Response of Soybean Pathogens to Glyceollin

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ABSTRACT

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Plants recognize invading pathogens and respond biochemically to prevent invasion or inhibit colonization in plant cells. Enhancing this response in crop plants could improve sustainable methods to manage plant diseases. To enhance disease resistance in soybean, the soybean phytoalexin glyceollin was assessed in soybean hairy roots of two soybean genotypes, Spencer and PI 567374, transformed with either soybean isoflavone synthase (*IFS2*) or chalcone synthase (*CHS6*) genes that were inoculated with the soybean pathogens *Diaporthe phaseolorum* var. *meridionales*, *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, and *Phytophthora sojae*. The hairy-root-transformed lines had several-fold decreased levels of isoflavone daidzein, the precursor of glyceollin, and considerably lower concentrations of glyceollin induced by pathogens measured 5 days after fungal inoculation compared with the nontransformed controls without phenolic transgenes. *M. phaseolina*, *P. sojae*, and *S. sclerotiorum* grew much more on *IFS2*- and *CHS6*-transformed roots than on control roots, although there was no significant difference in growth of *D. phaseolorum* var. *meridionales* on the transformed hairy-root lines. In addition, glyceollin concentration was lower in *D. phaseolorum* var. *meridionales*-inoculated transformed and control roots than roots inoculated with the other pathogens. Glyceollin inhibited the growth of *D. phaseolorum* var. *meridionales*, *M. phaseolina*, *P. sojae*, *S. sclerotiorum*, and three additional soybean pathogens: *Cercospora sojina*, *Phialophora gregata*, and *Rhizoctonia solani*. The most common product of glyceollin conversion or degradation by the pathogens, with the exception of *P. sojae*, which had no glyceollin degradation products found in the culture medium, was 7-hydroxyglyceollin.

Additional keywords: isoflavones, transformation.

The ability to recognize pathogenic or nonpathogenic fungi and respond biochemically to prevent invasion or inhibit pathogen colonization in plant cells is a well-established plant process that has been studied intensively (1,6,8,16). An incompatible interaction between plants and pathogenic fungi induces a resistance response that involves an increase in the activity of multiple local and systemic biochemical pathways, producing compounds that can directly restrict pathogen colonization. Plant antimicrobial compounds such as phytoalexins, synthesized in response to pathogen invasion, are capable of arresting or inhibiting colonization of pathogenic invaders (17).

Different tissues of soybean plants can produce the pterocarpan phytoalexin glyceollin in response to various pathogen attacks (8,16). Three isomers of glyceollin (I, II, and III) which arise from the common precursor, glycinol, were described in infected or stressed soybean plants, and their proportion may vary depending on plant organ, the reaction type, and environment, with glyceollin I being predominant in etiolated tissues (3). The biosynthesis of this compound occurs via the isoflavonoid branch of the phenylpropanoid pathway. In our previous studies, we found that the growth of the fungal pathogen *Fusarium virguliforme*, the cause of soybean sudden death syndrome (SDS) on soybean plant roots or *Agrobacterium rhizogenes*-transformed soybean hairy roots, was inversely proportional to the glyceollin content. This trend was evident when we analyzed soybean genotypes with different levels of SDS resistance (10) and also hairy roots established from these genotypes (11,13). In addition, hairy roots transformed with soybean chalcone synthase (*CHS6*) or isoflavone synthase (*IFS2*) genes synthesized significantly lower levels of isoflavone daidzein, the precursor of glyceollin synthesis, than nontransformed control cultures and, as a consequence, produced lower levels of glyceollin in response to *F. virguliforme* inoculation, resulting in higher *F. virguliforme* root colonization (12). These results indicated that glyceollin biosynthesis was involved in limiting colonization of this pathogen. The accumulation of glyceollin in the hairy roots in response to *F. virguliforme* invasion in that study was consistent with the response to invasion by *Phytophthora sojae* (1–3,6,8).

The objective of this study was to determine the effect of glyceollin on the colonization and growth of eight soybean pathogens, some of which cause soybean diseases to which satisfactory resistance has not been found.

MATERIALS AND METHODS

Soybean pathogens. We studied the following pathogens: *Cercospora sojina*, *Colletotrichum truncatum*, *Diaporthe phaseolorum* var. *meridionales*, *Macrophomina phaseolina*, *Phialophora gregata*, *Phytophthora sojae*, *Rhizoctonia solani* (AG-4), and *Sclerotinia sclerotiorum*. The diseases they cause and the plant parts most commonly attacked are listed in Table 1. All of the pathogens used in this study were obtained from active cultures maintained at the Soybean Pathogen Collection Center at the University of Illinois, Urbana on potato dextrose agar (PDA) or V8 juice agar (V8A). These pathogens were selected because they

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represent some of the most common and important diseases on soybean in the Midwest.

Hairy-root cultures. Hairy-root cultures of two soybean genotypes (cv. Spencer, *F. virguliforme* susceptible, and PI 567374, partially resistant to *F. virguliforme*) were established after transformation with *A. rhizogenes* strain K599 (Stephen Farrand, University of Illinois, Urbana) possessing either the empty vector pILTAB-357 to generate control roots or *CHS6* or *IFS2* genes in sense orientation, as described by Lozovaya et al. (12). These hairy-root lines, with the transgenes, are listed in Table 2.

Pathogen growth on soybean-transformed hairy roots. Pathogen growth on hairy roots and on the medium containing the soybean phytoalexin glyceollin was measured as follows. A 35-mm-wide section of hairy-root solid culture medium was removed from across the center of each petri dish. Three roots with multiple branches from 2-week-old cultures were placed in the center of each petri dish so that both ends of the root sections were in contact with the medium and spread across and over the section empty of medium. A 1-mm^3 cubic section was cut with a cork borer from the margin of an actively growing colony, cultured on 1.5% water agar, and placed on the center of each of three roots placed across the area devoid of medium in each petri dish, followed by incubation in dark at 25°C for 5 days. The pathogen growth observed along the hairy-root sections was measured with the aid of a stereoscope at ×40 magnification at the same time of day on multiple days (every 24 h) until the pathogen overgrew the hairy-root culture. Pathogen growth along each hairy root was measured with a ruler. The number of measurements varied according to the growth rate of each pathogen and stopped when the pathogen overgrew the hairy roots. The mean growth per day of each pathogen on the three hairy roots placed in each dish was calculated and was the experimental unit statistically analyzed. Five petri dishes or replications of each hairy-root line–pathogen combination were arranged in a completely randomized design.

