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Biochemical Response of Soybean Roots to *Fusarium solani* f. sp. *glycines* Infection

V. V. Lozovaya,* A. V. Lygin, S. Li, G. L. Hartman, and J. M. Widholm

ABSTRACT

The soil-borne fungus *Fusarium solani* (Mart.) Sacc. f. sp. *glycines* (FSG) infects soybean [*Glycine max* (L.) Merr.] roots and causes the disease sudden death syndrome (SDS). The biochemical response of soybean roots to FSG infection, which has not been studied before, was investigated by comparing FSG-inoculated and noninoculated roots of two partially resistant ('PI 520733' and 'PI 567374') and susceptible ('Spencer') genotypes. Activity of phenylalanine ammonia-lyase (PAL), the first enzyme in the phenylpropanoid biosynthetic pathway, was increased in inoculated roots of all three genotypes. The phytoalexin glyceollin increased to much higher levels in inoculated roots of the partially resistant cultivars PI 520733 and PI 567374 than in the susceptible Spencer. The changes in phenolic metabolism were localized in lesion-containing areas of roots rather than in the new portion growing under the FSG inoculum. No clear correlation was found between the glyceollin precursor daidzein (4′,7-dihydroxyisoflavone) and its conjugates and glyceollin levels in root tissues; however, isoflavone levels increased only in roots of inoculated plants of partially resistant lines, even though constitutive isoflavone levels were higher in the susceptible control. The FSG growth on potato dextrose agar medium was inhibited by increasing concentrations of glyceollin. Induction of lignin synthesis was found in the inoculated roots of all three lines, with the highest rate of lignification observed in roots of the partially resistant genotypes, especially PI 567374. These studies show for the first time that FSG inoculation of soybean roots in soil induces the phenylpropanoid pathway to synthesize isoflavones, the phytoalexin glyceollin, and lignin, indicating that these compounds may be involved in the partial resistance response.

Plants have been able to develop an array of constitutive and inducible mechanisms to defend themselves against pathogens. The most common defense reactions in pathogen-challenged plants include the generation of active oxygen species (oxidative burst), the hypersensitive response, the induced accumulation of pathogenesis-related proteins and antimicrobial phytoalexins, and the synthesis of callose and various wall-bound phenolic compounds (Hahlbrock and Scheel, 1989; Dixon et al., 1994; Robbins et al., 1995; Koch et al., 1998). These common responses are part of the incompatible interaction involving a resistant plant and an avirulent pathogen and are under genetic control.

Many phytoalexins, particularly those produced by legumes, are flavonoids that are products of phenylpropanoid metabolism. Flavonoid and isoflavonoid compounds are important components in the host plant's defensive arsenal, but direct proof of the phytoalexin hypothesis is lacking (Dixon and Steele, 1999). Increased lignification at the site of infection represents an additional inducible defense that can inhibit the infection process and pathogen proliferation, and is often associated with phytoalexin production (Dixon et al., 1994).

There are many root-invading fungal pathogens, but for most plant–pathogen root interactions, the biochemical activities that occur during root infection are not well studied, in part because of the difficulty of working with roots and root pathogens. Severe yield losses occur in soybean because of SDS (Rupe and Hartman, 1999), which is caused by the soil-borne fungus FSG (Rupe, 1989; Rupe and Hartman, 1999). The fungus is localized in roots and lower parts of stems and has not been isolated from leaves (Roy, 1997). Foliar symptoms have been attributed to fungal toxins produced in the roots and translocated to the leaves (Baker and Nemec, 1994; Roy, 1997). Sudden death syndrome has become a widespread and consistent problem in the USA and in other countries because of the lack of soybean genotypes with adequate resistance. Some sources of SDS partial resistance have been identified (Huang and Hartman, 1998), but even these sources of resistance exhibit only decreased foliar response while the roots are still infected, resulting in reduced plant health. The hypersensitive type of resistance has not been reported (Jin et al., 1996).

Plant cell walls are known to be a barrier to the entry of many microorganisms. The walls contain many components, including phenolic compounds that consist of phenylpropanoid units that are found as both conjugated acids and, more commonly, lignin alcohols. Phenolic acids are precursors for the synthesis of lignin (Lewis and Yamamoto, 1990) and phenylpropanoid phytoalexins (Kessmann et al., 1990). Deposition of phenolics into the cell wall during pathogen infection is an important defense mechanism, either because of a hypersensitive reaction of entire cells or for local wall reinforcement due to deposition of papillae (Vance et al., 1980; Bolwell et al., 1985; Barber et al., 1989; Bruce and West, 1989; Wallace and Fry, 1994; Franke et al., 1998). Lignin is one of the most abundant biopolymers on earth and is important for the defense of vascular plants because of its strengthening ability and resistance to enzymatic degradation (Bruce and West, 1989; Lange et al., 1995; Huang and Hartman, 1998; Egea et al., 2001). A close relationship between lignification and disease resistance has been demonstrated in a number of experiments. Resistant plants accumulate lignins more rapidly and/or exhibit enhanced lignin deposition as compared with susceptible plants (Nicholson and Hammerschmidt, 1992; Walter, 1992). In addition, the use of specific chemical inhibitors of lignification led to the inhibition of the hypersensitive response in wheat plants challenged by *Puccinia graminis* Pers.:Pers., rendering resistant plants

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Abbreviations: AIR, alcohol-insoluble residue; FSG, *Fusarium solani* f. sp. *Glycines*; PAL, phenylalanine ammonia-lyase; Phe, L-phenylalanine; SDS, sudden death syndrome; TGA, thioglycolic acid.

