metabolism.

Aminooxyacetic acid (AOA), aminooxyphenylpropionic acid (AOPP) and benzyloxycarbonyl aminooxyphenylpropionic acid (N-BOC-AOPP) have been reported to inhibit the enzyme phenylalanine ammonia-lyase in several plants (3,4,21). The

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herbicide, glyphosate (*N*-phosphonomethyl glycine), has been reported to inhibit aromatic amino acid synthesis in several species (2,5,10,15). Since synthesis of the isoflavonoid phytoalexin, glyceollin, depends on the availability of phenylpropanoid precursors, the above compounds were tested for ability to inhibit glyceollin accumulation and resistance expression in soybean leaves. A preliminary communication has been published (12).

MATERIALS AND METHODS

Phenylalanine, tyrosine, and AOA were obtained from Sigma Chemical Co., St. Louis, MO 63178. Samples of AOPP and N-BOC-AOPP were kindly supplied by M. Legrand (Institute de Biologie Moleculaire et Cellulaire, du CNRS, Strasbourg, France), and N. Amrhein (Ruhr-Universität, Bochum, Germany) or synthesized according to the method of Briggs and Morley (7). Glyphosate (96.5%) was a gift from E. G. Jaworski (Monsanto, St. Louis, MO 63166).

Soybean (*Glycine max* (L.) Merr. 'Harosoy' plants unless otherwise stated) were grown and bacteria were cultured and maintained as described previously (16). Some bacterial isolates were grown on minimal agar medium containing 40.0 mM K_2HPO_4, 11.0 mM KH_2PO_4, 0.4 mM $MgSO_4$, 7.57 mM $(NH_4)_2SO_4$, 0.125 M glucose, 15 g of agar (Difco) per liter. A complete medium contained the same ingredients plus casamino acids and yeast extract (17).

Primary leaves of 12-day-old soybean plants were inoculated as described before (14) with a hand sprayer; approximately 60% of the surface area of each leaf was infiltrated with water suspensions of bacteria at 8×10^7 cells per milliliter. The average leaf surface area was 9.0 cm^2 and average leaf fresh weight was 0.11 g. Soybean leaves were similarly infiltrated with 1 mM sodium iodoacetate (IOA) (17).

Plants were treated with inhibitors by placing cut epicotyls into 0.5 mM sodium phosphate buffer, pH 6.85, containing the appropriate concentration of each test compound. Treatments were performed 4 hr after infiltration of leaves with bacteria or IOA, when all water-soaking had disappeared from the leaf tissue. In some experiments with glyphosate, uninoculated plants were pretreated with phenylalanine or tyrosine or both amino acids at 500 μg/ml each in 0.1 $\times$ nutrient solution (11), pH 7.0. In such cases, cut epicotyls of 12-day-old plants were placed into the amino acid solution or nutrient solution alone for 2 days before infiltration with bacteria. Four hours after infiltration, plants were placed in nutrient solution alone, nutrient solution containing amino acids and glyphosate, or nutrient solution containing glyphosate only.

Bacterial populations in soybean leaves were estimated by rinsing weighed leaves with distilled water and grinding at full speed in a Sorvall omnimixer for 30 sec with 100 ml of sterile distilled water. The extracts were stirred for 10 min, filtered through fluted filter paper, and the filtrates were plated using standard serial dilution techniques onto complete agar medium (17). In early experiments, bacterial populations were estimated on complete agar medium alone. However, to ensure that only populations of the inoculated bacteria were estimated in soybean leaves, glyphosate-resistant or kasugamycin-resistant mutants of *P. syringae* pv. *glycinea* were infiltrated into leaves and subsequent plate counts were made on minimal agar medium supplemented with 1.0 mg glyphosate per milliliter, or complete agar medium (17) supplemented with 70 μg kasugamycin (Sigma) per milliliter, respectively. Resistant mutants were obtained by plating 10^8–10^9 wild-type bacterial cells onto glyphosate-supplemented minimal agar medium or kasugamycin-supplemented complete agar medium and selecting spontaneously resistant colonies. Single colony isolates were reselected several times on glyphosate or kasugamycin media and tested in soybean plants to ensure that mutants gave the same plant reactions as the wild types.

Glyceollin was extracted from soybean leaves by the facilitated diffusion technique and quantitated by tlc-UV spectrometry (16).

