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Inhibition of Aflatoxin Formation in Aspergillus Species by Peanut (Arachis hypogaea) Seed Stilbenoids in the Course of Peanut–Fungus Interaction

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Inhibition of Aflatoxin Formation in *Aspergillus* Species by Peanut (*Arachis hypogaea*) Seed Stilbenoids in the Course of Peanut−Fungus Interaction

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Supporting Information

ABSTRACT: Common soil fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, are opportunistic pathogens that invade preharvest peanut seeds. These fungi often produce carcinogenic aflatoxins that pose a threat to human and animal health through food chains and cause significant economic losses worldwide. Detection of aflatoxins and further processing of crops are mandated to ensure that contaminated agricultural products do not enter food channels. Under favorable conditions, the fungus-challenged peanut seeds produce phytoalexins, structurally related stilbenoids, capable of retarding fungal development. The purpose of the present study was to evaluate the potential influence of peanut phytoalexins on fungal development and aflatoxin formation in the course of peanut−fungus interaction. The present research revealed that during such interaction, aflatoxin formation was completely suppressed in *A. flavus* and *A. parasiticus* strains tested, when low concentrations of spores were introduced to wounded preincubated peanuts. In most of the experiments, when fungal spore concentrations were 2 orders of magnitude higher, the spores germinated and produced aflatoxins. Of all experimental seeds that showed fungal growth, 57.7% were aflatoxin-free after 72 h of incubation. The research provided new knowledge on the aflatoxin/phytoalexin formation in the course of peanut−fungus interaction.

KEYWORDS: *peanut, groundnut, Arachis hypogaea, phytoalexin, stilbenoid, arachidin, aflatoxin, aflatoxin inhibition, Aspergillus, Aspergillus flavus, Aspergillus parasiticus, norsolorinic acid*

INTRODUCTION

Common soil fungi, *Aspergillus flavus* and *Aspergillus parasiticus*, are opportunistic pathogens that invade peanut seeds at different stages of their development. These fungi often produce highly toxic, carcinogenic aflatoxins, predominantly aflatoxin B_1 (**5**, Figure 1), that pose a threat to human and animal health through food chains and cause significant economic losses worldwide.[1−9]

Under favorable temperature and adequate water activity level, fungal conidia, the asexual spores of a fungus, germinate and form hyphae, the branching filaments that form the mycelium of a fungus. The formation of hyphae occurs after absorption of water through the walls of fungal spores, activation of the cytoplasm, and nuclear division. Fungi obtain nutrients through the action of enzymes secreted into the substrate on/in which they grow. Components of the fermented substrate are absorbed directly through the entire surface of hyphal walls of a fungus. Further fungal development may or may not lead to the formation of its dedicated reproductive structures, conidial heads (Figure 2C,D).[10,11] *Aspergillus* invasion triggers accumulation of numerous structurally related, biologically active prenylated stilbenoids in peanut, as a plant defense mechanism.[12−25] These stilbenoids are considered peanut phytoalexins with antifungal properties and are an essential natural defensive factor in preharvest aflatoxin contamination.[14,23,26] The positive role of stilbenoids as defensive compounds against *Aspergillus* in peanut has been evaluated in 2-year field experiments.[27] Sufficient water content in seeds is crucial for efficient production of the phytoalexins.[15,19] Several compounds, including caffeic acid and resveratrol, the compounds formed in peanut seeds, were demonstrated to reduce aflatoxin production in peanut with or without affecting *Aspergillus* growth and normal mycelial development.[28−31] A significant reduction or virtually complete suppression of aflatoxin production was revealed when selected *Aspergillus* toxigenic species were fed with major individual peanut stilbenoids, arachidin-1, arachidin-3, and chiricanine A, added to the growth medium.[26] However, it was not clear whether fungal growth and aflatoxin inhibition by the de novo synthesized stilbenoids occurs in the course of peanut−fungus interaction.

The purpose of the present study was to evaluate the potential influence of peanut phytoalexins on *Aspergillus* development and aflatoxin formation in the course of peanut−fungus interaction.

MATERIALS AND METHODS

Reagents, Materials, and Basic Apparatus. HPLC-grade solvents used in the preparation of mobile phases and extractions of peanut seeds and fungal colonies were obtained from Fisher (Suwanee, GA). Formic acid (88% ACS grade) was also purchased from Fisher. HPLC-grade H_2O was prepared with a ZD20 four-bowl Milli-Q water system (Millipore).

Reference Compounds. Pure individual stilbenoids *trans*-arachidin-1 (**1**), *trans*-arachidin-2, *trans*-arachidin-3 (**2**), and *trans*-3′-

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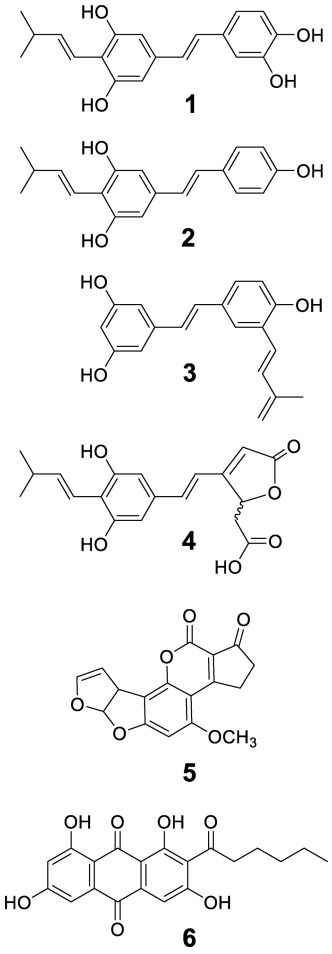

Figure 1. Structures of major peanut prenylated polyphenolic compounds, aflatoxin B_1, and norsolorinic acid: **1**, *trans*-arachidin-1; **2**, *trans*-arachidin-3; **3**, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; **4**, SB-1; **5**, aflatoxin B_1; **6**, norsolorinic acid.

