Induction of the Soybean Phytoalexins Coumestrol and Glyceollin by *Aspergillus*

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Several isoflavonoid phytoalexins produced by soybeans are known to be estrogenic, with potential beneficial health effects in humans. Increased production of phytoalexins by the soybean plant will facilitate research efforts in this area. In this study, phytoalexin induction and accumulation in soybean cotyledon tissue was observed using four species of *Aspergillus*: *A. sojae*, *A. oryzae*, *A. niger*, and *A. flavus*. All four *Aspergillus* species tested elicited phytoalexin accumulation in living soybean cotyledons. Results from a time course study indicated that maximum concentrations of the phytoalexin glyceollin, 955 μg/g fresh weight (fw), occurred at day 3 in soybean cotyledon tissue inoculated with *A. sojae*. Other *Aspergillus* species caused an accumulation of glyceollin at significantly lower levels. A maximum concentration of coumestrol of 27.2 μg/g fw was obtained from soybean cotyledons inoculated with *A. niger*. Soybean phytoalexins induced by food-grade *A. sojae* and *A. oryzae* allowed the collection of higher concentrations of phytoalexins for further examination in several in vitro and in vivo biological studies conducted to determine potential estrogenic activities.

Keywords: *Phytoalexin; isoflavone; glyceollin; coumestrol; Aspergillus; A. sojae; A. oryzae; A.niger; A. flavus; phytoestrogen*

INTRODUCTION

Some species of the genus *Aspergillus* are recognized as the most widely distributed fungi encountered in foodstuffs, soils, and other materials. In addition to their function as major agents of decomposition and decay, Aspergilli are economically important. The enzymes that facilitate their biodegradative activities are harnessed in food fermentations and industrial processes (Bennett and Klich, 1992). Several Aspergilli produce toxic metabolites (mycotoxins) or cause serious diseases (mycoses). Among these, *Aspergillus flavus* produces aflatoxin B_1, the most potent naturally occurring carcinogenic substance known (Squire, 1981). Other Aspergilli are grown in controlled fermentations to harvest various small molecules of industrial importance. For example, *Aspergillus niger* is used in citric acid production (Bennett and Klich, 1992). Several Aspergilli are used to prepare fermented foods and beverages. *Aspergillus sojae* and *Aspergillus oryzae* are both widely used in the production of koji, the starting material for many fermented soybean food products, including soy sauce and miso (Liu, 1997; Luh, 1995).

Soybean phytochemicals called isoflavones, particularly genistein and daidzein, have been linked to many health benefits associated with soybeans (Carrol and Kurowska, 1995; Humfrey, 1998; Tham et al., 1998). One mechanism for many of these benefits is the ability of isoflavones to function as phytoestrogens, compounds that are structurally and functionally similar to 17β-estradiol. Coumestrol, a coumestan isoflavone, has estrogenic properties (Collins et al., 1997; Martin et al., 1978; Stahl et al., 1998; Whitten et al., 1992) and is considered a soybean phytoalexin, a low molecular weight antimicrobial compound that is synthesized de novo and accumulates in plants after exposure to microorganisms (Ebel, 1986; Kuc and Rush, 1985; Paxton, 1981, 1988, 1991). Much research on plant defense has focused on the soybean phytoalexins glyceollin I, II, and III shown in Figure 1 (Ayers et al., 1976a−c; Bailey and Mansfield, 1982; Graham et al., 1990; Graham and Graham, 1991; Lyne et al., 1976; Lyne and Mulheirn, 1978; Lyon and Albersheim, 1982). Studies have shown that glyceollin prevents the accumulation of aflatoxin B_1 in cultures of *A. flavus* (Song and Karr, 1993), and glyceollin concentrations can be increased with the application of certain herbicides on

