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Glyceollin I in Soybean-Cyst Nematode Interactions[1]

Spatial and Temporal Distribution in Roots of Resistant and Susceptible Soybeans

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ABSTRACT

Accumulation of the phytoalexin glyceollin I in roots of soybean (*Glycine max* [L.] Merr.) following inoculation with race 1 of *Heterodera glycines* Ichinohe, the soybean cyst nematode (SCN), was determined in a whole-root system by high performance liquid chromatography (HPLC) and in a cross-section system by a radioimmunoassay procedure. In the whole-root system, roots were harvested from controls and nematode-inoculated seedlings immediately after inoculation and at 2-day intervals for 8 days. The roots were extracted with ethanol, and the extracts were subjected to HPLC. Glyceollin I was not detected in roots of either resistant cultivar Centennial or susceptible cultivar Ransom immediately after inoculation with SCN but steadily accumulated in large quantity in roots of Centennial. Accumulation of glyceollin I in roots of Ransom following nematode inoculation was minimal. In the cross-section system, 3-day-old soybean seedlings were inoculated with juvenile nematodes, and root segments containing a single nematode were dissected from inoculated plants at 4-hour intervals under a dissecting microscope. The root segments were embedded in ice and cut into 16-micrometer sections with a cryostat microtome. The spatial and temporal distribution of glyceollin I was determined with a radioimmunoassay procedure specific for the phytoalexin. Glyceollin I was found to accumulate in tissues immediately adjacent to the head region of the nematode in Centennial but not in Ransom. Glyceollin I was detected 8 hours after nematode penetration, and the concentration increased steadily up to 0.3 micromole per milliliter in Centennial 24 hours after penetration.

Heterodera glycines Ichinohe, the SCN,[2] is one of the most destructive pests in many soybean-growing regions. In the 16 southern states of the United States, soybean yields valued between $83 and $166 million were lost to this pest annually between 1984 and 1987 (25–27). This soil-borne nematode infects roots of susceptible soybeans and disrupts symbiotic nitrogen fixation by suppressing nodulation and reducing nitrogen-fixing efficiency (2, 18). As a result, infected soybeans are usually stunted and chlorotic due primarily to nitrogen deficiency.

The soybean cyst nematode has many infraspecific races (9, 28, 29, 32). Responses of soybean cvs, breeding lines, and plant introductions to the inoculation of various races of this nematode have been investigated (17, 20, 22, 31). These results indicated that suppression of nodulation by SCN was determined by the soybean cv-nematode race interaction. For example, SCN race 1 reproduces well and severely suppresses nodulation and nitrogen fixation in cvs Lee 68, Lee 74, and Ransom, whereas it reproduces poorly and causes only mild damage to cvs Peking, Pickett, and Centennial (17, 31). The resistance mechanisms, however, have not been studied.

The resistance of a soybean cv to SCN is dependent on factors other than its ability to impede nematode penetration. Reports have indicated that there is no significant difference between the rates of penetration of resistant and susceptible soybeans by the juveniles (7, 17, 30). Only when juveniles penetrate roots, migrate through cortical tissues, and become sedentary in steles are sharp differences manifested between resistant and susceptible plants. On susceptible plants, cellular hypertrophy and cell-wall dissolution occur 1 d after nematode penetration, and syncytia become well established in 2 to 6 d. On resistant plants, nematode infection causes a hypersensitive response that is characterized by necrosis at the stele and the cortex surrounding the invading nematode. Although syncytia form within 2 to 3 d after infection, many degenerate within 5 d and associated juveniles die (7).

Phytoalexins are secondary metabolites produced in certain plants in response to microbial infection and/or abiotic stress. Phytoalexins called glyceollins are frequently synthesized and accumulated in soybeans in response to inoculation with incompatible pathogens and are associated with resistance against certain fungal, bacterial, viral, and nematode diseases (14–16). Although glyceollins are generally considered as antimicrobial compounds and may play a role in disease resistance, few thorough studies have been made regarding the timing, location, and quantity of glyceollins accumulated. One of the exceptions is the *Phytophthora megasperma* f.sp. *glycinea*-soybean interaction where spatial and temporal distribution of glyceollin I in an incompatible combination was determined by a radioimmunoassay procedure (10).

