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Quantitative Localization of the Phytoalexin Glyceollin I in Relation to Fungal Hyphae in Soybean Roots Infected with *Phytophthora megasperma* f. sp. glycinea[1]

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ABSTRACT

A radioimmunoassay specific for glyceollin I was used to quantitate this phytoalexin in roots of soybean (*Glycine max* [L.] Merr. cv Harosoy 63) after infection with zoospores of either race 1 (incompatible) or race 3 (compatible) of *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin. The sensitivity of the radioimmunoassay and an inmmunofluorescent stain for hyphae permitted quantitation of phytoalexin and localization of the fungus in alternate serial cryotome sections from the same root. The incompatible interaction was characterized by extensive fungal colonization of the root cortex which was limited to the immediate vicinity of the inoculation site. Glyceollin I was first detected in extracts of whole roots 2 hours after infection, and phytoalexin content rose rapidly thereafter. Significant concentrations of glyceollin I were present at the infection site in cross-sections (42 micrometers thick) of such roots by 5 hours, and exceeded 0.6 micromoles per milliliter (EC_{50} *in vitro* for glyceollin I) by 8 hours after infection. Longitudinal sectioning (14 micrometers thick) showed that glyceollin I accumulated particularly in the epidermal cell layers, but also was present in the root cortex at inhibitory concentrations. No hyphae were observed in advance of detectable levels of the phytoalexin and, in most roots, glyceollin I concentrations dropped sharply at the leading edge of the infection. In contrast, the compatible interaction was characterized by extensive unchecked fungal colonization of the root stele, with lesser growth in the rest of the root. Only small amounts of glyceollin I were detected in whole root extracts during the first 14 hours after infection. Measurable amounts of glyceollin I were detected only in occasional cross-sections of such roots 11 and 14 hours after infection. The phytoalexin was present at inhibitory concentrations in the epidermal cell layers, but the inhibitory zone did not extend appreciably into the cortex. Altogether, these data support the hypothesis that the accumulation of glyceollin I is an important early response of soybean roots to infection by *P. megasperma*, but may not be solely responsible for inhibition of fungal growth in the resistant response.

The production of phytoalexins is a well-documented response of plants to infection with a wide variety of microorganisms (7). The various lines of evidence supporting a role for these antimicrobial compounds in the resistance of plants to disease have been discussed in recent reviews (13, 27).

One important facet in ascertaining the significance of phytoalexins in plant disease resistance is the quantitative knowledge of their spatial and temporal distribution within plant tissue at or near infection sites. Several groups of workers have used fluorescence and UV microspectrophotometry to show localization of phytoalexins in plant cells immediately adjacent to infection sites (21, 28, 29). Moesta *et al.*, using laser microprobe mass analysis, demonstrated an abrupt rise in phytoalexin content in the vicinity of the infection boundary (33). Unfortunately, since quantitation of phytoalexins using any of these techniques is difficult, if not impossible, these reports could not establish whether or not the phytoalexins were present in these cells at toxic concentrations.

A disease that has received considerable attention as a model host-pathogen system is root and stem rot of soybean (*Glycine max*) cause by *Phytophthora megasperma* f. sp. *glycinea*. In the field, this pathogen most commonly infects soybean plants under conditions of water-logged soils (12). The natural infective propagules are zoospores, and the most frequent site of infection is the plant root. Young seedlings are most severely damaged. Most studies on the biochemistry of this plant-pathogen interaction have relied on infecting mechanically wounded hypocotyls with small pieces of mycelia (2, 11, 14, 23, 30, 34, 38–40), an exception being the experiments reported by Ward and coworkers (36, 37), where zoospore-infected, unwounded etiolated hypocotyls were utilized. Zoospore infection of soybean roots was employed by Eye *et al.* for pathogenicity and race determinations (17), and by Slusher *et al.* (35) and, more recently, Beagle-Ristaino and Rissler (9) for histopathological studies.

We chose to use an inoculation system which mimics as closely as possible, under laboratory conditions, the natural infection process. That is, young unwounded soybean seedlings were inoculated at the roots with zoospores of *P. megasperma*. Using a radioimmunoassay specific for the soybean phytoalexin, glyceollin I (32), we report here the localization and quantitation of this phytoalexin in relation to the location of fungal hyphae in infected soybean roots. A preliminary report of a portion of this work has been published (20).

MATERIALS AND METHODS

Soybean Seedlings. Seeds of soybean (*Glycine max* [L.] Merr. cv Harosoy 63) were obtained from R. I. Buzell (Harrow, Ontario, Canada). The seeds were hand-selected for soundness, surface-sterilized with 0.75% NaOCl for 5 min and germinated aseptically on wet filter paper in Petri plates (10–15 seeds/plate) in the dark at 25°C. Two- or 3-d-old seedlings were used for

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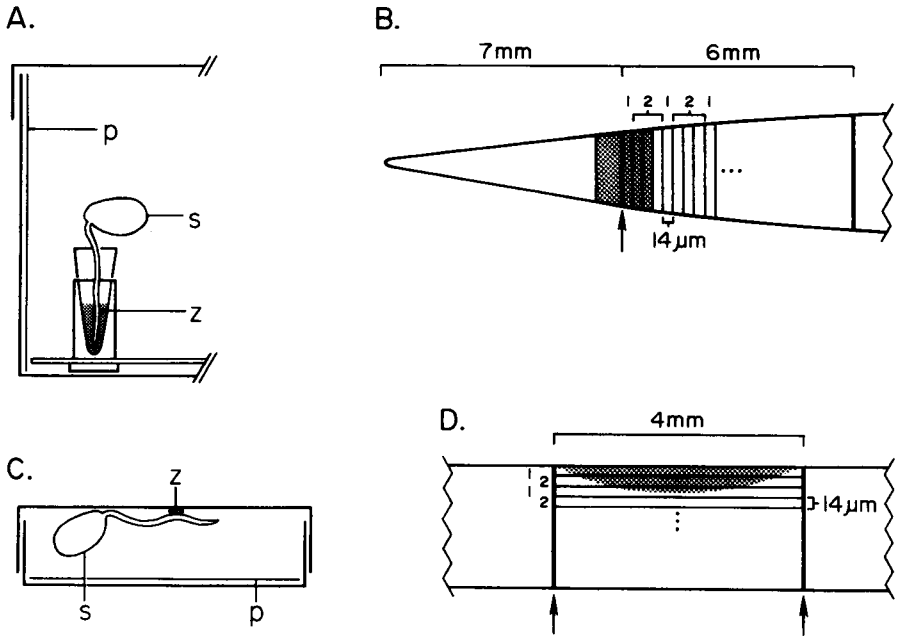

FIG. 1. Inoculation of roots of soybean seedlings with zoospores of *P. megasperma* f. sp. *glycinea*, and subsequent cryotome sectioning. A, Dip inoculation. Two-d-old seedlings (s) were placed in excised conical ends of 1.5-ml Eppendorf test tubes containing 100 μl zoospore suspension (z) (about 10^4 zoospores). The seedlings were incubated in covered glass dishes lined with wet filter paper (p) as described in the text. B, Cryotome sectioning of dip inoculated roots. After incubation, the lower 7 mm of the root were discarded. The next 6 to 8 mm were embedded and sectioned into 14-μm thick cryotome sections at −20°C. Every fourth section (1) was stained for fungal hyphae (31). The three intervening sections (2) were combined, extracted with 10% (v/v) methanol, and the glyceollin I quantitated by radioimmunoassay. The shaded region represents the primary infection zone. The arrow indicates the excision point referred to in Figures 5 to 8. C, Droplet inoculation. Three-d-old soybean seedlings (s) were affixed to the underside of the lid of a Petri plate. A 5-μl droplet of zoospore suspension (z) containing about 500 zoospores was placed between the root and the Petri plate lid. A piece of wet filter paper (p) was placed in the bottom of the Petri plate to maintain humidity. The seedlings were incubated as described in the text. D, Cryotome sectioning of droplet inoculated roots. After incubation, a 2- to 4-mm piece of root (arrows) centering on the infection site (shaded region) was excised and embedded. Cryotome sections 14 μm thick were prepared as above. Alternate sections were stained for hyphae (1) or extracted with 10% (v/v) methanol for glyceollin I quantitation (2).

experiments.

Fungal Cultures. *Phytophthora megasperma* Drechs. f. sp. *glycinea* Kuan and Erwin races 1 and 3 were obtained from B. L. Keeling (Stoneville, MS) and were grown as described (1). Zoospores were obtained from 6-d-old cultures according to a published method (16).

