Effect of Spores of Saprophytic Fungi on Phytoalexin Accumulation in Seeds of Frog-Eye Leaf Spot and Stem Canker-Resistant and -Susceptible Soybean (*Glycine max* L.) Cultivars

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Two saprophytic fungi (*Mucor ramosissimus* and *Rhizopus* sp.) were tested for their ability to induce phytoalexin production by seeds of frog-eye leaf spot and stem canker-resistant and -susceptible soybean (*Glycine max* L.) cultivars. Only *M. ramosissimus* was shown to elicit a response and qualitative differences in phytoalexin accumulation were found between the susceptible and resistant cultivars. Glyceollins I, II, and III and glycinol were isolated from the susceptible cultivar, whereas Glyceollins I, II, and III, glycinol, glyceocarpin, genistein, isoformononetin, and *N*-acetyltyramine accumulated in the resistant cultivar in response to the same fungal elicitor. Genistein was found to be an inducibly formed isoflavonoid instead of a constitutive metabolite in the resistant cultivar, whereas *N*-acetyltyramine is described for the first time as a soybean phytoalexin. All the compounds, except genistein, showed fungitoxic activity against *Cladosporium sphaerospermum*. Spectral data of the pterocarpan phytoalexins, genistein, and *N*-acetyltyramine are also given in this work.

Keywords: *Glycine max; soybean; phytoalexins; Mucor ramosissimus; Rhizopus sp.*

INTRODUCTION

The study of defense mechanisms in plants has revealed that plant resistance against pathogenic infection is effective at diverse levels in the parasite−host interactions, including chemical and physical barriers, either preformed or induced by the invading organism. Many plant species synthesize a wide range of defense compounds in response to microbial infection. Among them are phytoalexins, which are low-molecular-weight compounds synthesized de novo by the plant in response to pathogen infection or environmental stimuli (Paxton, 1981; Kuć, 1997).

Phytoalexin production by soybean (*Glycine max* cv. L.), induced by either abiotic or biotic elicitors, has been extensively investigated in recent years (Davis et al., 1986; Liu et al., 1992; Schmidt et al., 1992; Miller et al., 1994; Graham and Graham, 1996; Scharff et al., 1997; Pueppke et al., 1998). Among the latter, several reports are available concerning the action of phytopathogenic fungi or their products on the synthesis of defense compounds in this species (Sutton and Deverall, 1984; Morris et al., 1991; Wyss et al., 1991; Favaron et al., 1992; Waldmüller et al., 1992; Umemoto et al., 1997). On the other hand, there are few reports on saprophytic fungi efficiency in eliciting phytoalexin production in soybean (Braga et al., 1986; Cordeiro Neto and Dietrich, 1992). In the cases where such studies have been undertaken, however, only detection of a phytoalexin response based on fungitoxic activity of inoculated leaves or a quantitative assay of glyceollin induction in inoculated cotyledons were considered.

In the present work, we have aimed at not only investigating the phytoalexin response of seeds of two soybean cultivars (Dourados and Bragg, resistant and susceptible, respectively, to frog-eye leaf spot and stem canker) upon inoculation with spores of two saprophytic fungi, *Mucor ramosissimus* cv. Samutsevitsch and *Rhizopus* sp., but also finding whether a correlation between phytoalexin accumulation and frog-eye leaf spot- and stem canker-resistance could be established. Frog-eye leaf spot and stem canker, caused by *Cercospora sojina* cv. Hara and *Diaporthe phaseolorum* cv. (Cooke & Ellis) Sacc, respectively, are two severe fungal diseases of soybean, the latter being responsible for significant yield losses of crops in Brazil, mainly in the central-western regions.

The fungitoxic potential of the phytoalexins eventually produced by the two cultivars against the phytopathogenic fungus *Cladosporium sphaerospermum* cv. Penz. was also evaluated in this work.

MATERIALS AND METHODS

Seeds of soybean cultivars Dourados and Bragg, frog-eye leaf spot and stem canker-resistant and -susceptible, respectively, were provided by EMBRAPA/CPAO (Dourados, MS, Brazil).

Fungal Material. The fungi inducer [*Mucor ramosissimus* cv. Samutsevitsch (URM 3087) and *Rhizopus* sp.] and detector [*Cladosporium sphaerospermum* cv. Penz. (SPC 491)] were grown, in the dark at 25 ± 2 °C, in Petri disks containing a culture medium of potato, dextrose, and agar. The fungi were cultivated for 8 and 12 days, respectively, to achieve adequate sporulation.