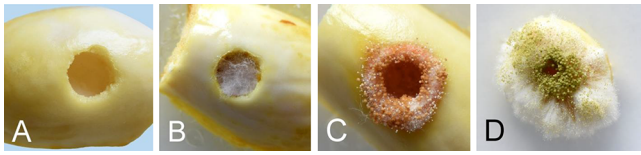

Figure 2. Wounded peanut seeds with typical levels of fungal colonization after 72 h of incubation at 30 °C: plate A, control seeds without fungus and experimental seeds without any signs of fungal infection; plate B, experimental seeds with fugal mycelium *A. flavus* NRRL 3357 retained within the drilled hole; plate C, experimental seeds with moderate-to-heavy fungal colonies of *A. parasiticus* NRRL 6111 showing orange conidial heads and mycelium structures; plate D, experimental seeds with heavy fungal colonies of *A. flavus* NRRL 3357 showing green conidial heads and white mycelium structures. All peanut seeds with the fungal colonization levels shown in plates B–D were marked as seeds with fungal growth in Table 1 and were not subjected to smudging.

isopentadienyl-3,5,4′-trihydroxystilbene (IPD) (**3**) (Figure 1) were obtained as described[18] except that preparative HPLC was used as a final purification step rather than preparative TLC. The separations were performed using a 100 mm × 19 mm i.d., 5 μm XTerra Prep RP18 OBD column (Waters); the column temperature was 40 °C. The isocratic mobile phase used was composed of 55% CH_3CN, 3% of 2% HCOOH in H_2O, and 42% of H_2O; the flow rate was 8.0 mL/min.[25] Chiricanine A was prepared as described.[25] SB-1 (**4**) was obtained according to the described procedure.[32] Aflatoxins B_1 (**5**, Figure 1), B_2, G_1, and G_2 were purchased from Sigma. Norsolorinic acid (98% pure) (**6**, Figure 1) was made available from the secondary fungal metabolite collection of the National Peanut Research Laboratory, ARS, USDA (Dawson, GA). The identities of the reference compounds were confirmed by APCI-MS/MS (MS^2) and UV spectroscopy (the equipment used is listed in the HPLC-DAD–MS Analysis section). These data are given in parentheses as $[M + H]^+$ values followed by UV absorption maxima wavelengths: *trans*-resveratrol (*m/z* 229; 305 and 317 nm), *trans*-arachidin-1 (**1**) (*m/z* 313; 339 nm), *trans*-arachidin-2 (*m/z* 297; 308 and 322 nm), *trans*-arachidin-3 (**2**) (*m/z* 297; 335 nm), IPD (**3**) (*m/z* 295; 295 nm), SB-1 (**4**) (*m/z* 345; 363 nm), chiricanine A (*m/z* 281; 311 nm), chiricanine B (*m/z* 297; 234, 301, and 311 nm), arahypin-1 (*m/z* 281; 328 nm), arahypin-2 (*m/z* 331; 306 and 317 nm), arahypin-3 (*m/z* 331; 306 and 320 nm), arahypin-4 (*m/z* 315; 311 nm), arahypin-5 (*m/z* 295; 233, 267, and 334 nm), arahypin-6 (*m/z* 607; 272 and 340 nm), arahypin-7 (*m/z* 623; 271 and 347 nm), arahypin-10 (*m/z* 295; 230, 274, and 315 nm), arahypin-13 (*m/z* 279; 230, 274, and 315 nm), arahypin-14 (*m/z* 313; 236 and 305 nm), arahypin-15 (*m/z* 313; 229, 307, and 321 nm). The above results were in agreement with published data.[33]

Fungal Material. The fungi used included three strains of *Aspergillus* that differed from each other by their aflatoxigenic potential: *A. flavus* NRRL 29487, a high aflatoxin producer; *A. flavus* NRRL 3357, a moderate aflatoxin producer; *A. parasiticus* NRRL 6111 (color mutant), a norsolorinic acid and low aflatoxin producer. The strains, as well as the information on the fungal toxigenic potential, were made available from the fungal collection and the database of the National Peanut Research Laboratory (NPRL), ARS, USDA (Dawson, GA).

