Effects of Glyphosate on Glyceollin Production and the Expression of Resistance to *Phytophthora megasperma* f. sp. *glycinea* in Soybean

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ABSTRACT

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The phenylalanine ammonia-lyase inhibitors aminooxyacetate and aminooxyphenyl propionate did not block resistance expression or glyceollin accumulation in soybean hypocotyls inoculated with an incompatible race of *Phytophthora megasperma* f. sp. *glycinea*. However, low, nonphytotoxic concentrations of the herbicide glyphosate (*N*-phosphonomethyl glycine) effectively inhibited both processes. The results support other data indicating that glyceollin accumulation accounts for the expression of resistance to incompatible races of the fungus.

Additional key words: herbicides, phenylpropanoid pathway, phytoalexin.

Chemical inhibitors are useful for assessing the involvement of targeted metabolic steps in certain physiological processes. For firm conclusions to be reached, however, a high degree of inhibitor specificity is desirable. The induced production of the phytoalexin glyceollin after infection of soybean tissues by several incompatible plant pathogens appears to be involved in the expression of disease resistance (5–7,14). One line of evidence leading to this conclusion is the observation that inhibitors of transcription or protein synthesis in plant cells block production of the phytoalexin by otherwise resistant plants, which thus become susceptible (8,14). It is difficult to ascertain, however, whether the observed physiologic response is due solely to inhibition of glyceollin production or to other processes that also may require primary plant metabolism. Inhibitors that more specifically affect glyceollin production, but not primary metabolism, would therefore be useful to more thoroughly assess the role of glyceollin in disease resistance.

In this paper we report that low, nonphytotoxic concentrations of the herbicide glyphosate (*N*-phosphonomethyl glycine) are potent inhibitors of glyceollin accumulation in soybean hypocotyls and concomitantly block the expression of resistance to an incompatible race of *Phytophthora megasperma* f. sp. *glycinea*. A preliminary communication (3) has been published.

MATERIALS AND METHODS

Soybean (*Glycine max* (L.) Merr. 'Harosoy 63') plants were grown in 10-cm-diameter pots in lighted growth chambers as previously described (9). In some experiments, the near-isogenic cultivar Harosoy was used. Six-day-old seedlings were excised at the soil line and the cut ends of five seedlings were placed into each of several 4.0-ml vials containing water or various concentrations of aqueous chemical solutions adjusted to pH 6–7. The cotyledons and upper portion of the hypocotyls were above the vials. In some experiments, the chemicals were prefed for 24 or 48 hr before the plants were inoculated, but generally the plants were inoculated immediately after excision. The hypocotyls were wounded 1 cm below the cotyledons with a razor blade, care being taken to ensure that the wounds were superficial and did not extend to the vascular tissue. The epidermal wounds (~1 cm long) were inoculated with mycelial fragments of races 1 (incompatible) or 7 (compatible) of *P. megasperma* f. sp. *glycinea*. The fungi were grown on V-8 juice broth for 48 hr prior to inoculation, and inoculated plants were incubated at 22 C under glass jars in a water-saturated atmosphere in a lighted growth chamber. Seedlings were harvested at intervals for determination of disease reaction, fungus growth, and glyceollin content.

Chemicals supplied to the excised seedlings were obtained as follows: aminooxyacetic acid (AOA), phenylalanine, and tyrosine were from Sigma Chemical Co., St. Louis, MO 63178; aminooxyphenyl propionate (AOPP) synthesized according to Briggs and Morley (2) was obtained from N. Amrhein and M. Legrand; and glyphosate (96.5%) was a gift from E. Jaworski, Monsanto Chemical Co., St. Louis, MO 63166.

Fungus growth in inoculated seedlings was determined by staining freehand sections with 0.2% rose bengal according to Yoshikawa et al (14). Glyceollin was extracted from 'whole hypocotyl' segments or excised inoculation areas with 95% ethanol and the mixture of three naturally occurring isomers was quantitated by TLC-UV spectrometry (14). Inoculation sites were excised with razor blades, and tissue sections ~0.5 mm thick were cut parallel to the inoculated surface. Sections from 35–40 plants were weighed and pooled for glyceollin extraction.