Glyceollin purification. The soybean phytoalexin glyceollin—a mixture of glyceollins I (≈80%), II (≈10%), and III (≈10%)—was produced in bulk from soybean cotyledons infected with *P. sojae* using a modified method based on the work of Ayers et al. (2). Soybean seed were soaked in water for 4 h, sliced into quarters, and inoculated with *P. sojae* race 1. Glyceollin was extracted and purified from the infected seed 48 h after inoculation.

Glyceollin was extracted with 95% ethanol followed by partial purification using liquid chromatography on silica gel (column size: 35 cm in length and 2.5 cm in diameter). Glyceollin was eluted with 200 ml of solvent (hexane-ethyl acetate, 60:40, vol/vol), the column fractions with high absorbance at 285 nm were collected, and the solvents were evaporated using rotor evaporator under reduced pressure. The purification of glyceollin was carried out using thin-layer chromatography (2). The purity of glyceollin obtained (not less than 95%) was confirmed by high-pressure liquid chromatography (HPLC).

Pathogen growth on glyceollin-amended culture media. A purified mixture of glyceollins I, II, and III dissolved in 1.5 ml of absolute ethanol was added to 500 ml of cooled PDA (Difco Laboratories, Sparks, MD), or V8A, containing 100 ml of V8 juice, 1.5 g of $CaCO_3$, and 7.5 g of Bacto agar per 500 ml, before agar solidification to make a final concentration of 75 or 150 μM. A control treatment was prepared by adding 1.5 ml of absolute ethanol without glyceollin to the medium. Two experiments were conducted, one with PDA media amended with 75 μm glyceollin and an agar media control without glyceollin, and another with three treatments: V8A amended with 75 or 150 μm glyceollin and a V8A control without glyceollin.

In the first experiment with glyceollin-amended PDA, there were nine dishes or replicates with glyceollin and eight dishes without glyceollin for each pathogen tested. In the second experiment with two levels of glyceollin in V8A and V8A control without glyceollin, there were six replicate dishes for the control, two replicate dishes for the 75-μM treatment, and eight replicate dishes for the 150-μM treatment. The dishes were inoculated with 4-mm-diameter plugs cut with a cork borer from the margins of actively growing pathogen cultures placed in the center of each plate with the mycelium side down in direct contact with the medium. The cultures were incubated at 24°C in the dark. Colony diameter was measured once at the same time of day on multiple days (every 24 h). The number of measurements taken for each pathogen was determined by its growth rate and was stopped when the pathogen overgrew the medium in each dish.

Extraction and measurement of glyceollin and products of glyceollin degradation. Extraction, measurement, and identification of glyceollin contents in hairy roots were carried out according to the procedure described previously (13). A sample of glyceollin (provided by Dr. Gijzen, Agriculture and Agri-Food Canada, Ontario, Canada) was used as a standard in glyceollin measurements. After pathogen growth measurements were recorded, a section of glyceollin-amended culture medium (3.5 cm wide) fully covered by a colony was transferred to a centrifuge tube and extracted with 5 ml of ethyl acetate after vortexing for 1 min. The ethyl acetate layer was separated by centrifuging (15,000 × *g* for 20 min) and the ethyl acetate supernatant was collected. This extraction procedure was repeated twice for each

TABLE 2. Hairy-root lines used in pathogen growth tests and isoflavone concentration in these lines[a]

Soybean genotype	Phenolic gene expressed	Isoflavone concentration (μmol/g FW)
PI 563374	None (control)	2.7
PI 563374	*CHS6* sense (soybean)	0.6
PI 563374	*IFS2* sense (soybean)	0.3
Spencer	None (control)	5.8
Spencer	*CHS6* sense (soybean)	1.2
Spencer	*IFS2* sense (soybean)	0.7

[a] Hairy-root cultures of two soybean genotypes, cv. Spencer and PI 567374, established after transformation with *Agrobacterium rhizogenes* strain K599 (Stephen Farrand, University of Illinois, Urbana), possessing either empty vector pILTAB-357, to generate control roots, or *CHS6* or *IFS2* genes, involved in isoflavone biosynthesis, in sense orientation (12).

TABLE 1. List of soybean pathogens tested in this study and diseases caused by these pathogens

Pathogen	Disease	Plant parts colonized
Cercospora sojina	Frogeye leaf spot	Foliage
Colletotrichum truncatum	Anthracnose	Leaves, petioles, stem
Diaporthe phaseolorum var. *meridionales*	Southern stem canker	Stem
Macrophomina phaseolina	Charcoal rot	Roots and stem
Phialophora gregata	Brown stem rot	Roots and vascular system
Phytophthora sojae (race 1)[a]	Phytophthora root and stem rot	Roots and stem
Rhizoctonia solani (AG-4)[b]	Rhizoctonia damping-off and root rot	Seedlings and roots
Sclerotinia sclerotiorum	Sclerotinia stem rot	Primary stem

[a] *P. sojae* race 1 (virulence formula: *Rps2*, *Rps3c*, and *Rps7*, which are compatible soybean Phytophthora root and stem rot resistance genes).
[b] AG-4 is anastomoses group 4, a subgroup within *R. solani*.

sample and extracts were combined and evaporated to dryness using a rotary evaporator. The residue was dissolved in 80% methanol and glyceollin and glyceollin derivatives were analyzed by HPLC and liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) under previously described conditions (12). Extraction from the medium without pathogen was done as above to determine the initial level of glyceollin. Positive LC-MS measurements were acquired using an LCQ Deca XP electrospray ionization/ion trap mass spectrometer.