Chemicals. All chemicals and solvents were of analytical quality. Sodium [^{125}I]iodide (481–629 MBq/μg) was purchased from Amersham (Braunschweig, F.R.G.). Glyceollin I was supplied by P. Moesta of our laboratory, and standard solutions were prepared using an extinction coefficient of 10,300 (1) at 285 nm. Normal rabbit serum was obtained from Paesel (Frankfurt, F.R.G.), fluorescein isothiocyanate-conjugated anti-rabbit IgG from Sigma (Munich, F.R.G.), and Tissue Tek II O.C.T. from Miles Laboratories (Naperville, IL). Antisera to *P. megasperma* (31) were a gift from E. Ziegler (Aachen, F.R.G.). Antisera to glyceollin I were those whose preparation was described previously (32).

HPLC. HPLC was performed on a 9- × 250-mm silica gel column (Lichrosorb Si 60 5 μm) (Merck, Darmstadt, F.R.G.) at a flow-rate of 2.5 ml/min. Total pterocarpans were fractionated using a solvent mixture of hexane:isopropanol (9:1, v/v). Separation of the glyceollin isomers was accomplished with a solvent mixture of hexane:isopropanol (92:8, v/v). Column effluent was monitored at 280 nm.

Iodination of Glyceollin I. Purified glyceollin I was labeled with ^{125}I using essentially the method of Greenwood *et al.* (19). To a glass test tube, the following were added in rapid succession: 10 μl of 0.1 M sodium phosphate (pH 7.5), 2.8 μl Na ^{125}I (0.148 nmol) as supplied, 20 μl of glyceollin I (1.48 nmol) dissolved in methanol, and 10 μl of chloramine T (2.8 mg/ml in 0.1 M sodium phosphate, pH 7.5). This solution was mixed gently and allowed to stand at room temperature for 2 min. Subsequently, 100 μl of cysteine (5 mg/ml) and 100 μl of KI (1 mg/ml), each dissolved in 0.1 M sodium phosphate (pH 7.5), were added, the solution again gently mixed, and then extracted three times with 0.5 ml of chloroform. The combined chloroform phase was dried over anhydrous sodium sulfate, applied to a SepPak silica cartridge (Waters, Koenigstein, F.R.G.), and the iodinated glyceollin I eluted from the cartridge with 8 ml of chloroform. Uniodinated glyceollin I remained bound to the cartridge. The eluate was evaporated to dryness and redissolved in 50% (v/v) methanol in 0.05 M sodium acetate (pH 6.2). An average yield of 82.6% was calculated based on the recovery of ^{125}I in labeled product in three experiments. The labeled glyceollin I was stored at −20°C until needed, and was diluted to about 2 × 10^5 cpm/ml with 50% (v/v) methanol in 0.05 M sodium acetate (pH 6.2) for use in the radioimmunoassay.

Radioimmunoassay for Glyceollin I. The radioimmunoassay for glyceollin I was carried out exactly as described (32) except that the buffer was 0.05 M sodium acetate (pH 6.2) throughout.

Root Inoculation Procedures. All operations were carried out in a laminar flow hood using sterilized equipment. Unwounded soybean seedlings were infected with zoospores of *P. megasperma* by either dip or droplet inoculation.

Dip Inoculation. Individual 2-d-old soybean seedlings having roots at least 2.5 cm long were each placed upright in excised conical ends of 1.5 ml Eppendorf plastic test tubes containing

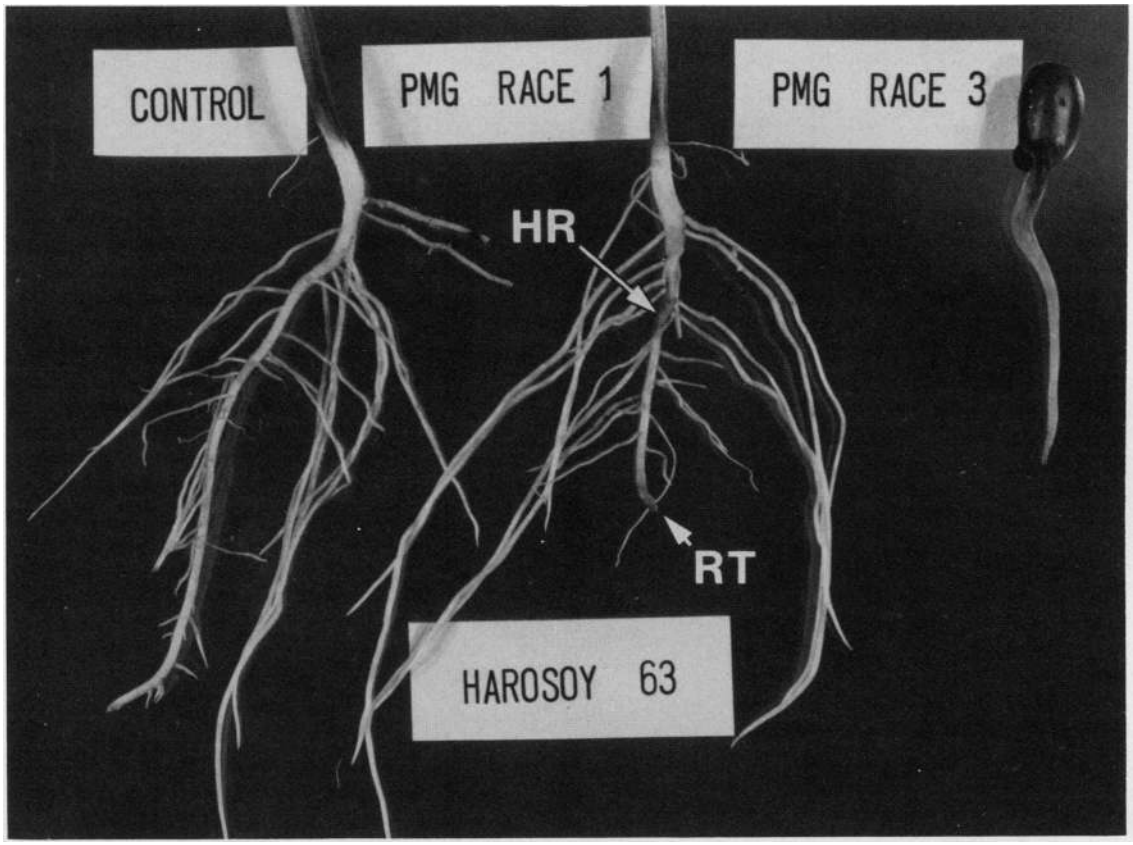

FIG. 2. Soybean roots (cv Harosoy 63) 4 d after inoculation with zoospores of race 1 or race 3 of *P. megasperma* f. sp. *glycinea*. Two-day-old seedlings were dip-inoculated with about 10^4 zoospores for 2 h, and then planted in sterile vermiculite with the cotyledons at the surface. Control was treated with sterile, distilled H_2O. The hypersensitive ring necrosis (HR) and the nongrowing primary root tip (RT) are marked.

100 μl of zoospore suspension (about 10^4 zoospores) (Fig. 1A). Control seedlings were placed in 100 μl of sterile distilled H_2O. The seedlings were incubated at 100% RH in the dark at 25°C for 2 h. Subsequently, the seedlings were transferred to fresh cones containing 100 μl of sterile tap water and incubated as above for varying lengths of time. After incubation, the lowest 7 to 8 mm of root were excised and discarded (Fig. 1B). This point of excision was approximately located at the meniscus of the zoospore suspension. Since the zoospores primarily swim near the surface of the solution and attach themselves to the root extension zone (J. Golecki, personal communication), the excision point is within the primary infection zone. The next 6 to 8 mm of root were excised and embedded vertically, upside down in Tissue Tek II O.C.T. compound. Out of each treatment series, three to five seedlings were planted in sterile vermiculite such that their cytoledons were at the surface. The plants were then incubated in a growth chamber (14-h day, Osram HQIL 400 w lamps, 22,500 to 23,900 lux; 10-h night) at about 75% RH and 28°C. Disease readings were taken 4 d after planting.

Droplet Inoculation. Three-d-old soybean seedlings were affixed to the underside of a Petri plate lid using transparent tape. A 5-μl droplet of zoospore suspension (about 500 zoospores) was placed between the root and the Petri plate lid (Fig. 1C). The seedlings were then incubated at 100% RH in the dark at 25°C for varying lengths of time. Segments of root tissue 2 to 4 mm in length centered on the infection site were excised (Fig. 1D) and embedded horizontally, infection side up, in Tissue Tek II O.C.T. compound.