The strains were grown on potato-dextrose agar (PDA) medium for 6 days at 29 °C under luminescent light to promote conidia formation. The spores of each isolate were harvested by adding 10 mL of water with Tween 20 (two drops/L) and filtering through glass-fiber wool. Concentrations of spores in the suspension was determined with the help of a Scientific Hemocytometer (Fisher, cat. #02-671-52B). The spore suspension was diluted with distilled sterile H_2O to the needed concentrations of 1.7×10^3/mL, 1.7×10^4/mL, and 1.7×10^5/mL. Prepared spore suspensions were used for the experiments without delay. At the time of application, spores were maintained suspended with the help of a mini Teflon-coated magnetic stirrer. To ensure the viability of fungal spores, 3 μL of spore suspension from each dilution vial, e.g., 5, 50, and 500 spores, were applied in the marked square sections of a PDA agar Petri plate (Figure 3A). The photo shows growth of the *A. parasiticus* NRRL 6111 isolate in all marked sections of the plate after incubation at 30 °C for 72 h.

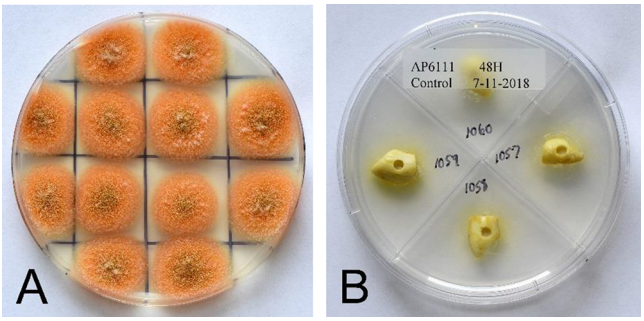

Figure 3. Plate A, at the beginning of the experiment, 3 μL of spore suspension from each dilution vial, e.g., 5, 50, and 500 spores, was applied in the middle of a marked square section of a PDA agar Petri dish. The number of marked sections (288) was equal to the number of seeds that received fungal spores. The photo shows the growth of the *A. parasiticus* NRRL 6111 isolate in all marked sections of the plate after incubation at 30 °C for 72 h. Similar growth was observed for all spore concentrations of all fungal isolates tested. Plate B, wounded seeds on water agar incubated at 30 °C for 48 h (control).

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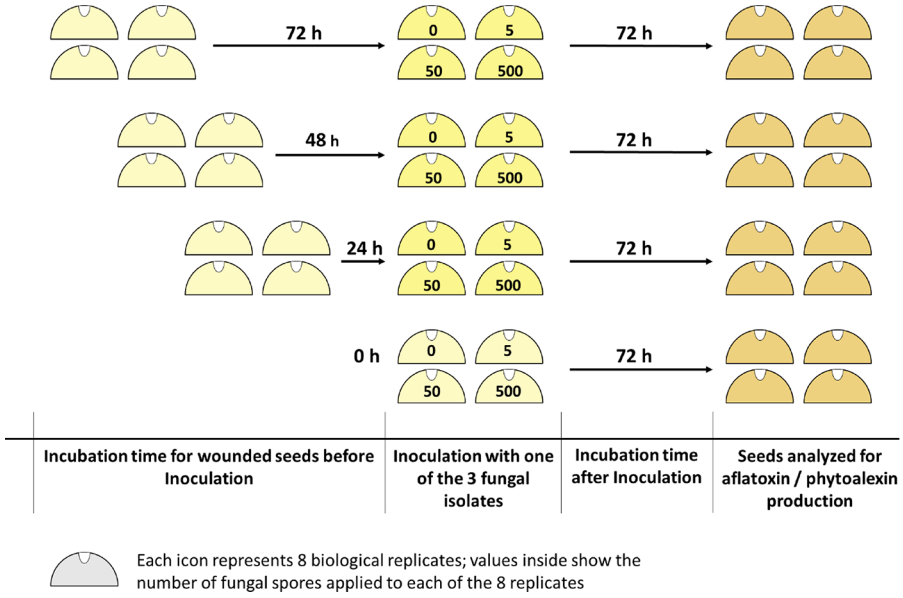

Figure 4. Flowchart of the experimental setup for the elucidation of changes in aflatoxin and phytoalexin formation in the course of peanut−fungus interaction for each of the *Aspergillus* species tested.

Plant Material. Viable seeds of a popular Georgia-O6G peanut cultivar (harvested in 2017) with acceptable disease resistance were chosen for the research. The nondestructive hull-scrape method was used to determine the maturity of the pods.[34] Only seeds from brown and black mature pods were used.

Experimental Setup. Peanut pods were manually cracked, and whole seeds with skins were surface sterilized as described,[35] but with some changes to the procedure. In brief, the seeds were submersed for 5 min in 75% ethanol followed by 5 min in 1% sodium hypochlorite (NaClO), rinsed once with sterile distilled H_2O, and sterilized one more time for 5 min in 1% NaClO followed by rinsing four times with sterile distilled H_2O. Then the seeds were placed in a beaker with sterile distilled H_2O and left for 16 h at 23 ± 2 °C without light. The next day the seeds were rinsed with sterile H_2O twice. The skins were removed from the seeds by gloved hands under axenic conditions. The deskinned seeds were manually separated into cotyledons. The embryos were removed by hand, and 3 mm of the cotyledon from the embryo side was cut off with a scalpel. Then, with the help of a 3 mm drill bit, a 2.5−3 mm deep hole was made in the middle of the outer surface of the seed (Figure 2A). After that, the seeds were additionally sterilized for 10 s in 75% EtOH and rinsed with H_2O twice. Then the seeds were placed on a sterile paper towel, blotted, and allowed to dry under laminar flow for 2 min. The seeds were placed on Difco (BD, Franklin Lakes, NJ) 1.5% water agar in Fisher 100 × 15 mm compartmentalized Petri quad plates (Figure 3B). Each sector of the plate accommodated one seed on 6.5 mL of solidified agar. A total of 384 seeds were divided into 3 groups; 128 seeds were used for experiments with each fungal strain. According to the setup (Figure 4), each group of 128 seeds was subdivided into 4 groups, 32 seeds in each. Two Petri dishes with four seeds each served as eight biological replicates per test. In addition, 8 original dry seeds and 22 seeds hydrated for 16 h were used as controls. These seeds were kept frozen at −80 °C before the aflatoxin and phytoalexin analysis.

