Glyceollin Accumulation in Soybean Lines Tolerant to *Phytophthora megasperma* f. sp. *glycinea*

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ABSTRACT

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Wounded and unwounded hypocotyls and tap roots of soybean lines that have high or low tolerance to *Phytophthora megasperma* f. sp. *glycinea* were tested to determine whether glyceollin accumulation could account for tolerance. No correlation was shown. After inoculation into the lateral root area with compatible races, four types of lesions in the tap root were recognized. These were: no rot, 1–10 mm rot, rot to the hypocotyl-root junction, and rot into the hypocotyl. Only plants with hypocotyl rot died within 10 days; others were stunted and recovered. Time course of accumulation and concentration of glyceollin in tap roots suggests that glyceollin does not play a role in soybean tolerance. No fungitoxic compounds other than glyceollin were detected by TLC bioassay of infected roots.

Additional key words: phytoalexin, resistance, root rot.

Two disease reaction types occur in soybean hypocotyls inoculated with *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin (hereafter referred to as *P. megasperma*). These types are resistant, which is typified by a hypersensitive reaction, and susceptible, which is characterized by ensuing growth of *P. megasperma* in the plant tissues (5,10,27). The resistant reaction is race-specific and involves fungal elicitation of necrosis in the tissues surrounding the inoculation site coincident with the rapid synthesis of a mixture of fungitoxic glyceollin isomers (1,2,10,27). Presumably, the tissue necrosis and toxicity of induced phytoalexins prevent the spread of *P. megasperma* beyond the infection court. In contrast, the pathogen in the susceptible reaction is not delimited by tissue necrosis and only nontoxic concentrations of glyceollin are produced; this allows the pathogen to grow and spread through the plant.

Many susceptible soybean lines grown in the field yield well, even though infected with *P. megasperma*. This has been called field resistance, rate-reducing resistance, field tolerance, or tolerance (14,18,19,21,23,24). To describe this susceptible type, we use the term *tolerance* as defined by Mussell (14). If roots are properly inoculated, plants can be screened for this tolerant reaction either in an infested field or in the greenhouse (6,7,9,18,23). The amount of disease present forms a continuum from no apparent effect on yield (high tolerance) to death of the plant (low tolerance). The genetics of the mechanism appear to be quantitative and controlled by relatively few genes (24). A cultivar rating system for tolerance has been developed (23). For ease of description we call these high-tolerant (HT) or low-tolerant (LT) soybean lines. Conceptually, HT lines will grow and yield well despite being susceptible to and infected by *P. megasperma*. Tolerance is race-nonspecific (19,22,23) and, although not completely independent from resistance, the presence of an allele for resistance does not strongly influence it (22,24). Further, it is not known whether this root tolerance of young seedlings, demonstrated by using a method of Schmitthenner and Hilty (18), is controlled in the same manner as age-resistance expressed in the hypocotyl (13,16,20,25,26). LT soybean lines grow and yield poorly when infected by races of *P. megasperma* to which they are not resistant. Recently, Tooley and Grau (21) devised a test showing differences in tolerance of soybean

TABLE 1. Summary of soybean lines used and their known resistance or tolerance to races of *Phytophthora megasperma* f. sp. *glycinea*

	Tolerance level	
Resistance	High	Low
No resistance	A76-304005	Corsoy
Races 1-2	Voris 295	Harosoy 63
Races 1-3, 6-9	L60-347	Corsoy 79

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TABLE 2. Summary of symptoms caused by *Phytophthora megasperma* f. sp. *glycinea* on soybean from inoculation to 7 days

Wounded	Unwounded
Hypocotyl	
i[y] = Dark brown lesion, hypersensitive reaction. No apparent disease.	i = No symptoms or minor flecks. No apparent disease.
c = Watersoaked, brown spreading lesion. Collapse, drying, and death in 48 hr.	c = Same as wounded, except first symptoms noticeable at 48 hr and death at 72 hr.
Roots	
i = Brown area where inoculated, no apparent disease.	i = No symptoms or minor flecks. No apparent disease.
c = Four lesion types[z]	c = No distinct lesion but internal spread as in wounded A, B, C, D. Earliest symptoms delayed 48 hr erupting to surface 20–30 mm above inoculation area. Plant with type D lesions die in 7 days.
A) Brown, watersoaked lesion. No spread. No death.	
B) Brown, watersoaked lesion. Spread 1–10 mm. No death.	
C) Brown, watersoaked lesion. Spread to root-hypocotyl junction. No death.	
D) Brown, watersoaked lesion. Spread into hypocotyl and cotyledons. Death in 7 days.	