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susceptible to pathogen attack (Moerschbacher et al., 1990).

The accumulation of the phenylpropanoid phytoalexin glyceollin in infected tissues of different plant organs was responsible, at least in part, for the resistance of soybean seedlings toward *Phytophthora sojae* M.J. Kaufmann & J.W. Gerdemann (Zahringer et al., 1978; Bhandal et al., 1987; Graham et al., 1990; Morris et al., 1998; Mohr and Cahill, 2001). The fungitoxicity of the glyceollin isomers to the *P. sojae* mycelial growth or to zoospore germination was demonstrated in vitro (Bhattacharyya and Ward, 1985). Glyceollin accumulated in soybean cell suspension cultures treated with *Pseudomonas siringae* pv. *glycinea* harboring an avirulence gene (Guo et al., 1998) or with *P. sojae* culture filtrate or cell walls (Bhandal et al., 1987). Glyceollin also accumulated in soybean roots inoculated with the soybean cyst nematode (Huang and Barker, 1991).

The biochemical events that occur in soybean roots infected with FSG have not been reported. Thus, the objective of our research was to determine the alterations in phenolic compounds that may provide resistance to the FSG. This should indicate what changes are likely to be effective in imparting *F. solani* f. sp. *glycine*s resistance and will describe for the first time how soybean plants react at the biochemical level to SDS disease development. This information will help us better understand the plant response by FSG infection to develop strategies to improve plant defenses by altering the amount and composition of the plant's phenylpropanoid compounds via genetic engineering.

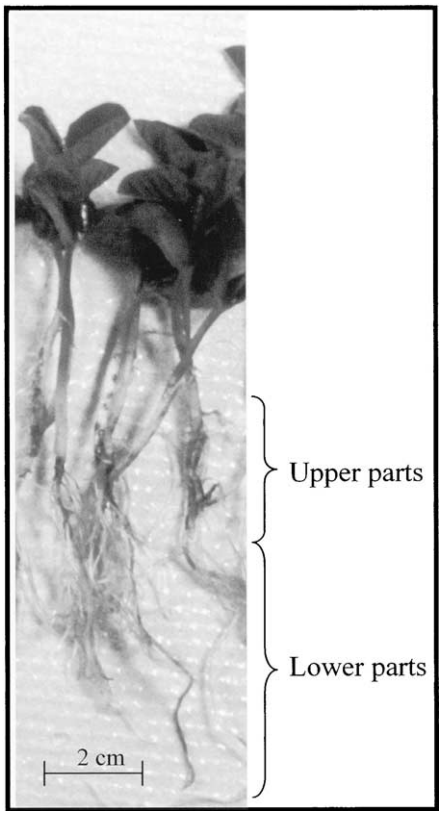

Fig. 1. Plants of partially resistant genotype 'PI 567374' inoculated with *Fusarium solani* f. sp. *glycines* (FSG) 7 d after planting.

MATERIALS AND METHODS

Plant Materials, Inoculation, and Samples

Seeds of a susceptible soybean cultivar (Spencer) and two partially resistant plant introductions (PI 520733 and PI 567374) were inoculated by sowing seeds [*Sorghum bicolor* (L.) Moench] 2 to 3 cm directly above FSG-infested (Mont1 isolate) sorghum seeds in flats in the greenhouse following previously reported methodology (Hartman et al., 1997). Noninoculated plants sown in other flats containing noninoculated sorghum seeds were used as controls. To obtain roots, plants were topped and flats were soaked in water and roots were washed clean of soil. Seven days after planting, fresh roots were either used for PAL enzyme assay and radiolabeling experiments or kept frozen until analysis. From each genotype, 0.4-g samples were used for assay of PAL activity and 1.0-g samples for each phenolic (isoflavone or lignin) analysis in two or three replications. The experiments were performed three times and similar results were obtained each time. The data presented are from one representative experiment. In some of these experiments the phenolic compounds were analyzed in whole-root samples that consisted of a mixture of tap and lateral roots. Since the most severe damage to inoculated roots occurred in the tap root areas contacting the sorghum seeds (Fig. 1), we then separated the roots into the *upper parts* that included the lesions in the FSG-inoculated roots and the rest designated as *lower parts* (Fig. 1).

Root Growth and Dry Weight Estimation

To evaluate early root growth of the three genotypes, seeds were germinated in soil above FSG-infested or uninfested sorghum seeds or in soil without sorghum seeds as described above. There were five plants for each genotype per treatment in three replications. Seedling growth and dry weights of whole seedlings were measured 5 d after sowing.