Statistical analyses. Analysis of variance (ANOVA) of pathogen growth on hairy-root cultures and agar media was performed with the aid of JMP (version 7; SAS Institute, Cary, NC). Fungal growth measurements on hairy-root cultures were transformed by $\log_{10}(x + 1)$ prior to analysis to correct for the lack of independence between the treatment means and their variances. Protected F tests of the significance of the means were conducted and the least significant difference between means for pathogen growth on the soybean hairy-root lines was calculated when the ANOVA indicated there were significant differences among the treatments. Single degree-of-freedom contrasts were performed to determine the significance of differences between glyceollin treatments on fungal growth of each pathogen on agar media.

RESULTS AND DISCUSSION

Pathogen growth on hairy roots of soybean lines with different capacity to produce glyceollin. In fungal growth tests, we used soybean hairy-root lines that were transformed with soybean *IFS2*, the gene that encodes the enzyme of the first reaction in the isoflavonoid synthesis branch of the phenylpropanoid pathway, or soybean *CHS6*, the gene which controls the first step of the metabolic branch resulting in the synthesis of flavonoids. These lines had markedly decreased levels of total isoflavones, ≈10-fold lower than the nontransformed controls (Table 2), apparently resulting from post-transcriptional gene silencing, as was described in our previous studies (10–13). Pathogen colonization on transformed and nontransformed control hairy roots was evaluated with: *D. phaseolorum* var. *meridionales*, *M. phaseolina*, *P. sojae*, and *S. sclerotiorum.* We selected these pathogens for testing because of their economic importance and, in the cases of *S. sclerotiorum* and *M. phaseolina*, the lack of complete resistance available in soybean. Because variances of the tests were homogeneous (Bartlett's test, $P > 0.05$), and the effects of each of the two soybean genotypes were similar, Figure 1 shows detransformed means of the growth (millimeters per day) of these four pathogens on soybean hairy roots, either nontransformed (control) or expressing soybean *IFS2* or *CHS6* transgenes in sense orientation, across the three experiments and the two soybean genotypes. Although the overall growth of the pathogens on PI 567374 and Spencer was similar, the effects of the transgenes present in the hairy roots on pathogen growth were highly significantly different ($P < 0.001$). There was a significant transgene–pathogen interaction ($P < 0.05$) for fungal growth (millimeters per day); however, overall, growth was higher on hairy roots transformed with the *IFS2* sense gene than hairy roots transformed with the *CHS6* sense gene, which had higher growth than the nontransformed controls, except for *D. phaseolorum* var. *meridionales*. Growth of *M. phaseolina* was the most affected by the presence of the transgenes in the hairy roots compared with the other pathogens, whereas there was no significant difference in growth of *D. phaseolorum* var. *meridionales* on the test hairy-root lines.

We previously reported that very low amounts of the phytoalexin glyceollin accumulated in these transformed hairy roots in response to the *F. virguliforme* infection (11–13), which resulted in markedly more fungal growth on the glyceollin-deficient roots compared with nontransformed control roots. We also found that the amounts of glyceollin released into the hairy-root culture medium 3 days after root inoculation with *F. virguliforme* were comparable with glyceollin concentrations within infected hairy-root tissues (11). In this study, we also analyzed isoflavone and glyceollin levels in hairy-root tissues 5 days after inoculation with *D. phaseolorum* var. *meridionales*, *M. phaseolina*, *P. sojae*, and *S. sclerotiorum* (Fig. 2). As was found in our previous experiments

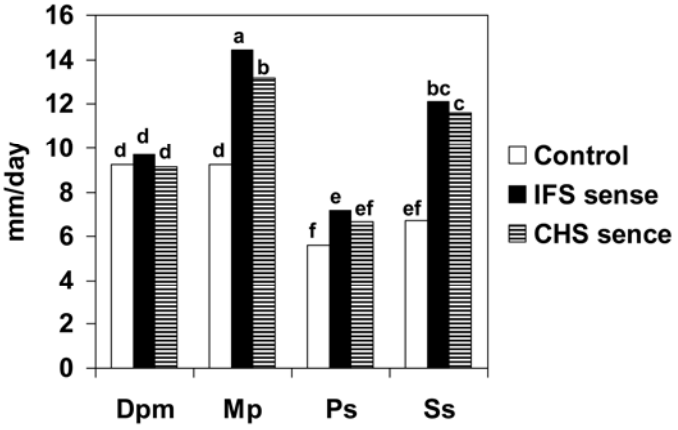

Fig. 1. Effects of hairy-root lines, with or without transgenes controlling soybean isoflavonoid biosynthesis, on the growth of four soybean pathogens. The transgenes were isoflavone synthase (*IFS2*) or chalcone synthase (*CHS6*), genes involved in the isoflavonoid biosynthesis pathway in soybean, in sense orientation. Results for the soybean lines PI 567374 and Spencer, transformed with the same genes, were pooled before analysis because there was no marked difference in their effects on pathogen growth. Mean growth rate in colony area per day for *Macrophomina phaseolina* (Mp) at 2 days, *Diaporthe phaseolorum* var. *meridionales* (Dpm) and *Sclerotinia sclerotiorum* (Ss) at 3 days, and *Phytophthora sojae* (Ps) at 4 days after inoculation. Bars with common letters were not significantly different using least significant difference ($P = 0.05$).