The embedded sections from both inoculation procedures were immersed in liquid N_2 slowly to avoid cracking the embedding material. Embedded tissues were stored at −70°C.

Identification of Pterocarpan Phytoalexins Produced in Infected Soybean Roots. Soybean seedlings were dip-inoculated with zoospores of either race 1 or race 3 of *P. megasperma* and incubated 24 to 28 h as described above. The roots were excised and immediately frozen in liquid N_2. Ten to twelve roots (about 0.5 g fresh weight) were combined and pulverized for 30 s in a Teflon vessel (5 ml) containing a tungsten carbide ball (5 mm diameter) using a Mikrodismembrator II (Braun, Melsungen, F.R.G.). The frozen root powder was immediately suspended in 10 ml of methanol. When the methanolic suspensions had come to about room temperature, the insoluble material was removed by centrifugation and the supernatant evaporated to dryness under reduced pressure at 30°C. The residue was taken up in 2 ml of chloroform and applied to a SepPak silica cartridge, and the cartridge washed with 5 ml of chloroform. Subsequently, the cartridge was eluted with 3 ml of chloroform:methanol (1:1, v/v), and the eluate evaporated to dryness under reduced pressure at 30°C. The residue was redissolved in 100 μl of ethanol, and the pterocarpans separated and quantitated by HPLC.

Extraction and Quantitation of Glyceollin I in Single Whole Roots. Soybean seedlings were infected with zoospores of *P. megasperma* using the dip inoculation procedure. After incubation, the entire root was excised and immediately frozen in liquid N_2. Frozen roots were stored at −70°C until used. Single frozen roots were pulverized as above. The resulting frozen root powder was suspended in 4 ml of methanol. When the methanolic suspensions had come to about room temperature, the insoluble material was removed by centrifugation. The supernatants were evaporated to dryness at 30°C under a stream of N_2. The residues were each taken up in 1 ml of methanol, and 0.1-ml aliquots of these solutions were diluted with 0.9 ml of distilled H_2O. Serial

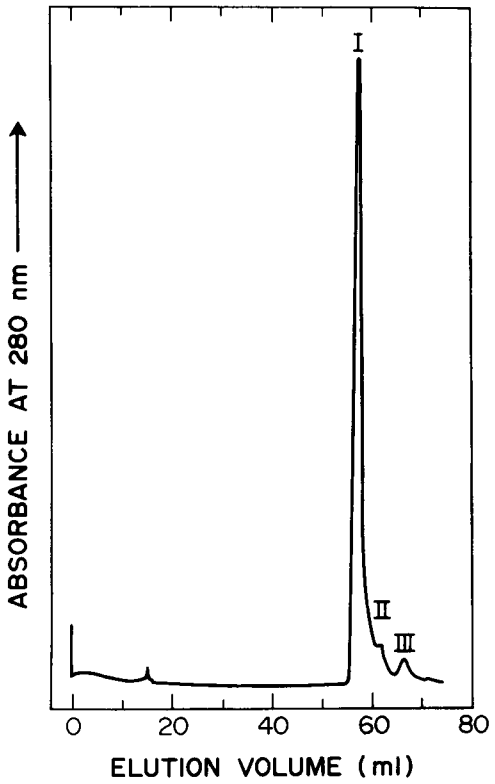

FIG. 3. Chromatography of an extract prepared from soybean roots 24 to 28 h after dip inoculation with race 1 of *P. megasperma* f. sp. *glycinea*. Preparation of the extract is described in the text. Chromatography on a Merck Lichrosorb Si 60 column (5 μm silica; 9 mm i.d. × 250 mm) at ambient temperature in hexane:isopropanol (92:8, v/v) permitted the separation of the glyceollin isomers I, II, and III.

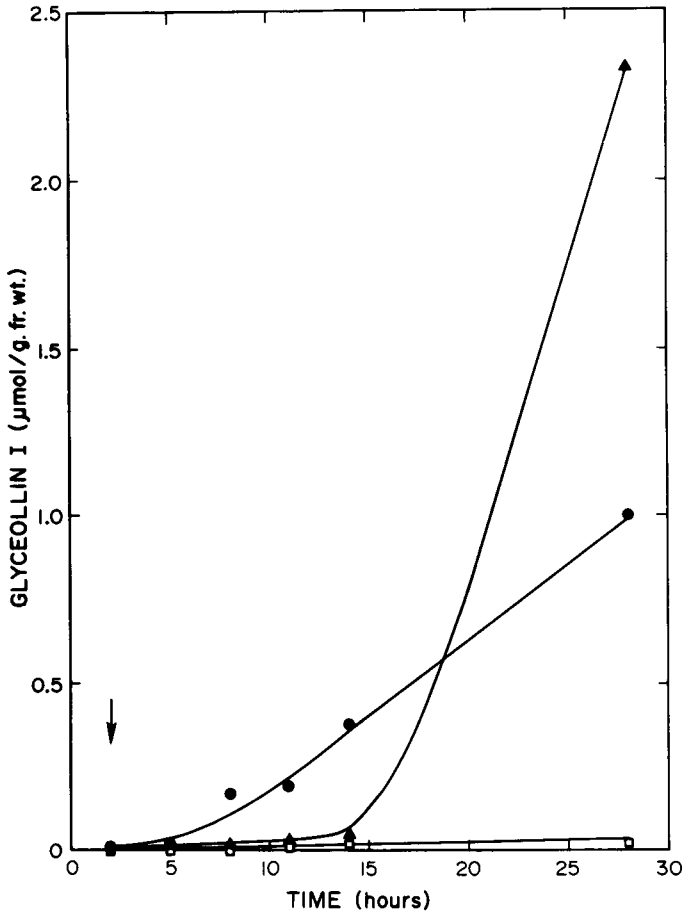

FIG. 4. Quantitation of glyceollin I by radioimmunoassay in single whole roots after dip inoculation with zoospores of *P. megasperma* f. sp. *glycinea* race 1 (●) or race 3 (▲), or treatment with sterile deionized H_2O (□). Each point represents the average of three roots. The data for the individual roots are shown in Table I. The arrow indicates the time when the seedlings were transferred from the zoospore suspension to sterile water.

1:10 dilutions of the resulting solutions were made using 10% (v/v) methanol in distilled H_2O. The amount of glyceollin I present in these solutions was determined using the radioimmunoassay.

Quantitation of Glyceollin I and Localization of Fungal Hyphae in Embedded Soybean Root Segments. Embedded soybean root segments were cut into 14 μm thick sections using a cryotome (Reichert-Jung, Heidelberg, F.R.G.) at −20°C. Every fourth section from dip-inoculated roots (Fig. 1B), and every other section from droplet-inoculated roots (Fig. 1D) were affixed to microscope slides that had been coated with Haupt's adhesive (18). The areas of these sections were determined under a microscope and the volumes of the sections calculated. Subsequently, the sections were examined immunohistochemically for the presence of fungal hyphae as described previously (31). The three intervening sections from dip-inoculated roots were combined (Fig. 1B) and extracted with 250 μl of 10% (v/v) methanol in distilled H_2O. Each intervening section from droplet-inoculated roots (Fig. 1D) was extracted with either 0.5 or 1 ml of 10% (v/v) methanol in distilled H_2O. The amounts of glyceollin I present in these extracts was determined directly using the radioimmunoassay. The concentrations of glyceollin I present in each thin section were calculated using a computer program written by the authors. The radioimmunoassay was able to measure glyceollin I concentrations in the sections as low as 0.01 μmol/ml.

The recovery of glyceollin I from cryotome sections was estimated by adding known amounts of purified glyceollin I to cryotome cross-sections (either 14 or 42 μm thick) from uninoculated soybean roots. Average recoveries of 90% from 14 μm thick sections, and 85% from 42 μm thick sections were obtained. The data presented in this paper have not been corrected.

RESULTS

Infections of Soybean Roots with Zoospores of *P. megasperma*. Infection of unwounded soybean roots with zoospores of *P. megasperma* was reproducibly carried out using either of the inoculation procedures described and the expected race specificity of the host-pathogen interaction was observed (Fig. 2). The incompatible interaction was characterized by a darkening of the infection site that was observed as early as 5 h after infection. The darkened area was identifiable as a hypersensitive ring necrosis on seedling roots 4 d after dip-inoculation (Fig. 2). In some instances, the primary root had ceased growing by this time (Fig. 2). The seedlings developed numerous secondary roots and could not be distinguished from water-treated plants in their aboveground growth.