Bioautography Assays. The detection of fungitoxic activity was performed by bioautograms on silica gel plates, as

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10.1021/jf991146o CCC: $19.00
Published on Web 07/22/2000

described elsewhere (Homans and Fuchs, 1970), employing spores of *Cladosporium sphaerospermum* cv. Penz. as detecting fungus.

Elicitation and Extraction of Phytoalexins. Seeds (250 g) from each cultivar were incubated in water at 22 °C for 4 h, then cut into four pieces (keeping the embryo intact) and inoculated, in separate experiments, with aqueous suspensions of *Mucor ramosissimus* and *Rhizopus* sp., containing ca. 5×10^7 spores/mL. Following incubation for 72 h at 30 °C, the seeds were extracted separately with ethanol for 48 h (Ayers et al., 1976). The ethanolic extracts thus obtained were concentrated under reduced pressure. Water (300 mL) was added to the residue and the resulting aqueous extracts were successively partitioned with hexane (2 × 300 mL), ethyl ether (2 × 300 mL), and *n*-butanol (2 × 300 mL). Bioautography was performed with the hexane, ether, and *n*-butanol solubles obtained in each experiment. Two control treatments were employed in all experiments, comprising noninoculated seeds of each cultivar and spore suspensions of *M. ramosissimus* and *Rhizopus* sp., which were extracted with ethanol.

Isolation and Characterization of Phytoalexins. *From cv Bragg.* The ethereal phase, obtained from partition of the ethanolic extract of the seeds inoculated with spores of *M. ramosissimus*, was subjected to chromatographic separation on a silica gel column (60H, 50 g), on gradient elution with hexane−ethyl acetate−methanol mixtures (60:15:1 to 30:20:10), collecting 64 fractions of 20 mL each. The eluates from the fractions which were shown to contain aromatic and/or fungitoxic compounds were purified as follows: the fractions eluted with hexane−ethyl acetate−methanol (60:40:5) were separated by column chromatography over Sephadex LH-20 polymer beads using methanol as a solvent. Further purification by semipreparative HPLC [Shimadzu LC 6AD; column, Shim-pack SIL, 20 mm × 25 cm, 5 μm particle size; eluant, hexane−2-propanol (96:4), 8 mL/min.; detection 254 nm] afforded glyceollins I [7 mg], II [12 mg], and III [17 mg] (Lyne et al., 1976; Ingham et al., 1981).

One of the fractions eluted with hexane−ethyl acetate−methanol (50:50:10), after column chromatography on Sephadex LH-20 with methanol as eluant, yielded glycinol [28 mg] (Ingham et al., 1981).

From cv Dourados. The ethereal phase, obtained from the partition of the ethanolic extract of the seeds inoculated with spores of *M. ramosissimus*, was chromatographed over silica gel column (60H, 50 g), eluted with hexane−ethyl acetate−methanol mixtures with increasing polarity (60:15:1 to 40:60:15), to give 21 100-mL fractions. Fractions with the same thin-layer chromatography profile were combined and, among them, fractions 9 and 10, 11 and 12 [eluted with hexane−ethyl acetate−methanol (60:30:3)], 14, 15 [eluted with hexane−ethyl acetate−methanol (60:40:5)], 16 and 17, and 18 [eluted with hexane−ethyl acetate−methanol (50:50:10)] were shown to contain aromatic and/or fungitoxic compounds. Fractions 11 and 12 were analyzed by HPLC [Shimadzu LC 6AD; column, Shim-pack SIL, 4.6 mm × 25 cm, 5 μm particle size; eluant, hexane−2-propanol (97:3), 1 mL/min; detection 254 nm] and shown to contain glyceollins I, II, and III when compared with authentic samples obtained from cv. Bragg.

The other fractions (9 and 10, 14, 15, 16 and 17, and 18) were further purified by Sephadex LH-20 CC eluting with methanol, to give, respectively, glyceollin I (34 mg), glyceocarpin [8 mg] (Ingham et al., 1981) and genistein (5 mg), isoformononetin (15 mg), glycinol (30 mg), and *N*-acetyltyramine (27 mg).