[y] Abbreviations: i = incompatible and c = compatible.
[z] High-tolerant lines have more lesions of the A and B type than low-tolerant lines.

TABLE 3. Frequency (%) of each lesion type 144 hr after wound inoculation of tap roots of soybean with high or low tolerance to *Phytophthora megasperma* f. sp. *glycinea*

Lesion type[y]	High tolerance A76-304005	Low tolerance Corsoy
A	33[z]	11
B	6	7
C	32	11
D	28	71

[y] For lesion types, see Table 2.
[z] Mean of three experiments with a total of 400 plants for each line. $LSD_{P=0.05} = 4\%$.

lines based on the restriction of fungal movement from infected cotyledons into the hypocotyl.

The purpose of this work was to determine whether glyceollin plays a role in the expression of tolerance of soybeans to *P. megasperma* and to further investigate a reported anomaly (10) that levels of glyceollin elicited in roots did not mimic those elicited in hypocotyls. A preliminary report of this research has been published (15).

MATERIALS AND METHODS

Pathogen growth and inoculum preparation. Stock cultures of *P. megasperma* races 1, 4, and 7 were stored on V-8 juice agar at 10 C. To maintain aggressiveness, they were inoculated into appropriate susceptible lines of soybean at 4-mo intervals via a root technique (18). The fungus was reisolated on V-8 PBNC medium (formulated as specified by Schmitthenner [17] but modified to contain 5 mg of benomyl, 40 mg of pentachloronitrobenzene, and 20 mg of iprodione per liter) from the front of advancing rot that reached beyond the hypocotyl-root junction. For routine inoculum, a piece of mycelium was transferred to V-8 juice agar containing only 1.2% agar and grown 10 days at 24 C under dim light. This culture was then forced through a 0.8-mm (18-gauge) syringe needle and the resulting brei was used as inoculum within 1 hr.

Plant growth. Seeds of soybean cultivars Corsoy, Corsoy 79, Harosoy 63, Voris 295, and the experimental lines A76-304005 (Iowa Agric. Home Econ. Exp. Stn.) and L60-347 (Illinois Agric. Ext. Stn.) were harvested from seed-increase plots at the OARDC in Wooster and stored at room temperature. Known resistance and tolerance levels of these lines are shown in Table 1.

Seeds were sown in wet vermiculite in a growth chamber set for a 14-hr day (23,000 lux, 25 C) and 10-hr night (20 C). The pots were watered with a standard nutrient solution 24 hr after planting and daily thereafter with deionized water. Six days after planting the hypocotyl hook had straightened, the cotyledons were generally free of the seed coats, and small lateral roots were evident below the hypocotyl-root junction (VC stage of growth [3]).

For hypocotyl studies, the plants were left growing in the original vermiculite. For root inoculations, however, 6-day plants were gently lifted from the vermiculite, rinsed with tepid water, and arranged on slant boards similar to those described by Kendall and Leath (11), except that polyester greenhouse wicking material was used as absorbent backing material instead of perlite-filled cloth bags.

Inoculation techniques. *Wounded hypocotyl.* A flat teasing needle was used to open a 1-cm slit, starting 0.5 cm below the cotyledonary node (5). Within about 1 min, 0.1 ml of inoculum was placed directly into the slit.

Unwounded hypocotyls. A 1 × 2-cm piece of Parafilm was wrapped loosely around the hypocotyl 0.5 cm below the cotyledonary node and held in place by a tight twist in the corner of the Parafilm, leaving an open end in the Parafilm toward the base of the plant. Inoculum was then placed up and under the Parafilm, totally surrounding the hypocotyl for a 1-cm inoculation zone. All hypocotyl-inoculated plants were placed in a lighted mist chamber (25 C) for 12 hr and then returned to the growth chamber.