The role of glyceollins in soybean-cyst nematode interactions has not been investigated. For glyceollins to be important in resistance to SCN, infected soybean tissues must rapidly accumulate sufficient quantity of these phytoalexins to exert detrimental effects on the invading nematode at the sites of infection. The objectives of this research were to

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[2] Abbreviation: SCN, soybean cyst nematode.

determine: (a) whether there is a differential accumulation of glyceollin I in soybeans resistant and susceptible to SCN; and (b) its spatial and temporal distribution at and near the sites of SCN infection. The biological effects of glyceollin I on SCN will be published elsewhere.

MATERIALS AND METHODS

Source of *H. glycines* Race 1 and Soybean Seeds

A culture of race 1 of *H. glycines* from North Carolina as defined by Golden *et al.* (9) was reared on Lee 68 soybean (*Glycine max* [L.] Merr.) in a greenhouse as previously described (11). Second-stage juveniles, collected from hatching eggs from mature cysts according to the procedure described (11), were used as inocula. Seeds of soybean cvs Ransom (susceptible to race 1 of *H. glycines*) and Centennial (resistant to race 1 of *H. glycines*) were obtained from N. C. Foundation Seed Producers Association (Raleigh, NC) and Dr. D. P. Schmitt of North Carolina State University (currently at University of Hawaii, Honolulu), respectively.

Extraction and Quantification of Glyceollins in Whole Root Systems

Soybean seeds of Ransom and Centennial were surface-sterilized in 0.5% sodium hypochlorite for 3 min, rinsed, and germinated in vermiculite in a greenhouse. Three-day-old seedlings were transplanted to flat trays containing 212-μm quartz sand (Whitehead Brothers Co., Florham Park, NY) and were inoculated with either 0 or 600 juveniles/seedling. Inoculated seedlings were harvested immediately and at 2-day intervals for 8 d, and roots were excised from plants, washed free of sand, and blotted dry. From each treatment and harvest, three roots were stained for nematode penetration (4), and nine roots were weighed and frozen in liquid nitrogen and stored at −70°C until use for quantification of glyceollins. The experiment was repeated twice.

Glyceollins were extracted from soybean roots according to the procedure described by Moesta and Grisebach (23) with some modifications. Roots (2–3 g fresh weight) were extracted with boiling ethanol (1:10 [w/v]) twice, and washed with cold ethanol once (1:10 [w/v]). The ethanol extracts and washings were combined, centrifuged to remove debris, and dried under vacuum. The residues were taken up in 2 mL of chloroform and applied to Sep-Pak silica cartridges (Waters Associates, Milford, MA). Nonpolar components were washed out of the cartridges with 5 mL of chloroform. Isoflavones were eluted from the cartridges with 3 mL of chloroform-isopropanol (1:1 [v/v]), and dried under nitrogen (23). They were then taken up in a small amount of hexane-isopropanol-methanol (94:4:2 [v/v]) and subjected to HPLC with a Perkin-Elmer LC-4 system equipped with a silica column (250 mm × 4.6 mm, Econosphere silica, 5-μm particle size, Alltech Associates, Deerfield, IL). Hexane-isopropanol-methanol was used as the mobile phase at a flow rate of 2.5 mL/min. The effluent was monitored at 285 nm. Fractions containing glyceollins I, II, and III were identified, and their concentrations calculated with a standard curve and a SP4270 integrator (Spectra-Physics, San Jose, CA). The standard curve was developed from a mixture of glyceollins I, II, and III obtained from Dr. N. T. Keen of University of California-Riverside. Molar extinction coefficients of 10,300, 8,700, and 9,600 at 285 nm were used to calculate the concentrations of glyceollins I, II, and III, respectively (1, 19).