Genistein: ^{1}H NMR (Bruker DPX-300, 300 MHz, acetone-d_6): 6.33 (1H, d, J = 2.0 Hz, H-6), 6.47 (1H, d, J = 2.0 Hz, H-8), 6.96 (2H, d, J = 8.6 Hz, H-3′, 5′), 7.44 (2H, d, J = 8.6 Hz, H-2′, 6′), 8.17 (1H, s, H-2).

N-Acetyltyramine: ^{1}H NMR (Bruker DPX-300, 300 MHz, acetone-d_6): 1.89 (3H, s, −OCOCH_3), 2.67 (2H, t, J = 8.0 Hz, H-7), 3.34 (2H, t, J = 8.0 Hz, H-8), 6.75 (2H, d, J = 8.4 Hz, H-2,6), 7.04 (2H, d, J = 8.4 Hz, H-3,5). ^{13}C NMR (75 MHz, acetone-d_6): 22.2 (−OCO*C*H$_3$), 34.2 (C-7), 41.2 (C-8), 115.6 (C-2,6), 130.2 (C-3,5), 130.9 (C-4), 154.8 (C-1), 173.3 (−*O*COCH$_3$).

Table 1. ^{13}C NMR Data for Pterocarpan Phytoalexins 1−5 (75 MHz, $CDCl_3$, TMS $\delta = 0$)[a]

C	1	2	3	4	5
1	130.8	128.4	126.4	133.2	131.7
2	111.0	116.9	121.5	110.7	115.2
3	153.9	154.6	143.6	161.9	154.1
4	110.2	104.7	98.2	103.8	102.6
4a	150.3	155.4	155.2	157.1	156.1
6	69.7	69.7	69.9	70.6	69.8
6a	76.5*	76.7*	76.6	76.7	75.9
6b	119.7	120.2	120.2	121.4	120.6
7	124.0	124.2	124.1	125.1	124.2
8	108.8	108.7	108.7	108.9	108.0
9	160.6	160.8	160.7	160.7	161.1
10	98.7	98.8	98.7	98.6	97.8
10a	158.7	158.4	158.6	159.6	159.8
11a	84.9	84.8	85.3	85.9	85.2
11b	112.3	112.2	111.8	113.3	112.2
12	116.4	121.5	33.8		27.6
13	129.2	129.4	86.8		123.0
14	76.2*	76.6*	161.2		130.3
15	27.7**	28.0**	112.3		25.1
16	27.8**	28.2**	17.1		17.0

[a] *, **Assignments within the same column may be interchangeable.

ESI-MS (Micromass Platform II single quadrupole mass spectrometer, ionization in positive ion mode) *m*/*z* (relative intensity): 180 $[M + H]^+$ (100).

Spectral data of isoformononetin (^{1}H NMR, 300 MHz; ^{13}C NMR, 75 MHz; acetone-d_6) and genistein (^{13}C NMR, 75 MHz, acetone-d_6) were in accordance with previous reports (Ingham et al., 1981; Markham and Chari, 1982). ^{13}C NMR data of the pterocarpan phytoalexins **1**−**5** are depicted in Table 1.

RESULTS AND DISCUSSION

Rhizopus sp. was ineffective as a phytoalexin elicitor agent in seeds of both cvs. Bragg and Dourados, as indicated by bioautographic assays, as well as by thin layer chromatographic analysis of the hexane, ethereal, and *n*-butanol phases, when compared with the same material obtained from noninoculated seeds.

On the other hand, not only did spores of *Mucor ramosissimus* induce a phytoalexin response in soybean seeds, but also the elicited accumulation of phytoalexins varied for each cultivar tested. The ethanolic extracts obtained from *M. ramosissimus*-inoculated seeds of both cultivars yielded, after partition procedures, the hexane, ethereal, and *n*-butanol phases. Of these, only the ethereal phases were found to contain aromatic substances, indicative of the presence of soybean phytoalexins, as shown by their ^{1}H NMR spectra. Likewise, bioautography performed with these materials revealed that fungitoxic activity rested only in the ethereal solubles.

Four phytoalexins were produced by the *Mucor*-inoculated susceptible cultivar. They were isolated after a series of chromatographic separations and readily identified as glyceollins I (**1**), II (**2**), and III (**3**), and glycinol (**4**), well-known induced soybean metabolites (Ingham, 1982) [Figure 1].