Glyphosate was dissolved to 3.3 mg·ml^{-1} in 50% ethanol and aliquots were added to standard petri plates along with sufficient 50% ethanol only to make 300 μl total solvent per plate. Ten milliliters of molten V-8 juice agar were immediately added to the plates and the contents were thoroughly mixed before solidification occurred. Then plugs of mycelium of races 1 or 7 of *P. megasperma* f. sp. *glycinea* were placed in the center of the plates and the cultures were incubated at 25 C. Colony diameters were determined daily for 6 days to assess fungal growth rates.

Experiments were performed at least three times and representative data are shown.

RESULTS

Excised Harosoy 63 soybean seedlings placed in water and inoculated with incompatible race 1 of *P. megasperma* f. sp. *glycinea* produced an incompatible reaction similar to that observed by inoculation of intact seedlings, characterized by considerable host cell death and pigmentation at the inoculation site coupled with high accumulation of glyceollin (Table 1).

Incubation of seedlings with various concentrations of AOA up to 1 mg·ml⁻¹ did not reverse the expression of resistance. Aminooxyphenyl propionate at 400 μg·ml⁻¹ led to an intermediate plant reaction with variable degrees of fungus colonization of individual plants but considerable host cell necrosis, pigmentation, and glyceollin accumulation (Table 1). However, neither chemical completely blocked resistance expression or glyceollin accumulation at any concentration tested.

Glyphosate supplied to excised seedlings at ≥4 μg·ml⁻¹ completely blocked the expression of resistance to race 1 of the fungus after 48 hr, and these plants exhibited disease symptoms and glyceollin levels similar to plants inoculated with compatible race 7 (Table 1). At 1.0 and 0.2 μg·ml⁻¹, glyphosate led to intermediate plant reactions and glyceollin accumulation, and variable numbers of these plants exhibited slight to severe rotting symptoms. At 0.04 μg·ml⁻¹ or less, glyphosate led to incompatible plant reactions, but glyceollin accumulation was somewhat reduced relative to the water controls inoculated with the incompatible race. Significantly, glyphosate concentrations up to 15 μg·ml⁻¹ in the vials containing cut seedlings did not produce detectable phytotoxicity during the 48 hr in which the experiments were conducted.

Administration of phenylalanine and tyrosine (400 μg·ml⁻¹ each) with glyphosate (10 μg·ml⁻¹) at the time of inoculation with race 1 resulted in a completely compatible host reaction and glyceollin levels similar to inoculated plants of the genetically compatible cultivar Harosoy. However, prefeeding the aromatic amino acids for 24–48 hr before plants were supplied glyphosate and inoculated led to a completely incompatible plant reaction and high glyceollin accumulation occurred (Table 1).

When glyphosate (15 μg·ml⁻¹) was supplied to inoculated, excised seedlings at 6 hr or less after inoculation, completely compatible plant reactions were observed after 48 hr and glyceollin levels were very low (Table 2). Administration of glyphosate at 10–23 hr led to progressively higher glyceollin levels at 48 hr postinoculation, but these plants exhibited intermediate disease symptoms varying from slight to considerable water-soaking and rotting.

Microscopic measurement was employed to critically assess the development of the fungus in inoculated hypocotyls (Fig. 1). Confirming the work of Yoshikawa et al (14), growth of race 1 was identical until 8 hr after inoculation in both cultivar Harosoy and the incompatible cultivar Harosoy 63. Growth in the resistant plants supplied only water ceased at about 9–12 hr, but

TABLE 1. Effects of aminooxyphenyl propionate and glyphosate on plant reaction and glyceollin production by excised soybean hypocotyls inoculated with race 1 of *Phytophthora megasperma* f. sp. *glycinea*