After incubation at 30 °C for 0, 24, 48, or 72 h (Figure 4), all experimental seeds in each group were inoculated on the same day with 0, 5, 50, and 500 fungal spores by applying 3 μL of the appropriate spore concentration to the bottom of the drilled hole (Figure 2A). Inoculated seeds were further incubated at 30 °C without light for 72 h. Each cotyledon after incubation was visually rated for fungal colonization and photographed. Seeds that did not show fungal growth were cut into two pieces through the center of the hole, and each half of a cotyledon was smudged five or six times along a PDA-agar plate (one plate per cotyledon) so that agar touched the inside walls of the drilled hole (Figure 5A−C). To prevent potential bacterial growth, the PDA

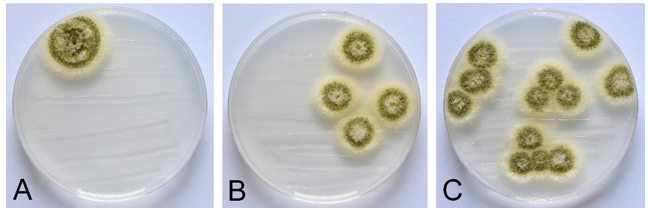

Figure 5. Petri dishes with five or six "smudges" made from side-to-side on PDA agar with single experimental cotyledons (one cotyledon per one Petri dish) that did not show any signs of fungal growth after incubation for 72 h at 30 °C: plate A, smudges were made with cotyledons with 5 spores initially applied to the seed wound; plate B, 50 spores; plate C, 500 spores.

medium was impregnated with the antibiotics carbenicillin and kanamycin, 100 and 50 mg/L, respectively. After smudging, each cotyledon was placed into a preweighed 2 mL Bead Raptor vial (reinforced polypropylene with a silicon O-ring) vial with six 2.8 mm ceramic beads (both from Omni International, Inc., Kennesaw, GA) and kept frozen at −80 °C before the analysis. To extract aflatoxins and phytoalexins, 0.7 mL of MeOH was added to each vial, and the sample was pulverized in an Omni Bead Raptor 24 (Omni International, Inc., Kennesaw, GA) for 20 s. Then the vials were centrifuged in a model 6765 Corning LSE Mini microcentrifuge (Corning, Inc., Tewksbury, MA) for 5 min, and the supernatant was filtered through a Pasteur pipet with a glass-fiber plug firmly placed in its tip into a Thermo screw thread autosampler vial (catalog # 03-375-17CA) with a matching cap (catalog # 03-397-05). For aflatoxin analysis, 100 μL of the filtrate was transferred into a Waters UPLC 700 μL polypropylene 12 × 32 mm vial (catalog # 186005221) preloaded with 300 μL of MeOH, vortexed for 5 s, and subjected to analysis by UPLC. Phytoalexins in the extract were analyzed by HPLC/MS without dilution.

HPLC-DAD−MS Analysis. Separations of the seed extracts were performed using a tandem HPLC−MS Surveyor system equipped with MS Pump Plus, Autosampler Plus, a PDA Plus Detector (Thermo Electron Corp., San Jose, CA) covering the 210−600 nm range, and a 100 mm × 4.6 mm i.d., 3.5 μm XSelect HSS C18 analytical column (Waters). H_2O (A), MeOH (B), and 2% HCOOH in H_2O (C) were used in the following gradient: initial conditions, 59% A/40% B/1% C, changed linearly to 10% A/89% B/1% C in 11 min, changed to 0% A/99% B/1% C in 0.01 min, held isocratic for 3 min, and then changed to initial conditions in 0.01 min and held for 4 min before the next

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injection. The flow rate was 1.2 mL/min. The column was maintained at 40 °C. MS analysis was performed using a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with an APCI 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 1000. The heated capillary temperature was 250 °C, APCI vaporizer temperature 380 °C, sheath gas flow 60 units, auxiliary gas flow 5 units, capillary voltage 53 V, and source voltage 4.5 kV. In MS^2 analysis, the $[M + H]^+$ ions observed for each chromatographic peak in full-scan analysis were isolated and subjected to source collision-induced dissociation (CID) using He buffer gas. In all CID analysis, the isolation width, relative fragmentation energy, relative activation Q, and activation time were 1.4, 30 or 35%, 0.25, and 30 ms, respectively. Concentrations of all identified stilbenoids in the extracts were calculated by reference to peak areas (calibration curves) of corresponding pure standards at their UV absorption maxima or based on their published coefficients of molar extinction.[33] Other stilbenoids were suggested to have the same coefficient of molar extinction as arachidin-1 (**1**). Their concentrations throughout the text, expressed in mg/g, are given for the comparison purpose only and may not represent the true concentrations of these compounds.