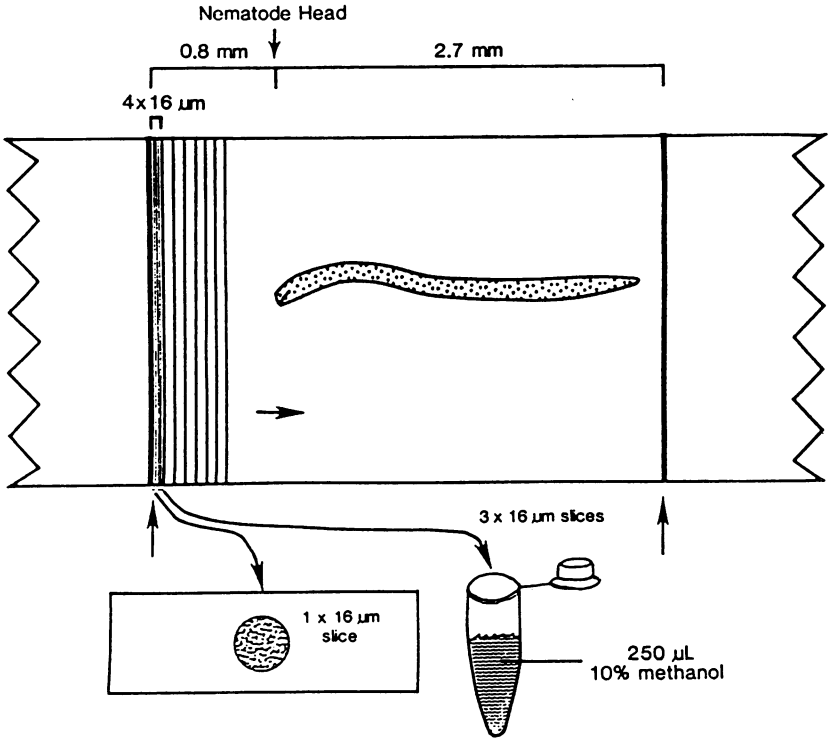

Figure 1. Cryostat microtomy of a root segment containing a single, second-stage juvenile of *H. glycines*. The root segment was dissected from the inoculated soybean root and was embedded in an ice block. Sections of 16 μm in thickness were cut with a cryostat microtome at −20°C. The first of every four sections was mounted on a microscope slide for location and orientation of the nematode. The remaining three sections were placed in an Eppendorf tube containing 250 μL of 10% methanol for glyceollin extraction.

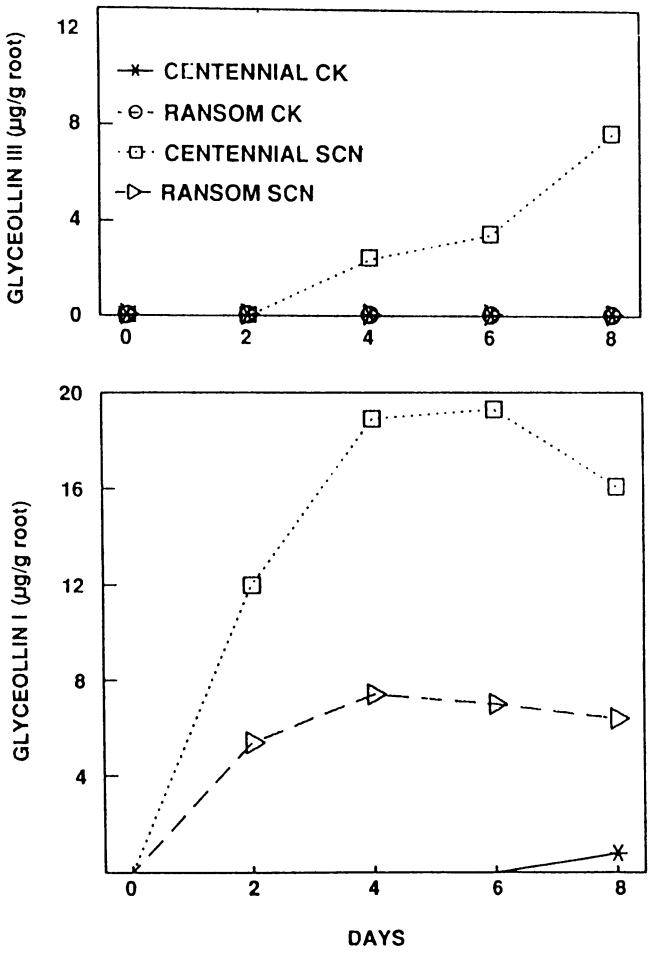

Figure 2. Accumulation of glyceollins I and III in the roots of resistant cv Centennial and susceptible cv Ransom following the inoculation of race 1 of *H. glycines*. No glyceollin II was detected in either cv.

Quantification and Localization of Glyceollin I in Sections of Nematode-Infected Roots

Inoculation Procedure

Three-day-old Ransom and Centennial soybean seedlings, germinated in wet paper towels from sterilized seeds, were transplanted to flat trays of acid-washed, sterilized, 212-µm quartz sand. Roots of each seedling were inoculated with either 0 or 200 juveniles of race 1 of *H. glycines*. One hour after inoculation, all seedlings were removed, washed, and replanted into fresh, acid-washed and sterilized quartz sand to synchronize nematode penetration. Six samples were harvested from each treatment at 4-h intervals for 24 h. The experiment was repeated twice.

