Production of Phytoalexins in Peanut (*Arachis hypogaea*) Seed Elicited by Selected Microorganisms

Victor S. Sobolev*

National Peanut Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Post Office Box 509, Dawson, Georgia 39842, United States

ABSTRACT: Under favorable conditions, the peanut plant demonstrates appreciable resistance to fungal invasion by producing and accumulating phytoalexins, antimicrobial stilbenoids. This mechanism for resistance is little understood, yet it is crucial for breeding and genetically modifying peanut plants to develop new cultivars with fungal resistance. The dynamics of phytoalexin production in peanut seeds and embryos challenged by selected important fungi and bacteria was investigated. Different biotic agents selectively elicited production of major peanut stilbenoids, resveratrol, arachidin-1, arachidin-3, and SB-1. *Aspergillis* species, compared to other biotic agents, were more potent elicitors of stilbenoids. Embryos demonstrated significantly higher production of stilbenoids compared to cotyledons and may serve as a convenient source of genetic material in isolating genes for peanut plant defense enhancement.

KEYWORDS: *peanut, Arachis hypogaea, Leguminosae, groundnut, phytoalexin, stilbenoid, biotic elicitor, Aspergillus, Bacillus, Rhizobium, Cladosporium, Saccharomyces, Candida*

■ INTRODUCTION

Peanut or groundnut (*Arachis hypogaea*) is an economically important leguminous plant. Contamination of peanuts with aflatoxins is an important food safety issue and threatens the competitiveness of this important crop in the world market. Aflatoxins are potent carcinogens produced mainly by the fungus *Aspergillus flavus*. Peanut plants are most susceptible to invasion by *A. flavus* under conditions leading to drought stress.[1] However, when water activity exceeds 0.95, which is normally observed in irrigated fields, peanut plants demonstrate an appreciable resistance to fungal invasion.[2] This resistance has been attributed to phytoalexins.[2−6]

Peanut phytoalexins are low-molecular-weight, stilbene-derived antimicrobial compounds (Figure 1) that are synthesized and accumulate in the plant after exposure to exogenous stimuli.[5,7,8] Some flavonoids and fatty acid-related compounds are considered peanut phytoalexins as well.[5,9] However, the role of compounds other than stilbenoids in plant resistance is unknown and requires additional research.

There is ample evidence that rapid accumulation of sufficient concentrations of phytoalexins in the vicinity of the pathogen attack is inhibitory to pathogen growth and is critical for plant defense.[8,10,11] Phytoalexins accumulate to inhibitory concentrations corresponding to pathogen repression.[5,12] The role of stilbenoids as defensive compounds against aflatoxigenic fungi in peanut has also been evaluated in field experiments designed to elucidate the relationship between aflatoxin contamination, plant drought stress, pod damage, and phytoalexin production.[2,6] These experiments supported the hypothesis that peanut stilbene phytoalexins are an important natural resistance factor in preharvest aflatoxin contamination. Such evidence is based on observations that (1) stilbenes are naturally produced in field-damaged peanuts; (2) stilbenes possess biological activity against *A. flavus* and *Aspergillus parasiticus*; and (3) aflatoxin contamination does not occur until peanuts lose the capacity for phytoalexin production as a result of drought-induced seed dehydration.[2] Effective chemical control measures are available for fungal leaf spot diseases but not for infection of peanut seeds by *Aspergillus* species in the soil. Therefore, the natural phytoalexin-based mechanism of peanut resistance is a promising strategy for control of aflatoxin contamination.[4,6]

The relationship between fungal pathogen and plant host is a complex interaction involving pathogenicity of the fungus and resistance by the plant. During the initial stages of infection, the fungal pathogen produces proteins and carbohydrates that elicit a plant response,[13,14] which involves the production and accumulation of phytoalexins.[15−17] Early studies show a strong correlation between the tolerance of fungal strains to phytoalexins and their pathogenicity on the plant host.[18] However, after decades of research, little progress has been made in understanding this mechanism. Nevertheless, this information may be crucial for developing new cultivars with higher levels of fungal resistance.[19] Study of the dynamics of elicited phytoalexin biosynthesis in peanut seeds is important for better understanding and potential manipulation of this natural mechanism of the plant's resistance to pests.[19] At present, the information on the differences between peanut genotypes[6] in their ability to produce phytoalexins is used in a breeding program at the U.S. National Peanut Research Laboratory in Dawson, GA. The ultimate goal of this program is to develop more effective strategies for controlling aflatoxins in peanut.

Medical importance and health benefits of stilbenoids, particularly resveratrol (**1**) from peanut and other plants, have been widely reported.[20−23] Recent systematic studies on
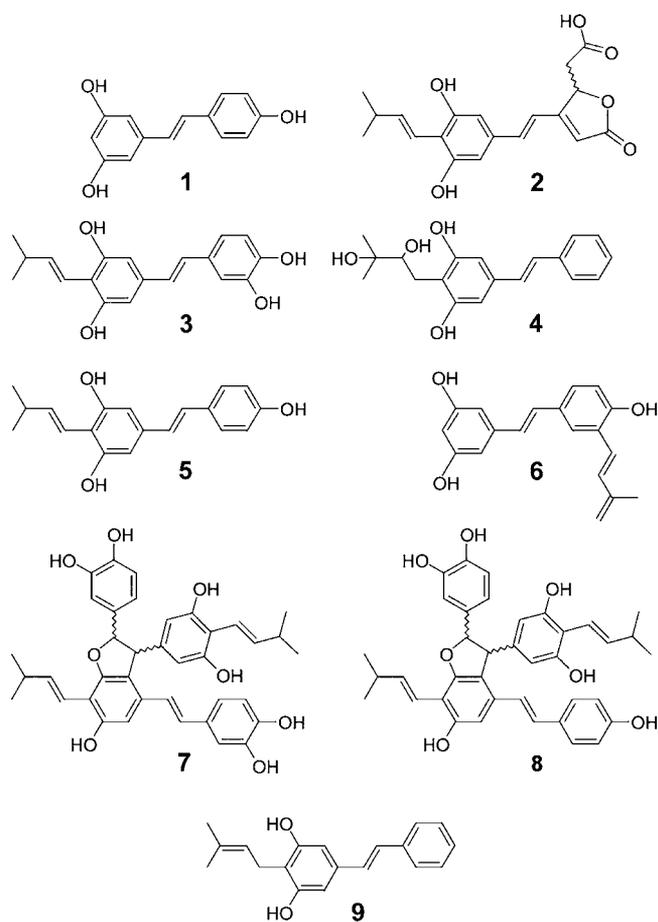
Figure 1. Major stilbene phytoalexins found in tested peanut genotypes. **1**, *trans*-resveratrol; **2**, SB-1; **3**, *trans*-arachidin-1; **4**, arahypin-4; **5**, *trans*-arachidin-3; **6**, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; **7**, arahypin-7; **8**, arahypin-6; **9**, chiricanine A.

the biological activity of all identified peanut stilbenoids[8,24−26] established not only high antifungal activity against several economically important fungal species but also strong antioxidative, cytotoxic, and anti-inflammatory properties utilizing human cell lines. Several research groups recently reported improvements in the production of bioactive peanut stilbenoids by whole or sliced seeds[24,27] and by hairy root cultures.[26,28] In addition, the authors suggested approaches to obtain appreciable quantities of natural bioactive stilbenoids to satisfy the growing demand of numerous veterinary and medical institutions. Continued research on phytoalexin biosynthesis in peanuts may thus also lead to the development of new peanut cultivars enriched with bioactive stilbenoids desired for human consumption.[8]

The objective of this research was to study the dynamics of phytoalexin production by seeds and embryos of an important U.S. peanut cultivar, Georgia 06G, challenged by selected significant fungi and bacteria.

