Age-Related Changes in Specificity and Glyceollin Production in the Hypocotyl Reaction of Soybeans to *Phytophthora megasperma* var. *sojae*

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ABSTRACT

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Intact, 6-day-old soybean hypocotyls (*Glycine max* [L.] Merr.) increased in resistance to inoculation with zoospores of *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. (*Pms*) from the top (youngest part) to the bottom (oldest part). The interaction with the compatible race at the top of the hypocotyl, was characterized by watersoaking and unrestricted tissue colonization with low glyceollin production whereas at the middle and bottom of the hypocotyl, the interaction was necrotic and glyceollin production was increased. This change in host response corresponded to a difference in tissue age of 1–2 days. The incompatible interaction, which typically was necrotic with high glyceollin production at the top of the hypocotyl, became even more incompatible lower down, where there was a reduction in both necrosis and glyceollin production. With cultivar Altona and *Pms* races 4 (incompatible) and 6 (compatible) the numbers of appressoria formed and penetrations were similar regardless of race or inoculation site. Similarities in symptoms and in the time-course of glyceollin production suggested that similar mechanisms govern the incompatible response whether it occurred on the incompatible host in the normal way, or on the compatible host at resistant-responding sites on the lower parts of the hypocotyl.

Phytophthora rot of soybeans (*Glycine max* [L.] Merr.) has been used by several authors as a model system for the study of race-specific mechanisms of resistance (1–3,8,9,15). Typically, infection with races of the causal fungus, *Phytophthora megasperma* Drechs. var. *sojae* Hildeb. (*Pms*), results in limited hypersensitive necrosis and rapid phytoalexin (glyceollin) production in incompatible interactions and in either reduced or delayed glyceollin production and unrestricted tissue colonization in compatible interactions. This system is controlled by major host genes, each conferring resistance to one or more races of *Pms* and accounts satisfactorily for the disease reaction in young seedlings (10). Paxton and Chamberlain (12), however, observed that soybean plants became increasingly resistant to *Pms* as they matured, despite greatly reduced glyceollin production, and suggested that additional mechanisms of resistance may develop in older tissues. Keen (8) confirmed that resistance increases with age, but, by relating glyceollin measurements to the amount of infected tissue, concluded that the highest glyceollin concentrations occurred in older plants. Recently, we observed that even young soybean tissues rapidly develop resistance more typical of older plants (14). In 6-day-old seedlings only the upper one-third of the hypocotyl gave a characteristic race-specific response when inoculated with zoospores of *Pms* races.

This paper describes the effect of maturation of young hypocotyl tissues on the specificity of resistance and glyceollin production in response to *Pms* races.

MATERIALS AND METHODS

***Pms* races.** Isolates of *Pms* races 4 and 6 used here were described previously (14). They were grown routinely and for zoospore production on V-8 juice agar at 25 C in the dark. Procedures for zoospore production generally followed those of Ho and Hickman (7) and Eye et al (4) and have been described previously (11,14) except that an incubation temperature of 22 C has been found to be optimum during leaching. Zoospores in suspensions were counted with a haemocytometer and, unless otherwise indicated, adjusted to 1×10^5/ml with sterile distilled water.

Soybean cultivars. The soybean cultivar Altona (which is susceptible to *Pms* race 6 and resistant to race 4) was used routinely, but the hypocotyl reaction and glyceollin production of several other cultivars also was examined (Table 1). Seeds of all cultivars were obtained from R. I. Buzzell (Research Station, Agriculture Canada, Harrow, Ontario). Details of the growth of etiolated seedlings have been given in full previously (11,14).

Hypocotyl inoculation. Six-day-old seedlings were washed in running tap water, blotted dry, and transferred to glass trays (35 × 21 cm). Up to 20 seedlings were placed horizontally in each tray and held in position by slotted Plexiglas racks (1.2-cm wide), one just above the roots and the other immediately below the cotyledons. The roots were covered with a layer of cellucotton soaked with water. Hypocotyls were inoculated with 10-μl drops of zoospore suspension placed either at intervals throughout the length of the hypocotyl or more usually in three separate groups of three or four closely spaced drops at the top, middle and bottom of the hypocotyl (Fig. 1). Following inoculation, the trays were sealed with plastic film and incubated in the dark at 25 C for 24 hr or other periods as indicated below.