Phenylalanine Ammonia-Lyase Assay

The PAL activity was determined by measuring the conversion of L-phenylalanine (Phe) to *trans*cinnamic acid according to Edwards and Kessmann (1992) with a few modifications: 400 mg of fresh roots were ground in liquid nitrogen and extracted with 1600 μL of the extraction buffer, containing 0.05 *M* Tris-HCl (pH 8.8), 0.5% ascorbate, 10% glycerol, and 10 m*M* β-mercaptoethanol. One-hundred microliters of crude enzyme extract was combined with 400 μL of 0.05 *M* Tris buffer (pH 8.8), containing 0.2 m*M* Phe as substrate. The reaction mixture was incubated for 0, 30, 60, and 120 min at 37°C. The reaction was stopped by adding 100 μL of 0.5 *M* HCl. Cinnamic acid was extracted with 1 mL of toluene and the absorbance was measured at 290 nm with toluene as a blank.

Isoflavone Extraction and Analysis

Soybean root samples were ground in liquid nitrogen and extracted with five volumes of 80% methanol at 20°C with vigorous shaking for 12 h. The pellet obtained after centrifugation at 5000 g for 10 min was washed twice with chloroform: methanol (1:1, v/v) followed by 100% methanol, and the alcohol-insoluble residue (AIR) was dried at 65°C and used for measurements of lignin content. The supernatant from the initial extract was centrifuged at 12 000 g for 10 min and the supernatant filtered through a 0.45-μm membrane before HPLC analysis. Separations of isoflavones were achieved with

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a Waters HPLC system (Waters Corp., Milford, MA, USA)[1] with a 53 mm × 7 mm EPS C_{18} Alltech Rocket Column (Alltech Assoc., Deerfield, IL, USA) following a previously reported method (Graham, 1991a). A linear gradient composed of water (pH 2.8 with acetic acid) and acetonitrile was used. Following injection of 20 to 50 μL of sample, acetonitrile was increased from 0 to 12% across 6 min, from 12 to 23% across 12 min, and then increased from 23 to 100% across 26 min. The solvent flow was 2.5 mL min^{-1}. A Waters (Milford, MA) 996 photodiode array detector was used. Glyceollin standards were provided by Dr. Gijzen (Agriculture and Agri-Food Canada, ON, Canada). Isoflavone standards [daidzein, genistein (4′,5,7-trihydroxyisoflavone), glycitein (4′,7-dihydroxy-6-methoxyisoflavone), and their glucosides] were purchased from LC Laboratories (Woburn, MA, USA).

In Vitro *Fusarium solani* f. sp. *glycines* Growth Studies

Glyceollin as a mixture of Isomers I, II, and III was purified according to Ayers et al. (1976) and added after being dissolved in 100% ethanol to potato dextrose agar (PDA) (3.9%) medium to produce the final concentrations of 0, 25, 75, 150, and 215 μ*M*. The ethanol concentration in the medium including the control was 1% (v/v). The inoculum plugs, 4 mm in diameter, from the margin of 2-wk-old cultures of the FSG isolates (Mont1, I9, I49, I95, I98) were placed mycelial side down directly on top of the medium in the center of each plate (45-mm diam.). Inoculated and control (without mycelium) plates were kept in the dark at room temperature. Radial growth of FSG was measured 14 d after inoculation.

Labeling Experiments

Phenolic compounds were labeled by incubating soybean seedling roots in buffer (0.05 *M* MES, pH 5.4) containing ^{14}C-Phe (0.3 μCi mL^{-1} or 0.011 MBq mL^{-1}) for 2 h before rinsing and analysis.

Determination of Lignin Content

The lignin content was determined by derivatization with thioglycolic acid (TGA) by a modified method (Bruce and West, 1989; Bonello et al., 1993). Ten to fifteen milligrams of the AIR (described above) were placed in a 1.5-mL Eppendorf screw-cap vial and treated with 1 mL of 2 *M* HCl and 0.2 mL of TGA for 4 h at 95°C. After being cooled to room temperature, the mixture was centrifuged for 10 min at 12 000 g in a bench-top centrifuge. The supernatant was removed with a Pasteur pipette, and the remaining pellet was washed three times with distilled water. The pellet was suspended in 1 mL of 0.5 *M* NaOH and vigorously shaken overnight to extract the TGA lignin. Following centrifugation as above, the supernatant was decanted into a 2-mL Eppendorf vial, and the pellet was washed with 0.5 mL of 0.5-*M* NaOH. The combined alkali extract was acidified with 0.3 mL of concentrated HCl, and the lignin was allowed to precipitate at 4°C for 4 h. The mixture was centrifuged as above, the supernatant was removed with a Pasteur pipette, and the brown pellets were dissolved in 1 mL of 0.5 *M* NaOH and diluted to 10 mL with 0.5 *M* NaOH. The absorbance at 280 nm was measured in 0.5 *M* NaOH and the hydrolyzed, hydroxymethyl derivative of lignin (Aldrich Chem. Co., St. Louis, MO, USA) was used as standard. Another sample was neutralized and used for measurement of lignin radioactivity in a Tri-Carb 1600 TR Liquid Scintillation Analyzer (Packard BioScience Co., Meriden, CT, USA).