■ MATERIALS AND METHODS

General Experimental Procedures. HPLC-grade solvents were used in the preparation of mobile phases and separations (Fisher, Suwanee, GA). HPLC-grade H_2O was prepared with a ZD20 four-bowl Milli-Q water system (Millipore). ACS-grade MeOH was used for seed extraction (VWR, Suwanee, GA). Silica gel for column chromatography (Silica gel 60, 0.063−0.200 mm) was used for preparative separations (EM Science, Gibbstown, NJ).

Reference Compounds. *trans*-Resveratrol **1** (Figure 1) and *trans*-piceid (resveratrol 3-*O*-β-D-glucoside) were purchased from Sigma−Aldrich. Pure individual stilbenes, *trans*-arachidin-1 (**3**), *trans*-arachidin-3 (**5**), and *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (**6**) were obtained as described,[29] except that preparative HPLC was used as a final purification step rather than preparative TLC. HPLC separation was achieved by using isocratic mobile phases 2 and 3.[19] SB-1 (**2**), chiricanine A (**9**), arahypin-4 (**4**), arahypin-6 (**8**), and arahypin-7 (**7**) were obtained as described.[19,30,31]

Elicitors. Fungi were *Aspergillus flavus* (NRRL 3357; aflatoxin producer), *Aspergillus niger* (NPRL 326), *Aspergillus caelatus* (NPRL 25529), *Aspergillus nomius* (LA3-4; aflatoxin producer), and *Cladosporium* sp. (from a peanut seed, identified to the genus level by Dr. B. W. Horn of the NPRL, ARS, USDA); yeasts were *Saccharomyces cerevisiae* (NRRL Y-12632), *Candida lipolytica* (NRRL Y-1095), and commercial Fleischmann's RapidRise (purchased locally); bacteria were *Bacillus subtilis* (ATCC 6051) (Fisher, Suwanee, GA) and *Rhizobium leguminosarum* (WL 23711) (VWR, Suwanee, GA). H_2O_2 (5%) was prepared by dilution of 30% ACS-grade H_2O_2 (Fisher) with sterile H_2O.

Plant Material and Processing. Mature seeds of commercial runner-type cultivars Georgia 06G and Georgia Green (both from 2009 local harvest) were used in the experiments. Georgia 06G was used in all experiments. To keep the experiments manageable, Georgia Green was used with a limited number of elicitors: *A. flavus*, *Cladosporium* sp., *S. cerevisiae*, and *B. subtilis*. Sampling times in the experiments with Georgia Green were limited to 48 and 96 h.

Two groups of controls were used in the experiments. The first group consisted of the same number of intact embryos and testa-free cotyledons on potato dextrose agar (PDA) medium or on filter paper moistened with water. The second group was composed of sliced uninoculated embryos and cotyledons on PDA medium or on moistened filter paper. The plant material in this control group was wounded as described below. Petri dishes with all controls were incubated at 30 °C and sampled at 24-h intervals for 5 or 6 days. Extraction of metabolites was performed as described below for the biotic elicitors.

Analysis of phytoalexin biosynthesis promoted by biotic and abiotic elicitors was performed as follows. Peanut seeds were surface-sterilized for 20 s in EtOH/H_2O (7:3 v/v), followed by 1.5% NaOCl for 3 min and four sterile H_2O rinses. The seeds were imbibed in sterile H_2O for 16−18 h at room temperature, rinsed three times with sterile H_2O, and manually separated into embryos and testa-free cotyledons. The plant materials were then sterilized and rinsed again as described above. Half of the embryos and cotyledons were wounded as follows. The embryos were cut into two halves at the long axis. The inner part of the cotyledons from seeds of about equal shape and size was removed (about 1 mm thick) with a microtome knife and discarded. The weight of each cotyledon was 0.40 ± 0.04 g. For experiments with biotic elicitors, in triplicate, fungal spore suspensions (100 μL, 10^6/mL) or yeast and bacterial suspensions (1 mg of wet colony material in 10 mL of H_2O) were evenly distributed on the surface of potato dextrose agar (PDA) in a Petri dish. Wounded peanut cotyledons and embryos were placed wounded side down (12 per plate) on agar medium covered with a 3-day-old culture and incubated at 30 °C. At 24-h intervals for 7 days, 12 cotyledons and 12 embryos each were combined, lyophilized, ground with a mortar and pestle, and weighed. The cotyledon material was extracted with eight volumes (w/v, respectively) of MeOH and the embryo material with MeOH (0.3 mL) overnight. The extracts were filtered through a Pasteur pipet with a glass-fiber plug, and an aliquot of the filtrate (5−50 μL) was injected into a HPLC/MS system.

For experiments with an abiotic elicitor, the peanut cultivar Georgia 06G was used. Wounded embryos and cotyledons were prepared as described above and placed in Petri dishes with sterile filter paper to which one portion of 5% H_2O_2 (8 mL) was added. In alternative set of experiments, the plant material underwent multiple treatments with H_2O_2. Excess of H_2O_2 was removed with a pipet every 24 h and replaced with an equal volume of fresh H_2O_2.
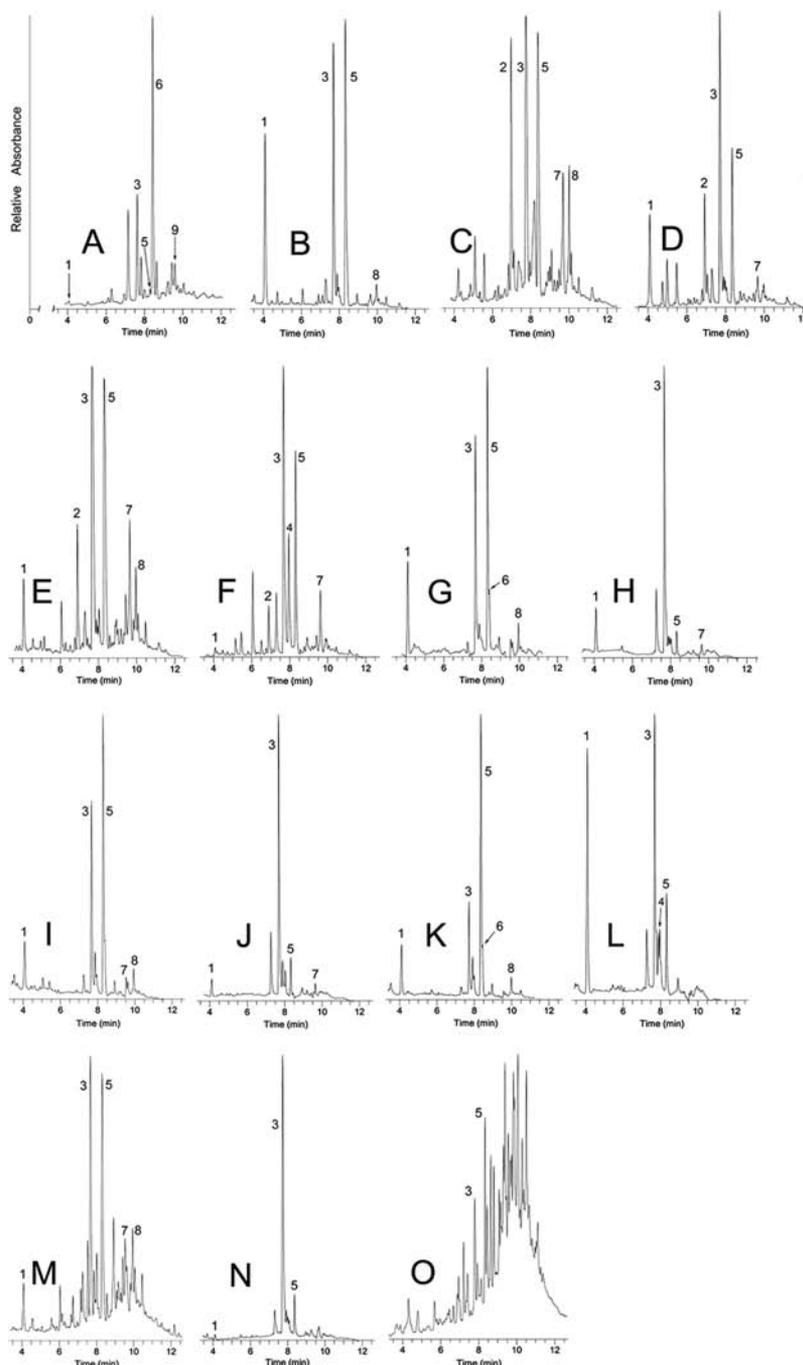
Figure 2. HPLC of extracts of peanut embryos and cotyledons treated with elicitors listed in the panels. **1**, *trans*-resveratrol; **2**, SB-1; **3**, *trans*-arachidin-1; **4**, arahypin-4; **5**, *trans*-arachidin-3; **6**, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; **7**, arahypin-7; **8**, arahypin-6; **9**, chiricanine A. Peaks are represented by total light absorbance from 200 to 800 nm. Chromatograms are scaled to the tallest peak. (A) Control, uninoculated sliced/wounded cotyledons, after 96 h of incubation; (B) *A. nomius*, embryos, after 24 h; (C) *A. nomius*, embryos, after 48 h; (D) *A. nomius*, cotyledons, after 48 h; (E) *A. niger*, embryos, after 48 h; (F) *A. niger*, cotyledons, after 48 h; (G) *Cladosporium* sp., embryos, after 48 h; (H) *Cladosporium* sp., cotyledons, after 48 h; (I) *B. subtilis*, embryos, after 48 h; (J) *B. subtilis*, cotyledons, after 48 h; (K) *R. leguminosarum*, embryos, after 48 h; (L) *R. leguminosarum*, cotyledons, after 48 h; (M) H_2O_2, embryos, after 96 h; (N) H_2O_2, cotyledons, after 96 h; (O) H_2O_2, embryos, after 168 h.