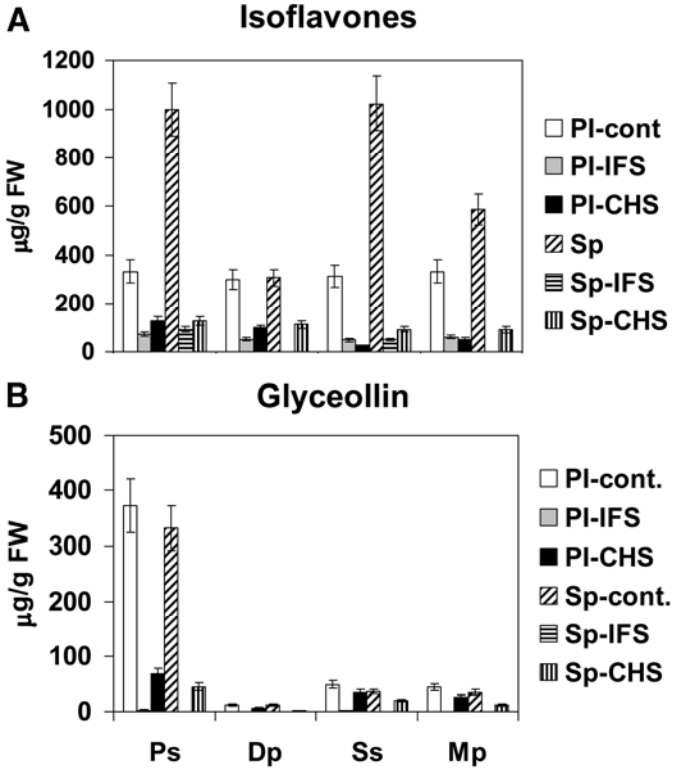

Fig. 2. A, Isoflavone and **B,** glyceollin levels in hairy roots after inoculation of soybean hairy roots transformed with two constructs harboring isoflavone synthase (*IFS2*) and chalcone synthase (*CHS6*) genes with four pathogens. Levels of phenylpropanoids were determined in hairy roots 5 days after inoculation. Error bars are standard errors.

(11), an expression of the *IFS2* and *CHS6* genes resulted in several-fold decrease of isoflavone concentrations in noninoculated roots, which deprived them of the precursors for phytoalexin synthesis (Table 2). The highest glyceollin concentrations in the control hairy roots were found after inoculation with *P. sojae* and the lowest glyceollin levels were in the *D. phaseolorum* var. *meridionales*-inoculated roots. Only trace amounts of glyceollin were detected in *IFS2*-transformed roots in all treatments. Glyceollin levels lower than those of the control were also found in hairy roots transformed with *CHS6* after *P. sojae*, *S. sclerotiorum*, and *M. phaseolina* inoculation, with the largest decrease (several-fold) in *P. sojae*-inoculated transformed roots.

Levels of glyceollin found in *D. phaseolorum* var. *meridionales*-inoculated transformed hairy roots in this study could be the result of a reduced rate of glyceollin biosynthesis in the roots or an increased rate of glyceollin degradation or conversion by pathogen or plant enzymes (21). The higher concentration of glyceollin found in *P. sojae*-inoculated control roots may be due to either high induction of glyceollin synthesis in roots or slow degradation or conversion by *P. sojae*. Further studies are needed to determine whether the glyceollin reduction found in this study was due to host plant or pathogen biochemical action.

Results of the effect of glyceollin on pathogen colonization found in this study are consistent with results of previous studies. An induced accumulation of large amounts of glyceollin in soybean roots infected by *R. solani* has been reported (20). The chemical induction of soybean resistance to *M. phaseolina* by sodium azide treatment was associated with increased glyceollin production (4). Presence of glyceollin in the culture medium reduced polygalacturonase activity in *S. sclerotiorum* culture filtrates and mycelial growth (15).

Results of this study indicated that the soybean plant capacity of accumulating glyceollin in response to pathogen inoculation may be an important part of innate or basal resistance to *P. sojae*, *S. sclerotiorum*, and *M. phaseolina*, in addition to the contribution to SDS partial resistance we previously found (10,12); however, glyceollin did not appear to play an important role in plant response to *D. phaseolorum* var. *meridionales* in this study.

Pathogen growth on glyceollin-amended culture medium. The effect of glyceollin in the culture medium on the growth of several soybean pathogens was tested using glyceollin concentrations that were effective in our previous experiments with *F. virguliforme* (8). These concentrations (75 and 150 μM) can be found in the rhizosphere of infected roots (12). The growth of pathogens *Cercospora sojina*, *D. phaseolorum* var. *meridionales*, *M. phaseolina*, *P. sojae*, *R. solani*, and *S. sclerotiorum* was significantly inhibited by 75 μM glyceollin in PDA, whereas growth *P. sojae* was not inhibited, in the first experiment testing pathogen growth on glyceollin-amended PDA (Table 3). In the second experiment, with two levels of glyceollin in V8A (75 and 150 μM) and V8A control without glyceollin, growth of *C. sojina*, *D. phaseolorum* var. *meridionales*, *M. phaseolina*, *P. sojae* (on V8A), and *R. solani* was significantly lower, whereas growth of *Colletotrichum truncatum* and *S. sclerotiorum* was not significantly reduced on V8A amended with 75 μM glyceollin compared with growth on the control (Table 4). Growth of *Cercospora sojina*, *D. phaseolorum* var. *meridionales*, *P. sojae*, and *R. solani* was significantly lower on V8A amended with 150 μM glyceollin compared with the 75-μM glyceollin level, whereas growth of *Colletotrichum truncatum*, *M. phaseolina*, and *S. sclerotiorum* was not significantly reduced on the media with the higher glyceollin concentration (Table 4). Differences in the significance of effect of glyceollin on fungal growth reflected the different growth rates of each pathogen on each culture medium. For instance, poor growth of *P. sojae* on PDA in the first experiment may have limited the detection of a significant effect of glyceollin on its growth. Poor growth of *P. sojae* on PDA has been previously reported (7). In the second experiment using V8A, poor growth may have also been responsible for the lack of significant differences in growth on V8A with or without glyceollin found for *C. truncatum* and *S. sclerotiorum*.