Aflatoxin Analysis. Extracts of peanut cotyledons were prepared as described above and subjected to aflatoxin analysis using a Waters Acquity UPLC instrument equipped with a matching UPLC H-class Quaternary Solvent Manager, UPLC Sample Manager, UPLC Fluorescent Detector (FLR), and an Acquity UPLC BEH C18 2.1 mm × 100 mm, 1.7 μm column. The mobile phase was composed of a water/MeOH/CH_3CN (64:22:14, v/v/v) mixture, and the flow rate was 0.3 mL/min. The column was maintained at 40 °C in the system column heater. The excitation and emission wavelengths used for aflatoxin quantification were 362 and 435 nm, respectively. Concentrations of aflatoxins were determined by reference to peak areas of corresponding commercial standards (calibration curve). The detection limit for aflatoxins G_1 and B_1 was 0.15 and 0.02 ng/g for aflatoxins G_2 and B_2.

Aflatoxin analysis in fungal colonies growing on smudged plates (Figure 5A–C) was performed as described[36] with the exception of using MeOH instead of a MeOH–H_2O mixture for the extraction of fungal colonies. The colonies were cut with a spatula and extracted with MeOH followed by purification on a minicolumn packed with basic aluminum oxide.

Statistical Analysis. Spearman's rank correlation coefficients were calculated and plotted using the corrplot R package.[37] Analysis of variance was performed on raw or transformed variables, and means were compared using the Tukey test. All statistical analysis was conducted in R software.[38]

RESULTS AND DISCUSSION

Our previous research demonstrated a significant reduction in aflatoxin production, when selected *Aspergillus* toxigenic species were fed with major peanut stilbenoids added to the growth medium.[26] On the basis of these findings, inhibition of aflatoxin formation in *Aspergillus* species by live peanut seeds was suggested. The complexity of the peanut seed–*Aspergillus* interaction in the field proposes the use of laboratory experimental models with both living organisms interacting under preset controlled conditions.

After preliminary trials of three experimental setups, including those previously used at the NPRL,[22,35] we chose a new setup with precisely wounded peanut seeds (Figure 2A) placed on non-nutritious agar (Figure 3B). Water agar was used to maintain a sufficient moisture level favorable for the seed physiology throughout the experiment, and a drilled cavity allowed for a compact area for spore application and provided a sufficiently humid environment for the applied spores.

The experimental flowchart is presented in Figure 4. Peanut seeds need some time to produce phytoalexins in response to

Table 1. Frequency of Fungal Infection and Aflatoxin Production as the Outcome of Peanut–Fungus Interaction after 72 h of Incubation at 30 °C

time before inoculation (h)	number of spores	total number of seeds[a]	number of seeds with fungal growth (%)	number of seeds with aflatoxin B_1 (%)
		A. flavus NRRL 29487		
72	5	8	3 (37.5)	0 (0.0)
72	50	8	7 (87.5)	0 (0.0)
72	500	8	8 (100.0)	0 (0.0)
48	5	8	6 (75.0)	0 (0.0)
48	50	8	5 (62.5)	1 (12.5)
48	500	8	8 (100.0)	5 (62.5)
24	5	8	6 (75.0)	2 (25.0)
24	50	8	8 (100.0)	5 (62.5)
24	500	8	8 (100.0)	8 (100.0)
0	5	8	8 (100.0)	8 (100.0)
0	50	8	8 (100.0)	6 (75.0)
0	500	7	7 (100.0)	7 (100.0)
		A. flavus NRRL 3357		
72	5	8	1 (12.5)	0 (0.0)
72	50	8	3 (37.5)	0 (0.0)
72	500	8	3 (37.5)	1 (12.5)
48	5	8	2 (25.0)	0 (0.0)
48	50	8	8 (100.0)	3 (37.5)
48	500	8	8 (100.0)	1 (12.5)
24	5	8	2 (25.0)	0 (0.0)
24	50	8	8 (100.0)	3 (37.5)
24	500	8	8 (100.0)	2 (25.0)
0	5	8	7 (87.5)	5 (62.5)
0	50	8	8 (100.0)	8 (100.0)
0	500	8	8 (100.0)	7 (87.5)
		A. parasiticus NRRL 6111		
72	5	8	0 (0.0)	0 (0.0)
72	50	8	2 (25.0)	1 (12.5)
72	500	8	5 (62.5)	1 (12.5)
48	5	8	3 (37.5)	2 (25.0)
48	50	8	1 (12.5)	0 (0.0)
48	500	8	6 (75.0)	2 (25.0)
24	5	8	1 (12.5)	1 (12.5)
24	50	8	8 (100.0)	5 (62.5)
24	500	8	6 (75.0)	0 (0.0)
0	5	8	5 (62.5)	0 (0.0)
0	50	8	8 (100.0)	0 (0.0)
0	500	8	8 (100.0)	1 (12.5)
total number of seeds (%)				**287 (100)**
number of seeds with fungal growth (% of total)				**201 (70.0)**
number of seeds with aflatoxin B1 (% of seeds with fungal growth)				**85 (42.3)**

[a]Total number of seeds excluding controls, which were not inoculated with fungal spores and did not show the presence of aflatoxins.