Klason lignin was determined gravimetrically according to the procedure described by Effland (1977) and was obtained as an insoluble residue after dissolution of wall polysaccharides and proteins by the treatment of AIR with 72% sulfuric acid for 2 h at 20°C, then diluted to 3% (v/v) with aqueous sulfuric acid and boiled for 4 h.

Data Analysis

Data presented in tables and figures are mean values of three independent biological replicates. The ANOVA and mean separations were completed using JMP (SAS Institute, Cary, NC).

RESULTS

Seedling Growth and *Fusarium solani* f. sp. *glycines* Inoculation

Since the FSG inoculation method requires the seedling roots to grow through a layer of FSG-infested sorghum seed placed in the soil 2 to 3 cm below the soybean seed, soybean seedling root growth was measured. The mean root lengths of the PI 520733, PI 567374, and Spencer genotypes were 8.6 cm at 5 d after planting and did not differ by entry or treatment. This would indicate that the roots of all three genotypes came into contact with the inoculum at the same time as the roots grew through the inoculum. The main dark-colored lesions were in the upper parts of infected soybean tap roots especially in the areas in contact with the sorghum inoculum (Fig. 1).

The root masses of Spencer were larger than those of PI 567374 and less than those of PI 520733 under all treatments used (Table 1). There were no differences in whole seedling weights within an entry regardless of the treatment (data not shown).

Root Isoflavone and Glyceollin Content and Phenylalanine Ammonia-Lyase Activity Analysis

The soybean plants were sampled 7 d after planting in these studies since there were no noticeable changes in phenolic compounds in inoculated roots 5 d after sowing (data not shown). It was also important to use plants at this early stage to be able to differentiate the

Table 1. Dry weight of roots of two partially resistant ('PI 520733' and 'PI 567374') and susceptible ('Spencer') genotypes not inoculated (control and sorghum control) or inoculated with *Fusarium solani* f. sp. *glycines* 5 d after planting.

Entry	Control	Sorghum control	Inoculated	Mean
		g		
PI 520733	1.2†	1.3	1.2	1.2
PI 567374	0.5	0.5	0.6	0.5
Spencer	0.8	0.7	0.8	0.8
LSD	0.32	0.16	0.18	
Mean	0.8	0.8	0.9	

† Means are weights of plants for each entry within each treatment using three replications.

[1] Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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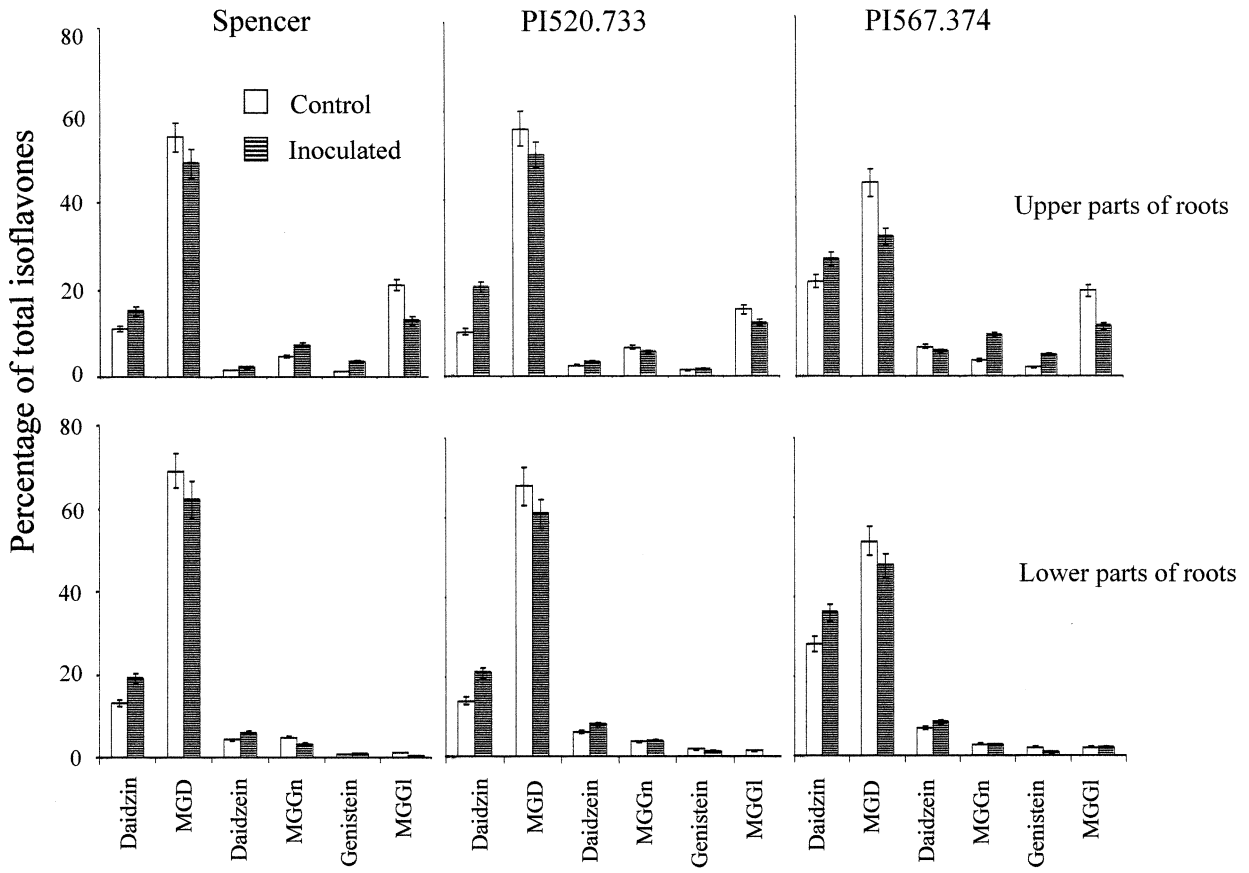