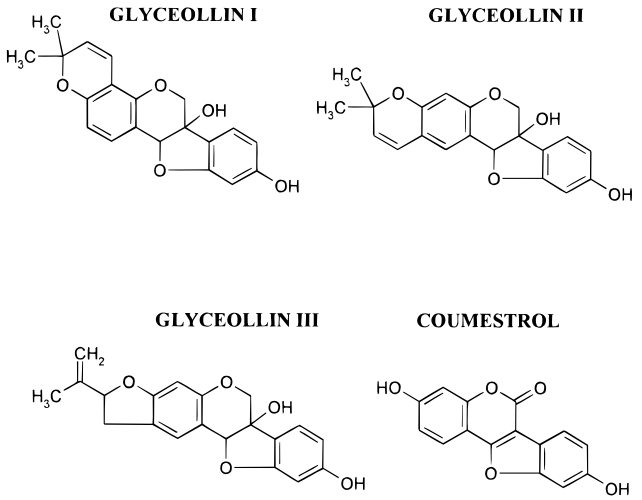

Figure 1. Structures of the soybean phytoalexins: glyceollins I−III and coumestrol.

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the soybean plant (Daniel et al., 1999). Other phytoalexin elicitors are classified as abiotic or biotic depending on origin (Darvill and Albersheim, 1984), and earlier soybean phytoalexin research focused on the fungal species *Phytophthora megasperma* var. *sojae* (Albersheim and Valent, 1978; Ayers et al., 1976a; Cheong et al., 1991; Ebel et al., 1976; Graham et al., 1990; Keen et al., 1971, 1983; Klarman and Gerdemann, 1963; Moesta and Griesbach, 1981; Sharp et al., 1984).

Because of the important implications of soybean isoflavones and phytoalexins for plant defense and human health, our research goals have been to examine the effect of fungi on phytoalexin induction. Although several fungal genera have been examined in our laboratory, the Aspergilli were chosen for the present study because of the potential application of *Aspergillus*-inoculated soybean extracts in several biological assays for the determination of estrogenic activity. The present work describes glyceollin and coumestrol induction in soybean cotyledons by four species of *Aspergillus*: *A. sojae*, *A. oryzae*, *A. niger*, and *A. flavus*. Of particular importance are the food-grade, nontoxin producing Aspergilli, *A. sojae* and *A. oryzae*, which currently are used in the production of several fermented soybean foods. A time course experiment using *A. sojae*-inoculated soybean cotyledons was conducted to determine incubation times necessary for maximum phytoalexin accumulation. A comparison study was also conducted to determine differences in the ability of the *Aspergillus* species examined to induce glyceollins I−III and coumestrol.

MATERIALS AND METHODS

Chemicals. Coumestrol was obtained from Indofine Chemical Co. (Somerville, NJ). Coumestrol was identified in soybean cotyledons by comparison with a known standard using UV−visible (UV−vis) spectrophotometry (Franke and Custer, 1994) and electrospray mass spectrometry. Glyceollins I, II, and III were isolated by use of a procedure developed in our laboratory. Soybean seeds (50 g) were sliced and inoculated with *A. sojae* spore suspension (3.4×10^7 ml^{-1}). After 3 days, phytoalexins were extracted from the inoculated seeds using 80% ethanol. Glyceollins I, II, and III were isolated using preparative scale high-performance liquid chromatography (HPLC) and confirmed by UV−Vis spectrophotometry (Osswald, 1985) and electrospray mass spectrometry. The solvents acetonitrile (HPLC grade) and ethanol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Water treated with a Millipore system was used during sample preparation procedures and HPLC analyses.

Fungal Cultures. *A. sojae* (SRRC 1125), *A. oryzae* (SRRC 302), *A. niger* (SRRC 60), and *A. flavus* (SRRC 13) cultures were grown at 25 °C in the dark on potato dextrose agar. Conidia were harvested from 5-day-old cultures of *A. sojae*, *A. niger*, and *A. flavus*. A 14-day-old culture of *A. oryzae* was used due to slower growth of the fungus. Conidia of each *Aspergillus* species were suspended in 15 mL sterile, distilled H_2O ($1.0-3.0 \times 10^7$ conidia mL^{-1}).