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Table 2. Comparison of Mean (Standard Error) Values for All Phytoalexins and Aflatoxin B_1 (Figures 6 and 7)[a]

	phytoalexins (μg/g)					
	SB-1	AR-1	AR-3	IPD	total	AFL B_1(ng/g)
			A. flavus NRRL 29487			
			Time (h)			
0	*178.0 a (112.4)	204.8 b (26.8)	*104.3 a (20.6)	*160.4 a (12.5)	1139.0 ab (100.1)	*4440.7 a (1194.1)
24	*255.4 a (220.0)	379.9 a (33.2)	*52.4 ab (10.5)	*103.4 b (8.2)	1309.1 a (125.2)	*939.3 b (538.3)
48	*130.2 b (154.3)	388.5 a (22.0)	*33.3 b (5.8)	*51.7 d (5.2)	945.9 b (92.8)	*159.5 c (81.1)
72	*108.2 b (106.5)	338.5 a (27.0)	*45.6 ab (6.4)	*62.9 c (3.8)	933.0 b (87.7)	*0.0 c (0.0)
			Number of Spores			
0	*0.0 c (0.0)	267.7 b (20.0)	*10.2 b (0.8)	*60.3 b (7.2)	565.7 c (54.7)	*0.0 c (0.0)
5	*102.0 b (19.0)	337.4 ab (21.6)	*54.9 a (9.7)	*94.3 a (11.8)	928.5 b (80.4)	*947.8 b (437.5)
50	*227.8 a (24.6)	361.7 a (30.7)	*72.9 a (12.7)	*111.0 a (12.7)	1323.9 a (87.4)	*625.8 b (187.7)
500	*292.6 a (29.7)	337.9 ab (39.8)	*78.8 a (15.2)	*95.9 a (7.5)	1350.7 a (111.8)	*3306.6 a (1142.2)
			A. flavus NRRL 3357			
			Time (h)			
0	**185.3 a (28.2)	290.5 b (24.8)	*157.7 a (27.4)	*94.6 a (8.9)	*1195.6 a (123.4)	*2334.0 a (630.9)
24	**177.2 a (31.6)	326.0 ab (22.0)	*101.6 ab (23.2)	*72.6 ab (6.4)	*1097.5 a (126.3)	*356.5 b (180.9)
48	**111.3 b (21.2)	398.4 a (27.6)	*113.9 a (21.4)	*55.6 bc (4.3)	*1096.3 a (97.5)	*199.1 b (105.5)
72	**72.0 c (21.4)	391.1 a (28.7)	*59.2 b (16.8)	*51.1 c (4.3)	*913.5 a (88.0)	*42.4 b (42.4)
			Number of Spores			
0	**0.0 d (0.0)	233.9 c (15.2)	*7.5 c (0.8)	*34.5 c (3.4)	*447.4 c (33.9)	*0.0 c (0.0)
5	**55.1 c (12.0)	335.6 b (20.4)	*49.6 b (8.9)	*65.0 b (5.8)	*801.1 b (49.4)	*383.6 bc (246.9)
50	**174.4 b (19.1)	389.3 ab (24.3)	*168.8 a (22.9)	*82.8 ab (7.6)	*1328.3 a (82.8)	*1432.2 a (531.8)
500	**293.9 a (26.1)	421.3 a (30.8)	*190.6 a (26.6)	*86.7 a (6.2)	*1615.8 a (106.5)	*1078.8 ab (408.2)
			A. parasiticus NRRL 6111			
			Time (h)			
0	*68.9 a (13.1)	433.0 ab (42.5)	*106.6 a (17.1)	*172.1 a (15.7)	*1300.2 a (125.1)	*131.2 a (131.2)
24	*42.9 b (10.0)	483.3 a (37.7)	*127.0 a (26.3)	*77.6 b (6.0)	*1168.7 ab (109.8)	*119.4 a (44.9)
48	*21.9 b (5.1)	393.6 b (25.5)	*68.8 a (14.1)	*84.8 b (11.5)	*887.7 b (77.1)	*76.6 a (47.2)
72	*10.8 c (3.2)	463.0 ab (24.6)	*58.4 a (14.5)	*81.5 b (5.7)	*973.8 ab (59.5)	*10.7 a (7.9)
			Number of Spores			
0	*0.0 d (0.0)	284.1 c (38.1)	*11.1 c (3.7)	*88.1 b (13.0)	*647.0 c (86.6)	0.0 a (0.0)
5	*24.1 c (7.5)	427.8 b (31.4)	*63.2 b (15.0)	*122.1 a (15.8)	*1047.8 b (106.3)	52.0 a (35.4)
50	*38.5 b (5.7)	481.9 ab (26.3)	*121.2 a (21.4)	*102.9 ab (10.2)	*1168.2 ab (80.4)	104.7 a (41.1)
500	*79.8 a (13.2)	556.0 a (26.3)	*151.5 a (17.9)	*104.3 ab (11.6)	*1399.3 a (81.5)	168.2 a (133.6)