Wounded roots. Roots were arranged on a slant board and gently scraped with a single-edge razor to open a 1-cm wound into the stele in the area of developing lateral roots 6 cm below the hypocotyl-root junction. Inoculum was placed directly on the wound and the 1-cm inoculation zone was protected from touching the slant board materials by a small strip of Parafilm. The Parafilm held the inoculum in position and also prevented leaching or diffusion of materials from the wound area.

Unwounded roots. A layer of inoculum was placed directly in contact with roots as described above (wounded), except that no wound was made.

After all root inoculations, the slant boards were irrigated with 50 ml of quarter-strength nutrient solution. Boards were stacked with plants upright and returned to the growth chamber with continuous irrigation from below. Control treatments consisted of similar inoculations with V-8 juice agar only.

Glyceollin analysis. The 1-cm inoculation zone was excised and weighed. Methyl ethyl ketone was added to the tissue at 10 ml/g fresh weight and the tissue was extracted by shaking at room temperature for 72 hr. The extract was filtered through a glass wool

TABLE 4. Glyceollin accumulation in hypocotyls of six high-tolerant or low-tolerant soybean lines 48 hr after slit-inoculation and in roots 96 hr after scraping and inoculation with *Phytophthora megasperma* f. sp. *glycinea*

Tissue	*P. megasperma* race	Soybean lines[y] Pair 1 A76-304005	Pair 1 Corsoy	Pair 2 Voris 295	Pair 2 Harosoy 63	Pair 3 L60-347	Pair 3 Corsoy 79	LSD at $P = 0.05$
Hypocotyl	1	102[z]	70	201	301	318	117	16
	4	51	30	50	50	27	19	8
	7	59	12	75	25	181	320	17
Root	1	180	70	53	50	54	31	9
	4	193	132	208	152	149	155	20
	7	195	181	194	124	36	38	21

[y] Pair 1 = no resistance; pair 2 = resistant to *P. megasperma* races 1-2; pair 3 = resistant to *P. megasperma* races 1-3 and 6-9. A76-304005, Voris 295, and L60-347 are high-tolerant types; Corsoy, Harosoy 63, and Corsoy 79 are low-tolerant types.
[z] Mean (micrograms per gram fresh weight) of three experiments, each with two replications. No glyceollin was detected in controls.

TABLE 5. Time course of glyceollin accumulation in wounded tap roots of 6-day-old soybeans after inoculation with virulent race 4 of *Phytophthora megasperma* f. sp. *glycinea*

Soybean line	Tolerance	Time after inoculation (hr) 48	72	96	120	144	168	192
A76-304005	High	71[z]	118	189	257	344	212	128
Corsoy	Low	75	112	132	116	84	60	58
Voris 295	High	73	104	204	131	106	98	81
Harosoy 63	Low	77	110	154	92	96	86	76
L60-347	High	51	105	149	87	83	83	83
Corsoy 79	Low	47	111	158	94	94	91	86
LSD at 0.05		9	NSD	21	17	17	12	20

[z] Mean (micrograms per gram fresh weight) of three experiments each with two replications. No glyceollin was detected in controls.

column, the tissue was reextracted ~1 hr with methyl ethyl ketone, and the combined filtrates were evaporated to dryness at 40 C with a stream of air. Extracted tissue was dried overnight at 100 C and weighed. Because of the difficulty of obtaining accurate fresh weights on rotted and small root samples, all calculations were based on an extracted dry weight of 8% of the fresh weight, as determined by preliminary experiments. The dried extract was dissolved in ethyl acetate:methanol (4:1, v/v) and spotted on TLC plates. TLC, elution from the silica gel, and quantitation by UV spectroscopy were conducted as described by others (1,8,10,27). The analysis procedure averaged 70% recovery (range 65–75%), based on samples with known glyceollin content or added glyceollin or formononetin, and data were corrected to reflect this.