Glyceollin determinations. *Diffusates.* Diffusate drops were removed by suction, collected in 15-ml graduated conical centrifuge tubes, and the total volume was recorded. Drops of distilled water were placed briefly on the inoculation sites to wash the surface. These were removed, combined with the diffusate and thoroughly mixed with 2.0 ml of ethyl acetate. After separation, 1.5 ml of the ethyl acetate extract was removed, dried under N_2 at 35 C, and the residue was redissolved in absolute ethanol. Glyceollin was calculated from the absorbance (285 nm) of the ethanol solution and the extinction coefficient (ϵ= 10,300) as described by Ayers et al (2). In agreement with these authors, we have found that glyceollin determined after thin-layer chromatography of diffusate extracts is approximately 25% of the value obtained in this way. Data are presented as μg glyceollin per milliliter of diffusate to compensate for variations in diffusate volume.

Total glyceollin. Diffusates were collected as above and the corresponding inoculated sections, plus 5 mm of additional tissue at each end, were cut from the hypocotyls. The tissue sections together with the diffusates were transferred to test tubes, covered with 4 ml of 95% ethanol and heated in a boiling water bath for 3 min. After the sections had steeped overnight at 10 C in the dark, the ethanol was replaced with 4 ml of fresh ethanol and the steeping was continued for another 24 hr. The ethanol extracts and

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an additional 2.0 ml used for rinsing were combined and reduced to dryness at 40 C on a rotary evaporator. The residue was extracted with 3 × 0.5 ml of ethyl acetate, the extract was transferred to small vials and the ethyl acetate was removed under N_2 at 35 C.

For thin-layer chromatography, the extract was redissolved in 100 μl of ethyl acetate and 25–50 μl applied to channels on Whatman LK6DF Silica gel plates (250 μm) and developed in benzene:methanol (95:8, v/v). Glyceollin (a mixture of isomers) was located by reference to a standard (supplied by P. Albersheim, Department of Chemistry, University of Colorado) and its fluorescence quenching in UV light. The silica gel bands containing the glyceollin were scraped from the plates and eluted in small glass columns with ethyl acetate. Glyceollin was determined as described for diffusates.

Dry weight of infected tissues. Tissue segments after extraction were used to obtain an estimate of the dry weight of infected tissue. Diseased tissue, as indicated by necrosis or discoloration and by light microscopic examination of representative samples was dissected from the segments, dried, and weighed. The minimum amount of tissue excised in highly incompatible interactions consisted of the surface cells to a depth of 0.5–1.0 mm. Where very small amounts of tissue were involved and especially where necrosis was not uniform throughout a lesion, the inclusion of some uninfected tissue was unavoidable. In compatible interactions, the entire hypocotyl segment was usually infected.

Appressorium formation and penetration. Following inoculation, hypocotyls were incubated for 10 hr. Infected areas were excised (~0.5–1.0 mm deep) and fixed overnight at 4 C in ethanol:acetic acid (3:1, v/v). The material was then rinsed with 95% ethanol. To determine the number of appressoria formed, the tissue was stained in lactophenol-cotton blue, mounted in 50% glycerin and viewed in the light microscope. Penetrations were counted in infected hypocotyl tissue after clearing in a saturated solution of chloral hydrate in distilled water and mounted in 50% glycerin.

RESULTS

Hypocotyls of cultivar Altona did not respond uniformly to inoculation with zoospores of *Pms* throughout their length (Fig. 1). The compatible race caused typical watersoaking only in the upper part of the hypocotyl (Fig. 1a). In the middle there was a transition from compatibility to hypersensitivity with increasing necrosis, and at the bottom the hypocotyl tissues were highly resistant, with light necrotic flecks as the only visible symptoms. There also was a gradient in the host reaction to the incompatible race 4 (Fig. 1b). Tissues at the top of the hypocotyl were uniformly necrotic and slightly sunken, but necrosis decreased progressively through the central region and became faint and barely discernible towards the bottom. When incubation was continued for longer than 24 hr, hypocotyls inoculated at the top with the compatible race rapidly disintegrated in a watery rot and the plants died. More variable results followed inoculation at the middle with the compatible race, but at the bottom or at all sites inoculated with the incompatible race, symptoms remained unchanged and the plants healthy over extended observation periods of up to 1 wk.