For the experiments with phytoalexin elicitation in uniformly sized plant material, in liquid medium, rectangular prisms (pieces with six faces that are rectangles) of equal size (2 × 2 × 1.5 mm) were cut from imbibed embryos and cotyledons and subjected to fungal stress as follows. Spores of *A. flavus* NRRL 3357 were used to inoculate 50 mL of Czapek-Dox (Difco, Detroit, MI) liquid medium and allowed to germinate and grow for 72 h at 28 °C. The culture was transferred to 550 mL of fresh liquid medium in a 1-L Erlenmeyer flask and incubated for 16 h at 28 °C. Initial *A. flavus* (NRRL 3357) biomass was

measured as packed-cell volume (PCV) of mycelium.[32] The initial *A. flavus* mycelium inoculum was measured as 2% PCV. The PCV was determined after centrifugation, for 5 min at 4930 relative centrifugal force (RCF), of 40 mL of the medium in 50 mL Falcon centrifuge tubes. The medium (600 mL) was evenly dispensed into 125-mL Erlenmeyer flasks. In each experiment, 20 pieces of surface-sterilized cuts from embryos and seeds were added to 125-mL Erlenmeyer flasks, each containing 33 mL of liquid medium with *A. flavus* culture, and incubated at 28 °C with agitation at 130 rpm. A similar

experimental setup but without the fungal culture served as a control. Each experiment was repeated in triplicate with two sampling times, 30 and 48 h. After incubation, the plant material was manually separated from fungal mycelium (its biomass reached 20% PCV after 48 h of incubation), rinsed with deionized water, blotted with paper towel, air-dried for 10 min under a laminar flow, weighed, and extracted with MeOH as described above.

HPLC-Diode Array Detector-Mass Spectrometry Analyses. Separations of plant material extracts were performed on a tandem HPLC-MS Surveyor system equipped with MS Pump Plus, Autosampler Plus, a PDA Plus Detector (Thermo Electron Corp., San Jose, CA), and a 100 mm × 4.6 mm i.d., 2 μm, TSK-gel super ODS analytical column (TosoHaas, Montgomeryville, PA). H_2O (A), MeOH (B), and 2% HCO_2H in H_2O (C) were used in the following gradient: initial conditions, 78% A/20% B/2% C, increased linearly to 0% A/98% B/2% C in 16 min, held isocratic for 4 min, and then changed to initial conditions in 0.01 min. The flow rate was 0.7 mL/min. The column was maintained at 40 °C.

MS analyses were performed on a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with an electrospray ionization (ESI) interface and operated with Xcalibur version 1.4 software (Thermo Electron Corp., San Jose, CA). The data were acquired in the full-scan mode (MS) from m/z 100 to 2000. Heated capillary temperature was 175 °C, APCI vaporizer temperature was 400 °C, sheath gas flow was 60 units, auxiliary gas flow was 5 units, capillary voltage was 10 V, and source voltage was 4.5 kV. In MS^2 analyses, the $[M + H]^+$ ions observed for each chromatographic peak in full-scan analyses were isolated and subjected to source collision-induced dissociation (CID) with He buffer gas. In all CID analyses, the isolation width, relative fragmentation energy, relative activation Q, and activation time were 1.2, 35%, 0.25, and 30 ms, respectively.

Concentrations of trans-resveratrol (**1**), trans-arachidin-1 (**3**), trans-arachidin-3 (**5**), trans-3′-isopentadienyl-3,5,4′-trihydroxystilbene (**6**), SB-1 (**2**), arahypin-4 (**4**), arahypin-6 (**8**), arahypin-7 (**7**), and chiricanine A (**9**) in the extracts were determined by reference to peak areas of corresponding pure standards at 317, 339, 334, 296, 364, 311, 339, 347, and 311 nm, respectively.

Data Analysis. Multiple comparisons of the various means were carried out by least significant difference (LSD) test at p = 0.01 using Statistix 7.0 (Analytical Software, Tallahassee, FL). Comparison of means of two groups of data was performed via t test; Mann−Whitney rank sum test was applied when normality test failed ($p < 0.05$).

RESULTS AND DISCUSSION

The present research was intended to study the action of selected biotic elicitors important to the peanut plant. Runner-type peanuts were chosen as the most accepted in major peanut-growing areas in the United States. Intact peanut embryos and embryo- and testa-free cotyledons were used as controls. Uninoculated embryos wounded by slicing and embryo- and testa-free cotyledons were used as a second set of controls, since wounded peanuts have been reported to produce stilbenoid **6** (Figure 1).[33]

The elicitors included important soil fungi from the genus *Aspergillus*.[12] *Cladosporium* species and yeasts (unicellular fungi) are not pathogenic to peanut but were chosen for comparison. *Rhizobium* spp. are essential synergetic nitrogen-fixing bacteria of peanut;[34] one of the *Rhizobium* strains, *R. leguminosarum*, was used in our tests. *B. subtilis* was identified as the cause of internal seed infection.[35] The growth of *B. subtilis* from a specific peanut seed prevented the development of fungal growth from that kernel. This inhibition suggests the possibility that the metabolic activity of *B. subtilis* may play a role in the inhibition of potential pathogens in the seed and developing seedling.[35,36] It was reasonable to test such a bacterium as an elicitor of stilbenoid biosynthesis. For comparison with the biotic elicitors, H_2O_2 was chosen as an

abiotic elicitor that is known to play a role in host−fungal interactions.[37]

Peanut embryos were reported to be microbe-free[38] and, therefore, represent an interesting object for research. Embryos have not been investigated in terms of phytoalexin production. However, it is reasonable to suggest that epicotyl/plumule emerging from the peanut embryo may have either no or limited physical barriers, such as exocarp or waxes, and must be protected by natural chemical defenses. A 20-fold higher concentration of *p*-coumaric acid in embryos compared to cotyledons has been reported.[39] This fact suggests that embryos may have the capacity to accumulate phytoalexins since *p*-coumarate is an immediate source of *p*-coumaroyl-CoA, an intermediate in the biosynthesis of resveratrol.[40]

Stilbenoid Production by Intact and Wounded Plant Material (Controls). During the course of experiments, intact control embryos and cotyledons produced some polar compounds, which were tentatively identified as bound and free phenolic acids on the basis of their UV and MS spectra (data not shown). These data were in agreement with previous findings.[39] However, stilbenoids were not detected in intact controls.