Parafilm (where used) surrounding 20 inoculated areas of each reaction and any exudate attached were extracted with 95% ethanol and treated as above. No glyceollin was found in these exudate samples.

Bioassay for fungitoxic products. TLC bioassay for fungitoxic products was made as described by Lazarovits et al (12). For each soybean cultivar, an extract equivalent to 500 mg wet weight of inoculation zone of tap roots that had been infected by *P. megasperma* for 96 hr was prepared as described above. Compounds were separated by two-dimensional chromatography on silica gel plates using toluene:chloroform:acetone (45:25:35, v/v) in the first direction and chloroform:acetone:conc NH_4OH (60:60:1, v/v) in the second direction. After 30 min of drying, encysted zoospores of *P. megasperma* (race 4) were applied to the plates. After a suitable incubation period the TLC plates were treated with charcoal as described by Lazarovits et al (12). The presence of inhibitory compounds was noted visibly, compared to known phytoalexins and isoflavonoids from soybean, and recorded, but the size of the inhibitory zones was not measured.

RESULTS

Visible symptoms. Injury symptoms and extent of rot exhibited in roots and hypocotyls, with wounded and nonwounded inoculations, are summarized in Table 2. All cultivars displayed a mixture of the four lesion types, but HT lines had more plants with nonspreading infection types than LT lines. Data typical for A76-304005 and Corsoy are shown in Table 3.

Glyceollin accumulation. All incompatible responses of hypocotyls had higher concentrations of glyceollin than compatible responses, after the 48-hr incubation period (Table 4). HT cultivars did not always have higher levels of glyceollin than LT cultivars. Susceptible, wounded hypocotyls had lower levels of glyceollin than incompatible, wounded hypocotyls and, generally, the HT lines had more glyceollin than LT lines. All glyceollin concentrations for compatible interactions were low.

Time course of glyceollin accumulation in wounded tap roots was determined by inoculating the six cultivars with *P. megasperma*, race 4 (all susceptible) and quantitating the amount of glyceollin in the lesion area for up to 192 hr (Table 5). For all cultivars except A76-304005, glyceollin concentration increased until 96 hr and then declined to values slightly higher than controls. Peak glyceollin accumulation in A76-304005 was not recorded until 144 hr.

Glyceollin accumulation for the six cultivars with wounded tap roots for a 96-hr period is shown in Table 4. In these tests, incompatible interactions resulted in low levels of glyceollin, in contrast to that found in the hypocotyls. Four of the six compatible interactions tested had higher concentrations of glyceollin in HT lines than in LT lines.

Preliminary investigation indicated that maximum accumulation of glyceollin in unwounded tissues occurred 72 hr after inoculation. Thus, all sampling of unwounded tissues was done at this time (Table 6). When inoculated with a virulent race of *P. megasperma*, hypocotyls of LT lines had significantly higher levels of glyceollin than did hypocotyls of HT lines. There was no recognizable pattern of glyceollin accumulation in unwounded root tissues; levels elicited appeared to be independent of the tolerance level.

No fungitoxic compounds other than glyceollin were found during TLC bioassay of roots inoculated with a virulent race of *P. megasperma*.

DISCUSSION

Wounded hypocotyls rapidly synthesized and accumulated glyceollin after inoculation with a virulent race of *P. megasperma*, as has been shown by others (5,9,10,16). The amount of glyceollin accumulated in wounded hypocotyls, however, did not appear to be influenced by the specific resistance allele or by the reported tolerance level. Within the HT-LT pair A76-304005-Corsoy, the HT line produced a significantly higher level of glyceollin than the LT line with all races of *P. megasperma* tested, but there was not a subsequent reduction in disease development. All plants of both cultivars died within 48 hr of being wound-inoculated in the hypocotyl.