Fig. 2. The percentage of various isoflavonoids of total isoflavone fractions extracted from either upper or lower root parts of 'PI 520733', 'PI 567374', and 'Spencer', either inoculated or not inoculated with *Fusarium solani* f. sp. *glycines* 7 d after planting. Bars represent standard errors of the means. Daidzin, 7-*O*-glucosyl daidzein; MGD, 6″-*O*-malonyl-7-*O*-glucosyl daidzein; MGGn, 6″-*O*-malonyl-7-*O*-glucoside genistein; MGGl, 6″-*O*-malonyl-7-*O*-glucosyl glycitein.

changes in root phenolic metabolism caused by the pathogen from those related to developmental control, since several-fold increases in total isoflavone levels in all soybean roots were found 2 wk after sowing (data not shown). These changes could relate to other roles of isoflavones such as an involvement in nodulation, for example. The FSG-inoculated roots also gradually became more necrotic at later stages.

The soybean isoflavones daidzein, genistein, and glycitein and their respective glycosyl (daidzin, genistin, and glycitin) and malonyl glycosyl (malonyl daidzin, malonyl genistin, and malonyl glycitin) conjugates were measured in soybean roots. The soybean root isoflavone fraction of all root samples (both upper and lower parts) consisted mainly of daidzein conjugates (>60% of the total isoflavones) for roots of the 7-d-old soybean seedlings (Fig. 2) as also reported by Graham (1991b) with the soybean genotype 'Williams'. Malonyl daidzin was the most abundant daidzein conjugate at Day 7 and its proportion of the total isoflavone fraction was greater in noninoculated than in inoculated plant root samples. The daidzin proportion increased in inoculated roots compared with controls. Genistein conjugates accounted for about 3 to 14% of the isoflavone fraction in young roots with higher proportions detected in the infected upper root parts. Malonyl glycitin was >10% of the total isoflavones in the upper parts of the roots and was higher in the controls. However, the malonyl glycitin proportion was much lower (2% or less of the total isoflavones) in the lower parts of the roots (Fig. 2).

Inoculation of soybean roots with FSG caused a doubling in PAL activity in the upper portion where the lesions occurred while no changes in PAL activity was seen in the lower root parts 7 d after planting (Table 2). The highest total isoflavone content was found in Spencer control roots but the content increased only in the PI upper parts of the roots upon FSG infection (Table 2).

Large increases in the concentration of the phytoalexin glyceollin were found only in the upper parts of FSG-inoculated roots of all soybean lines where symptoms occurred. Higher concentrations were detected in the PI root samples than in Spencer 7 d after sowing (Table 2). The lower portion of the infected roots where symptoms were much less visible did not show marked glyceollin accumulation compared with the corresponding controls (Table 2). Glyceollin was found only in very low and variable amounts in control plants. This variability might be caused by mechanical damage, which is difficult to avoid during the sampling.

Inhibition of *Fusarium solani* f. sp. *glycines* Growth by Glyceollin

Since glyceollin accumulated to higher levels in the infected roots of the partially resistant genotypes and

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Table 2. Effect of *Fusarium solani* f. sp. *glycines* (FSG) inoculation on phenylalanine ammonia-lyase (PAL) activity and isoflavone and glyceollin content in upper (show lesions) and lower (few lesions) parts of the roots of two partially resistant ('PI 520733' and 'PI 567374') and susceptible ('Spencer') genotypes 7 d after planting. Values are all mean ± SE.