Wounding by slicing was reported to be an effective stimulus of *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (**6**) production in peanut seeds.[33] The present study established that all wounded controls contained this phytoalexin after 48 h of incubation (Figures 2A and 3A,B). Embryos and cotyledons
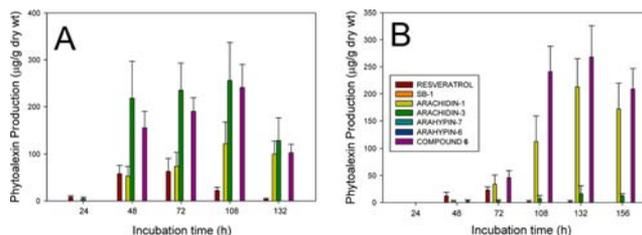
Figure 3. Production of phytoalexins in peanut embryos and embryo-free cotyledons in control experiments. The legend in panel B is applicable to panel A. (A) Control, uninoculated sliced/wounded embryos; (B) control, uninoculated sliced/wounded cotyledons.

were capable of producing overall comparable quantities of **6**. Concentrations of **6** in embryos were 0 ± 0, 156 ± 34, 190 ± 29, 241 ± 49, and 103 ± 18 μg/g of fresh tissue, respectively after 24, 48, 72, 108, and 132 h of incubation (Figure 3A). Concentrations of **6** in cotyledons were 0 ± 0, 3 ± 2, 46 ± 13, 241 ± 47, 268 ± 58, and 209 ± 38 μg/g of fresh tissue, respectively after 24, 48, 72, 108, 132, and 156 h of incubation (Figure 3B). Controls were the only samples that contained appreciable concentrations of **6**; minor quantities of **6** were found in the extracts of embryos incubated with the *Cladosporium* species, *B. subtilis*, and *R. leguminosarum* after 48 h of incubation (Figure 2G,K). The lack of SB-1 (**2**) metabolite in all controls was noticed during the experiments. Chiricanine A (**9**) was observed in cotyledon controls at very low concentrations after 72 and 96 h of incubation (Figure 2A). Controls with H_2O and PDA medium were not significantly different ($p > 0.05$, $n = 3$).

Stilbenoid Production Elicited by Fungi. Four *Aspergillus* species that are commonly found in peanut field soil, *A. flavus*, *A. niger*, *A. caelatus*, and *A. nomius*, as well as a *Cladosporium* species, were used for the experiments. All

Aspergillus species tested demonstrated prompt production and accumulation of numerous stilbene-derived compounds at high concentrations in embryos and cotyledons. Consistently, embryos showed significantly ($p < 0.05$, $n = 3$) higher production of stilbenoids compared to cotyledons (Figure 2C−F). *Aspergillus* species elicited the production of SB-1 (**2**) along with other major stilbenoids at high concentrations (Figure 2C−F; similar chromatograms for *A. flavus* and *A. caelatus* are not shown; see also Figure 4A−H). In contrast, the
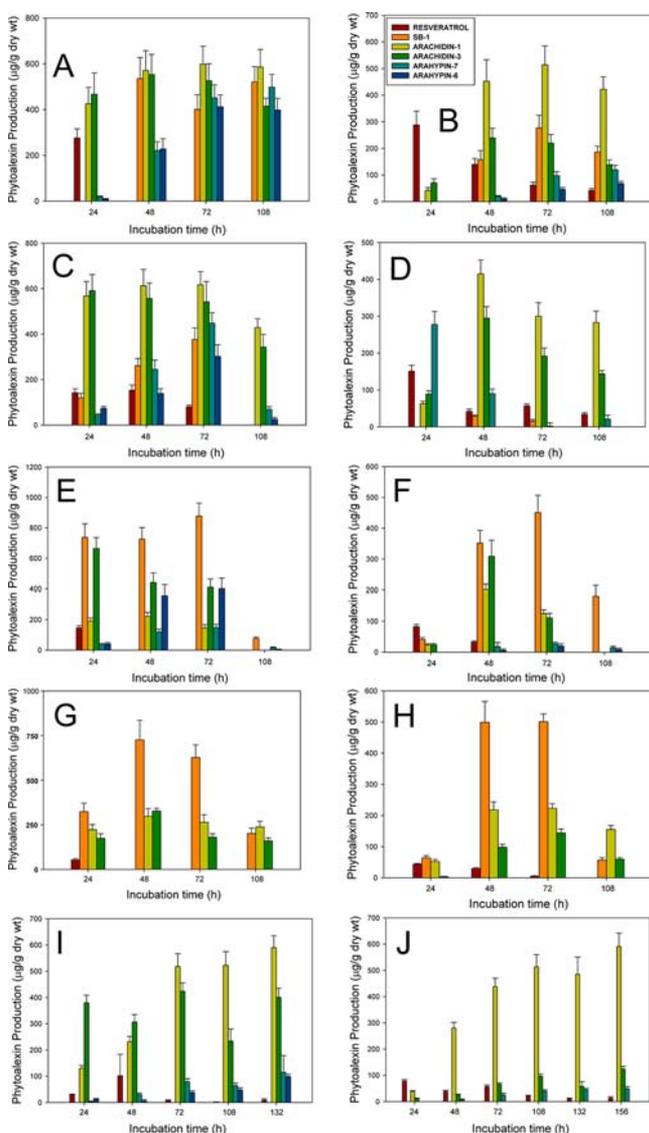
Figure 4. Production of phytoalexins in peanut embryos and embryo-free cotyledons elicited by selected fungal strains of *Aspergillus* and by a *Cladosporium* species. The legend in panel B is applicable to all panels. (A) *A. nomius*, embryos; (B) *A. nomius*, cotyledons; (C) *A. niger*, embryos; (D) *A. niger*, cotyledons; (E) *A. caelatus*, embryos; (F) *A. caelatus*, cotyledons; (G) *A. flavus*, embryos; (H) *A. flavus*, cotyledons; (I) *Cladosporium* sp., embryos; (J) *Cladosporium* sp., cotyledons.

Cladosporium species elicited production of fewer metabolites (Figure 2G,H). In all experiments, concentrations of resveratrol (**1**) versus concentrations of other stilbenoids were consistently higher in cotyledons compared to embryos. At the same time, exponential decay of resveratrol was observed in cotyledons with all fungal elicitors tested (Figure 4B,D,F,H). The most

prominent results were observed with *A. nomius* (Figure 4B): the nonlinear regression $y = ae^{-bx}$ was characterized by nearly perfect statistical data ($R^2 = 0.9969$; normality test passed, $P = 0.9416$). Resveratrol decay with the *A. flavus* strain (Figure 4H) fitted the same equation ($R^2 = 0.9050$; normality test passed, $P = 0.4819$). The reduction in resveratrol concentrations in the plant material was often accompanied by the concomitant rise of concentrations of other major stilbenoids (Figure 4). While cotyledons incubated with *Aspergillus* species demonstrated mixed results in terms of major stilbenoid production (Figure 4B,D,F,H), cotyledons incubated with the *Cladosporium* species showed significantly higher relative production of arachidin-1 (**3**) after 48 h (Figure 4J). The stilbenoid biosynthesis pattern in embryos and cotyledons with *A. nomius* and *A. niger* was similar, with comparable relative production of major stilbenoids (Figure 4A−D), while *A. caelatus* and *A. flavus* favored production of SB-1 (**2**) (Figure 4E−H). The latter was not detected at any time of incubation in embryos and cotyledons incubated with the *Cladosporium* species (Figures 2G,H and 4I,J). This phenomenon is not understood. SB-1 was suggested to be a peanut metabolite de novo, because it is closely related to but-2-en-4-olide biosynthesized by the legume *Pericopsis elata*.[30] Although the sequence of SB-1 formation from arachidin-1 was suggested,[30] it is not clear whether certain fungal species are capable of triggering the production of this compound in seeds or if SB-1 is a product of fungal detoxification.