These observations were extended to additional race-cultivar combinations and the concentration of glyceollin in diffusates from the top, middle, and bottom of the hypocotyl were determined (Table 1). In each case, the reaction at the top of the hypocotyl, was typical for the race-cultivar combination, but at the middle and the

TABLE 1. Host response and glyceollin production of soybean cultivars inoculated with races 4 or 6 of *Phytophthora megasperma* var. *sojae* at the top, middle, or bottom of the hypocotyls

Cultivar	Position inoculated[a]	Race 4		Race 6	
		Host[b] response	Glyceollin[c] (μg/ml)	Host response	Glyceollin (μg/ml)
Altona	T[d]	R	307	S	88
	M	R	175	SR	153
	B	VR	153	R	88
Tracy	T	R	263	R	350
	M	R	175	R	131
	B	VR	153	VR	153
Harosoy	T	S	44	S	88
	M		-	SR	219
	B	R	263	R	153
PI 171442	T	R	240	S	66
	M	R	197	SR	175
	B	VR	65	R	88
Harosoy 63	T	S	44		
	M	SR	307		
	B	R	219		

[a] Three drops of a zoospore suspension were placed at the top (T), middle (M), or bottom (B) of 20 hypocotyls.

[b] Reactions were graded by the degree of necrosis 24 hr after inoculation: S = susceptible, water-soaked lesions; SR = spreading necrotic lesions; R = resistant greater than 50% of area under drop was necrotic; VR = very resistant, ranging from flecking to no visible symptoms.

[c] Diffusates were extracted with ethyl acetate and the glyceollin determined from the absorbance at 285 nm of the ethyl acetate-soluble material. Values are expressed as micrograms of glyceollin per milliliter of diffusate collected.

[d] Response at the top is that expected for the race-cultivar combination.

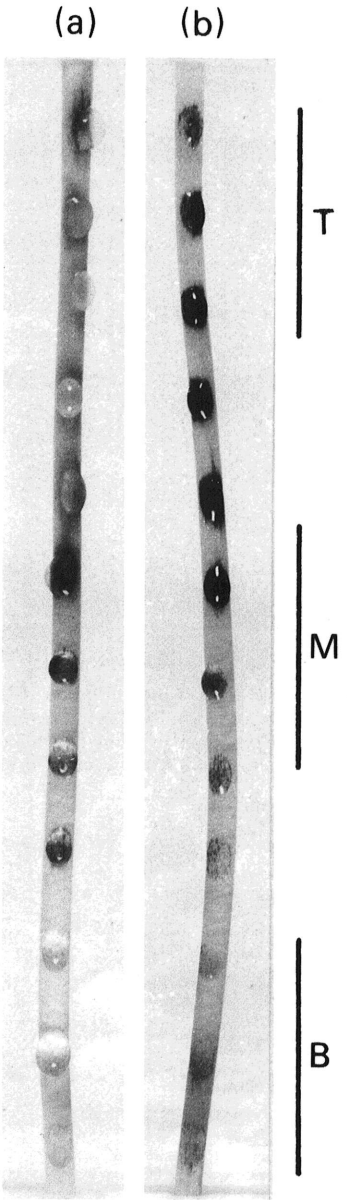

Fig. 1. Soybean hypocotyls (cultivar Altona) inoculated with droplets of zoospore suspensions of *Phytophthora megasperma* var. *sojae:* **a,** race 6 —compatible; **b,** race 4—incompatible. The letters T, M, and B indicate the top, middle, and bottom (respectively) segments of the hypocotyl used for comparisons and analysis in subsequent experiments.

bottom it became increasingly resistant. In response to the compatible race, glyceollin concentrations were predictably low at the top of the hypocotyl, but uncharacteristically high at the middle and the bottom. The reverse was true with the incompatible race, the highest glyceollin concentrations were always at the top.

With Altona, the atypical increase in glyceollin production that followed inoculation of the middle or bottom of the hypocotyl with the compatible race 6, was initiated early in the interaction (Fig. 2a). For the first 12–14 hr, glyceollin production following these inoculations was very similar to that at all three sites inoculated with the incompatible race 4 (Fig. 2b). Subsequently, the rate of glyceollin production declined at the middle and the bottom in interactions with both races, but continued to much higher levels in a typical incompatible reaction with race 4 at the top of the hypocotyl.