Phytophthora megasperma var. *sojae* (race 1) cultures were grown for 6 days at 25 °C in the dark on V8 juice agar. Zoospores (1×10^5 mL^{-1}) were produced using a procedure modified from that reported by Eye et al. (1978), by flooding (15 mL sterile water), and maintaining cultures on an orbital shaker (Lab-Line) for 3 days at 500 rpm.

Fungal Inoculations of Soybean Cotyledons. Seeds from commercial soybean variety Pioneer 95B41 were surface-sterilized for 3 min in 70% EtOH followed by a quick deionized-H_2O rinse and two 2-min rinses in deionized H_2O. Seeds were presoaked in sterile deionized H_2O for 5 h before placement in treatment chambers (three seeds/chamber). Each chamber consisted of a Petri dish (100 × 15 mm, four compartments), each compartment lined with two autoclaved filter papers (Whatman) moistened with distilled H_2O (500 μL). One seed was placed in a single compartment, then sliced in half. Fungal spore suspensions (10 μL) were applied to the cut surface of each appropriate seed. Control soybean seeds were sliced in half and received no fungal inoculum. All chambers were Parafilm sealed and stored at 25 °C in the dark for 3 days.

Time Course Study Using *A. sojae* for the Accumulation of Phytoalexins. Seeds from Pioneer 95B41 were used in a 6-day time course experiment to monitor induction of coumestrol and glyceollin with *A. sojae*. Seeds were surface-sterilized as described previously, then imbibed with deionized-H_2O for 5 h before placement in treatment chambers. Six separate treatment chambers (four seeds/chamber) were assembled as described above to provide analysis for each day during the course of the experiment. Imbibed seeds were sliced in half, treated with *A. sojae* spore suspension (10 μL), then stored at 25 °C in the dark for 3 days.

Soybean Cotyledon Sample Preparation. Phytoalexins were extracted from soybean cotyledons (0.3−0.6 g) homogenized (Tekmar Tissuizer) in 80% ethanol (1.5 mL). Homogenate was heated at 50 °C for 1 h, cooled, then centrifuged at 14 000*g* for 10 min. An aliquot (100 μL) of supernatant was analyzed by HPLC. All HPLC analyses were run in triplicate unless otherwise stated.

HPLC and Electrospray Ionization-Mass Spectrometric Analysis of Phytoalexins. HPLC analyses were performed on a Waters 600E System Controller combined with a Waters UV−vis 996 detector following established methods (Wang and Murphy, 1994). Coumestrol was monitored at a wavelength of 260 nm and glyceollins I−III were monitored at 285 nm. Separations were performed using a Vydac Multiring C_{18} (4.6 × 250 mm; 5 μm) reverse-phase column. A guard column containing the same packing was used to protect the analytical column. Elution was carried out at a flow rate of 1.0 mL/min with the following solvent system: A, acetic acid/water (pH = 3.0); B, acetonitrile, 0% B to 45% B in 17 min, then 45% B to 90% B in 10 min, followed by holding at 90% B for 6 min. Retention times for the phytoalexins were as follows: coumestrol (20.7 min), glyceollin III (23.3 min), glyceollin II (23.6 min), and glyceollin I (23.7 min). Calibration curves with high linearity were constructed for coumestrol and glyceollins I, II, and III using a series of diluted standards.

Mass spectral data were obtained for isolated isoflavonoids using a direct infusion technique at a flow rate of 3 μL/min using 50/50 acetonitrile/water (0.1% trifluoroacetic acid). The mass spectrometer used was a quadrupole ion trap (Finnigan LCQ instrument) equipped with a heated capillary electrospray interface. Positive ion mode was used with a sprayer needle voltage of 4 kV. The capillary temperature was 210 °C.