Microscopic cell death was observed by using a fluorescent vital staining technique (13).

RESULTS

Glyceollin accumulation in IOA-infiltrated leaves was not inhibited by treating plants with either AOPP or N-BOC-AOPP; to the contrary, at some concentrations these compounds appeared to stimulate glyceollin accumulation (Table 1). Glyceollin accumulation elicited by IOA was inhibited 64% in leaves of plants treated with 10 μg of AOA per milliliter. Higher concentrations of AOA were not tested because uninoculated control plants treated with 50 μg of AOA per milliliter collapsed after 3 days. Glyceollin accumulation elicited by IOA was inhibited 82% in leaves of plants treated with 10 μg of glyphosate per milliliter (Table 1) and no detrimental effects were observed in control plants treated only with this concentration of glyphosate. Control plants exhibited slight leaf chlorosis after 3 days of treatment with 50 μg of glyphosate per milliliter; therefore, 10 μg/ml treatments were used for the remainder of the study.

Glyceollin accumulation in leaves inoculated with incompatible race-1 bacteria was not inhibited by 10 μg of AOA per milliliter, but was inhibited 90% by 10 μg of glyphosate per milliliter (Table 2). Although glyceollin accumulation was inhibited, glyphosate did not affect bacterially induced hypersensitive leaf-cell necrosis. Fluorescent vital staining of leaf tissue that responded incompatibly (13) indicated that microscopic cell death, first observed at 10 hr after inoculation as small groups of dead cells, occurred both in glyphosate-treated and untreated plants. Macroscopically visible cell necrosis, apparent 24 hr after inoculation, was also similar in leaves of treated and untreated plants. However, the dark discoloration normally associated with hypersensitive necrosis was absent in glyphosate-treated leaves inoculated with incompatible race-1 bacteria.

Cell populations of incompatible *P. syringae* pv. *glycinea* race-1 were twofold higher after 72 hr in leaves of glyphosate-treated plants than in those of untreated plants (Table 3). In contrast,

TABLE 1. Glyceollin accumulation in soybean leaves infiltrated with the abiotic elicitor IOA and subsequently treated with glyphosate, AOA, AOPP, or N-BOC-AOPP

Treatment[a]	Inhibitor concentration (μg ml^{-1})	Glyceollin[b] (μg/g fresh wt)
None (buffer control)	...	1,000
Glyphosate	10	180
AOA	10	360
AOPP	10	1,070
AOPP	50	1,115
N-BOC-AOPP	10	1,380
N-BOC-AOPP	50	975

[a]Compounds dissolved in 0.5 mM phosphate buffer, pH 6.85, were fed through cut epicotyls 4 hr after infiltration with IOA (1 mM).
[b]Leaves were extracted 72 hr after infiltration with IOA; results represent means of two repeated experiments corrected for the estimated glyceollin content of water-infiltrated control leaves (~20 μg/g).

TABLE 2. Glyceollin accumulation in soybean leaves infiltrated with incompatible *Pseudomonas syringae* pv. *glycinea* race 1[a] and subsequently treated with glyphosate or AOA

Treatment[b]	Glyceollin[c] (μg/g fresh wt)	
	48 hr after inoculation	72 hr after inoculation
None (buffer control)	715	805
AOA	605	810
Glyphosate	80	75

[a]Inoculum adjusted to 0.1 $A_{500\ nm}$ (~8×10^7 colony-forming units per milliliter).
[b]Ten μg/ml concentrations of each compound in 0.5 mM phosphate buffer, pH 6.85, were fed through cut epicotyls 4 hr after infiltration with bacteria.
[c]Results represent means of two separate experiments corrected for the estimated glyceollin content of water-infiltrated control leaves (~20 μg/g).

compatible race-2 cell populations were twofold lower in leaves of glyphosate-treated than in those of untreated plants. Race-2 bacterial populations were 33-fold higher than race-1 populations in untreated leaves, but only eightfold higher than race-1 populations in leaves of glyphosate-treated plants (Table 3).

Cell populations of *P. syringae* pv. *glycinea* were also estimated in glyphosate-treated or untreated leaves of soybean cultivar Acme, which is compatible to both race 1 and race 2. At 72 hr after inoculation, race-2 cell populations were slightly higher than race-1 cell populations in treated and untreated cultivar Acme leaves (Table 4). However, these data indicated that the multiplication of both races was inhibited similarly in glyphosate-treated leaves of cultivar Acme compared to the untreated controls.