An advantage of the zoospore inoculation method used here is that it permits the standardization and comparison of inoculum levels (Fig. 3). In the compatible combination even the lowest level of inoculum caused extensive watersoaking and softening of tissue at the top of the hypocotyl 24 hr after inoculation. The middle of the hypocotyl was partly resistant (to race 6) at lower zoospore concentrations, but tended to be susceptible at the highest concentrations. The bottom of the hypocotyl remained firm and healthy at all concentrations, although there was a slight increase in epidermal necrosis at higher zoospore concentrations.

In the incompatible interaction with race 4 a minimum of 1×10^5 zoospores per milliliter of inoculum was required to produce a uniformly necrotic lesion at the top of the hypocotyl. In the middle, twice this number was needed to produce the same response, while at the bottom even this concentration caused only a slight browning.

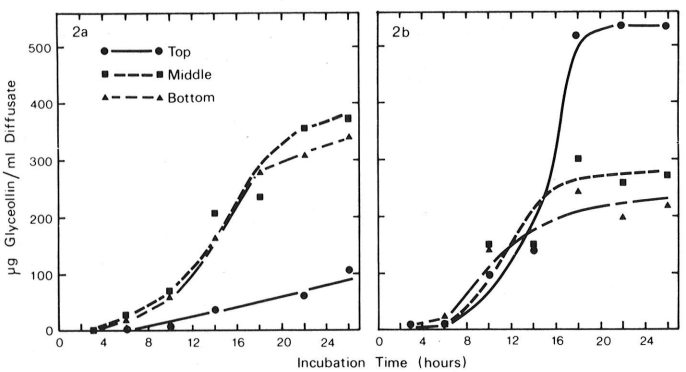

Fig. 2. Time-course of glyceollin accumulation in diffusates from soybean hypocotyls (cultivar Altona) inoculated with zoospore suspensions of *Phytophthora megasperma* var. *sojae* at the top, middle, and bottom. **a,** race 6—compatible; race 4—incompatible.

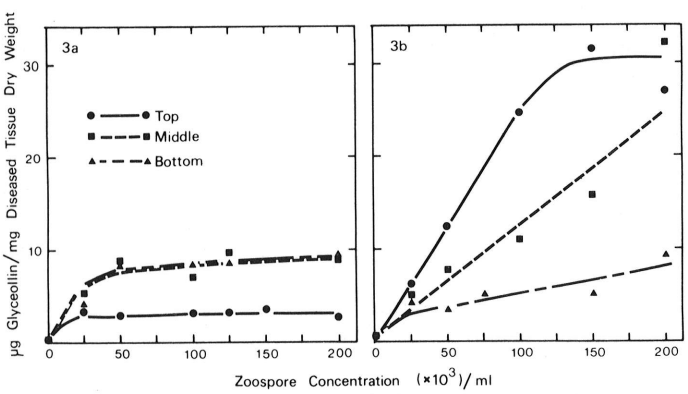

Fig. 3. The effect of inoculum concentration of glyceollin accumulation of soybean hypocotyls (cultivar Altona) inoculated with zoospore suspensions of *Phytophthora megasperma* var. *sojae* at the top, middle, and bottom. **a,** race 6—compatible; **b,** race 4—incompatible.

Zoospore concentration had no effect on glyceollin production in the compatible interaction at the top of the hypocotyl (Fig. 3a). At the middle and the bottom, glyceollin increased with the first increase in zoospore concentration but further increases were without effect. Quite different results were obtained with the incompatible race 4 (Fig. 3b). Glyceollin production at the top and middle of the hypocotyl was directly related to numbers of zoospores. At the top, for part of the concentration range a doubling in zoospore numbers caused a doubling in glyceollin concentration. At the bottom only small increases in glyceollin levels were associated with increases in inoculum concentration.

Sites at the top and the bottom of Altona hypocotyls inoculated with race 6 or race 4 were examined with the light microscope. No differences were found in the numbers of germinated encysted zoospores, appressoria formed or penetrations of the hypocotyl surface (Table 2).

DISCUSSION

Inoculation of intact hypocotyls with zoospores has several advantages over the procedures for wound inoculation with mycelium, more commonly employed (2,8,15). Inoculum can be standardized, the important stages of appressorium formation and penetration are not bypassed, and physiological and biochemical experiments are not compromised by wound responses for which adequate controls cannot be provided. Concerns over the last of these have been emphasized particularly by recent evidence that host cells contain constitutive elicitors of phytoalexins that are released following injury or death (5,6). An additional advantage of the method used here is that it has permitted the application of the drop diffusion technique to the study of glyceollin production in soybeans. This is rapid and simple and provides useful qualitative information (Table 1, Fig. 2).