The growth rate of *M. phaseolina*, *P. sojae*, and *S. sclerotiorum* on glyceollin-amended PDA appeared to agree with growth on the hairy-root transformants. Pathogen growth was sensitive to the amount of glyceollin present in the culture medium and growth was higher on transformed hairy-root lines that had low glyceollin production. Rapidly growing *D. phaseolorum* var. *meridionales* was very sensitive to the glyceollin in the medium; however, a difference in the growth of this fungus on hairy roots with different capacities for glyceollin synthesis was not observed. The lowest glyceollin concentration was found in roots inoculated with *D. phaseolorum* var. *meridionales* compared with all other pathogens tested, including fast-growing fungi such as *M. phaseolina* and *R. solani* (Fig. 2), which may indicate the low induction of phytoalexin synthesis caused by *D. phaseolorum* var. *meridio-*

TABLE 3. Colony growth of eight soybean pathogens on potato dextrose agar medium with or without 75 μM glyceollin

Pathogen	Area of growth (mm^2/day) per glyceollin level		Significance[a]
	Control	75 μm	
Cercospora sojina	3.1	1.7	***
Diaporthe phaseolorum var. *meridionales*	241.0	58.7	***
Macrophomina phaseolina	337.0	87.4	***
Phialophora gregata	7.3	1.9	***
Phytophthora sojae race 1[b]	1.4	1.4	n.s.
Rhizoctonia solani (AG-4)[c]	392.3	249.3	**
Sclerotinia sclerotiorum	155.9	78.1	***

[a] Significance of difference: ** and *** indicate $P < 0.01$ and 0.001, respectively, and n.s. = nonsignificant.
[b] *P. sojae* race 1 (virulence formula: *Rps2*, *Rps3c*, and *Rps7*, which are compatible soybean Phytophthora root and stem rot resistance genes).
[c] AG-4 is anastomoses group 4, a subgroup within *R. solani*.

TABLE 4. Comparisons of the effects of two levels of glyceollin and a control on growth of eight soybean pathogens on V8 agar

Pathogen	Area of growth (mm^2/day) per glyceollin level			Significance of differences[a]	
	Control	75 μM	150 μM	Control vs. 75 μM glyceollin	75 vs. 150 μM glyceollin
Cercospora sojina	1.8	1.0	0.4	**	***
Colletotrichum truncatum	34.8	30.5	28.8	n.s.	n.s.
Diaporthe phaseolorum var. *meridionales*	71.9	30.4	18.9	***	***
Macrophomina phaseolina	329.5	172.7	147.4	***	n.s.
Phytophthora sojae race 1[b]	53.8	39.2	20.5	**	***
Rhizoctonia solani[c]	534.3	267.9	154.4	***	***
Sclerotinia sclerotiorum	171.3	165.0	131.5	n.s.	n.s.

[a] Symbols: ** means differences were significant ($P < 0.01$), *** means the differences were highly significant ($P < 0.001$), and n.s. means the difference between the means in the comparison were nonsignificant ($P > 0.05$).
[b] *P. sojae* race 1 (virulence formula: *Rps2*, *Rps3c*, and *Rps7*, which are compatible soybean Phytophthora root and stem rot resistance genes).
[c] AG-4 is anastomoses group 4, a subgroup within *R. solani*.

nales pathogenesis. It is also possible that *D. phaseolorum* var. *meridionales* is capable of suppressing glyceollin synthesis in roots or the fungus may be able to detoxify the phytoalexin. One study found that, during infection of soybean by ascospores and mycelia of *S. sclerotiorum,* hyphal growth from mycelia was less affected than hyphal growth from ascospores by the glyceollin, and glyceollin accumulated in leaves at concentrations that were markedly below those required for prevention of mycelial growth in in vitro tests (186); however, results in this study provide evidence that, at least on PDA, growth of *S. sclerotiorum* was significantly lower on PDA amended with glyceollin than on the control without glyceollin.

Biodegradation of glyceollin. The success of soybean pathogens to infect a plant and colonize host tissue could depend upon their capacity to produce enzymes that can destroy or detoxify compounds invoked in the plant defense response. HPLC analysis of combined extracts from pathogen and culture medium (containing glyceollin) used for pathogen growth showed that all pathogens tested, except for *P. sojae*, which is a stramenopile and not a fungus, are capable of metabolizing glyceollin. Levels of glyceollin in medium fully colonized by each pathogen, except the stramenopile *P. sojae*, decreased dramatically compared with that in medium of the same area but without a pathogen tissue (Table 5). Glyceollin level in medium colonized by *R. solani* decreased to 20%; <2% of initial glyceollin concentration was detected in medium colonized by *Cercospora sojina*, *M. phaseolina*, and *S. sclerotiorum*; and even less for *D. phaseolorum* var. *meridionales*, which was very efficient in metabolizing glyceollin, because only trace amounts were detected in the medium. Importantly, ≈80% of glyceollin was still detected in samples with *P. sojae* (Table 5).

We identified potential products of glyceollin degradation or detoxification by soybean pathogens in the medium after incubation (Table 6). A major compound was found in the in-glyceollin-amended media that was not found in the control media following pathogen growth, apparently resulting from glyceollin degradation by pathogen enzymes. The compound with an R_T of 27.5 min (metabolite 1) was the main and most common product of glyceollin degradation by five of the pathogens tested here (*C. sojina*, *D. phaseolorum* var. *meridionales*, *M. phaseolina*, *Phialophora gregata*, and *R. solani*) (Table 6). Four derivatives of glyceollin were found in *S. sclerotiorum* samples, whereas *Phytophthora sojae* was the exception, because we did not detect any glyceollin conversion or degradation products in the culture medium of *P. sojae*, a stramenopile. The inability of *P. sojae* to metabolize glyceollin was also previously demonstrated by Yoshikawa et al. (21).

TABLE 5. Residual levels of glyceollin (percentage of initial glyceollin concentration, 150 μM) measured in a mixture of culture medium and pathogen mycelium after the agar media was fully colonized

Pathogen	Concentration (%)[a]
Colletotrichum truncatum	2
Diaporthe phaseolorum var. *meridionales*	Trace
Macrophomina phaseolina	<1
Phytophthora sojae race 1[b]	80
Rhizoctonia solani (AG-4)[c]	20
Sclerotinia sclerotiorum	<0.5

[a] Percentage of the glyceollin concentration remaining in the culture medium.
[b] *P. sojae* race 1 (virulence formula: *Rps2*, *Rps3c*, and *Rps7*, which are compatible soybean Phytophthora root and stem rot resistance genes).
[c] AG-4 is anastomoses group 4, a subgroup within *R. solani*.