Sectioning

Roots were washed and examined under a dissecting microscope. Segments, each 3.5 mm in length, containing a single nematode, were dissected and placed in depressions (14 mm long × 5 mm wide × 3 mm deep) of a flat silicone rubber embedding mold (Ladd Research Industries, Burlington, VT) filled with water. The root segments were oriented along the long axis of the depression and embedded in ice blocks by placing the embedding mold in a freezer.

The ice blocks were cut into 16-µm sections with a cryostat (Model CTD, International Equipment Co., Needham Heights, MA) at −20°C. The first of every four sections was mounted on a microscope slide for location and orientation of the nematode, and the remaining three sections were placed in a 1.5-mL Eppendorf centrifuge tube containing 250 µL of 10% methanol for extraction and radioimmunoassay of glyceollin I (Fig. 1).

Radioimmunoassay of Glyceollin I

Glyceollin I used in the radioimmunoassay was isolated from Centennial soybean seeds challenged with *Cladosporium cucumerinum* according to the procedure of Ayers *et al.* (1). The ethanol extract was filtered through Miracloth and dried under reduced pressure. The residues were taken up in a small volume of chloroform, passed through Sep-Pak columns, and subjected to HPLC as described above. Fractions containing glyceollin I were collected by a fraction collector and subjected to a second HPLC. The glyceollin I thus obtained had a UV-absorption spectrum in agreement with published data (12).

Conjugation of glyceollin I to BSA, preparation of antiserum against glyceollin-BSA conjugate, and iodination of glyceollin I with ^{125}I were carried out as described by Moesta *et al.* (24).

Extracts of root sections in 10% methanol were assayed radioimmunologically for glyceollin I in Eppendorf centrifuge tubes (1.5 mL capacity). Each tube contained 100 µL of extract, 100 µL of 0.01 M sodium phosphate buffer containing 0.15 M NaCl, and 0.1% (w/v) NaN_3 (pH 7.4), 50 µL of [^{125}I]-glyceollin I tracer (2×10^5 dpm) in 50% methanol in the phosphate buffer, and 100 µL of antiserum diluted 1:250 in the phosphate buffer. After incubation at 4°C for 72 h, 100 µL of normal rabbit serum diluted 1:10 in the phosphate buffer were added, followed by 0.5 mL of a saturated ammonium sulfate solution. The mixtures were incubated at room temperature for 30 min and centrifuged at 8,000 *g* for 10 min. The supernatant was discarded, and the pellets were resuspended in 0.75 mL of 50% saturated ammonium sulfate.

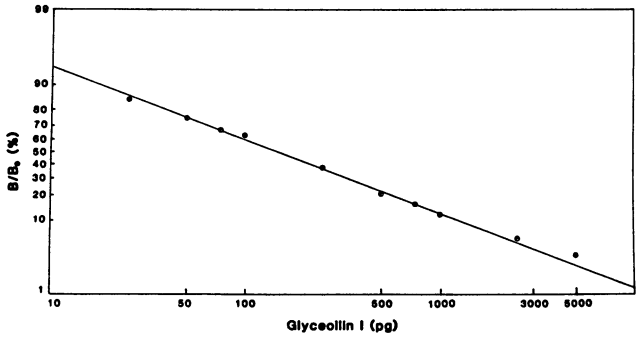

Figure 3. Standard curve for the radioimmunoassay of glyceollin I. B_0 = cpm of [^{125}I]-glyceollin I precipitated by anti-glyceollin I serum in the absence of exogenous glyceollin I. B = cpm of [^{125}I]-glyceollin I precipitated by anti-glyceollin I serum in the sample.