[a]Means followed by the same letter in the same column are not significantly different ($p < 0.05$). Analysis of variance was performed on raw or transformed variables, log or square root, marked * and **, respectively, but the values are presented as raw values in the table. Abbreviations: AR-1, *trans*-arachidin-1; AR-3, *trans*-arachidin-3; IPD, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; AFL B_1, aflatoxin B_1.

exogenous stimuli.[17,21,39] Therefore, wounded seeds were allowed up to 72 h of incubation (Figure 4) before exposing them to fungal spores, a stressful challenge that in most cases leads to increased production of stilbenoids.[21,22,39] At the beginning of the experiment, 3 μL of spore suspension from each dilution concentration, e.g., 5, 50, and 500 spores, was applied in marked sections of a PDA agar Petri dish (Figure 3A). The number of marked sections (288) was equal to the number of seeds that received fungal spores at matching concentrations. Figure 3A shows growth of the *A. parasiticus* NRRL 6111 isolate in all marked sections of the plate after incubation at 30 °C for 72 h. Similar growth was observed for all spore concentrations of all fungal isolates tested. This fact demonstrates the viability of fungal spores used for each individual seed at the time of the experiment. After 3 and 5 days of incubation at 30 °C, some colonies of each fungus tested were cut with a spatula from the marked sections of a PDA agar, extracted with MeOH, and purified.[36] The extracts of all fungal isolates showed the presence of aflatoxins at levels proportional to their NPRL toxigenic ratings.

Wounded seeds demonstrated different responses to the fungal presence that varied with time of incubation before inoculation with spores, number of spores applied, and the nature of the fungal strain used (Figure 2A–D). Of particular interest were the experimental seeds that were observed under 30× magnification and did not show signs of fungal growth (Figure 2A). Apparently, spore germination was temporarily suppressed, or the spores' vitality was destroyed permanently by the defensive peanut compounds. In order to ensure the nonviability/viability of these spores, all the seeds were smudged on PDA agar as described in the Materials and Methods and incubated. Panels A, B, and C of Figure 5 show an example of incubated plates with smudges made with cotyledons with initial 5, 50, and 500 spores applied to the seed wound, respectively. Apparently, some fungal spores from cotyledons were transferred onto the agar and germinated. Some fungal colonies were cut from the plates and prepared as described above for the aflatoxin analysis. All of the tested fungal colonies showed the presence of aflatoxins at concentrations comparable to those obtained from the marked sections. The result of this experiment allows one to suggest that wounded peanut seeds obtain appreciable fungistatic activity. Table 1 shows that about one-third (30%) of all experimental seeds did not demonstrate any visible fungal development.

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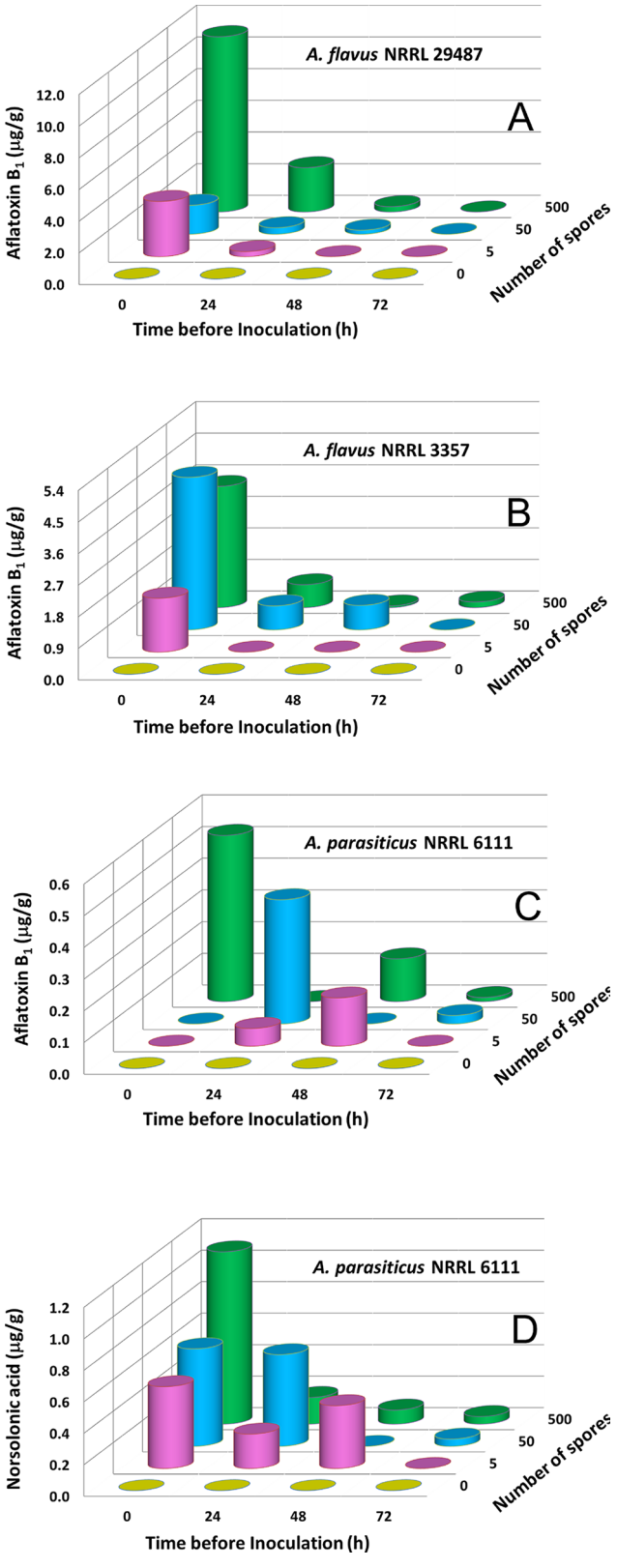