P. syringae pv. *glycinea* race 1 or race 2 did not grow on minimal agar medium supplemented with 1.0 mg glyphosate per milliliter. Neither race was inhibited, however, when the bacteria were grown on complete, casamino acids-supplemented agar medium (17) containing 1.0 mg glyphosate per milliliter. Populations of glyphosate-resistant mutants of both races were similar to wild-type bacteria in glyphosate-treated or untreated leaves (Table 3).

Pretreatment of plants with phenylalanine and tyrosine for 2 days before inoculation resulted in a 70% reversal of glyphosate-inhibited glyceollin accumulation in leaves infiltrated with incompatible race-1 bacteria (Table 5). Pretreatment with phenylalanine alone or both amino acids at the time of inoculation with race-1 also reversed inhibition, but less substantially. All other treatments resulted in less reversal of inhibition; tyrosine supplied at the time of inoculation was the least effective. Populations of race-1 bacterial cells in leaves of plants pretreated with phenylalanine and tyrosine and subsequently treated with glyphosate were similar to those in leaves of untreated plants or plants treated with amino acids alone when estimated 72 hr after inoculation (Table 6). As observed in the previous experiment (Table 3), race-1 cell populations in leaves of plants treated only with glyphosate were about twofold higher than those for all other treatments shown in Table 5.

TABLE 3. Cell populations of *Pseudomonas syringae* pv. *glycinea* race 1 or 2 in inoculated leaves of cultivar Harosoy soybean plants treated with buffer or glyphosate

Bacterial isolate[w]	Treatment[x]	Cfu[y] per leaf ($\times 10^7$)	Cfu per leaf $\log_{10}$ conversion[z]
Race 2	B	341	9.5326 a
Race 2 *gly*	B	319	9.5036 a
Race 2	G	184	9.2647 b
Race 2 *gly*	G	121	9.0821 b
Race 1	B	9.68	7.9861 c
Race 1 *gly*	B	7.30	7.8634 c
Race 1	G	23.2	8.3656 d
Race 1 *gly*	G	22.1	8.3446 d

[w] Race 2, compatible; race 1, incompatible; *gly*, glyphosate-resistant mutants that multiply on minimal medium containing 1.0 mg/ml glyphosate.
[x] Treatments were performed 4 hr after inoculation by placing cut epicotyls in 0.5 mM phosphate buffer, pH 6.85 (B), or in 10 μg of glyphosate per milliliter in buffer (G).
[y] Cfu = colony-forming units. Cell populations were estimated in leaves 72 hr after inoculation as described in Materials and Methods. Results represent means of three separate experiments with two replicates each; each replicate consisted of four leaves.
[z] Means with the same letter were not significantly different, $P = 0.01$, according to Duncan's multiple range test.

TABLE 4. Cell populations of *Pseudomonas syringae* pv. *glycinea* race 1 or 2 in inoculated leaves of compatible cultivar Acme soybean plants treated with buffer or glyphosate

Inoculum	Treatment[a]	Cfu[b] per leaf ($\times 10^7$)
Race 1	B	289
	G	206
Race 2	B	438
	G	303

[a] Treatments were performed 4 hr after inoculation by placing cut epicotyls in 0.5 mM phosphate buffer, pH 6.85 (B), or in 10 μg of glyphosate per milliliter in buffer (G).
[b] Cfu = colony-forming units. Cell populations were estimated in leaves 72 hr after inoculation as described in Materials and Methods. Results represent means of three separate experiments with two replicates each; each replicate consisted of four leaves.

DISCUSSION

The abiotic phytoalexin elicitor, IOA (17), was used in initial inhibitor studies with soybean leaves to exclude possible effects of test compounds on the metabolism of phytoalexin-eliciting bacteria. Glyphosate and AOA significantly inhibited glyceollin accumulation in response to IOA, but only glyphosate inhibited glyceollin accumulation in response to the incompatible race-1 bacteria (Tables 1 and 2). The ineffectiveness of AOA in this case is unexplained, but may have been due to metabolism of AOA by the bacteria, different mechanisms of phytoalexin inhibition by AOA and glyphosate, or different mechanisms of phytoalexin elicitation by IOA and incompatible bacteria.