The UV spectrum of metabolite 1 was identical to that of glyceollin I (λ_{max} = 284 nm; 289, 306, and 317 sh [shoulder]). LC-ESI-MS of this compound showed that the molecular ion is present at *m/z* 355 $[M+H]^+$ and the important fragment ion at *m/z* 337 (base peak), that corresponds to the loss of H_2O molecule. The existence of an ion with *m/z* 355 indicates that metabolite 1 has an additional hydroxyl group compared with glyceollin. This is in good agreement with previous data showing that conversion of phytoalexins by fungi in most known cases includes the addition of a hydroxyl group to the molecule (19). Tandem mass spectrometry (MS-MS) of the intensive fragment (at *m/z* 337) gave a number of fragments: at *m/z* 319 $[M+H-H_2O]^+$; 309 $[M+H-CO]^+$, with the highest intensity; 295 $[M+H-C_2H_2O]^+$ as a result of ring B fragmentation; 203 and 137 as a result of retro Diels-Alder fragmentation; and an ion at *m/z* 187 after the loss of a methyl group from the fragment at *m/z* 203.

The existence of fragments at *m/z* 203 and 137 showed that hydroxylation of glyceollin did occur in ring D and not in rings A and B because these parts of the molecule were not altered (Fig. 3). Hydroxylation of the aromatic E ring at carbon atom 7 occurred in pterocarpane maakiain through intermediate dihydroxy-8,9-methyldioxypterocarpan with hydroxyl group in 6a position by the fungi *Botrytis cinerea*, *Colletotrichum lindemuthianum, and S. trifoliorum* (14). These reported results and our MS data allowed us to assign the structure 7-hydroxyglyceollin for metabolite 1 (Fig. 3).

Metabolite 1 could be further metabolized. We found metabolite 2 in the growth medium of *M. phaseolina* and *Cercospora sojina* with an R_T of 24.4 min, λ_{max} = 284.2 nm, 289 and 301 sh. ESI-LC MS of this peak gave a low-intensity ion at m/z 517 and high-intensity ions at m/z 355 and 337. MS-MS of the ion at m/z 355 gave fragments at m/z 337 $[M+H-H_2O]^+$, 309 $[M+H-H_2O-CO]^+$, 295 $[[M+H-H_2O-C_2H_2O]^+$, and 283 $[M+H-H_2O-C_4H_8]^+$, resulting from fragmentation of dimethyl chromen ring; 203 after retro Dils-Alder fragmentation. MS-MS of the ion at m/z 337 gave fragments identical to those for metabolite 1 as described above. Based on these data, we assigned the structure for metabolite 2 as hexoside of 7-hydroxyglyceollin. Metabolites of

TABLE 6. Glyceollin metabolites found in the fungal growth medium containing 75 μM glyceollin following pathogen colonization

Fungus	Metabolites	Molecular mass	Metabolite structure	
			Aglycone	Sugar
Cercospora sojina	Metabolite 1	354	7-Hydroxyglyceollin	…
	Metabolite 2	517	7-Hydroxyglyceollin	Hexoside
Macrophomina phaseolina	Metabolite 1	354	7-Hydroxyglyceollin	…
	Metabolite 2	517	7-Hydroxyglyceollin	Hexoside
Rhizoctonia solani (AG4)[a]	Metabolite 1	354	7-Hydroxyglyceollin	…
Sclerotinia sclerotiorum	Metabolite 3	534	7-Hydroxyglyceollin	Aminohexoside
	Metabolite 4	515	Metoxyisoflavanone	Aminohexoside
	Metabolite 5	548	7-Hydroxy-methoxy glyceollin	Aminohexoside
	Metabolite 6	353	Metoxyisoflavanone	…
Phytophthora sojae (race 1)[b]	None	…	…	…
Phialophora gregata	Metabolite 1	354	7-Hydroxyglyceollin	…
Diaporthe phaseolorum var. *meridionales*	Metabolite 1	354	7-Hydroxyglyceollin	…

[a] AG-4 is anastomoses group 4, a subgroup within *R. solani*.
[b] *Phytophthora sojae* race 1 (virulence formula: *Rps2*, *Rps3c*, and *Rps7*, which are compatible soybean Phytophthora root and stem rot resistance genes).

phytoalexins are usually less toxic than the phytoalexin itself, though not always (19), and glycosylation could decrease the toxicity of metabolite to some pathogens.

The *S. sclerotiorum* samples contained four derivatives of glyceollin: metabolite 3 (R_T of 25.4 min, λ_{max} = 283.0, 288, 307, and 320 sh nm), metabolite 4 (R_T of 27.5 min, λ_{max} = 275.0 and 314 nm), metabolite 5 (R_T of 28.0 min, λ_{max} = 284.2, 288, 307, and 320 sh nm), and metabolite 6 (R_T of 29.2 min, λ_{max} = 274.7 and 315 nm).

Glyceollin and metabolites 3 and 5 had almost identical UV spectra, except that the shoulder at 320 nm with glyceollin was less definite than with the other two metabolites. LC-MS of metabolite 3 showed a pseudomolecular ion at m/z 534. MS-MS of this ion gave fragments at m/z 517 $[M+H-NH_3]^+$, 499 $[M+H-NH_3-H_2O]^+$, 355 $[M+H-NH_3\text{-Hexose}]^+$, 337 $[M+H-NH_3\text{-Hexose-}H_2O]$, and 203, which corresponds to the retro Dils-Alder fragmentation. We believe that this metabolite is aminohexoside of 7-hydroxyglyceollin. The ESI-LC-MS spectrum of metabolite 5 showed an intensive ion at m/z 548 and its MS-MS fragmentation produced ions at m/z 531 $[M+H-NH_3]^+$, 517 $[M+H-NH_3-CH_3OH]^+$, 499 $[M+H-NH_3-H_2O-CH_3OH]^+$, 369 $[M+H-NH_3\text{-Hexose}]^+$, and 337 $[M+H-NH_3\text{-Hexose-}CH_3OH]^+$. The similarity of UV spectra of metabolites 5 and 3, the difference in their molecular mass of 14, and the loss of the fragment, which could correspond to methyl hydroxyl, allow us to assume that the metabolite 5 structure is aminohexoside of methoxyderivative of 7-hydroxyglyceollin, although we cannot determine which hydroxyl group was methylated.