The compatible interaction was characterized by a slight browning of the root 8 to 12 h after infection. When subsequently planted, the seedlings ceased growing almost entirely and developed no secondary roots during the 4 d following infection (Fig. 2). Often fungal mycelia could be observed growing out of the cotyledons. The roots remained turgid and were easily cut with a razor blade. The extensive collapse and rotting previously

Table I. *Glyceollin I Content of Single Soybean Roots Infected with P. megasperma f. sp. glycinea*

Soybean seedlings (cv Harosoy 63) were dip-inoculated with 10^4 zoospores of either race 1 or race 3 of *P. megasperma* for 2 h and then transferred to sterile H_2O and incubated further. Controls were treated with sterile deionized H_2O. After incubation at 25°C in the dark, the roots were frozen in liquid N_2, individually pulverized, and the root powder extracted with methanol. The glyceollin I contents of the extracts were determined by radioimmunoassay. Each value represents one root, and is the average of four or six determinations.

Time	Glyceollin I		
	Control	Race 1	Race 3
h		*nmol/g fresh wt*	
	0.2	21	0.9
2 + 0	<0.1	2.4	<0.1
	1.1	0.2	0.7
	<0.1	40	3.1
2 + 3	<0.1	11	6.7
	11	23	9.6
	10	150	6.5
2 + 6	12	260	5.4
	6.3	100	55
	1.6	220	3.7
2 + 9	20	280	70
	4.5	94	21
	1.1	280	7.1
2 + 12	19	650	8.1
	59	210	140
	0.8	1200	3500
2 + 26	46	490	1600
		1300	1900

observed 24 to 48 h after inoculation of wounded soybean hypocotyls with pieces of mycelia of compatible races (14, 23, 30) was not observed in susceptible zoospore-infected roots.

Water-treated control seedlings rarely showed any discoloration at the inoculation site, and developed normally when subsequently planted (Fig. 2).

Relative Amounts of Phytoalexins Produced in Soybean Roots. Glyceollin I was the only prominent peak observed in HPLC analysis of extracts of infected soybean roots. Glyceollin I constituted approximately 90% of the pterocarpan phytoalexins produced in such roots (Fig. 3) regardless of which race of *P. megasperma* was used for the infection. Only small amounts (<2%) of glyceollins II and III and glycinol were found. Thus, the radioimmunoassay, which is specific for glyceollin I (32), detects and quantitates about 90% of the total phytoalexin content of soybean root tissue.

Glyceollin I Content of Single Whole Roots. The glyceollin I content of single whole roots was determined at various times after dip-inoculation with zoospores of either race 1 or 3 of *P. megasperma* or treatment with sterile water. The time course of glyceollin I accumulation is most clearly seen in Figure 4. The average glyceollin I content of control roots remained low (<0.03 μmol/g fresh weight) throughout the incubation. In the incompatible interaction, glyceollin I was detected in extracts of whole roots examined 2 h after infection, and rose steadily thereafter, reaching an average of 1 μmol/g fresh weight 28 h after infection. In contrast, in the compatible interaction, the average glyceollin I content of the roots remained at or just above the values for control roots throughout the first 14 h after inoculation. Only at 28 h after infection did large amounts of glyceollin I (2.3 μmol/g fresh weight average) accumulate in susceptible roots.

There was considerable root-to-root variability in the glyceollin I content data (Table I). For example, one race 1-infected root contained appreciable amounts of the phytoalexin (21 nmol/g fresh weight) 2 h after infection, while the amount present in another was barely above the detection limit off the assay. By 5 h after infection, all three roots infected with the incompatible race contained more glyceollin I than the roots infected with the compatible race. This pattern continued through the first 14 h of infection, although single race 3-infected roots contained glyceollin I in amounts approaching those present in the race 1-infected roots. By 28 h after infection, the situation was reversed, with all three roots from the compatible interaction containing more glyceollin I than any of the roots from the incompatible interaction.

Extent of Fungal Colonization of Infected Soybean Roots. The extent of fungal colonization of soybean root tissue was followed immunohistochemically in cryotome sections of the roots. Fungal hyphae were present at the surface of dip-inoculated roots, or had penetrated a few cell layers 2 h after inoculation with either race of the fungus. Three h later, both races had penetrated about half the radius of the root, and had not progressed noticeably along the axis of the root toward the cotyledons. At later times, growth of race 1 (incompatible) proceeded slowly primarily toward, but rarely penetrated the endodermis, with fairly uniform dense colonization of the root cortex in the lower parts of the root (Fig. 5a). Closer to the infection boundary, the hyphal density decreased markedly, and the hyphae were only found near the epidermis (Fig. 5c). A clear infection boundary was found in most roots (Figs. 7 and 8), Beyond which no race 1 hyphae were observed (Fig. 5d). Single hyphae were occasionally observed near the epidermis throughout the piece of root sectioned (Fig. 8B). In contrast, hyphae of race 3 (compatible) penetrated the endodermis, and grew rapidly up the vascular tissue such that the limits of hyphal growth along the root axis were not found within the piece of root taken for sectioning 8 h after inoculation or thereafter. Furthermore, race 3 appeared to colonize the cortex of the root less heavily than race 1. Rather, race 3 hyphae were heavily concentrated around the endodermis, particularly at later times (Fig. 6). Thus, the extent of root tissue colonization by the two races of *P. megasperma* could be clearly differentiated starting 8 h after dip-inoculation.

The infection progressed more slowly in the droplet-inoculated roots used for longitudinal sectioning. Fungal hyphae were present only on the root surface of such roots 5.5 h after inoculation. By 9 h after inoculation, race 1 had penetrated 0.2 to 0.4 mm, while race 3 hyphae were found in the root interior 0.5 to 0.7 mm from the root surface. At later times, race 3 hyphae were found within the stele of the roots, while race 1 hyphae were only rarely observed there.

Precisely determining the infection boundary using longitudinal sectioning was, in contrast to the cross-sectional analysis, impossible for two reasons. First, both races grew primarily in a longitudinal direction (data not shown). Thus, the hyphal tips had grown out of the piece of root taken for sectioning. Second, the infection droplet covered from 20 to 40% of the circumference of the soybean roots, a sizable arc relative to the thickness of the sections. Longitudinal sectioning was carried out transversely parallel to a tangent of the cylinder of root tissue (Fig. 1D). Growth of fungal hyphae toward the root center occurred radially as observed in root cross-sections (see above). Hyphae would therefore be observed in the root interior in a section more distal from the origin of sectioning than they had actually penetrated from the root surface. To precisely determine the depth of fungal penetration, cylindrical cryotome sections would