Appreciable concentrations of dimeric stilbenoids arahypin-7 (**7**) and arahypin-6 (**8**) were found at significantly higher ($p < 0.05$, $n = 3$) concentrations in embryos compared to cotyledons (Figure 4A−F,I,J); these stilbenoids were not quantitated in experiments with *A. flavus* due to insufficient chromatographic peak resolution. Arahypin-4 (**4**) was detected only in *A. niger*-challenged cotyledons (Figure 2F) at concentrations of 91 ± 36, 146 ± 39, 95 ± 23, and 72 ± 28 μg/g after 24, 48, 72, and 108 h of incubation, respectively. Tentatively, on the basis of UV spectra and MS fragmentation, small quantities of arachidin-3 glucoside were detected in embryos incubated for 24 h with the *A. nomius* strain. The following ions were observed: atmospheric-pressure chemical ionization (APCI) MS^2 m/z 459: m/z 297 [M + H − 162 (a hexose sugar)]. Another compound, which had MS data identical to a commercial sample of piceid (reservatrol 3-O-$β$-mono-D-glucoside), as well as matching retention time, was detected in the same set of samples. Its identity also should be considered tentative since this compound was partially coeluted with a phenolic acid, and its retention time was too short to distinguish between the two possible resveratrol glucosides. The following ions for this compound were observed: APCI-MS^2 m/z 391: m/z 229 [M + H − 162 (a hexose sugar)].

Stilbenoid Production Elicited by Yeasts. Yeasts elicited stilbenoid production in the plant material (Figure 5) at a slower rate compared to *Aspergillus* and *Cladosporium* species tested (Figure 4) but at a higher rate compared to the wounded controls (Figure 3). The stilbenoids produced were mainly represented by resveratrol (**1**), arachidin-1 (**3**), and arachidin-3 (**5**) (Figure 5). While the ratios of arachidin-1 and arachidin-3 with *S. cerevisiae* and Fleischmann's yeast in embryos and cotyledons were comparable (Figure 5C−F), the relative concentration of arachidin-1 to arachidin-3 was significantly ($p < 0.05$, $n = 3$) higher with *Candida lipolytica* (Figure 5A,B). Interestingly, resveratrol was not detected in embryos incubated with *C. lipolytica* (Figure 5A); in cotyledons, though,
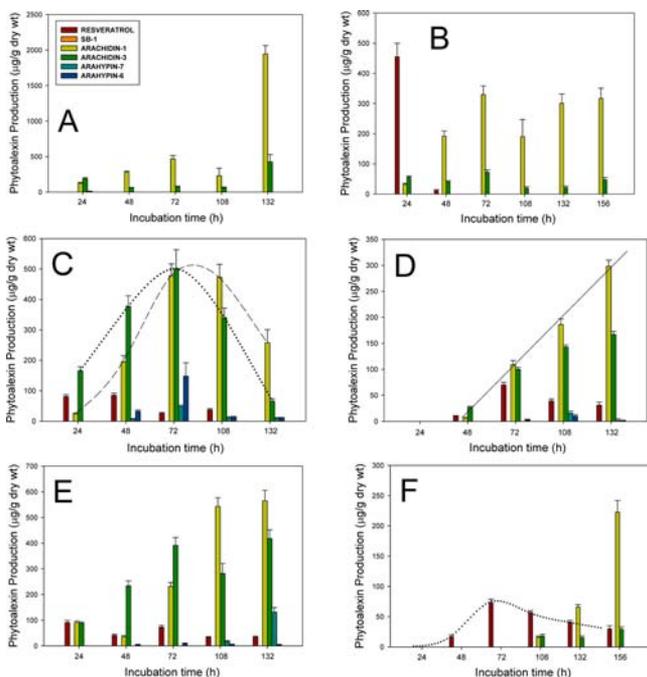
Figure 5. Production of phytoalexins in peanut embryos and embryo-free cotyledons elicited by yeast strains, *Saccharomyces cerevisiae* and *Candida lipolytica*, as well as a commercial Fleischmann's RapidRise yeast product. The legend in panel A is applicable to all panels. (A) *C. lipolytica*, embryos; (B) *C. lipolytica*, cotyledons; (C) *S. cerevisiae*, embryos; (D) *S. cerevisiae*, cotyledons; (E) Fleischmann's RapidRise yeast, embryos; (F) Fleischmann's RapidRise yeast, cotyledons.