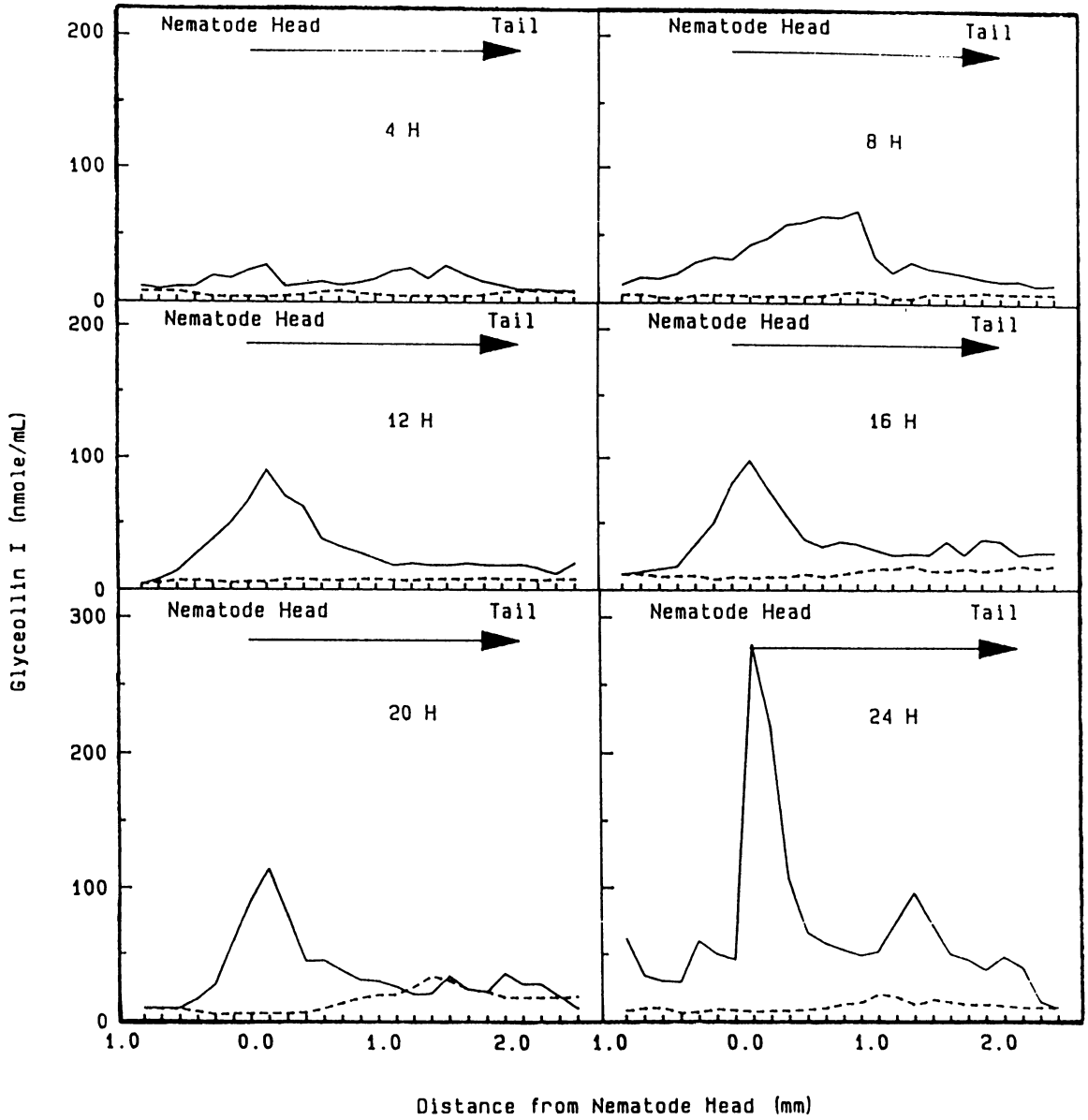

Figure 4. Spatial and temporal accumulation of glyceollin I in the roots of resistant cv Centennial (——) and susceptible cv Ransom (- - - -) soybeans inoculated with race 1 of *H. glycines*. Root segments (3.5 mm in length) containing a single nematode were dissected from the inoculated roots, embedded in ice blocks, and cut into 16-μm sections. The first of every four sections was mounted on a microscope slide for nematode orientation and the remaining three sections were extracted with 10% methanol and assayed for glyceollin I content with a radioimmunoassay procedure.

After centrifugation at 8,000 *g* for 10 min, the supernatant was discarded, and the pellet was resuspended in water and counted in a liquid scintillation counter (model 7500, Beckman Instruments, Irvine, CA). Triplicate samples were harvested and the experiment was repeated three times.

RESULTS

Glyceollin I in Whole-Root Systems

The glyceollin standard, a mixture of isomers I, II, and III, was separated into three components with retention times of 6.5, 7.0, and 7.4 min by HPLC under the conditions described in this study. They were identified as glyceollins I, II, and III, respectively, based on their UV-absorption spectra (12). A mobile phase consisting of hexane:isopropanol:methanol provided a better separation of these three glyceollin isomers than one consisting of only hexane and isopropanol (23, 24).