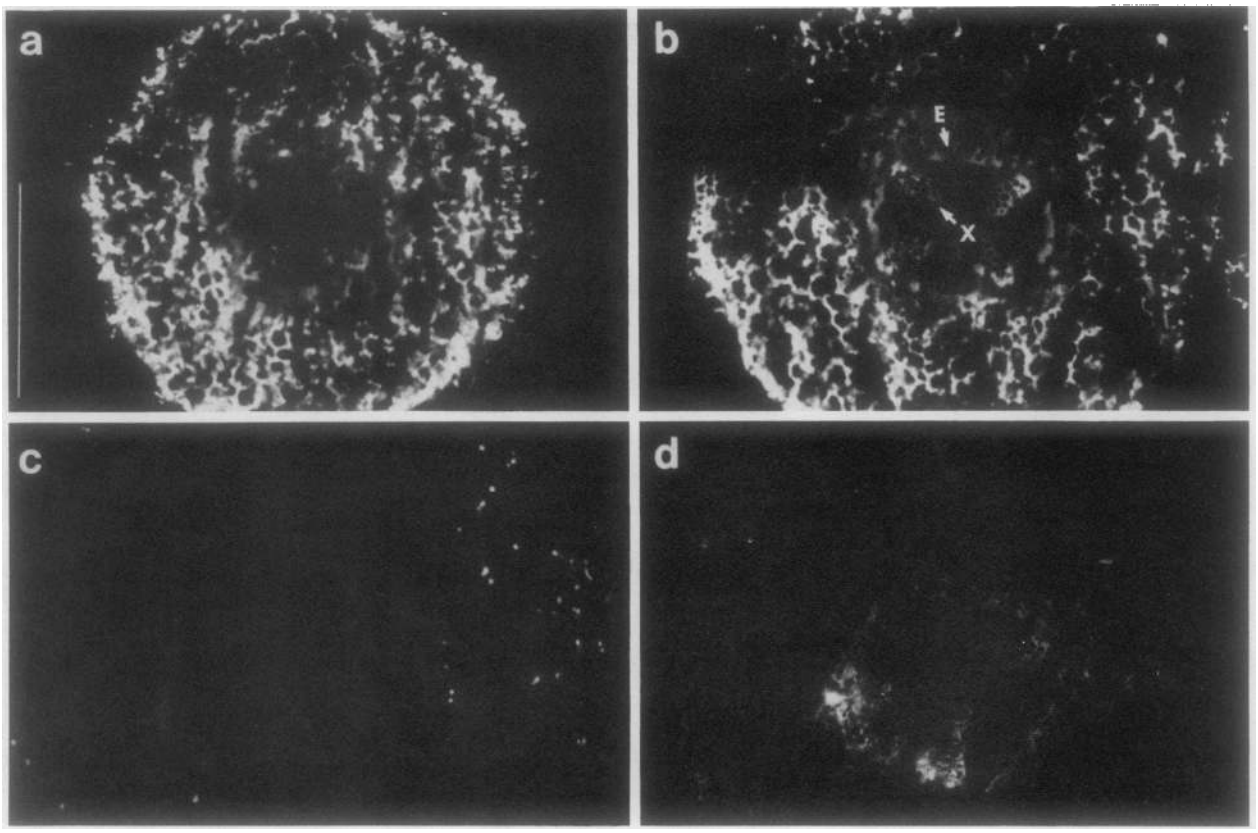

FIG. 5. Immunohistochemical localization of hyphae of *P. megasperma* f. sp. *glycinea* race 1 in cryotome cross-sections (14 μm thick) of soybean roots 11 h after dip inoculation. The position xylem vessels (X) and the endodermis (E) are noted. The bar represents 0.5 mm. The glyceollin I distribution for this root is shown in Fig. 7. a, Cross-section 0.45 mm from excision point (see Fig. 1B). b, Cross-section 1.8 mm from excision point. c, Cross-section 2.5 mm from excision point. d, Cross-section 5.1 mm from excision point.

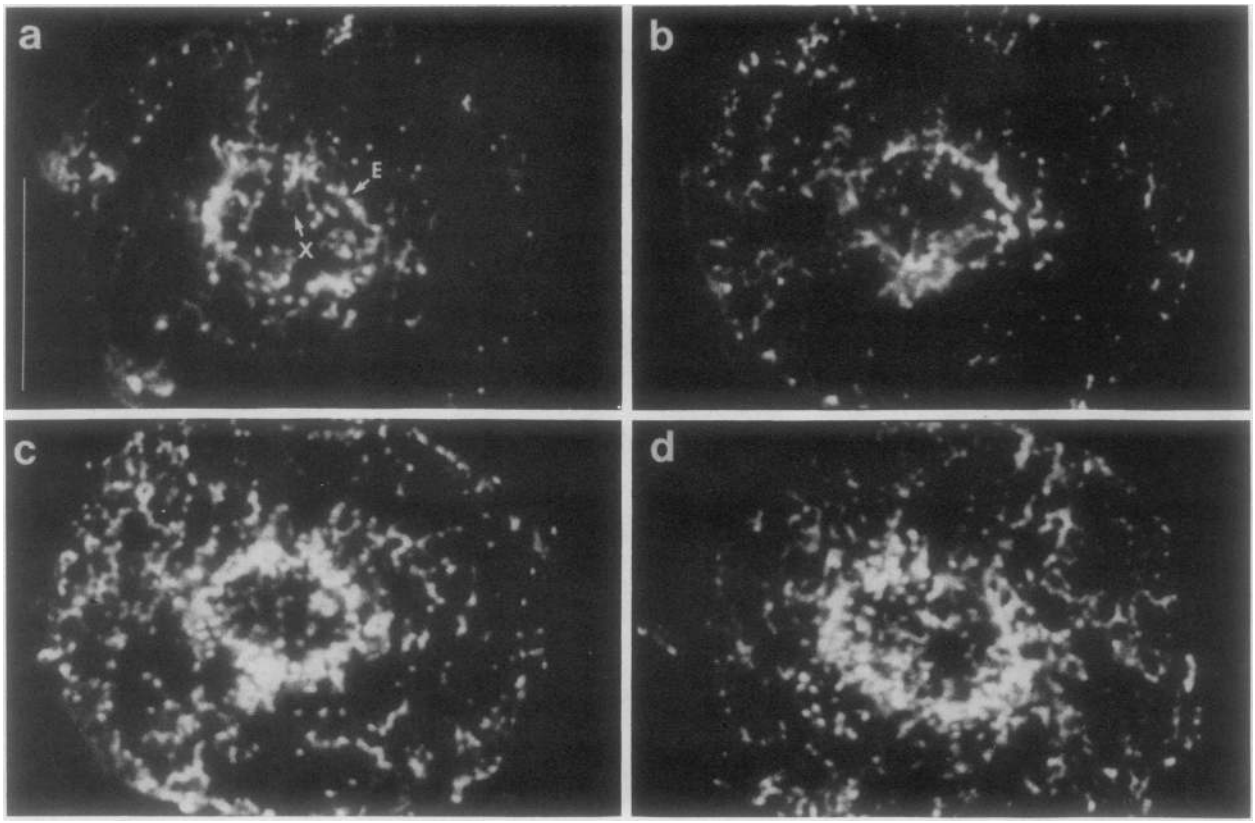

FIG. 6. Immunohistochemical localization of hyphae of *P. megasperma* f. sp. *glycinea* race 3 in cryotome cross-sections (14 μm thick) of soybean roots 11 h after dip inoculation. The position of xylem vessels (X) and the endodermis (E) are noted. The bar represents 0.5 mm. a, Cross-section 1.6 mm from excision point (see Fig. 1B). b, Cross-section 2.7 mm from excision point. c, Cross-section 3.5 mm from excision point. d, Cross-section 6.1 mm from excision point.

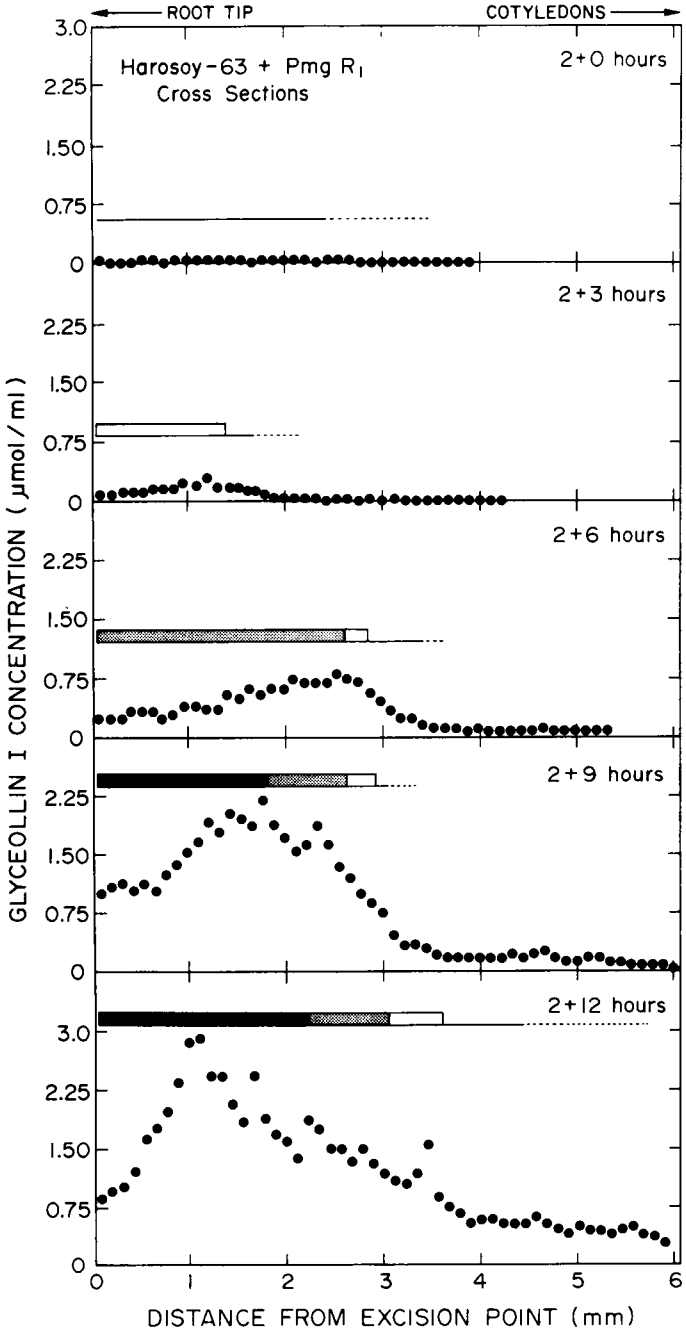

FIG. 7. Spatial and temporal course of glyceollin I accumulation along the axis of soybean roots after infection with race 1 of *P. megasperma* f. sp. *glycinea*. Seedlings (2 d old) were dip-inoculated with about 10^4 zoospores as described in the text. Glyceollin I was quantitated in extracts of cryotome cross-sections (3 × 14 μm thick; see Fig. 1B) by radioimmunoassay, and each point is the average of two determinations. Data are shown for every other triple section. The *in vitro* EC_{90} of glyceollin I against *P. megasperma* is 0.6 μmol/ml (26, 39). The extent of fungal colonization, determined immunohistochemically, is indicated by the bars: (■), >90% of cross-section colonized; (▩), 50 to 90% colonized; (□), 25 to 50% colonized; (——), a few hyphae; (– – –), single hyphae.

have to be made. This is technically not possible at present.