Treatment[a]	Plant reaction[b]	Glyceollin[c] (μg·g⁻¹ fresh weight)	
		Exp 1	Exp 2
Water control	None	10	25
Water + race 1	I	1,240	1,300
Water + race 7	C	220	···
AOPP 400 μg·ml⁻¹	Int	1,360	1,050
Glyphosate control 10 μg·ml⁻¹	None	20	10
Glyphosate 10 μg·ml⁻¹	C	220	130
Glyphosate 4 μg·ml⁻¹	C	440	420
Glyphosate 1 μg·ml⁻¹	Int	1,540	1,180
Glyphosate 0.2 μg·ml⁻¹	Int	590	780
Glyphosate 0.04 μg·ml⁻¹	I	920	960
Glyphosate 10 μg·ml⁻¹+phe+tyr[d]	I	1,370	1,150

[a] Excised Harosoy 63 seedlings were inoculated with race 1 in all cases except those stated and placed into the noted solutions; water and glyphosate control plants were wounded but not inoculated and incubated as the experimental plants; AOPP = aminooxyphenyl propionic acid.
[b] Plant reactions were rated at 48 hr after inoculation; I = incompatible, C = compatible, and Int = intermediate reaction; none = no visible plant response.
[c] Glyceollin extracted from excised whole hypocotyls at 48 hr after inoculation.
[d] Phenylalanine (phe) and tyrosine (tyr) (each at 400 μg·ml⁻¹ in water) were fed to excised seedlings for 48 hr in the growth chamber before the seedlings were inoculated and supplied glyphosate in the presence of the same concentrations of amino acids.

TABLE 2. Effects of glyphosate supplied to inoculated soybean seedlings at various times after inoculation with race 1 of *Phytophthora megasperma* f. sp. *glycinea*

Time glyphosate supplied (hr)[a]	Plant reaction[b]	Glyceollin[c] (μg·g⁻¹ fresh weight)
Uninoculated control	None	20
0	C	120
6	C	160
10	Int	865
14	Int	1,030
23	Int	1,745
Control—no glyphosate	I	1,780

[a] Excised Harosoy 63 plants were wounded and inoculated with race 1 and placed in water, then transferred to vials containing glyphosate at 15 μg·ml⁻¹ at the noted times. The uninoculated control was incubated only with water.
[b] Plant reactions were read at 51 hr after inoculation. Reactions are as in Table 1.
[c] Glyceollin was extracted from whole hypocotyls 47 hr after inoculation.
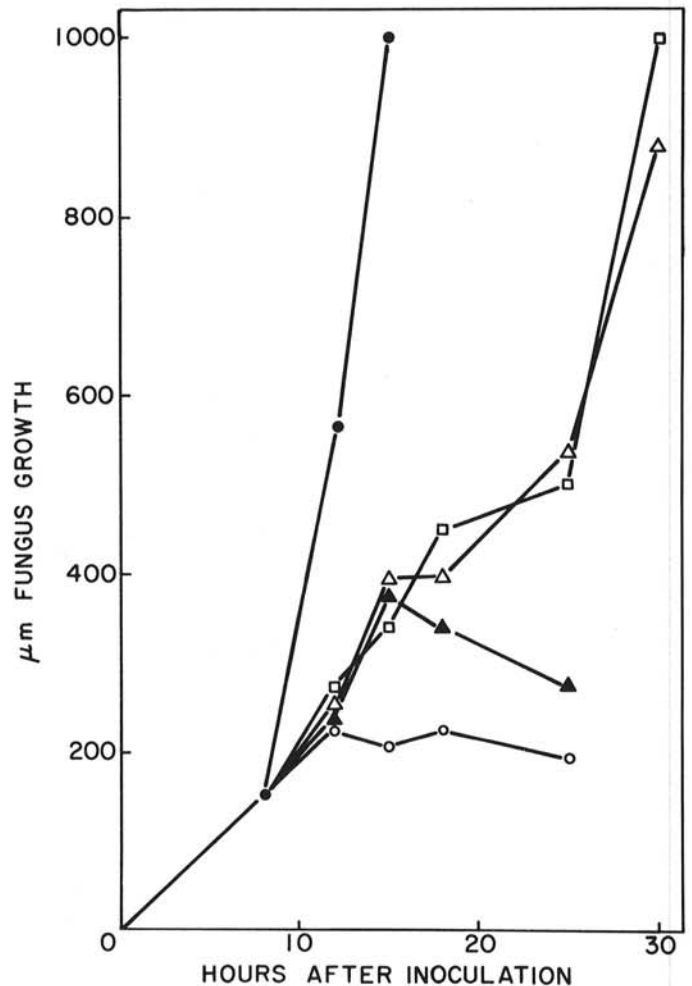