No glyceollin was detected in roots of control soybeans. Glyceollin I at a concentration of 5.5 μg/g root was found in cv Ransom 2 d after nematode inoculation. The concentration increased to 7.5 μg/g on the fourth day but decreased to 7 and 6.5 μg/g root on the sixth and eighth days, respectively. The concentration of glyceollin I in roots of SCN-inoculated Centennial was 12 μg/g root 2 d after inoculation. It reached

19 μg/g on the fourth day, and peaked at 23 μg/g at the sixth day and finally decreased to 16 μg/g root on the eighth day (Fig. 2). No glyceollin II was detected in any of the root samples throughout the study. Glyceollin III was found only in SCN-inoculated Centennial roots. The concentration reached 8 μg/g root at the eighth day after inoculation (Fig. 2).

Conditions for Radioimmunoassay of Glyceollin I

A standard curve for the radioimmunoassay of glyceollin I is shown in Figure 3. The detection limit was approximately 0.075 pmol (25 pg) of glyceollin I. A 50% displacement of [^{125}I]glyceollin I was achieved with 3.6 pmol. The useful measuring range of the assay was from 0.075 to 30 pmol.

Radioimmunoassay of Glyceollin I in Root Sections

Spatial and temporal accumulation of glyceollin I in serial cross-sections of soybean roots of the two cvs with and without nematode penetration is shown in Figure 4. Root sections of Ransom soybean, susceptible to race 1 of *H. glycines*, contained very low concentrations of glyceollin I throughout the 24-h experimental period. Root sections of the resistant Centennial soybean accumulated significant amounts of glyceollin I 8 h after nematode penetration. The concentrations reached 100 nm/mL at 12 h and 300 nm/mL 24 h after nematode penetration. The peaks of glyceollin I accumulation occurred in sections where nematode heads were located. There were variations in glyceollin I concentrations among roots and between experiments, but the pattern presented in Figure 4 was observed in all roots.

DISCUSSION

Glyceollins accumulated primarily in incompatible but not in compatible soybean-SCN combinations. These results are generally in agreement with those from other host plant-parasite interactions. For example, glyceollins accumulated 2 to 3 d after inoculation of incompatible Centennial soybean roots with the root-knot nematode, *Meloidogyne incognita*, but not in the roots of the compatible soybean, Pickett 71 (14).

Our results also showed that glyceollin I was the predominant phytoalexin produced in soybean roots in response to SCN stress. Glyceollin III was detected 4 d later than glyceollin I and in smaller quantities. No glyceollin II was detected throughout the study (Fig. 2). The relative concentrations of glyceollins I, II, and III detected in SCN-infected roots are similar to those accumulated in roots inoculated with *P. megasperma* f.sp. *glycinea* (3). Bhattacharyya and Ward (3) reported that relative proportions of glyceollins I, II, and III vary with soybean organs. Glyceollin I is the most abundant isomer in roots, whereas glyceollin III is the most abundant isomer in leaves. Due to low cross-reactivity of anti-glyceollin I serum toward glyceollins II and III and other structurally and metabolically related molecules (24), it is important to determine the isomers of glyceollin involved in incompatible reactions prior to the production of anti-glyceollin serum.

We also determined the accumulation of glyceollin I in sections of roots containing single nematodes by a radioimmunoassay technique. The radioimmunoassay procedure specific for glyceollin I developed by Moesta *et al.* (24) makes the quantification and localization of the phytoalexin possible at the cellular level (10). In this study, we utilized the procedure to determine the spatial and temporal distribution of glyceollin I in a root segment containing a single nematode.

Glyceollin I accumulated in tissues immediately adjacent to the head regions of the nematodes. This response suggests that head regions are the sites where elicitation of glyceollin production originates. It is well documented that oligosaccharides, glycoproteins, and proteins elicit glyceollin production in soybeans (5). Although the chemical composition of cuticles of *H. glycines* is not known, it is well established that cuticles of numerous free-living and plant parasitic nematodes are comprised in part of various carbohydrates and proteins (8, 13). The accumulation of glyceollins around head regions of penetrated nematodes suggests the occurrence of high concentrations of glyceollin elicitors at the nematode heads. Reports have suggested that the distribution of carbohydrates and glycoproteins is not uniform over the nematode body, but confined to specific areas. For example, the majority of cuticular Con A binding sites, mannose and mannoside residues, were on the head regions of *Caenorhabditis elegans* and *M. incognita* (21). The binding sites of fluorescent conjugates of Con A and agglutinins of soybean, wheat germ, *Lotus tetragonolobus*, and *Limulus polyphemus* were in the vicinity of the amphidial openings of viable second-stage juveniles of *M. incognita* and *M. javanica* (6). Whether the cuticular carbohydrates in the vicinity of the head region of *H. glycines* are elicitors of glyceollins remains to be investigated.

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