Quantitative Axial Distribution of Glyceollin I in Infected Soybean Roots. The quantitative distribution of glyceollin I along the axis of dip-inoculated soybean roots was determined by quantitating the phytoalexin in serial cryotome cross-sections of the roots. Such distributions in several roots at differing times after infection with the incompatible race 1 of *P. megasperma* are shown in Figure 7. Glyceollin I was first quantifiable in cross-sections 5 h after infection, being present in concentrations approaching the EC_{50} of this phytoalexin (0.17 μmol/ml) measured *in vitro* against *P. megasperma* (26). At later times, the phytoalexin concentrations in resistant roots reached or exceeded the *in vitro* EC_{90} (0.6 μmol/ml [26, 29]) (Figs. 7 and 8). The glyceollin I concentrations dropped sharply at the leading edge of the infection. In no resistant root were fungal hyphae seen in advance of measurable concentrations of the phytoalexin. In those roots where single hyphae were seen throughout the root, glyceollin I concentrations were at inhibitory concentrations in all cross-sections (Fig. 8B). Occasionally, significant concentrations of glyceollin I were detected in advance of the fungal hyphae (Fig. 8B).

Cryotome cross-sections were also prepared from roots dip-inoculated with the compatible race 3 at each of the time points shown in Figure 7. Low concentrations (<0.08 μmol/ml) of glyceollin I were detected in occasional cryotome cross-sections of roots 11 and 14 h following inoculation, but not earlier. No glyceollin I was detected in cross-sections of control roots at any time following inoculation.

The above results were obtained from single roots. In order to examine the root to root variability, the glyceollin I and hyphal distributions in three resistant roots at each of two time points after inoculation were determined (Fig. 8). As expected, there was some variation in the details of the distribution patterns probably due to biological variability of the soybean plants. The maximum concentrations of glyceollin I varied somewhat, as did the extent of fungal colonization. However, the basic patterns described above were observed in all roots.

Distribution of Glyceollin I in Longitudinal Root Sections. Additional information on the localization of glyceollin I in infected soybean roots was obtained from analyses of serial longitudinal cryotome sections of droplet-inoculated roots. The glyceollin I distributions for roots infected with either race 1 or race 3 of *P. megasperma* at varying times after inoculation are shown in Figure 9. The data show that the greatest accumulation of the phytoalexin occurs in the epidermal cell layers of the root. Low levels of glyceollin I were detected in the epidermal cell layers 5.5 h after infection with either race. In the incompatible (host-resistant) interaction, the concentrations of the phytoalexin in the epidermal cell layers rise dramatically at later times, going as high as 36 μmol/ml. Furthermore, the distance from the root surface at which the phytoalexin concentrations reach or exceed the EC_{90} for glyceollin I also increases. In contrast, the compatible (host-susceptible) interaction did not show such a dramatic rise in glyceollin I concentrations in the epidermal cell layers, nor did the EC_{90} boundary extend beyond 0.25 mm from the root surface at any time after infection.

The root to root variability of the longitudinal analyses was greater than that observed in the cross-sectional analyses (Figs. 10 and 11). When individual resistant and susceptible roots were compared, differences were not always apparent. However, when several roots of each type were compared, differences became clear. For example, maximum glyceollin I concentrations in the epidermal cell layers average 20 μol/ml in four resistant roots (Fig. 10) *versus* 6 μmol/ml in four susceptible ones (Fig. 11) 9 h after inoculation. The average maximum depth at which the phytoalexin concentrations exceed the EC_{90} is 0.4 mm in resistant roots (Fig. 10) *versus* 0.2 mm in susceptible ones (Fig. 11) at the same time.

DISCUSSION

The results reported in this paper describe the quantitative localization of a phytoalexin around invading fungal hyphae.

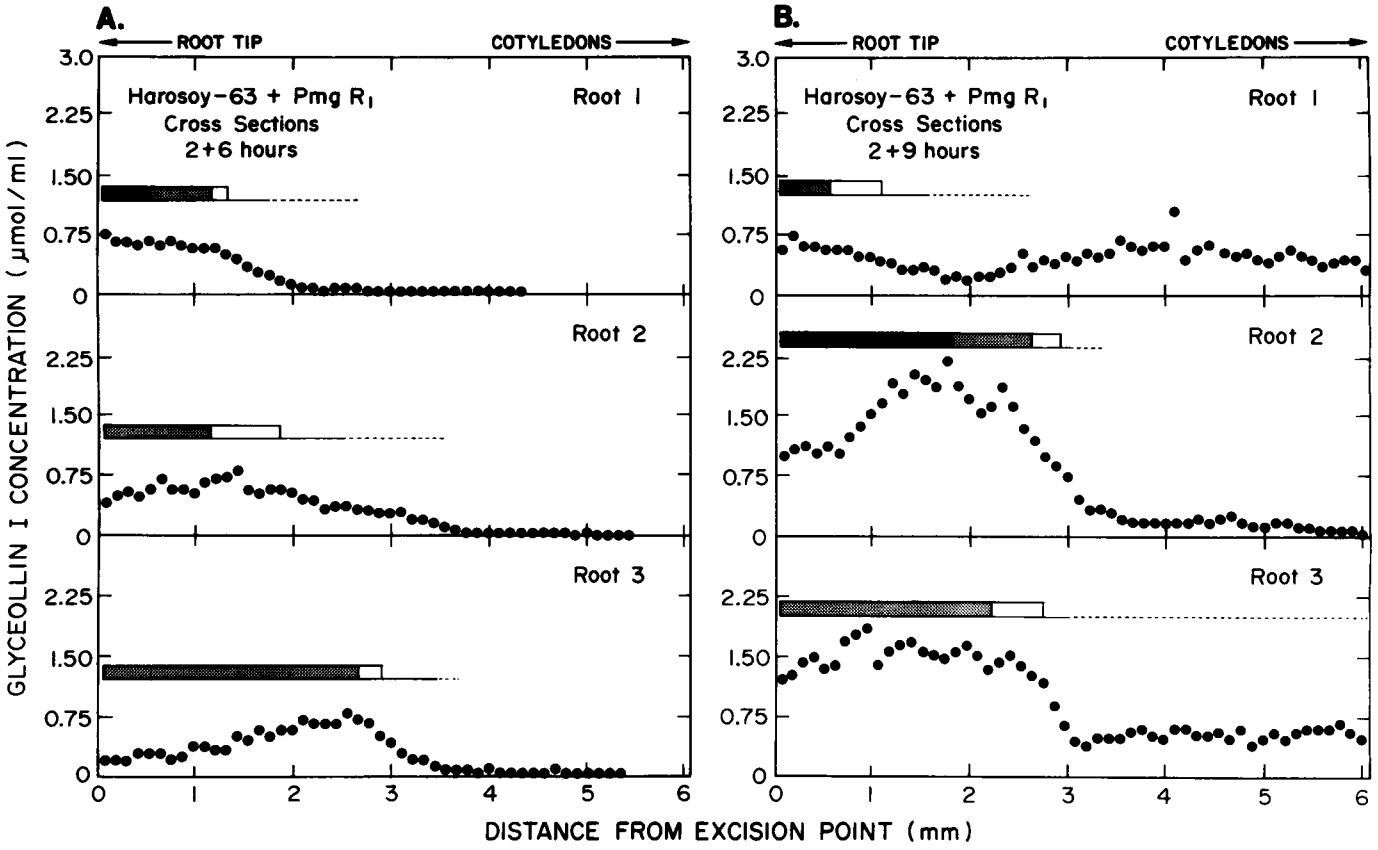

FIG. 8. Axial distribution of glyceollin I in three roots at each of two times (8 and 11 h) after dip inoculation with zoospores of race 1 of *P. megasperma* f. sp. *glycinea*. Details are the same as in Figure 8.