Sampled root sections	Genotype	Treatment	PAL activity	Total isoflavones	Daidzein	Glyceollin
			μmol h^{-1} mg^{-1} protein		μg g^{-1} FW	
Upper	Spencer	Control	87 ± 21	274 ± 26	5 ± 2	30 ± 12
		FSG	175 ± 28	247 ± 21	5 ± 1	109 ± 20
	PI 520733	Control	87 ± 13	149 ± 23	5 ± 3	13 ± 6
		FSG	200 ± 20	304 ± 34	8 ± 1	226 ± 62
	PI 567374	Control	107 ± 14	146 ± 16	16 ± 5	71 ± 13
		FSG	214 ± 51	213 ± 22	11 ± 5	211 ± 40
Lower	Spencer	Control	72 ± 2	339 ± 24	16 ± 3	8 ± 6
		FSG	81 ± 8	296 ± 5	17 ± 2	28 ± 9
	PI 520733	Control	92 ± 4	273 ± 23	17 ± 3	30 ± 14
		FSG	103 ± 6	237 ± 11	19 ± 1	23 ± 7
	PI 567374	Control	92 ± 22	136 ± 6	10 ± 2	10 ± 0
		FSG	103 ± 12	158 ± 18	13 ± 1	9 ± 9

may be secreted or leaked into the rhizosphere, as we found in experiments with FSG-inoculated hairy roots grown on plates (2004, unpublished data), we evaluated the effect of different glyceollin concentrations (25 to 215 μM) on FSG growth. The growth of Mont1 isolates was increasingly inhibited by glyceollin concentrations up to 150 μ*M* (Fig. 3), and 75-μ*M* glyceollin also inhibited the radial growth of four other FSG isolates by 15 to 30% (Fig. 4). The variable inhibition of the different isolates may indicate that different FSG isolates vary in their sensitivity to glyceollin.

Lignin Content and Synthesis from Carbon-14-Phe

To evaluate the effect of FSG inoculation on lignin content, the lignin quantity was measured using two different methods: Klason lignin represents the gravimetrically measured residue that remains after removal of other cell wall polymers by acidic treatments, and TGA lignin represents the lignothioglycolic acid complex extracted from AIR with TGA which is measured spectrophotometrically. Higher levels of both the TGA and Klason lignins were found in most FSG-inoculated roots compared with corresponding control roots of all three genotypes when whole roots were analyzed (Table 3). The TGA lignin contents were always lower than Klason lignin levels in all three genotypes and the differences found between inoculated and noninoculated roots were much less. When TGA lignin was analyzed in the upper parts of roots, FSG inoculation caused an increase in the lignin content of roots of all three genotypes, with the increase being lowest in Spencer (Table 4). The highest lignin content was detected in the upper parts of PI 567374-inoculated and noninoculated roots.

Even though some increase in the TGA lignin level was found in infected roots of Spencer and PI 520733 when compared with the control at 7 d after planting (Table 3), the amount of ^{14}C-Phe incorporation into lignin following a 2-h incubation was lower in infected roots of these genotypes compared with the control at this stage (Table 5). However, the PI 567374-inoculated roots did show an increase of label incorporation into TGA lignin and a related increase in TGA lignin specific radioactivity. More than 50% of ^{14}C-Phe remained in the incubation medium after the 2-h exposure, indicating that the substrate should not be limiting.

DISCUSSION

In our experiments, FSG infection resulted in disease symptoms that are spatially localized in the upper roots that would be in contact with the FSG-infested sorghum seeds. This fits with the data from these biochemical

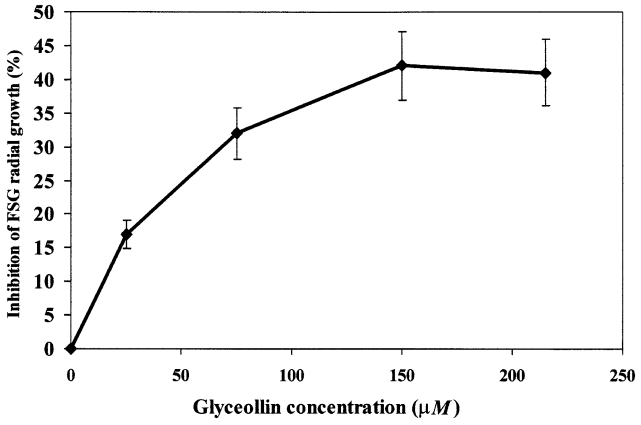

Fig. 3. Inhibition of radial growth of *Fusarium solani* f. sp. *glycines* (FSG; Mont1 isolate) measured after 14 d. Bars represent standard errors of the means.

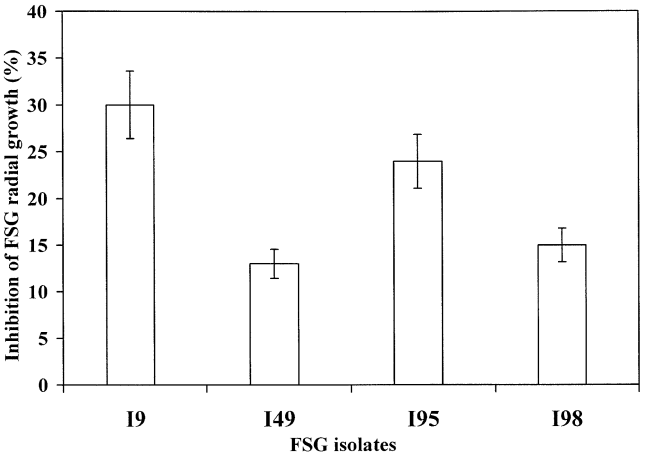

Fig. 4. Effect of 75-μ*M* glyceollin on the radial growth of four different *Fusarium solani* f. sp. *glycines* (FSG) isolates measured after 14 d. Bars represent standard errors of the means.