RESULTS AND DISCUSSION

The changes in isoflavonoid phytoalexin concentrations were investigated using cotyledons inoculated with four species of Aspergilli. Inoculated cut cotyledons after 3 days were generally characterized by dark brown necrotic tissue restricted to the area of contact with the inoculum droplet, which has been correlated with the presence of the phytoalexin glyceollin (Ayers et al., 1976a). The HPLC profile of noninoculated soybean tissue was quite different from *A. sojae*-inoculated cotyledon tissue (Figure 2). The more prevalent constitutive isoflavones daidzin, genistin, malonyldaidzin (MGD), malonylgenistin (MGG), daidzein, and genistein were observed in noninoculated cotyledon tissue in Figure 2A. The coumestan phytoalexin, coumestrol, and the pterocarpan phytoalexins, glyceollins I−III, were detected at high concentrations in inoculated cotyledons

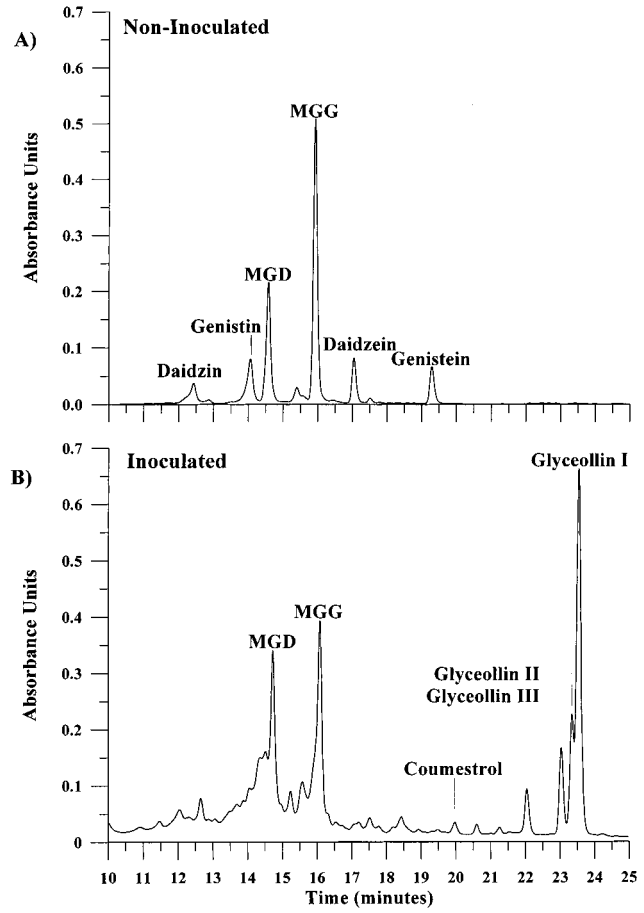

Figure 2. HPLC comparison between noninoculated and inoculated Pioneer 95B41 soybean cotyledons: (A) HPLC chromatogram of 3-day-old noninoculated cotyledons showing constitutive isoflavones, and (B) HPLC chromatogram of 3-day-old cotyledons inoculated with *A. sojae* detailing the induction of coumestrol and glyceollin isomers I, II, and III. The chromatograms were obtained by recording absorbance at 285 nm.

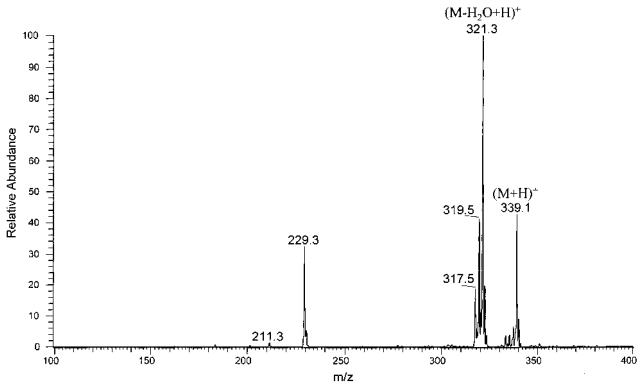

Figure 3. Positive ion ESI mass spectrum of glyceollin I isolated from soybean cotyledons inoculated with *A. sojae.*