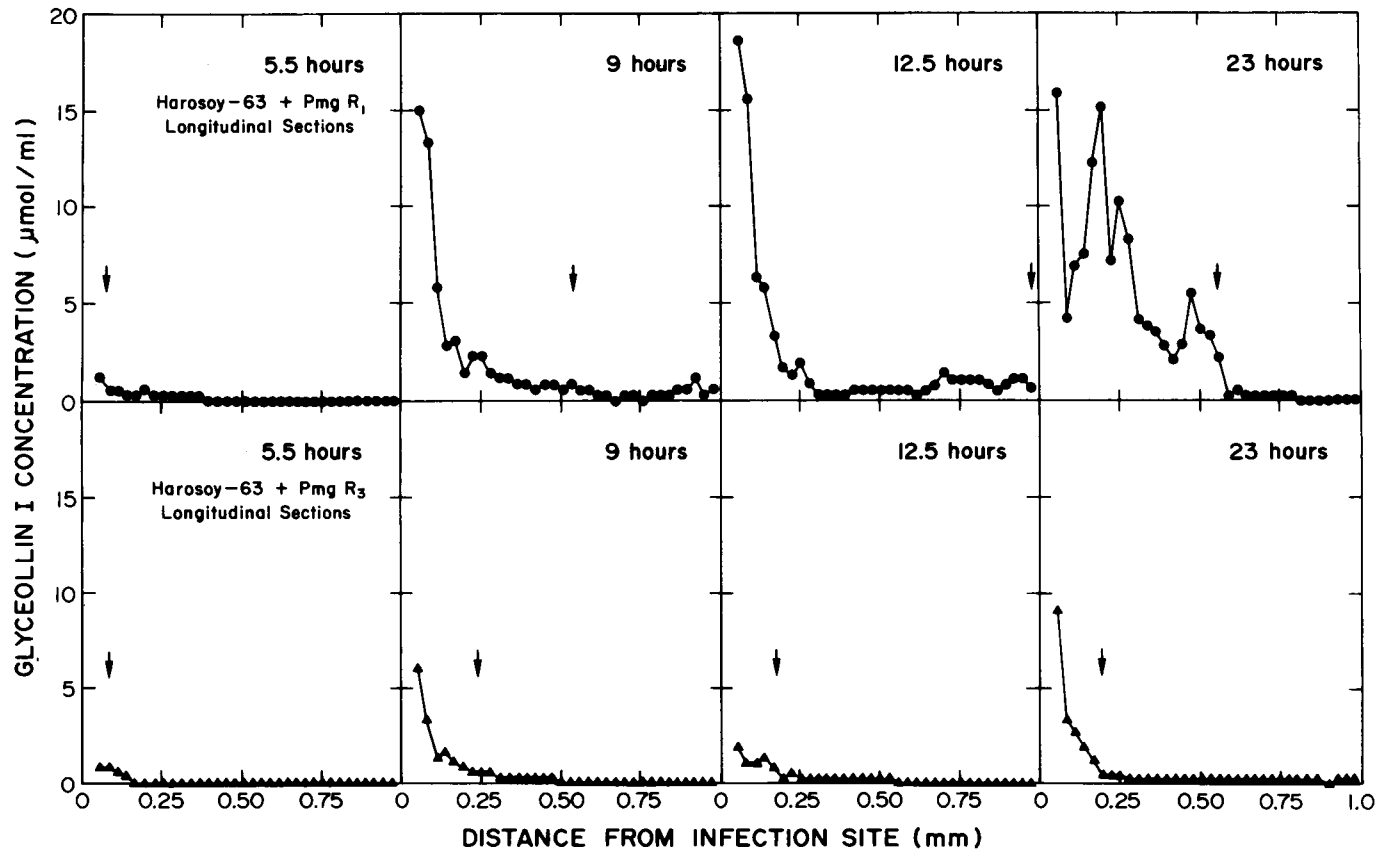

FIG. 9. Lateral spatial and temporal course of glyceollin I accumulation in single soybean roots infected with race 1 or race 3 of *P. megasperma* f. sp. *glycinea*. Seedlings (3 d old) were droplet-inoculated with about 500 zoospores as described in the text. Glyceollin I was quantitated in extracts of cryotome longitudinal sections (15 μm thick; see Fig. 1D) by radioimmunoassay, and each point is the average of two determinations. Arrows indicate the greatest distance from the infection site at which the glyceollin I concentrations reached or exceeded the *in vitro* ED_{90} (0.6 μmol/ml). The average diameter of the roots was about 2 mm.

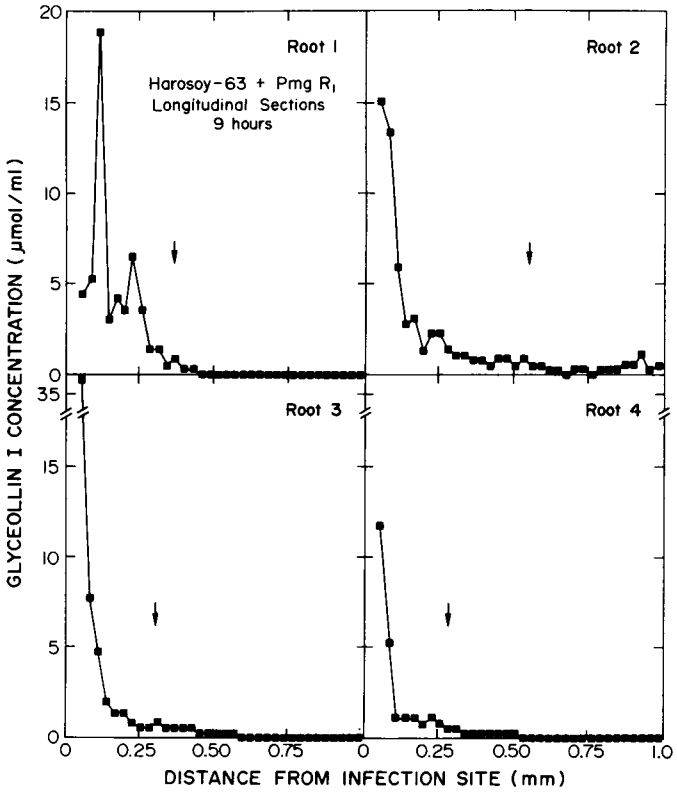

FIG. 10. Lateral distribution of glyceollin I in four soybean roots 9 h after droplet inoculation with zoospores of race 1 of *P. megasperma* f. sp. *glycinea*. Details are the same as in Figure 9. Fungal hyphae had penetrated 0.2 to 0.4 mm in these roots.

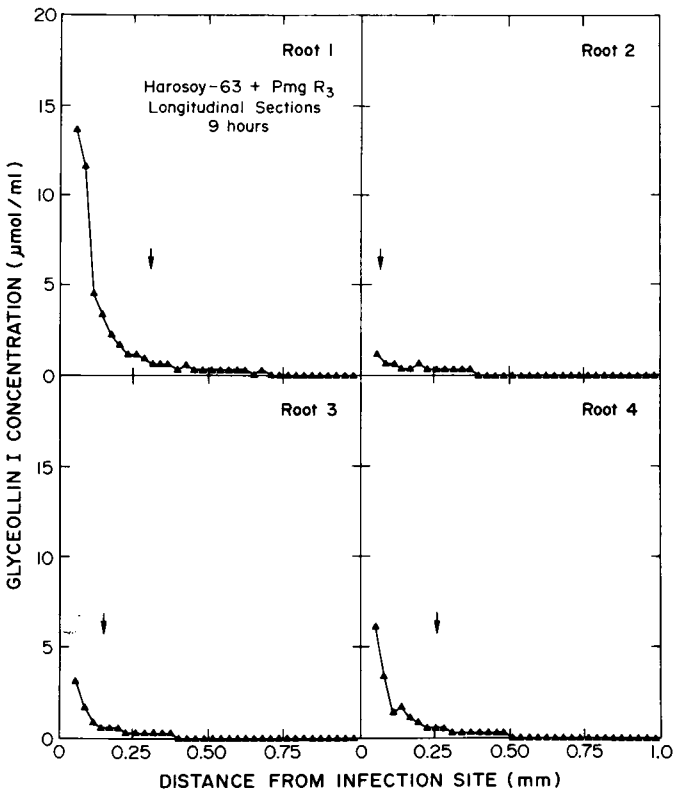

FIG. 11. Lateral distribution of glyceollin I in four soybean roots 9 h after droplet inoculation with zoospores of race 3 of *P. megasperma* f. sp. *glycinea*. Details are the same as in Figure 9. Fungal hyphae had penetrated 0.5 to 0.7 mm in these roots.