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Table 3. Lignin content of whole roots of two partially resistant ('PI 520733' and 'PI 567374') and susceptible ('Spencer') genotypes either inoculated or not with *Fusarium solani* f. sp. *glycines* (FSG) 7 d after planting.

Cultivar	Treatment	Klason lignin	TGA† lignin	TGA lignin as % of Klason lignin
		— μg mg^{-1} AIR‡ (±SE) —		%
Spencer	Control	129 ± 32	106 ± 1	82
	FSG	218 ± 19	113 ± 3	52
PI 520733	Control	133 ± 13	81 ± 8	61
	FSG	202 ± 15	117 ± 8	58
PI 567374	Control	118 ± 37	112 ± 3	95
	FSG	266 ± 10	114 ± 6	43

† TGA, thioglycolic acid.
‡ AIR, alcohol-insoluble residue.

studies that show marked differences between the upper roots of susceptible and partially resistant lines in PAL activity, glyceollin levels, and TGA lignin accumulation, while there were little or no differences found in the lower roots (Table 2). Both the absence and existence of correlations between PAL and accumulation of glyceollin have been reported in studies with *P. sojae* (Partridge and Keen, 1977; Yoshikawa et al., 1978; Yoshikawa et al., 1979). The accumulation in infected tissues of the phenylpropanoid phytoalexin glyceollin was interpreted to be, at least in part, the cause of the resistance of soybean seedlings to *P. sojae* (Keen et al., 1972; Ayers et al., 1976; Zahringer et al., 1978). It was also emphasized that daidzein conjugate pools in soybean organs are more than sufficient to provide substrates for the synthesis of the glyceollin that accumulates in infected tissues (Graham et al., 1990). However, we found that following FSG inoculation the glyceollin concentration in roots could be comparable with the concentration of total isoflavones mainly consisting of daidzein derivatives (Table 2), indicating that the pool of daidzein might limit glyceollin synthesis. One also needs to consider the fact that isoflavones are not the final products but can be converted into other compounds (such as glyceollin, for example) and can be secreted or leaked by roots into the rhizosphere upon pathogen (elicitor) treatment. Thus, the measured isoflavone content in roots is affected by the rate of synthesis and their conversion to other compounds and secretion.

Isoflavone conjugates (newly formed or preexisting in cells) can be readily hydrolyzed by vacuole- or cell-wall-associated isoflavone-specific β-glucosidases to form the aglycone in soybean (Graham and Graham, 1991; Hsieh and Graham, 2001). The aglycone might then be used as a precursor for glyceollin or coumestrol synthesis, for example, or to be secreted into the rhizosphere. The availability of the daidzein aglycone to serve as substrate has been reported to be important for the rapid synthesis of glyceollin (Graham and Graham, 1991, 1996). The ability to rapidly release aglycones from their conjugates appeared to be one of the factors involved in the resistance to *P. sojae* during the incompatible infection of soybean cotyledons (Graham et al., 1990). However, as we report here (Table 2 and Fig. 2) and also as reported by Zacharius and Kalan (1990) with soybean suspension cultures, there was no correlation between the amount of glyceollin accumulated and the daidzein aglycone content. It has been assumed that the differential regulation of enzymes that control conjugation or deconjugation of isoflavones and efficient isoflavone aglycone incorporation into the branch leading to glyceollin production are very critical for successful defense (Abbasi and Graham, 2001).

Table 4. Effect of *Fusarium solani* f. sp. *glycines* (FSG) infection on thioglycolic acid lignin content in the upper parts of soybean roots of two partially resistant ('PI 520733' and 'PI 567374') and susceptible ('Spencer') genotypes 7 d after planting.

Cultivar	Control	FSG treated	Increase in FSG treated
	— μg mg^{-1} AIR† (mean ± SE) —		%
Spencer	67 ± 1	76 ± 3	13
PI 520733	60 ± 7	89 ± 5	48
PI 567374	76 ± 2	97 ± 4	28

† AIR, alcohol-insoluble residue.

Table 5. Effect of *Fusarium solani* f. sp. *glycines* (FSG) infection on incorporation of ^{14}C-Phe into thioglycolic acid (TGA) lignin by whole roots of two partially resistant ('PI 520733' and 'PI 567374') and susceptible ('Spencer') genotypes 7 d after planting. The incubation period was 2 h.

Cultivar	Control	FSG treated
	— cpm mg^{-1} TGA lignin × 10^{-3} (±SE)† —	
Spencer	44.6 ± 0.3	25.6 ± 0.8
PI 520733	62.6 ± 4.0	36.2 ± 0.1
PI 567374	57.1 ± 12.0	71.6 ± 3.1

† The actual numbers were multiplied by 10^{-3} to obtain the reported numbers.