Fig. 1. Growth of race 1 *Phytophthora megasperma* f. sp. *glycinea* into hypocotyls of Harosoy (compatible) or Harosoy 63 (incompatible) soybean plants in the presence or absence of glyphosate. (●——●), cultivar Harosoy supplied only water; (○——○) cultivar Harosoy 63 supplied only water; (□——□) cultivar Harosoy 63 supplied glyphosate (10 μg·ml⁻¹) at the time of inoculation; (▲——▲) Harosoy 63 seedlings prefed phenylalanine and tyrosine (400 μg·ml⁻¹ each) for 24 hr before inoculation and introduction of glyphosate (10 μg·ml⁻¹) and phenylalanine and tyrosine (400 μg·ml⁻¹ each); (△ —— △) Harosoy 63 seedlings prefed water only for 24 hr before inoculating and supplying glyphosate at 10 μg·ml⁻¹.

colonization of the susceptible cultivar continued rapidly until the entire hypocotyl was colonized after ~24 hr. Fungus growth in Harosoy 63 hypocotyls treated with glyphosate was inhibited at 9–12 hr, but resumed at about 18 hr to a rate similar to that in the susceptible cultivar. Therefore, it appeared that resistance expression was not completely abolished by glyphosate within a short period after inoculation. Plants pretreated with phenylalanine and tyrosine before inoculation and glyphosate treatment exhibited restricted fungus growth similar to control plants pretreated with water (Fig. 1). Similar results to those in Fig. 1 were obtained when cultivar Harosoy 63 plants were inoculated with race 7 as a genetically compatible control (*unpublished*).

To determine whether glyphosate inhibited the production of glyceollin within a short period after inoculation, excised inoculation sites were extracted and glyceollin was quantitated (Table 3). Confirming the data of Yoshikawa et al (14), Harosoy 63 plants inoculated with incompatible race 1 and incubated with water contained much higher glyceollin levels at 9–11 hr than did those inoculated with compatible race 7. Interestingly, however, glyphosate did not greatly inhibit glyceollin accumulation at these early stages in response to race 1 (Table 3).

When incorporated into V-8 juice agar medium up to 100 $\mu g \cdot ml^{-1}$, glyphosate did not significantly inhibit the growth of races 1 or 7 of *P. megasperma* f. sp. *glycinea*. In a typical experiment, the average diameter of colonies of race 1 in the presence of 0, 10, and 100 $\mu g \cdot ml^{-1}$ glyphosate was 7.3, 7.8, and 7.7 cm, respectively, after 4 days.

DISCUSSION

Glyphosate was a potent inhibitor of glyceollin accumulation and resistance expression in soybean plants inoculated with *P. megasperma* f. sp. *glycinea,* but had no detectable effects on fungus growth in culture at 25 times the concentration required to entirely break resistance expression. Its effects, therefore, were concluded to be entirely on plant metabolism. Significantly, the relatively low levels of glyphosate used in the experiments did not produce visible phytotoxicity symptoms on the excised soybean seedlings for at least 3 days after treatment.

Amrhein et al (1) showed that glyphosate is a specific and potent inhibitor of the higher plant conversion of shikimate to chorismate and that it therefore leads to a reduction in phenylalanine biosynthesis. This mode of action is consistent with our observed inhibition of glyceollin accumulation; phenylalanine is required for its synthesis (10), but we have not confirmed this by monitoring phenylalanine levels in glyphosate-treated plants. However, phenylalanine antagonism as a mode of action for glyphosate is supported by our observation that prefeeding phenylalanine and tyrosine to glyphosate-treated plants restored both glyceollin accumulation and resistance expression (Table 1, Fig. 1). This was also noted for glyphosate-inhibited phytoalexin production in soybean leaves inoculated with *Pseudomonas syringae* pv. *glycinea* (4). Therefore, glyphosate may be a useful inhibitor of resistance mediated by phytoalexin production in other plants that utilize the phenylpropanoid pathway.