These experiments were possible because of three developments: a specific radioimmunoassy for the major soybean phytoalexin, glyceollin I (32); an immunofluorescent stain for the fungal hyphae (31); and an inoculation system (Fig. 1A) that mimics the natural one as closely as possible under laboratory conditions. The sensitivity of the radioimmunoassay and the immunofluorescent stain allowed quantitation of phytoalexin and localization of hyphae in the same root. In this work, the comparisons between the compatible and incompatible interactions were made using a single soybean cultivar, thus avoiding potential difficulties (3) arising when these two different plant responses are compared using different soybean cultivars (23, 39). The use of the zoospore-root inoculation system avoids artefacts arising from wounding and allows fungal inocula to be reproducibly quantitated. This infection system consistently yielded the expected pathogenicity pattern of races 1 and 3 on soybean cv Harosoy 63 (Fig. 2), as well as on cv Harosoy and cv Wells II (data not shown). Furthermore, the infected roots accumulated the pterocarpan phytoalexins (Fig. 3) previously identified in other soybean tissues (22). Thus, we conclude, in contrast to others (25), that *P. megasperma*-infected soybean roots are a useful system for studying this host-pathogen interaction.

Wounded hypocotyls inoculated with mycelia were used in previous studies on glyceollin accumulation in the soybean-*P. megasperma* interaction (2, 23, 30, 39). In these studies, glyceollin was first detected in whole hypocotyl segments between 8 and 12 h after inoculation. Significant differences in phytoalexin accumulation between compatible and incompatible interactions were observed at later times. Yoshikawa *et al.* (39) examined and compared glyceollin accumulation in 0.25 mm thick longitudinal sections from two soybean cultivars infected with race 1 of *P. megasperma*, one giving an incompatible, the other a compatible response. It was concluded that toxic levels of glyceollin were present in the incompatible interaction at a time when fungal growth appeared to slow or stop (9–12 h after inoculation). Based on these and other data (38, 40), Keen and Bruegger (24) proposed a time course for the incompatible interaction in which glyceollin begins to accumulate 6 to 7 h, and reaches EC_{90} concentrations 8 h after inoculation.

In the present study, glyceollin I was shown to be present in some roots 2 h, and in most roots 5 h after dip inoculation with an incompatible race (Table I) suggesting that glyceollin I accumulation is a more rapid plant response than previously thought (24). The rapid increase in glyceollin I acumulation observed beginning 5 h after inoculation (Fig. 4) correlates well with recent studies on the induction of the biosynthetic pathway for this phytoalexin. Börner and Grisebach reported induction of phenylalanine ammonia-lyase and chalcone synthase, two early enzymes in the pathway, as early as 6 h, and maximum enzyme activities between 12 and 15 h after inoculation of wounded hypocotyls with mycelia (11). More recently, the *in vitro* translational activities of mRNA for these two enzymes, and 4-coumarate CoA-ligase, as well as the relative amount of chalcone synthase mRNA were shown to increase by 3 h, and reach a maximum 6 h after inoculation in the same system (20, 34). Ebel *et al.* (15, 34), using elicitor-induced soybean cell suspension cultures, reported similar induction time courses. Further studies using a natural infection system and more sensitive techniques would refine our understanding of the induction and regulation of this pathway particularly at early times in the host-pathogen interaction.

The distribution of glyceollin I within infected roots was determined in the axial (using dip-inoculated roots) and the longitudinal (using droplet-inoculated roots) directions. The for-

mer analysis showed that local phytoalexin concentrations high enough to significantly inhibit hyphal growth were present in cross-sections containing hyphae in the incompatible interaction at 5 h, and exceeded the EC_{90} 8 h after infection (Figs. 7 and 8). At the infection boundary, hyphae were found only near the epidermis where the majority of the phytoalexin accumulates (Figs. 9 and 10). The decline in phytoalexin concentration at the infection boundary seen in most incompatible roots (Figs. 7 and 8) was also observed in infected hypocotyls (33, 39). Occasionally, substantial phytoalexin concentrations were present in advance of any detectable fungal hyphae (*e.g.* Fig. 8B, Root 1), a sign that molecular signals of fungal or plant origin, or a combination of both (13), may be moving ahead of the infection boundary. In sharp contrast, there was not an inhibitory concentration of the phytoalexin in cross-sections containing hyphae in the compatible (host-susceptible) interaction throughout the first 14 h after infection. Differences in the extent of fungal colonization between the two interactions were seen 8 h after dip-inoculation, but not earlier. Altogether, the glyceollin I distribution pattern along the root axis is consistent with the hypothesis that phytoalexins play an important role in halting fungal growth in the incompatible (host-resistant) interaction.

The analysis above does not fully take into account possible unequal distribution of the phytoalexin within the cross-sections. Longitudinal sectioning, undertaken in an effort to address this question, demonstrated that most of the phytoalexin accumulated in the cell layers near the epidermis in both the incompatible and the compatible interactions (Figs. 9–11). Inhibitory concentrations were present in these cell layers 5.5 h after droplet inoculation (Fig. 9) before significant fungal penetration was detected. Thus, phytoalexin accumulation is a very rapid response of epidermal cells to the presence of the fungus. Hyphae of the compatible race 3 did not elicit as dramatic a rise in phytoalexin levels as did the incompatible race 1, and had penetrated beyond the inhibitory zone 9 h after inoculation (Figs. 10 and 11). At later times, the compatible hyphae predominantly colonized the root stele with lesser hyphal growth in the rest of the root (Fig. 6). Stössel *et al.* (36) also observed increased hyphal colonization toward the center of zoospore-infected soybean hypocotyls in a compatible interaction. The fact that the root stele is the primary site of fungal growth in the compatible interaction while the epidermal cell layers are the primary site for glyceollin I accumulation could explain why growth of the compatible race remained unchecked despite the high phytoalexin content of whole roots at 28 h (Fig. 4; Table I). In contrast, hyphae of the incompatible race 1 had penetrated the root cortex 9 h after inoculation but were still located in tissue containing inhibitory glyceollin I concentrations (Fig. 10). Cross-sectional analysis showed that race 1 penetrated as far as the endodermis at later times (Fig. 5a). Thus, race 1 colonized the entire root cortex in the vicinity of the infection site despite inhibitory concentrations of the phytoalexin, although growth was clearly slower than that of the compatible race 3. The inability of race 1 to colonize the root stele as race 3 did (Figs. 5 and 6) may have been due to weakening of the incompatible fungus by the continual proximity of high phytoalexin concentrations. Alternatively, one can envision a model where phytoalexins form a second layer of defense behind the structural barrier of the root surface. In this model, both races are capable of penetrating through the primary zone of phytoalexin accumulation in the epidermal cells. The incompatible race then triggers another as yet unidentified plant response which prevents colonization of the root stele and hence leads to resistance. The compatible race avoids triggering this response and hyphal growth remains unchecked.

The pattern of glyceollin I accumulation in whole roots (Fig. 4) is reminiscent of that observed in infected hypocotyls of *Phaseolus vulgaris* (6, 10), except that the time scale for soybean roots is much compressed. In the *P. vulgaris-Colletotrichum lindemuthianum* interaction it has been proposed that compatibility is associated with the ability of the pathogen to maintain biotrophic growth for an extended period of time as compared with the incompatible interaction where a rapid necrosis (hypersensitivity) occurs (6, 8). It has been proposed (4, 5, 27) that the rapid necrosis of plant cells in an incompatible interaction may be the trigger which activates the plant's defense responses, including phytoalexin accumulation. In the present study, visible browning at the site of inoculation at 5 to 8 h in the incompatible interaction, and the absence of tissue collapse even 4 d after infection in the compatible interaction were noted. These observations suggest that a detailed histopathological examination of both the compatible and incompatible soybean-*P. megasperma* interactions at early times, particularly with reference to the timing and localization of host cell death and/or host cell wall changes (*e.g.* lignification, callose deposition, hydroxyproline-rich glycoprotein synthesis), would provide useful information about the importance of these plant responses in the outcome of this host-pathogen interaction.

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