Jaworski (15) proposed that the herbicidal activity of glyphosate was due to inhibition of aromatic amino acid biosynthesis because growth inhibition of *Lemmna gibba* and *Rhizobium japonicum* by

TABLE 5. The effect of pretreatment with phenylalanine, tyrosine, or both on glyphosate inhibition of glyceollin accumulation in soybean leaves infiltrated with incompatible *Pseudomonas syringae* pv. *glycinea* race 1

		Glyceollin[c] (μg/g fresh wt)	
Pretreatment[a]	Treatment[b]	48 hr after inoculation	72 hr after inoculation
NS	NS	705	800
NS	Glyphosate	80	75
NS	Tyr+glyphosate	125	135
NS	Phe+glyphosate	140	230
NS	Phe+Tyr+glyphosate	160	290
Tyr	Tyr+glyphosate	135	170
Phe	Phe+glyphosate	230	300
Phe+Tyr	Phe+Tyr+glyphosate	225	510

[a] Plants were fed amino acids (500 μg/ml) in nutrient solution, or nutrient solution alone (NS) through cut epicotyls for 48 hr prior to infiltration with bacteria; Phe = phenylalanine; Tyr = tyrosine.
[b] Plants were fed amino acids (500 μg/ml) and/or glyphosate (10 μg/ml) in nutrient solution, or nutrient solution alone (NS) through cut epicotyls 4 hr after infiltration with bacteria; Tyr = tyrosine; Phe = phenylalanine.
[c] Results represent means of two repeated experiments corrected for the glyceollin content of water-infiltrated control leaves (~20 μg/g).

TABLE 6. Cell populations of *Pseudomonas syringae* pv. *glycinea* race 1 in inoculated leaves of cultivar Harosoy soybean plants pretreated with phenylalanine (Phe) and tyrosine (Tyr), or nutrient solution, and subsequently treated with phenylalanine and tyrosine and/or glyphosate, or nutrient solution alone

Pretreatment[w]	Treatment[x]	Cfu[y] per leaf ($\times 10^7$)	Cfu per leaf[z] $\log_{10}$ conversion
NS	Glyphosate	19.2	8.2839 a
Phe+Tyr	Phe+Tyr+glyphosate	8.9	7.9496 b
Phe+Tyr	Phe+Tyr	9.3	7.9682 b
NS	NS	9.1	7.9606 b

[w] Plants were fed amino acids (500 μg/ml) in nutrient solution, or nutrient solution alone (NS) through cut epicotyls for 48 hr prior to infiltration with bacteria.
[x] Plants were fed amino acids and/or glyphosate (10 μg/ml) in nutrient solution, or nutrient solution alone (NS) 4 hr after infiltration with bacteria.
[y] Cfu = colony-forming units. Results represent means of three separate experiments with two replicates each; each replicate consisted of four leaves.
[z] Means with same letter were not significantly different, $P = 0.01$, according to Duncan's multiple range test.

glyphosate was overcome by the addition of aromatic amino acids. Amrhein et al (2) recently demonstrated that glyphosate inhibited the conversion of shikimate to chorismate in buckwheat hypocotyls and cultured cells of *Galium mollugo* L. Amrhein et al (5) also provided evidence indicating that glyphosate inhibits the enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Glyphosate may have acted similarly in soybean leaves, thereby reducing phenylpropanoid pool sizes and consequently inhibiting the biosynthesis of the isoflavonoid phytoalexins. The observed reversal of glyphosate-inhibited glyceollin accumulation in plants pretreated with phenylalamine and tyrosine supported this contention (Table 5).

Populations of incompatible *P. syringae* pv. *glycinea* race 1 were twofold higher in glyphosate-treated leaves than in the untreated controls (Table 3). However, this increased bacterial multiplication was not observed in glyphosate-treated leaves when the ability to accumulate glyceollin was restored by pretreating plants with phenylalanine and tyrosine. These results give some support to previous data that indicate glyceollin accumulation may in part be causally related to the expression of resistance in soybean leaves to incompatible bacteria (14,16,17,19). However, inhibition of glyceollin accumulation by glyphosate did not result in a complete reversal of plant reaction from incompatible to compatible; compatible race-2 cell populations were still eightfold higher in glyphosate-treated leaves and 15-fold higher in untreated leaves than were race-1 cell populations in glyphosate-treated leaves.