resveratrol also disappeared rapidly (Figure 5B). In contrast, with *S. cerevisiae* and Fleischmann's yeast, resveratrol was present in samples up to 132 and 156 h of incubation, respectively (Figure 5D,F). The peak production of resveratrol was recorded in cotyledons at 72 h of incubation with *S. cerevisiae* strain and Fleischmann's yeast (Figure 5D,F). The production was characterized by a normal log function, $y = ae(-0.5[\ln(x/x_0)/b]^2)$ ($R^2 = 0.9262$; normality test passed, $P = 0.9987$), with *S. cerevisiae* (Figure 5D) and Fleischmann's yeast (Figure 5F) ($R^2 = 0.9534$; normality test passed, $P = 0.9640$). No detectable quantities of resveratrol were found in cotyledons with Fleischmann's yeast up to 48 h of incubation (Figure 5F). In contrast, after 24 h *C. lipolytica* elicited the production of 455 μg/g resveratrol (Figure 5B), which is more than 6 times higher than the highest production elicited by *S. cerevisiae* and Fleischmann's yeast in cotyledons after 72 h of incubation (Figure 5D,F). Distribution patterns similar to resveratrol were also observed for arachidin-1 and arachidin-3 concentrations. Logarithmic peak production of arachidin-1 (same equation as above; $R^2 = 0.9888$; normality test passed, $P = 0.9929$) and arachidin-3 (same equation as above; $R^2 = 0.8446$; normality test passed, $P = 0.9580$) was observed in embryos with *S. cerevisiae* (Figure 5C). The dotted line in Figure 5C shows the logarithmic function of arachidin-3 distribution; the dashed line shows a similar function for arachidin-1. Interestingly, accumulation of arachidin-1 in cotyledons with *S. cerevisiae* (Figure 5D) from 48 to 132 h was characterized by a linear regression ($R^2 = 0.9950$; normality test passed, $P = 0.4390$). Overall relative arachidin-1 and arachidin-3 production in cotyledons with *C. lipolytica* (Figure 5B) was similar to that of cotyledons with the *Cladosporium*
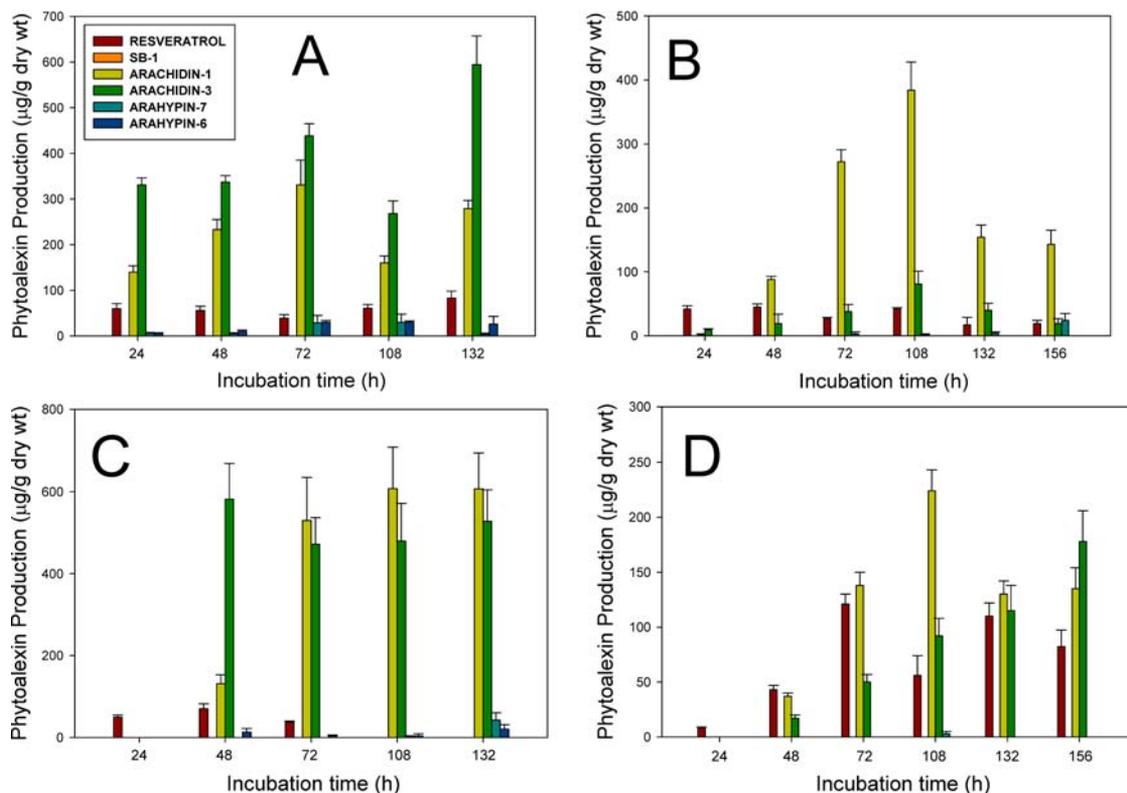
Figure 6. Production of phytoalexins in peanut embryos and embryo-free cotyledons elicited by bacterial strains, *Bacillus subtilis* and *Rhizobium leguminosarum*. The legend in panel A is applicable to all panels. (A) *B. subtilis*, embryos; (B) *B. subtilis*, cotyledons; (C) *R. leguminosarum*, embryos; (D) *R. leguminosarum*, cotyledons.

species (Figure 4J). Arahypin-4 (**4**) was also found in *C. lipolytica*-challenged cotyledons at concentrations of 24 ± 3, 39 ± 11, 24 ± 15, and 0 ± 0 μg/g, respectively, after 24, 48, 72, and 108 h of incubation.

Stilbenoid Production Elicited by Bacteria. Similar to the yeasts tested, *B. subtilis* and *R. leguminosarum* elicited production of resveratrol (**1**), arachidin-1 (**3**), and arachidin-3 (**5**) in embryos and cotyledons (Figures 2I–L and 6A–D). Insignificant quantities of dimeric stilbenoids **7** and **8** were also detected (Figures 2I–K and 6A–C); compounds **7** and **8** were not quantitated in experiments with *R. leguminosarum* due to insufficient chromatographic peak resolution. Relatively higher concentrations of arachidin-1 were, however, found in cotyledons (Figures 2J,L and 6B,D), while embryos favored synthesis of arachidin-3 (Figures 2I,K and 6A,C). Cotyledons responded to incubation with *B. subtilis* similarly to those incubated with the *Cladosporium* species, with resveratrol showing a gradual decline in its amounts. Also, SB-1 (**2**) was not present, and the ratios of major stilbenoids in both tests were comparable (Figures 2H,J, 4J, and 6B). Resveratrol was present in all embryo and cotyledon samples throughout the experimental time (Figure 6A,B). Incubation of cotyledons with *R. leguminosarum* established the production of major stilbenoids at concentrations that cannot be reasonably explained (Figure 6D). The stilbenoid profile in embryos with *R. leguminosarum* resembled that in embryos with *S. cerevisiae*; however, the peak production of arachidin-3 was delayed with *R. leguminosarum* for 24–36 h (Figures 5C and 6C).

Stilbenoid Production Elicited by H_2O_2. Despite the fact that H_2O_2 was quickly decomposed by seed enzymes, the seed material was sufficiently challenged to produce appreciable concentrations of stilbenoids. A single treatment of embryos and cotyledons with H_2O_2 triggered production of stilbenoids as shown in Figures 2M–O and 7. Arachidin-1 (**3**) was
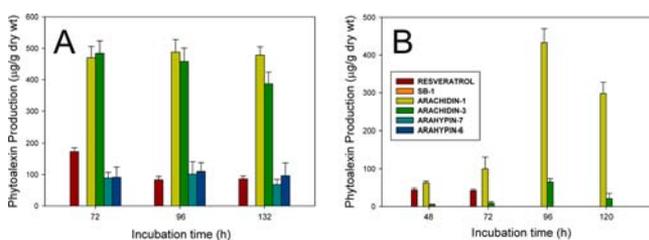
Figure 7. Production of phytoalexins in peanut embryos and embryo-free cotyledons elicited by H_2O_2. The legend in panel B is applicable to panel A. (A) H_2O_2, embryos; (B) H_2O_2, cotyledons.

detected in embryos and cotyledons at significantly ($p < 0.05$, $n = 3$) higher concentrations relative to other stilbenoids (Figures 2M,N and 7). Like other elicitors, with the exception of the *Aspergillus* species, H_2O_2 did not elicit SB-1 (**2**) production in the plant material used. High production of major stilbenoids in the seed material within 72 h after treatment with H_2O_2 (Figure 7) indicates the high potency of H_2O_2 as an elicitor of common stilbenoids. Under similar incubation conditions, the stilbenoid composition and content in wounded controls was significantly ($p < 0.05$, $n = 3$) lower (Figure 3). In the case of embryos, multiple treatments with H_2O_2 caused formation of numerous unidentified compounds, in addition to known stilbenoids during 96–288 h of incubation (Figure 2M,O). Most likely, the

majority of those compounds are derived from the condensation and/or oxidation of common peanut stilbenoids.[31]

Total Stilbenoid Production Elicited in Embryos and Cotyledons by Biotic and Abiotic Elicitors. The results on total stilbenoid production by embryos and cotyledons challenged by the elicitors tested are summarized in Figure 8.
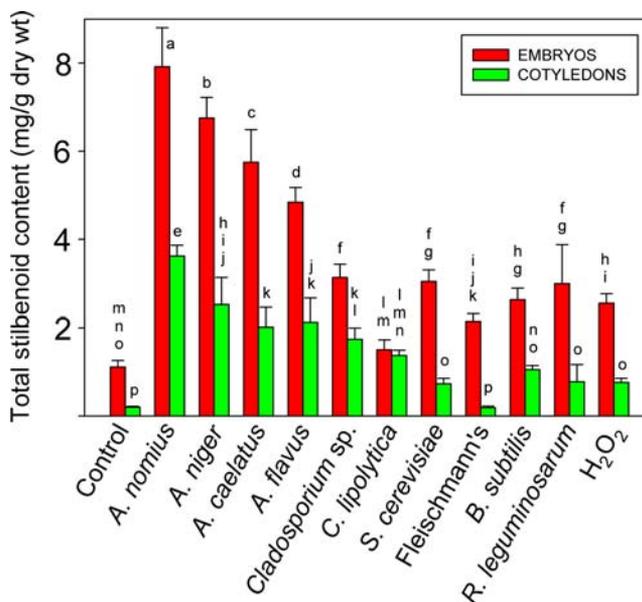
Figure 8. Total stilbenoid production by embryos and cotyledons challenged by biotic and abiotic elicitors. Combined data were collected from 24 to 108 h. Means of total phytoalexin production followed by the same letter are not significantly different ($p > 0.01$, $n = 3$).