Glyphosate is a more specific inhibitor of glyceollin accumulation than previously used agents that affect primary plant metabolism (8,14). Since it has been shown to function as a phenylpropanoid pathway inhibitor, however, protein synthesis would also be expected to be eventually inhibited by glyphosate due to decreased phenylalanine supply. Thus, although our experiments with glyphosate are consistent with the role of glyceollin in the expression of resistance to *P. megasperma* f. sp. *glycinea,* the possibility that other unknown defense mechanisms requiring protein synthesis or phenolic precursors may also have been affected by glyphosate cannot be discarded. Inhibitors affecting phenolic biosynthesis after the phenylalanine step would circumvent the first of these possibilities, but we have not found suitable agents of this type. The phenylalanine ammonia-lyase inhibitors AOA and AOPP partially blocked resistance to TMV in tobacco (11) and *Puccinia coronata* var. *avenae* in oat leaves (Mayama and Tani, *personal communication*), but neither chemical efficiently inhibited glyceollin accumulation or resistance expression in soybean hypocotyls (Table 1) or leaves (3). Whether this was due to insensitivity of soybean phenylalanine ammonialyase or to lack of uptake and/or translocation of the inhibitors to infection sites in hypocotyls is unknown.

Several observations from the glyphosate experiments are relevant to interpreting the role of glyceollin in resistance expression to *P. megasperma* f. sp. *glycinea*. First, colonization by the incompatible fungus race was initially restricted in glyphosate-treated plants, but growth then resumed at a rate similar to that in a genetically susceptible cultivar (Fig. 1). Second, plants accumulated significant amounts of glyceollin when glyphosate was applied at 10 or more hours after inoculation, but some of the treated plants subsequently developed disease symptoms considered to be intermediate in reaction type (Table 2). Finally, glyceollin production at the localized infection site was not severely inhibited by glyphosate at short periods after inoculation (Table 3). Accordingly, it is presumed that endogenous phenylalanine pools in the hypocotyls were sufficient to permit early, but not subsequent, glyceollin accumulation. These observations indicate that the initial burst of glyceollin production at the infection site inhibits growth of the incompatible fungus at about 9 hr, as noted by Yoshikawa et al (14). However, a few incompatible hyphae may escape this inhibition zone and penetrate more deeply into the plant tissue, as observed by Stössel et al (12), where they would be expected to initiate a second round of glyceollin accumulation. However, we have shown that such secondary production of glyceollin does not occur in the presence of glyphosate (Table 1); this may explain the initial inhibition followed by a resumption of pathogen growth in glyphosate-treated hypocotyls (Fig. 1, Table 2).

Some authors have been concerned about the observation that pathogen growth may occur under the proper experimental conditions in plant tissues that contain high bulk amounts of phytoalexins (13). For instance, in our experiments, glyceollin levels were very high when glyphosate was supplied to inoculated plants at 14 and 23 hr after inoculation with incompatible race 1 (Table 2) and when glyphosate was supplied initially at 1 $\mu g \cdot ml^{-1}$ (Table 1), but the fungus nevertheless resumed growth in some but not all of the plants. Therefore, it appears that one or more fungus hyphae may escape from the relatively high local concentrations of the phytoalexin, providing that subsequent glyceollin production in adjacent host tissue is blocked by glyphosate. This interpretation, coupled with the observations of Yoshikawa et al (14), emphasizes the need to critically assess localized phytoalexin concentrations in plant tissues colonized by advancing hyphal tips in order to adequately interpret their role in resistance expression.

TABLE 3. Glyceollin levels in excised hypocotyl tissues after treatment of seedlings with water or glyphosate and inoculation with either races 1 or 7 of *Phytophthora megasperma* f. sp. *glycinea*

Treatment[a]	Glyceollin ($\mu g \cdot g^{-1}$ fresh weight)[b]		
	Exp 1	Exp 2	Exp 3
Wounded-incubated with water	20	⋯	10
Race 1-incubated with water	350	114	240
Race 1-incubated with glyphosate	290	102	210
Race 7-incubated with water	50	22	10

[a] Harosoy 63 seedlings were excised at the soil level, wounded and inoculated where indicated with race 1 (incompatible) or race 7 (compatible) of *P. megasperma* f. sp. *glycinea*. The plants were incubated in vials containing either water or glyphosate at 15 $\mu g \cdot ml^{-1}$.