Results were quite different when roots were wounded and inoculated. In root tissue, less phytoalexin accumulated in incompatible than in compatible reactions. This anomaly was

TABLE 6. Glyceollin accumulation 72 hr after inoculation of high-tolerant or low-tolerant soybean lines inoculated with *Phytophthora megasperma* f. sp. *glycinea* without wounding

Tissue	*P. megasperma* race	Soybean lines: Pair 1[y] A76-304005	Pair 1[y] Corsoy	Pair 2 Voris 295	Pair 2 Harosoy 63	Pair 3 L60-347	Pair 3 Corsoy 79	LSD at $P = 0.05$
Hypocotyl	1	63[z]	175	0	0	0	0	9
	4	65	300	92	205	26	90	15
	7	70	191	110	154	0	0	10
Root	1	53	36	0	0	0	0	18
	4	63	62	45	39	53	59	13
	7	85	62	73	123	0	0	12

[y] Pair 1 = no resistance; pair 2 = resistant to *P. megasperma* races 1-2; pair 3 = resistant to *P. megasperma* races 1-3 and 6-9. A76-304005, Voris 295, and L60-347 are high-tolerant types; Corsoy, Harosoy 63, and Corsoy 79 are low-tolerant types.
[z] Mean (micrograms per gram fresh weight) of three experiments, each with two replications. No glyceollin was detected in controls.

reported earlier by Keen and Horsch (10) with roots infected with zoospores and may be the converse of that shown by Kaplan and Keen (8) with roots infected by nematodes. It is difficult to compare our work to those studies because of experimental differences. If there is no degradation of phytoalexin, as suggested by Borner and Grisebach (1), and leaching of compounds away from the wound site was prevented (Parafilm wrap), 96 hr may be the appropriate sampling time for roots, as indicated by time course studies of glyceollin accumulation. The reason for glyceollin decline after 96 hr is not known. We classified root rot progression into four lesion types (Table 2). In only one of the four types was the fungus restricted to the original wound site. By 96 hr after inoculation, the fungus had grown beyond the original inoculation site in three of the four lesion types. Thus, if high glyceollin levels were present in the inoculation zone, the accumulation was too slow to stop the spread of the pathogen. We found no new fungitoxic compounds in the inoculation zone of compatible interactions in the root. It is possible that fungitoxic compounds were present in lesion type A. These would not be detected in our experiments because we sampled all plants inoculated, which included a mixture of the four lesion types. The variable response to infection shown by roots when inoculated in the developing lateral root zone is a considerable barrier to further work of this type. We have also seen a nonuniform response of the compatible reaction in other studies in which we used zoospores as inoculum (A. F. Olah, *unpublished*). We believe it would be necessary to have soybean lines that give uniform lesion types to further clarify the role of fungitoxic compounds in the tolerant reaction, and we are now in the process of producing such clonal material.

When tissues were inoculated without wounding, the incompatible reaction was characterized by a lack of detectable glyceollin, in contrast to that found with wounding. Because our samples contained a high proportion of symptomless tissue (in effect, only the epidermal cells had the opportunity to become infected), it was possible that an intense cellular response by the surface cells was masked by a preponderance of nonreacting cells throughout the internal tissues of the inoculation zone. This difference between surface and internal response of the hypocotyl has been noted previously (20,27). With compatible reactions in the unwounded hypocotyl, the LT lines of the high-low tolerant pair always produced significantly more glyceollin. This may have been the response to elicitation from necrosis of tissue, as reported by Flood and Milton using alfalfa infected by *Verticillium albo-atrum* (4) and not as race-specific elicitation. Others have indicated (20,27) that within 72 hr *P. megasperma* had grown a considerable distance through compatible tissues.

Presently, it seems clear that the hypocotyl response after wounding and inoculation is an all-or-nothing phenomenon that depends on the presence of a resistance allele. In contrast, tolerance level shown in the greenhouse and field cannot be accounted for by measured levels of glyceollin elicited. Whether glyceollin accumulation in wound-inoculated roots is related to tolerance levels is unclear due to the mixed reactions found. Unwounded, inoculated tissue (which perhaps most closely approximates a natural situation) appears to accumulate glyceollin as a consequence of tissue necrosis. The root response was significantly less than that of the hypocotyl, on a weight basis. The time course of glyceollin accumulation and the ultimate levels of phytoalexin accumulated during the incompatible reaction were different in the roots reaction (at least in the lateral root zone). On the basis of this work, we do not believe that glyceollin accumulation can account for tolerance of soybean to *P. megasperma*.

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