Metabolites 4 and 6 had identical UV spectra, with a maximum absorption corresponding to that of isoflavanones. For example, similar UV spectrum with maximums of 277 and 309 nm were reported for 2′-hydroxydihydrodaidzein (9). Metabolite 4 gave a pseudo-molecular ion at m/z 515, which fragmented to ions at m/z 497 $[M+H-H_2O]^+$ and 353 $[M+H\text{-hexoside}]^+$ as result of the loss of hexose and very intensive ion 335 $[M+H\text{-hexoside-}H_2O]^+$. Metabolite 6 produced a low-intensity ion at m/z 353 and an intensive ion at m/z 335. Further fragmentation of that ion for both metabolites was identical and gave ions 317 $[M+H-H_2O]^+$, 303 $[M+H-H_2O-CH_3]^+$, 299 $[M+H-2H_2O]$, 285 $[M+H-H_2O-CO]^+$, and 267 $[M+H-2H_2O-CH_3]^+$. Such fragmentation indicates the existence of a methoxy group in both metabolites. Based on our MS and UV spectral data, their most probable structure should be described as methoxy derivatives of isoflavanone (Fig. 3). Thus, other routes of glyceollin degradation could occur through formation of the isoflavanones, although these derivatives were not the major products of fungal decomposition of glyceollin and were detected here only in *S. sclerotiorum* samples. Similar decomposition of the pterocarpanoid phytoalexins medicarpin and maackiain via the opening of the pterocarpanoid ring E was reported for *Nectria haematococca* isolate MP VI T-30 (5).

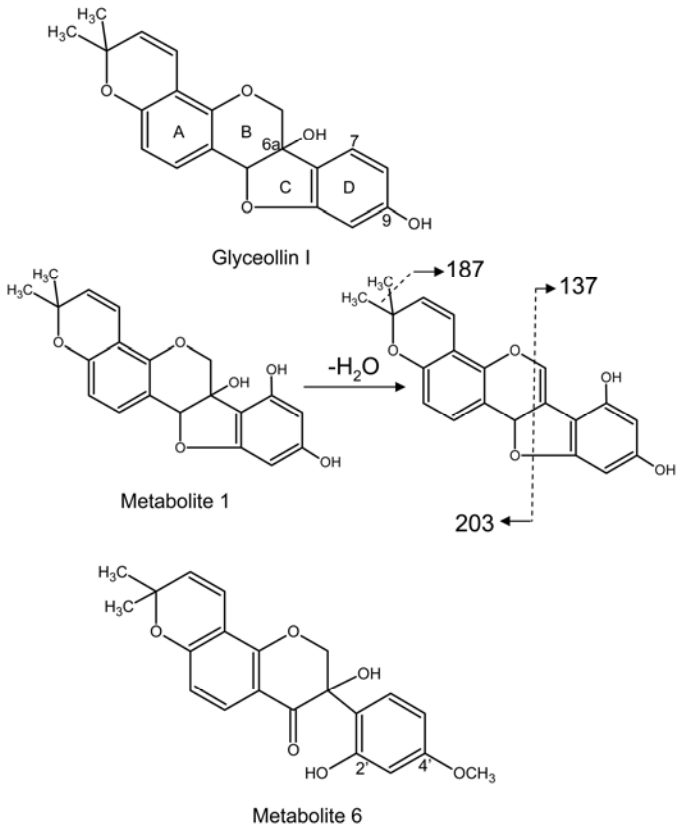

Fig. 3. Structure of glyceollin I and glyceollin's metabolites identified by liquid chromatography mass spectrometry.

We did not purify the products of glyceollin conversion; therefore, it was not possible to accurately measure their levels; however, because the structure of 7-hydroxy glyceollin and glyceollin and their absorption properties are very similar, we carried out the approximate estimation of their concentrations in the same way as was done for the glyceollin. Based on this assumption, we evaluated the amounts of derivatives of 7-hydroxy glyceollin in medium after growth of *R. solani* as 20% of initial glyceollin level and 80% for *M. phaseolina*, 40% for *S. sclerotiorum*, 70% for *C. sojina*, and 20% for *D. phaseolorum* var. *meridionales*. We were also unable to exclude the possibility that a portion of the glyceollin metabolites were further converted into polar compounds, which were not separated from the solid agar medium. Our estimation presented above indicated that fast-growing *D. phaseolorum* var. *meridionales* and *R. solani* had a higher ability to degrade of glyceollin.

Different mechanisms of phytoalexin degradation could help pathogens overcome basal plant defenses if the pathogen efficiency to convert phytoalexins into less inhibitory metabolites is high enough.

Another important aspect of glyceollin metabolism during pathogenesis is the differential effect of pathogens on glyceollin degradation by plant enzymes. Biodegradation of glyceollin by soybean hypocotyls, as measured by the decrease in glyceollin content, was markedly less in the hypocotyls infected with an incompatible race of *P. sojae* than in the hypocotyls infected with a compatible race of *P. sojae* (21). Most importantly, the stramenopile did not appear to have the capability to directly degrade glyceollin, in both the compatible *P. sojae* race-infected hypocotyls and in vitro tests in these experiments, although noninoculated wounded hypocotyls also had glyceollin present at a low level that did not accumulate in the tissues, apparently due to degradation by hypocotyl enzymes. Even though we found that glyceollin could be directly metabolized by most of the pathogens tested, glyceollin degradation by plant enzymes might also have occurred.