[b] Excised inoculation sites were prepared at 11 hr (experiment 1), 9 hr (experiment 2) and 10 hr (experiment 3) after inoculation; 35–40 plants were used for each experimental determination.

LITERATURE CITED

1. Amrhein, N., Deus, B., Gehrke, P., and Steinrücken, H. C. 1980. The site of the inhibition of the shikimate pathway by glyphosate. II. Interference of glyphosate with chorismate formation *in vivo* and *in vitro*. Plant Physiol. 66:830-834.

2. Briggs, M. T., and Morley, J. S. 1979. Polypeptides. 17. Aminooxy-analogues of aspartame and gastrin C-terminal tetrapeptide amide. Pages 2138–2143 in: J. Chem. Soc. Perkin Trans. 1. Am. Chem. Soc., Washington, DC.

3. Holliday, M. J., and Keen, N. T. 1981. The role of phytoalexins in resistance of soybean to bacteria and fungi—Inhibition of glyceollin synthesis by glyphosate. (Abstr.) Phytopathology 71:881.

4. Holliday, M. J., and Keen, N. T. 1982. The role of phytoalexins in the resistance of soybean leaves to bacteria: Effect of glyphosate on glyceollin accumulation. Phytopathology 72:1470-1474.

5. Kaplan, D. T., Keen, N. T., and Thomason, I. J. 1980. Association of glyceollin with the incompatible response of soybean roots to *Meloidogyne incognita*. Physiol. Plant Pathol. 16:309-318.

6. Keen, N. T. 1978. Phytoalexins: Efficient extraction from leaves by a facilitated diffusion technique. Phytopathology 68:1237-1239.

7. Keen, N. T., and Bruegger, B. B. 1977. Phytoalexins and chemicals that elicit their production in plants. Pages 1–20 in: Host Plant Resistance to Pests. P. Hedin, ed. Am. Chem. Soc. Symp. Ser. 62.

8. Keen, N. T., Érsek, T., Long, M., Bruegger, B., and Holliday, M. 1981. Inhibition of the hypersensitive reaction of soybean leaves to incompatible *Pseudomonas* spp. by blasticidin S, streptomycin or elevated temperature. Physiol. Plant Pathol. 18:325-337.

9. Keen, N. T., Sims, J. J., Erwin, D. C., Rice, E., and Partridge, J. E. 1971. 6a-Hydroxyphaseollin: An antifungal chemical induced in soybean hypocotyls by *Phytophthora megasperma* var. *sojae*. Phytopathology 61:1084-1089.

10. Keen, N. T., Zaki, A. I., and Sims, J. J. 1972. Biosynthesis of hydroxyphaseollin and related isoflavanoids in disease-resistant soybean hypocotyls. Phytochemistry 11:1031-1039.

11. Massala, R., Legrand, M., and Fritig, B. 1980. Effect of α-aminooxyacetate, a competitive inhibitor of phenylalanine ammonia-lyase, on the hypersensitive resistance of tobacco to tobacco mosaic virus. Physiol. Plant Pathol. 16:213-226.

12. Stössel, P., Lazarovits, G., and Ward, E. W. B. 1980. Penetration and growth of compatible and incompatible races of *Phytophthora megasperma* var. *sojae* in soybean hypocotyl tissue differing in age. Can. J. Bot. 58:2594-2601.

13. Ward, E. W. B., and Lazarovits, G. 1981. Influence of temperature on the disease reaction of soybean hypocotyls inoculated with *Phytophthora megasperma* var. *sojae*. (Abstr.) Phytopathology 71:911.

14. Yoshikawa, M., Yamauchi, K., and Masago, H. 1978. Glyceollin: Its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol. Plant Pathol. 12:73-82.