The interpretation of data obtained by using biosynthetic inhibitors depends on the selectivity of the inhibitor employed. Since glyphosate has been reported to inhibit bacterial multiplication as well as plant metabolism (10,15), the toxicity of glyphosate to *P. syringae* pv. *glycinea* was examined. Bacterial growth was completely inhibited on minimal medium containing glyphosate, but was unaffected by the herbicide when the medium was supplemented with casamino acids. Therefore, as observed in cultures of *Escherichia coli* (10), growth inhibition on minimal medium may have been due to the inhibition of aromatic amino acid biosynthesis by glyphosate. Glyphosate also inhibited bacterial growth in planta as indicated by the twofold reduction in compatible race-2 cell populations in glyphosate-treated leaves. Because multiplication of a glyphosate resistant race-2 isolate was also similarly reduced in glyphosate-treated leaves (Table 3), it was concluded that the growth inhibition was not due to direct glyphosate toxicity but to some nonspecific effect of glyphosate on the compatible response. The nature of this effect is unknown, but may involve disruption of compatible host or pathogen physiology by means other than inhibition of phenylpropanoid metabolism (6).

The observed inhibition of compatible bacteria in glyphosate-treated leaves (Tables 3 and 4) suggested that the in planta growth of incompatible bacteria may also have been affected by glyphosate. Furthermore, if incompatible race-1 bacteria were inherently more sensitive than race-2 bacteria to the glyphosate effects in planta, this might explain why race-1 bacteria did not cause a completely compatible response in cultivar Harosoy when glyceollin accumulation was inhibited by glyphosate. This possibility seems unlikely, however, because race-1 and race-2 bacteria were inhibited similarly in glyphosate-treated, compatible cultivar Acme leaves (Table 4), whereas incompatible race-1 bacterial cell populations were eightfold lower than compatible race-2 populations in glyphosate-treated leaves of cultivar Harosoy. Therefore, it appears probable that some other mechanism(s), in addition to glyceollin accumulation, must in part account for the resistance observed in incompatible leaves treated with glyphosate.

It is significant in this regard that hypersensitive host-cell necrosis proceeded normally in glyphosate-treated inoculated leaves. This raises the possibility that host cell death per se may be associated with reduced bacterial multiplication, especially in the case of the confluent hypersensitive necrosis studied here, in which a large proportion of leaf tissue becomes necrotic (13). It would be of interest in future work to monitor bacterial populations in glyphosate-treated leaves inoculated with low concentrations of incompatible bacteria that produce only microscopically visible areas of host cell necrosis (13). This approach should eliminate the possible adverse effects of massive host cell necrosis on the growth of incompatible bacteria and more closely resemble the interactions between leaves and bacteria in nature.

The translation inhibitor blasticidin S blocked glyceollin accumulation and hypersensitive host-cell death in soybean leaves (13,17), therefore indicating that both processes require protein synthesis. Our experiments demonstrate that they are distinct biochemical processes, however, because glyphosate blocked glyceollin accumulation, but not hypersensitive cell necrosis. This indicates that glyceollin accumulation was not the cause of host-cell death in soybean leaves as suggested by reports that phytoalexins are toxic to the host cells that produce them (9,20,23,24). To the contrary, our data support the possibility that host-cell death may normally function as a signal for the initiation of phytoalexin production in soybean leaves.

Certain herbicides predispose plants to disease (1), and this has been attributed to inhibition of host defenses in some cases (22). The fact that phytoalexin accumulation in soybean leaves was inhibited by sublethal doses of glyphosate raises the possibility that increased disease severity could occur with some crop plants due to the presence of low levels of residual herbicides.

The finding that glyphosate inhibits isoflavonoid phytoalexin accumulation, but not hypersensitive cell death, in soybean may provide a means to more critically test the role of phytoalexins in other host-pathogen systems. For example, inhibition of glyceollin accumulation by glyphosate rendered genetically resistant soybean hypocotyls susceptible to *Phytophthora megasperma* f. sp. *glycinea* (18). It might be useful to search for other inhibitors that block specific enzymatic steps in phytoalexin biosynthesis, since they would produce results less ambiguous than those resulting from the use of general metabolic inhibitors (8,17,26,27).

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