The results reported appear to confirm those of Paxton and Chamberlain (12) and Keen (8) who also concluded that aging soybean tissue becomes more resistant to *Pms*. However, although resistance to *Pms* races is governed by major genes, our observations indicate that resistance and susceptibility are neither mutually exclusive nor absolute alternatives. There appears to be a continuum ranging from very susceptible (race 6 at the top of the hypocotyl) to very resistant (race 4 at the bottom), with some overlap between the response to race 6 at the bottom and that to race 4 at the top. In a segment of this range (race 6 near the middle of the hypocotyl) it is possible to change the response from resistance to susceptibility by increasing the inoculum level. The earlier work also differs from ours in that tissues were wound-inoculated and differences in age were greater. In 6-day-old hypocotyls, the distance from the top to the middle, where transition from compatibility to incompatibility occurs, represents a growth period of 1–2 days. Paxton and Chamberlain (12),

TABLE 2. Zoospore germination, appressorium formation, and penetration at the top and the bottom of 6-day-old soybean hypocotyls (cultivar Altona) following inoculation with race 6 (compatible) or race 4 (incompatible) of *Phytophthora megasperma* var. *sojae*

	Race 6		Race 4	
	Top	Bottom	Top	Bottom
Ungerminated cysts[a]	3.1 ± 3.7	0.9 ± 1.3	2.5 ± 3.0	4.7 ± 3.3
Germ tubes without appressorium	2.2 ± 2.0	2.5 ± 0.9	3.2 ± 2.3	7.0 ± 5.4
Appressoria	44.8 ± 4.4	46.6 ± 1.9	44.5 ± 4.1	38.5 ± 6.1
Penetrations[b]	49.5 ± 1.0	48.5 ± 2.4	49.0 ± 2.0	47.8 ± 1.7

[a] Fifty encysted zoospores at each inoculation site on each of four hypocotyls were examined for germination and appressorium formation. Values are means and standard deviations for the four hypocotyls. Infected areas were stained with lactophenol-cotton blue.

[b] An additional 50 appressoria were examined after clearing infected tissue in chloral hydrate, to determine penetrations.

however, compared plants 7–21 days old and it is unlikely that the development of the woody and sclerified tissues which they observed is a factor here. Furthermore, we did not find age-related differences in appressorium formation or penetration to suggest that mechanical barriers are involved (Table 2). The similarities between the reactions to the compatible race at resistant parts of the hypocotyl and to the incompatibile race suggest that the mechanisms involved also may be similar. Thus, it seems reasonable to conclude that within 1–2 days of their formation, changes occur in susceptible hypocotyl cells which enable them to react to virulent races of *Pms* as if they were avirulent. Schemes explaining resistance in terms of the recognition of race-specific cell-wall glucans or glycoproteins may have to take such changes into consideration (9,13).

Increased glyceollin production was associated with age-related resistance to the compatible race 6. Amounts in diffusates frequently were similar to those in diffusates from the incompatible race 4 interaction at the top of the hypocotyl (Table 1). During the first 12–14 hr following inoculation the similarities in rate of glyceollin production in Altona at all resistant-responding sites also suggests that common mechanisms are involved regardless of tissue age or specificity of the *Pms* race. However, the effect of inoculum level on glyceollin production was not the same for both races, only for race 4 at the top and middle of the hypocotyl were the two correlated. Evidently this was related to the ability to cause necrosis at the different inoculation sites. Twice as many race 4 zoospores were required to cause uniform necrosis in the middle of the hypocotyl as at the top, while at the bottom there was only a slight increase in necrosis between lowest and highest inoculum levels. With race 6, the middle of the hypocotyls became susceptible as inoculum levels increased, so increases in glyceollin were not to be expected.

Glyceollin production decreased with tissue age in the incompatible race 4 interactions, in spite of increased resistance. Glyceollin production also decreased from the middle to the bottom of the hypocotyl in some resistant-responding race 6 interactions. Such highly resistant sites were characterized by hypersensitive flecks and very limited necrosis and hence glyceollin production appears to be more closely correlated with necrosis than with resistance. Similarly neither glyceollin production nor necrosis appear to be correlated with penetration, for numbers of penetrations were similar at all sites examined.

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