shown in Figure 2B. Coumestrol was characterized by its distinct UV−vis absorption spectrum and has been well characterized by mass spectral techniques (Setchell and Welsh, 1987; Mazur and Adlercreutz, 1998). For the confirmation of the glyceollin isomers, several analytical techniques were used including UV−vis absorption and electrospray ionization (ESI) mass spectrometry. The positive ion ESI mass spectrum of glyceollin I is shown in Figure 3. The base peak in the spectrum is the ion at *m*/*z* 321 detailing the loss of one water molecule, and the molecular ion at *m*/*z* 339 represents protonated

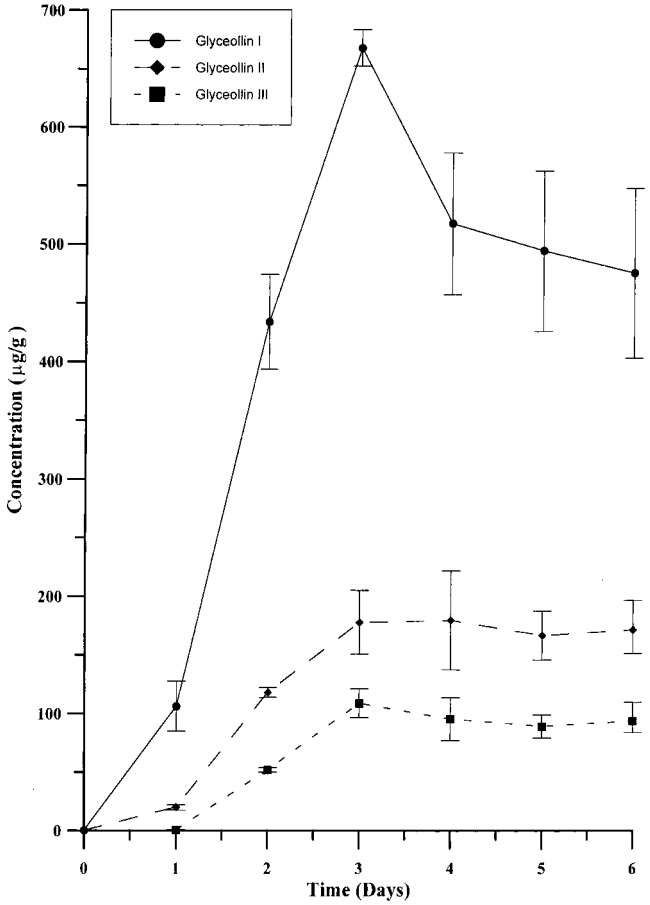

Figure 4. Time course for the induction of the glyceollin isomers I, II, and III from Pioneer 95B41 cotyledon tissue inoculated with *A. sojae.* The values represent mean MU ± SD for three independent experiments. Glyceollin was not detected in noninoculated controls.

glyceollin $(M + H)^+$. Similar mass spectra were obtained for all three glyceollin isomers.

To further characterize the accumulation of glyceollin in soybean cotyledons inoculated with *Aspergillus*, a time course experiment was conducted using *A. sojae* (Figure 4). The predominant isomer induced in cotyledon tissue by *A. sojae* was glyceollin I. Maximum levels of all three isomers occurred at day three: 668 μg/g fw glyceollin I, 178 μg/g fw glyceollin II, and 109 μg/g fw glyceollin III. The proportion of glyceollin isomers relative to glyceollin III was 6:2:1. After day 3 a slight decrease in glyceollin I concentration was observed, however, glyceollin II and III concentrations decreased to a much lesser degree.