As a toxic compound, the glyceollin that accumulates in lesion areas can help prevent the spread of fungi through the tissue. Glyceollin may also be secreted or leaked into the rhizosphere, which could also inhibit fungal growth. Glyceollin may be degraded by enzymes from both the plant and fungus. Thus, there is likely to be an underestimation of the scale of glyceollin synthesis when its content is determined only in plant tissue. Kneer et al. (1999) showed that increased levels of genistein in root exudates correlated with greater amounts of genistein in the elicitor-treated *Lupinus luteus* root tissues. Thus, it is possible that since the glyceollin levels were markedly higher in infected roots of PI 520733 and PI 567374 than in Spencer, the secretion of glyceollin could be greater in the PIs than in Spencer.

We demonstrated that glyceollin directly inhibits FSG hyphal growth in vitro, and this inhibition was concentration dependent. We also found that different isolates of FSG vary in their sensitivity to glyceollin when tested in vitro. Other reports have shown that fungal growth can be inhibited by glyceollin, as for example, the inhibition of *P. sojae* growth at the infection site on hypocotyls of the incompatible soybean cultivar Harosoy 63 upon the rapid accumulation of glyceollin (Yoshikawa et al., 1978). This high glyceollin accumulation did not occur in the infected hypocotyls of the near-isogenic compatible cultivar Harosoy. Various isolates of *P. sojae* also showed a differential sensitivity to glyceollin (Bhattacharyya and Ward, 1985). The glyceollin concentrations (25–215 μ*M*) that inhibited FSG growth in vitro (Fig. 3, 4) were comparable with those found in the FSG-inoculated roots (100–200 μg mL^{-1}, that is equal to 300–600 μ*M*) (Table 2) in our experiments. These concentrations are in

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the range of ED_{50} (effective dose 50%) values (25–100 μg mL^{-1} equal to 75–300 μ*M*) found in in vitro studies of glyceollin activity against *P. sojae* (Lazarovits and Ward, 1982; Stossel, 1983; Bhattacharyya and Ward, 1985). Thus, our results suggest that glyceollin accumulation might interfere with FSG progression as described for *P. sojae*, by inhibiting fungal growth. The higher glyceollin level in PI 520733 and PI 567374 roots compared with susceptible roots at early stages of disease development could account for the partial resistance of these plant introductions to FSG infection.

Another known defensive mechanism in plants is the deposition of lignin in cell walls. This provides an effective barrier to mechanical penetration by fungi, physically shields the wall polysaccharides from degradation by fungal enzymes, and restricts diffusion of enzymes and toxins from the fungus to the host and of water and nutrients from the host to the fungus (Ride, 1978). The incompatible interaction of soybean leaves and hypocotyls inoculated with *P. sojae* was characterized by deposition of phenolic compounds and lignin, in addition to the accumulation of glyceollin, resulting from pathogen-induced stimulation (Mohr and Cahill, 2001).

We found the Klason lignin content to be higher than the TGA lignin, and the differences were usually higher in inoculated roots than in the controls (Table 3). This might be explained by the difference in the lignin extractability, since the amount of TGA extract can depend on lignin structure and FSG inoculation might affect this structure, thus resulting in decreased TGA extract levels. The FSG infection caused an increase in lignin content (Tables 3) especially in the infected upper portion of the roots (Table 4). However, FSG induced an increase in TGA lignin synthesis rate (from ^{14}C-Phe) and lignin specific radioactivity only in PI 567374, while both Spencer- and PI 520733-infected roots had lignin-specific activity lower than control roots (Table 5). The lignin specific activity was still higher in PI 520733 than in Spencer. These results indicate that FSG infection leads to greater activation of lignin synthesis in the PI 567374 roots than in Spencer roots 7 d after planting. Decreased incorporation of ^{14}C-Phe into TGA lignin of infected tissues could be due to the capacity of FSG to degrade lignin, as we have found in other studies (2004, unpublished data). It is also possible that infected roots may synthesize less-extractable lignins.

This study shows that the PAL activity increases and phenylpropanoids accumulate in the upper portion of the roots as a consequence of FSG infection, indicating the importance of de novo synthesis of phenolic compounds (isoflavones, glyceollin, and lignin) in the soybean plant–FSG interactions in the rhizosphere. The difference between the partially resistant (PI 520733 and PI 567374) and susceptible (Spencer) plant responses to FSG infection indicates that the capacity to produce glyceollin rapidly and at high levels could be the critical factor that determines the plant's ability to combat FSG similar to the soybean response to another pathogen, *P. sojae*. The induction of the defense lignin synthesis during infection development could be another biochemical response important for plant resistance to FSG attack. Apparently, this defense mechanism is more effective in roots of PI 567374 than in PI 520733 (Table 5). Specific alterations of lignin structure may also be critical but this was not investigated.

From a practical standpoint, the genetic manipulation of the phenylpropanoid pathway could be a plausible and efficient approach to enhance soybean resistance to FSG, especially since there are no field treatments for SDS control or commercial varieties with adequate SDS root resistance. This approach could lead also to resistance to other soil-borne pathogens.

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