Data, collected from 24 to 108 h, are shown for direct comparison of the results. Means of total phytoalexin production followed by the same letter are not significantly different ($p > 0.01$, $n = 3$). Embryos demonstrated the ability to produce significantly higher levels of stilbenoids elicited by diverse biotic elicitors and H_2O_2, with the exception of *C. lipolytica* (Figure 8). All tested fungi from the genus *Aspergillus*, compared to other biotic agents, were more potent elicitors of stilbenoids. Production of stilbenoids triggered by yeasts and bacteria were comparable (Figure 8). H_2O_2 as an elicitor did not behave significantly different than Fleischmann's yeast and *B. subtilis*.

Stilbenoid Production Elicited by a Fungus in Uniformly Sized Plant Material. From all the elicitors tested in the course of present research, a fungal elicitor was chosen for these experiments. It has been demonstrated that phytoalexin production in peanut seeds depends on depths from wounds infected by fungal pathogens.[12] Therefore, to ensure that the difference in size of seeds and embryos did not lead to an incorrect conclusion about the ability of embryos to produce significantly higher concentrations of phytoalexins compared to cotyledons, rectangular prisms of equal size were cut from embryos and cotyledons and subjected to fungal stress. Liquid agitated medium was chosen for this experiment since it provided uniform access of the fungus to surfaces of the cut material. Total stilbenoid production after 30 h of incubation by embryos was 591 ± 95 mg/g (wet weight) compared to 136 ± 21 mg/g by cotyledons. Similar results were obtained after 48 h of incubation, 441 ± 65 and 186 ± 28 mg/g

for embryos and cotyledons, respectively. At both incubation times, 30 and 48 h, total stilbenoid production in embryos was significantly higher compared to cotyledons ($p < 0.05$, $n = 3$). Total production of stilbenoids in cotyledons at 30 and 48 h was not significantly different ($p > 0.05$, $n = 3$). Total stilbenoid content in control embryos was 0.06 ± 0.01 and 0.08 ± 0.01 mg/g, and 0.07 ± 0.01 and 0.09 ± 0.02 mg/g in cotyledons at 30 and 48 h, respectively. Those values were significantly ($p < 0.05$, $n = 3$) lower compared to the experimental values. This experimental setup allowed us to acquire results similar to those obtained from the agar-based procedures described above and to conclude that challenged peanut embryos are capable of significantly higher production of stilbenoids compared to cotyledons.

The dynamics of phytoalexin production by seeds and embryos of a common United States peanut cultivar, Georgia 06G, challenged by selected important fungi and bacteria, was investigated. Under the experimental conditions employed, there was no significant ($p > 0.05$, $n = 3$) difference between the above cultivar and the Georgia Green cultivar (data not shown) in terms of phytoalexin composition and concentrations.

Some peanut stilbenoids have been suggested to be biosynthesized by the peanut plant from the structurally simple stilbenoid resveratrol (**1**).[12] The results of present research agree with those suggestions, as resveratrol was detected at high concentrations at early stages of incubation followed by its exponential decline. Notably, *Aspergillis* species were more potent elicitors of stilbenoids compared to other elicitors tested; this may indicate the evolutionary affiliation of the *Aspergillus* species with the peanut plant that is capable of recognizing these fungi as pathogens. Such recognition could lead to the intensive biosynthesis of phytoalexins at high concentrations.

Higher stilbenoid concentrations were accumulated with longer incubation times, and the composition varied significantly among different elicitors. Different biotic agents selectively elicited production and accumulation of major peanut stilbenoids resveratrol (**1**), arachidin-1 (**3**), arachidin-3 (**5**), and SB-1 (**2**). Some elicitors were associated with the production of either arachidin-1 or arachidin-3 as a dominant stilbenoid (Figure 2H,J,K,N), which may find applications in production of these bioactive stilbenoids for veterinary/medical research. Significant quantities of **6** were detected only in the control cotyledons and embryos. In addition to the major metabolites, during the course of the experiments, arahypin-4 (**4**), stilbenoid (**6**), and chiricanine A (**9**) were observed in some sample extracts. Virtually all experiments with and without elicitors revealed some major unidentified peaks that likely belong to unknown stilbenoids, based on their characteristic UV absorption spectra and MS fragmentation (not shown).

Among the elicitors tested, embryos demonstrated their ability to synthesize an array of stilbenoids de novo at significantly higher concentrations compared to cotyledons. Therefore, embryos may serve as a more convenient source of genetic material for peanut enhancement compared to cotyledons. Continued research on phytoalexin biosynthesis in peanuts may lead not only to new resistant peanut cultivars but also to cultivars deliberately enriched with desired bioactive stilbenoids suitable for human consumption.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone (229) 995-7446; fax (229) 995-7416; e-mail vsobolev@nprl.usda.gov.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Hill, R. A.; Blankenship, P. D.; Cole, R. J.; Sanders, T. H. Effect of soil moisture and temperature on preharvest invasion of peanuts by the *Aspergillus flavus* group and subsequent aflatoxin development. *Appl. Environ. Microbiol.* **1983**, *45*, 628−633.

(2) Dorner, J. W.; Cole, R. J.; Sanders, T. H.; Blankenship, P. D. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. *Mycopathologia* **1989**, *105*, 117−128.

(3) Paxton, J. D. Biosynthesis and accumulation of legume phytoalexins. In *Mycotoxins and Phytoalexins*; Sharma, R. P., Salunkhe, D. K., Eds.; CRC Press: Boca Raton, FL, 1991; pp 485−499.

(4) Cole, R. J.; Dorner, J. W. Peanut phytoalexins. In *Mycotoxins and Phytoalexins*; Sharma, R. P., Salunkhe, D. K., Eds.; CRC Press: Boca Raton, FL, 1991; pp 501−509.

(5) Subba Rao, P. V.; Strange, R. N. Chemistry, biology, and role of groundnut phytoalexins in resistance to fungal attack. In *Handbook of Phytoalexin Metabolism and Action*; Daniel, M., Purkayastha, R. P., Eds.; Marcel Dekker: New York, 1995; pp 199−227.

(6) Sobolev, V. S.; Guo, B. Z.; Holbrook, C. C.; Lynch, R. E. Interrelationship of phytoalexin production and disease resistance in selected peanut genotypes. *J. Agric. Food. Chem.* **2007**, *55*, 2195−2200.

(7) Paxton, J. Phytoalexins: a working redefinition. *Phytopathol. Z.* **1981**, *101*, 106−109.

(8) Sobolev, V. S.; Khan, S. I.; Tabanca, N.; Wedge, D. E.; Manly, S. P.; Cutler, S. J.; Coy, M. R.; Becnel, J. J.; Neff, S. A.; Gloer, J. B. Biological activity of peanut (*Arachis hypogaea*) phytoalexins and selected natural and synthetic stilbenoids. *J. Agric. Food Chem.* **2011**, *59*, 1673−1682.

(9) Sobolev, V. S.; Neff, S. A.; Gloer, J. B. New pterocarpenes elicited by *Aspergillus caelatus* in peanut (*Arachis hypogaea*) seeds. *Phytochemistry* **2010**, *71*, 2099−2107.