These results show that *A. sojae* induced the biosynthesis and accumulation of glyceollins I, II, and III at the cut cotyledon surface. The mechanism for glyceollin induction has been characterized for the soybean fungal pathogen *P. megasperma* var. *sojae* (Albersheim and Valent, 1978; Ayers et al., 1976b; Cheong et al., 1991; Darvill and Albersheim, 1984; Ebel, 1976; Sharp et al., 1984; Keen et al., 1983). Several experiments were conducted in our laboratory to induce phytoalexins in soybean cotyledons inoculated with *P. megasperma* var. *sojae*, which yielded a total glyceollin (glyceollins I, II, and III) concentration of 335 μg/g fw. This result agrees with yields obtained by Graham et al. (1990). Our studies show that total glyceollin yields obtained with *P. megasperma* var. *sojae* were much lower (285% lower) than those induced by the nonpathogenic fungus *A. sojae*

Table 1. Phytoalexin Concentrations (μg/g Fresh Weight) in 3-Day-Old Cotyledons[a]

		inoculated			
phytoalexin	noninoculated[b]	*A. sojae*	*A. oryzae*	*A. niger*	A. flavus
coumestrol	N/D[c]	22.5 ± 2	17.8 ± 3	27.2 ± 8	17.2 ± 4
total glyceollin[d]	N/D	955 ± 40	660 ± 112	623 ± 101	576 ± 76

[a] Cotyledons from soybean variety Pioneer 95B41; each value is the average of three measurements. [b] Wounded (cut cotyledons), water-treated controls. [c] ND, not detected. [d] Glyceollins I, II, and III.

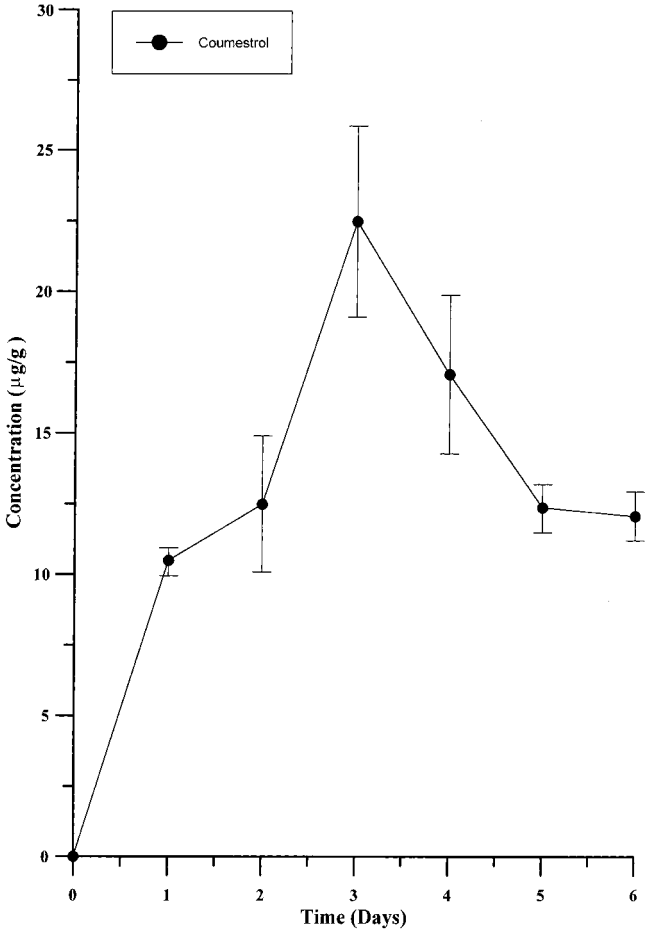

Figure 5. Time course for the induction of coumestrol from Pioneer 95B41 cotyledon tissue inoculated with *A. sojae*. The values represent mean MU ± SD for three independent experiments. Coumestrol was not detected in noninoculated controls.

(955 μg/g fw). A branched hexa-β-glucosyl glucitol was the smallest elicitor-active β-glucan fragment isolated from the fungal cell walls of *P. megasperma* var. *sojae* (Albersheim and Valent, 1978; Ayers et al., 1976c; Cheong et al., 1991; Ebel, 1976; Sharp et al., 1984).