The same isolation procedures were applied to the ethereal phase obtained from inoculated seeds of the resistant cultivar and, besides compounds **1**−**4** (also produced by cv. Bragg), four additional phytoalexins were isolated: the pterocarpan glyceocarpin (**5**), the isoflavones genistein (**6**) and isoformononetin (**7**) [Ingham, 1982], and *N*-acetyltyramine (**8**) [Figure 1].

Genistein, although considered a constitutive soybean metabolite in plantulae of other resistant isolines

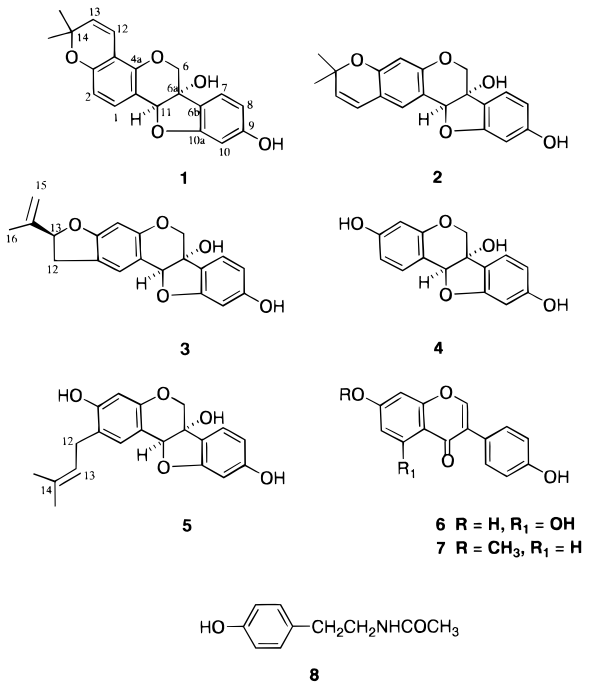

Figure 1.

(Graham et al., 1990), is actually an inducibly formed compound in the case of the resistant cultivar Dourados, considering that it was produced following inoculation of seeds with spores of *M. ramosissimus* and was not detected in noninoculated seeds.

Also worthy of mention is that *N*-acetyltyramine, a shikimate-derived metabolite, which was produced only by the resistant soybean cultivar, is being reported as a soybean phytoalexin for the first time.

When evaluated for their fungitoxic potential by bioautography experiments employing *Cladosporium sphaerospermum*, all phytoalexins isolated, except genistein, showed fungitoxic activity. Although genistein was inactive in this test, it is reported as a fungicidal agent against *Phytophthora sojae* (Rivera-Vargas, 1993).

CONCLUSION

The results obtained in the present work show that the ability of saprophytic fungi in eliciting phytoalexin synthesis in soybean seeds is dependent on the genus or species of fungi employed, a phenomenon probably related to the chemical nature of elicitors, either present in or produced by such fungi.

Mucor ramosissimus, unlike *Rhizopus* sp., was shown to be an efficient elicitor of phytoalexin synthesis in seeds of both tested cultivars. There were also significant qualitative differences between the susceptible and resistant cultivars with respect to their phytoalexin response to the same fungal elicitor. While seeds of the frog-eye leaf spot- and stem canker-susceptible cultivar produced four inducible isoflavonoids, after inoculation with spores of *M. ramosissimus*, the resistant cultivar accumulated, in addition to the phytoalexins synthesized by the susceptible cultivar, another four compounds, including *N*-acetyltyramine, not yet reported as a phytoalexin in soybean.

These results suggest that the qualitative differences in phytoalexin response between the susceptible and resistant cultivars are likely to play an important role among the diverse factors that induce soybean resistance to frog-eye leaf spot and stem canker diseases.

ACKNOWLEDGMENT

Thanks are due to Instituto de Química-USP, Brazil, for the ESI mass spectum of *N*-acetyltyramine and to M. Sc. Antonio Carnielli, EMBRAPA/ CPAO, MS, Brazil, for providing seeds of soybean cvs. Bragg and Dourados.

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Received for review October 20, 1999. Revised manuscript received May 18, 2000. Accepted May 21, 2000. Financial support from CNPq-PADCT, PIBIC-CNPq, and CPq-PROPP-UFMS is gratefully acknowledged.

JF991146O