(10) Mansfield, J. W. The role of phytoalexins in disease resistance. In *Phytoalexins*; Bailey, J. A., Mansfield, J. W., Eds.; John Wiley: New York, 1982; pp 153−288.

(11) Strange, R. N. Resistance: the role of the hypersensitive response and phytoalexins. In *Pests and Pathogens: Plant Responses to Foliar Pathogens*; Ayers, P. G., Ed.; Biosis Scientific: Oxford, U.K., 1992; pp 39−56.

(12) Sobolev, V. S. Localized production of phytoalexins by peanut (*Arachis hypogaea*) kernels in response to invasion by *Aspergillus* species. *J. Agric. Food Chem.* **2008**, *56*, 1949−1954.

(13) Cline, K.; Wade, M.; Albersheim, P. Host-pathogen interactions: XV. Fungal glucans which elicit phytoalexin accumulation in soybean also elicit the accumulation of phytoalexins in other plants. *Plant Physiol.* **1978**, *62*, 918−921.

(14) Onuorah, O. M. Isolation of pathogen-synthesized fraction that elicits phytoalexins in lucerne (*Medicago sativa* L.). *Acta Biol. Hung.* **1987**, *38*, 247−256.

(15) Gonzalez-Lamothe, R.; Mitchell, G.; Gattuso, M.; Diarra, M. S.; Malouin, F.; Bouarab, K. Plant antimicrobial agents and their effects on plant and human pathogens. *Int. J. Mol. Sci.* **2009**, *10*, 3400−3419.

(16) Jeandet, P.; Delaunois, B.; Conreux, A.; Donnez, D.; Nuzzo, V.; Cordelier, S.; Clement, C.; Courot, E. Biosynthesis, metabolism, molecular engineering, and biological functions of stilbene phytoalexins in plants. *Biofactors* **2010**, *36*, 331−341.

(17) Pedras, M. S.; Yaya, E. E. Phytoalexins from Brassicaceae: news from the front. *Phytochemistry* **2010**, *71*, 1191−1197.

(18) Cruickshank, I. A. M. Studies on phytoalexins. 4. Antimicrobial spectrum of pisatin. *Aust. J. Biol. Sci.* **1962**, *15*, 147−159.

(19) Sobolev, V. S.; Neff, S. A.; Gloer, J. B. New stilbenoids from peanut (*Arachis hypogaea*) seeds challenged by an *Aspergillus caelatus* strain. *J. Agric. Food Chem.* **2009**, *57*, 62−68.

(20) Aggarwal, B. B.; Bhardwaj, A.; Aggarwal, R. S.; Seeram, N. P.; Shishodia, S.; Takada, Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res.* **2004**, *24*, 2783−2840.

(21) King, R. E.; Bomser, J. A.; Min, D. B. Bioactivity of resveratrol. *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 65−70.

(22) Athar, M.; Back, J. H.; Tang, X.; Kim, K. H.; Kopelovich, L.; Bickers, D. R.; Kim, A. L. Resveratrol: a review of preclinical studies for human cancer prevention. *Toxicol. Appl. Pharmacol.* **2007**, *3*, 274−283.

(23) Pezzuto, J. M. Resveratrol as an inhibitor of carcinogenesis. *Pharmaceut. Biol.* **2008**, *46*, 443−573.

(24) Chang, J.-C.; Lai, Y.-H.; Djoko, B.; Wu, P.-L.; Liu, C.-D.; Liu, Y.-W.; Chiou, R. Y.-Y. Biosynthesis enhancement and antioxidant and anti-inflammatory activities of peanut (*Arachis hypogaea* L.) arachidin-1, arachidin-3, and isopentadienylresveratrol. *J. Agric. Food Chem.* **2006**, *54*, 10281−10287.

(25) Djoko, B.; Chiou, R. Y. Y.; Shee, J. J.; Liu, Y. W. Characterization of immunological activities of peanut stilbenoids, arachidin-1, piceatannol, and resveratrol on lipopolysaccharide-induced inflammation of RAW 264.7 macrophages. *J. Agric. Food. Chem.* **2007**, *55*, 2376−2383.

(26) Abbott, J. A.; Medina-Bolivar, F.; Martin, E. M.; Engelberth, A. S.; Villagarcia, H.; Clausen, E. C.; Carrier, D. J. Purification of resveratrol, arachidin-1, and arachidin-3 from hairy root cultures of peanut (*Arachis hypogaea*) and determination of their antioxidant activity and cytotoxicity. *Biotechnol. Prog.* **2010**, *26*, 1344−1351.

(27) Rudolf, J. R.; Resurreccion, A. V. A. Elicitation of resveratrol in peanut kernels by application of abiotic stresses. *J. Agric. Food Chem.* **2005**, *53*, 10186−10192.

(28) Condori, J.; Sivakumar, G.; Hubstenberger, J.; Dolan, M. C.; Sobolev, V. S.; Medina-Bolivar, F. Induced biosynthesis of resveratrol and the prenylated stilbenoids arachidin-1 and arachidin-3 in hairy root cultures of peanut: Effects of culture medium and growth stage. *Plant Physiol. Biochem.* **2010**, *48*, 310−318.

(29) Sobolev, V. S.; Cole, R. J.; Dorner, J. W.; Yagen, B. Isolation, purification, and liquid chromatographic determination of stilbene phytoalexins in peanuts. *J. AOAC Int.* **1995**, *78*, 1177−1182.

(30) Sobolev, V. S.; Deyrup, S. T.; Gloer, J. B. New peanut (*Arachis hypogaea*) phytoalexin with prenylated benzenoid and but-2-enolide moieties. *J. Agric. Food Chem.* **2006**, *54*, 2111−2115.

(31) Sobolev, V. S.; Neff, S. A.; Gloer, J. B. New dimeric stilbenoids from fungal-challenged peanut (*Arachis hypogaea*) seeds. *J. Agric. Food Chem.* **2010**, *58*, 875−881.

(32) Dixon, R. A. Isolation and maintenance of callus and cell suspension cultures. In *Plant Cell Cultures: A Practical Approach*; Dixon, R. A., Ed.; IRL Press: Oxford, U.K., 1985; pp 1−20.

(33) Cooksey, C. J.; Garratt, P. J.; Richards, S. E.; Strange, R. N. A dienyl stilbene phytoalexin from *Arachis hypogaea. Phytochemistry* **1988**, *27*, 1015−1016.

(34) Elkan, G. H. Biological nitrogen fixation in peanuts. In *Advances in Peanut Science*; Pattee, H. E., Stalker, H. T., Eds.; American Peanut Research and Education Society, Inc.: Stillwater, OK, 1995; pp 286−300.

(35) Pettit, R. E.; Taber, R. A.; Foster, B. G. Occurrence of *Bacillus subtilis* in peanut kernels. *Phytopathology* **1968**, *58*, 254−255.

(36) Balhara, M.; Ruhil, S.; Dhankhar, S.; Chhillar, A. K. Bioactive compounds hold up - *Bacillus amyloliquefaciens* as a potent bio-control agent. *Nat. Prod. J.* **2011**, *1*, 20−28.

(37) Stipanovic, R. D.; Mace, M. E.; Bell, A. A.; Beier, R. C. The role of free radicals in the decomposition of the phytoalexin desoxy-hemigossypol. *J. Chem. Soc., Perkin Trans.* **1992**, *1*, 3189−3192.

(38) Hale, M. G. Loss of organic compounds from roots. I. Cultural conditions for axenic growth of peanut, *Arachis hypogaea* L. *Plant Soil* **1969**, *31*, 463−472.

(39) Sobolev, V. S.; Horn, B. W.; Potter, T. L.; Deypup, S. T.; Gloer, G. B. Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth. *J. Agric. Food Chem.* **2006**, *54*, 3505−3511.

(40) Hahlbrook, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347−369.