Glyceollin was found to inhibit the growth of a range of soybean pathogens in this study. Rapid glyceollin biosynthesis during infection may be an important component of basal or innate resistance to pathogens in soybean. Because pathogens capable of degrading phytoalexins may be able to avoid or limit the effects of these plant defense compounds, we also investigated the capacity of important soybean pathogens to degrade glyceollin, which aided their ability to colonize soybean plants and could contribute important knowledge leading to increasing host defenses through manipulation of constitutive or inducible host phytoalexin production.

Thus, breeding and genetic engineering targeted to enhance the capacity of glyceollin production in response to pathogen invasion or the capacity to prevent glyceollin degradation by pathogens could increase overall plant resistance to multiple diseases in soybean. It is conceivable that new soybean cultivars with

stronger innate resistance would have a superior ability to accumulate the antibiotic glyceollin in response to general pathogen invasion, and not just to specific pathogens only. Cultivars with such enhanced innate resistance could help stabilize soybean production in the presence of diseases.

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LITERATURE CITED

1. Albersheim, P., and Valent, B. S. 1978. Host-pathogen interaction in plants. Plants, when exposed to oligosaccharides of fungal origin, defend themselves by accumulating antibiotics. J. Cell Biol. 78:627-643.
2. Ayers, A., Ebel, J., Finelli, N., Berger, N., and Albersheim, P. 1976. Quantitative assay of elicitor activity and characteristics of the elicitor present in the extracellular medium of cultures of *Phytophthora megasperma* var. *sojae*. Plant Physiol. 57:751-759.
3. Bhattacharyya, M. K., and Ward, E. W. B. 1985. Differential sensitivity of *Phytophthora megasperma* f. sp. *glycinea* to glyceollin isomers. Physiol. Mol. Plant Pathol. 27:299-310.
4. Chakraborty, B. N., and Purkayastha, R. P. 1988. Alteration in glyceollin synthesis and antigenic patterns after chemical induction of resistance in soybean to *Macrophomina phaseolin*a. Can. J. Microbiol. 33:835-840.
5. Denny, T. P., and VanEtten, H. D. 1982. Metabolism of the phytoalexins medicarpin and maackiain by *Fusarium solani*. Phytochemistry 21:1023-1028.
6. Ebel, J., and Grisebach, H. 1988. Defense strategies of soybean against the fungus *Phytophthora megasperma* f. sp. *glycinea*: a molecular analysis. Trends Biochem. Sci. 13:23-27.
7. Erwin, D., and Ribeiro, O. 1996. Phytophthora Diseases Worldwide. American Phytopathological Society Press, St. Paul, MN.
8. Graham, T. L., and Graham, M. Y. 1991. Glyceollin elicitors induce major but distinctly different shifts in isoflavonoid metabolism in proximal and distal soybean cell populations. Mol. Plant-Microbe Interact. 4:60-68.
9. Ingham, J. L. 1982. Phytoalexins from the Leguminosae. Pages 21-80 in: Phytoalexins. J. A. Baily and J. W. Mansfield eds. Wiley, New York.
10. Lozovaya, V. V., Lygin, A. V., Li, S., Hartman, G. L., and Widholm, J. M. 2004. Biochemical response of soybean roots to *Fusarium solani* f. sp. *glycines* infection. Crop Sci. 44:819-826.
11. Lozovaya, V. V., Lygin, A. V., Zernova, O. V., Li, S., Hartman, G. L., and Widholm, J. M. 2004. Isoflavonoid accumulation in soybean hairy roots upon treatment with *Fusarium solani.* Plant Physiol. Biochem. 42:671-679.
12. Lozovaya, V. V., Lygin, A. V., Zernova, O. V., Ulanov, A. V., Li, S., Hartman, G. L., and Widholm, J. M. 2007. Modification of phenolic metabolism in soybean hairy roots through down regulation of chalcone synthase or isoflavone synthase. Planta 225:665-679.
13. Lozovaya, V. V., Lygin, A. V., Zernova, O. V., and Widholm, J. M. 2005. Genetic engineering of plant disease resistance by modification of the phenylpropanoid pathway. Plant Biosyst. 139:20-23.
14. Macoy, C. A., and Smith, I. M. 1979. Phytoalexin production and degradation in relation to resistance of clover leaves to *Sclerotinia* and *Botritis* spp. Physiol. Plant Pathol. 14:99-111.
15. Marciano, P. 1994. In vitro effect of the phytoalexin glyceollin I on polygalacturonase and oxalic acid production of *Sclerotinia sclerotiorum*. Phytoparasitica 22:101-108.
16. Paxton, J. D. 1974. Phytoalexins, phenolics and other antibiotics in roots resistant to soil-borne fungi. Pages 185-192 in: Biology and Control of Soil-Borne Plant Pathogens. G. W. Bruehl, ed. American Phytopathological Society, St. Paul, MN.
17. Paxton, J. D., and Groth, J. 1994. Constraints on pathogens attacking plants. Crit. Rev. Plant Sci. 13:77-95.
18. Sutton, D. C., and Deverall, B. J. 1984. Phytoalexin accumulation during infection of bean and soybean by ascospores and mycelium of *Sclerotinia sclerotiorum*. Plant Pathol. 33:377-383.
19. Vanetten, H. D., Matthews, D. E., and Smith, D. A. 1982. Metabolism of phytoalexins. Pages 181-217 in: Phytoalexins. J. A. Baily and J. W. Mansfield, eds. Wiley, New York.
20. Wyss, P., Boller, T., and Wiemken, A. 1992. Testing the effect of biological control agents on the formation of vesicular arbuscular mycorrhiza. Plant Soil 147:159-162.
21. Yoshikava, M., Yamauchi, K., and Masago, H. 1979. Biosynthesis and biodegradation of glyceollin by soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. Physiol. Plant Pathol. 14:157-169.