Previous studies have indicated that coumestrol concentrations were highest in the soybean hull (seed coat) and increased with increasing germination time (Lookhart et al., 1979). The time course accumulation of coumestrol in cotyledon tissue inoculated with *A. sojae* is shown in Figure 5. Detectable concentrations of coumestrol were not observed in noninoculated soybean cotyledon tissue during the 6-day period of this study; several factors may contribute to this phenomenon. Lookhart et al. (1979) observed varietal differences in coumestrol concentrations particularly in soybean varieties containing lower levels of this isoflavonoid. The addition of a fluorescence detection method could offer increased sensitivity for the detection of lower coumestrol levels (Murphy, 1981; Franke and Custer, 1994). A steady increase in coumestrol concentrations in *A. sojae*-inoculated cotyledons was observed from day 1 (10.8 μg/g fw) through day 3 (22.5 μg/g fw). After day 3, the coumestrol concentration decreased to 12.5 μg/g fw. Maximum concentrations of coumestrol occurred at day 3, which correlated with the time when maximum glyceollin concentrations were detected.

We report above the results for the soybean variety Pioneer 95B41. However, several different soybean varieties were examined using *A. sojae*-inoculated cotyledons to test for a variety-specific response, including Buckshot 66, Asgrow, Vinton 81 (tofu variety), and Corsoy (tofu variety). Although minor quantitative differences were observed, the induction of coumestrol and glyceollins I−III closely paralleled the results obtained with inoculated cotyledons from Pioneer 95B41. Thus, data from other varieties were not included in this report because of their redundancy.

To compare the effect of different *Aspergillus* species on the induction and accumulation of phytoalexins in soybean cotyledon tissue, several Aspergilli (*A. sojae*, *A. oryzae*, *A. niger*, and *A. flavus*) were examined. The results detailing the accumulation of the phytoalexins coumestrol and glyceollin are presented in Table 1. Coumestrol and glyceollin were not detected in noninoculated 3-day-old control cotyledons. Of the *Aspergillus* species tested, cotyledons inoculated with *A. sojae* accumulated the highest concentration of total glyceollin. The two other food-grade Aspergilli examined, *A. oryzae* and *A. niger*, produced significantly lower levels of glyceollin, approximately 31% and 35% lower, respectively. A wild-strain *Aspergillus*, *A. flavus*, was tested to determine the effect of this species on the induction of phytoalexins. Cotyledons inoculated with *A. flavus* produced the lowest concentration of glyceollin, however, this concentration was sufficient to inhibit the biosynthesis of aflatoxin B_1 (Song and Karr, 1993). In all cotyledons inoculated with *Aspergillus*, glyceollin I was the predominant isomer (67%), followed by glyceollin II (22%), and glyceollin III (11%). Also shown in Table 1 are the coumestrol concentrations detected in soybean cotyledons inoculated with these four Aspergilli. The maximum concentration of coumestrol was observed using *A. niger*. Lower levels of coumestrol were detected from cotyledons inoculated with *A. sojae*, *A. oryzae*, and *A. flavus*.

Our study has shown that the phytoalexins glyceollin (I, II, and III) and coumestrol can be induced in soybean cotyledons inoculated with a variety of Aspergilli. *A. sojae* stimulated continuous glyceollin production in cotyledons for 6 days, reaching a peak concentration of 955 μg/g fw at day 3. *A. oryzae*, *A. niger*, and *A. flavus* also induced glyceollin production, to a slightly lesser degree by day 3 than that observed with *A. sojae*. *A. niger* was the best inducer of coumestrol in our cotyledon assay. Although the health effects of coumestrol have been characterized, the effects of glyceollin on human health are unknown. Several experiments are currently underway to examine the phytoestrogenic activity of

glyceollin using extracts obtained from *A. sojae*-inoculated soybean cotyledons.

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