Figure 6. Production of aflatoxin B_1 and norsolorinic acid by *Aspergillus* species applied to wounded peanut seeds 0, 24, 48, and 72 h prior to their inoculation with 0, 5, 50, and 500 fungal spores: plate A, *A. flavus* NRRL 29487; plate B, *A. flavus* NRRL 3357; plate C, *A. parasiticus* NRRL 6111; plate D, production of norsolorinic acid by *A. parasiticus* NRRL 6111. The results were obtained after 72 h of incubation of infected seeds at 30 °C. Each bar presents data from eight experiments; statistical analysis is shown in Table 2.

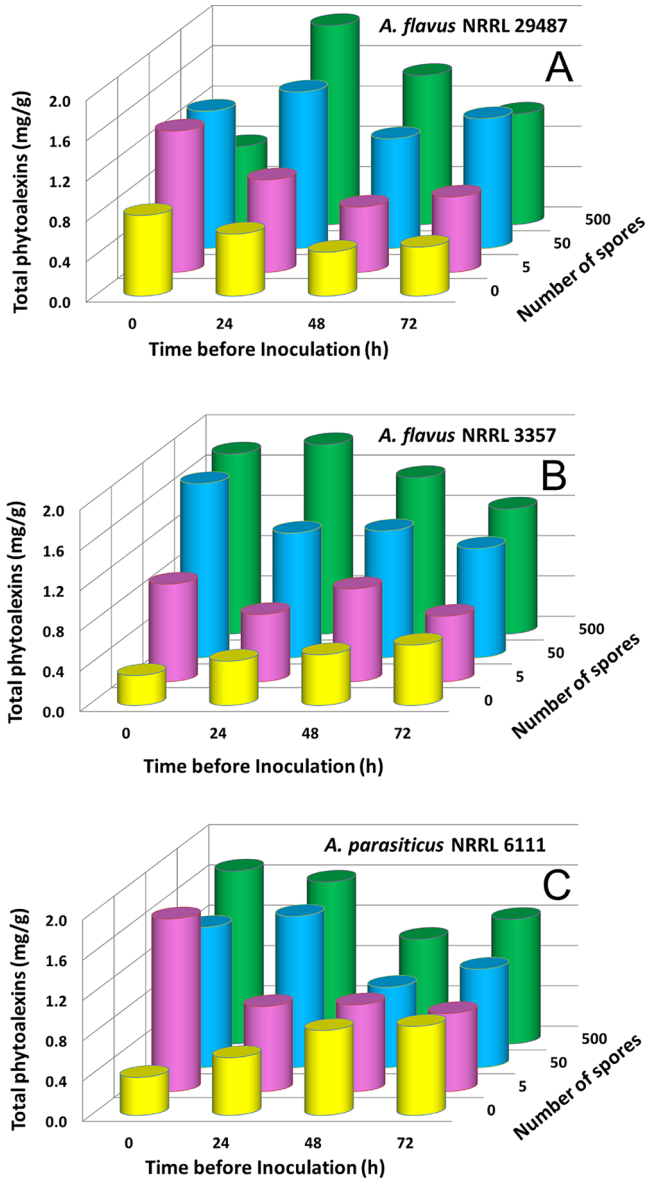

Figure 7. Total phytoalexin production by wounded peanut seeds incubated for 0, 24, 48, and 72 h prior to their inoculation with 0, 5, 50, and 500 fungal spores: plate A, *A. flavus* NRRL 29487; plate B, *A. flavus* NRRL 3357; plate C, *A. parasiticus* NRRL 6111. The results were obtained after 72 h incubation of infected seeds at 30 °C. Each bar presents data from eight experiments; statistical analysis is provided in Table 2.

Important results were obtained also from the aflatoxin and phytoalexin data analysis. At the end of the experiment (Figure 4), the data on production of aflatoxin B_1 and norsolorinic acid by the *Aspergillus* species tested, as well as production of major phytoalexins by the seeds, were statistically processed (Table 2) and are presented in a graphical format (Figure 6A–D). Aflatoxin B_2 was also detected, but the insignificant production of this toxin by the *A. flavus* strains and aflatoxins B_2 and G_2 by the *A. parasiticus* strain used in present research was not accounted here due to the simplicity of the presentation. Production of aflatoxin B_2 is shown in Figure A of the Supporting Information.

Higher aflatoxin production by the strains positively correlated with higher spore concentration and negatively correlated with longer incubation time before spore application

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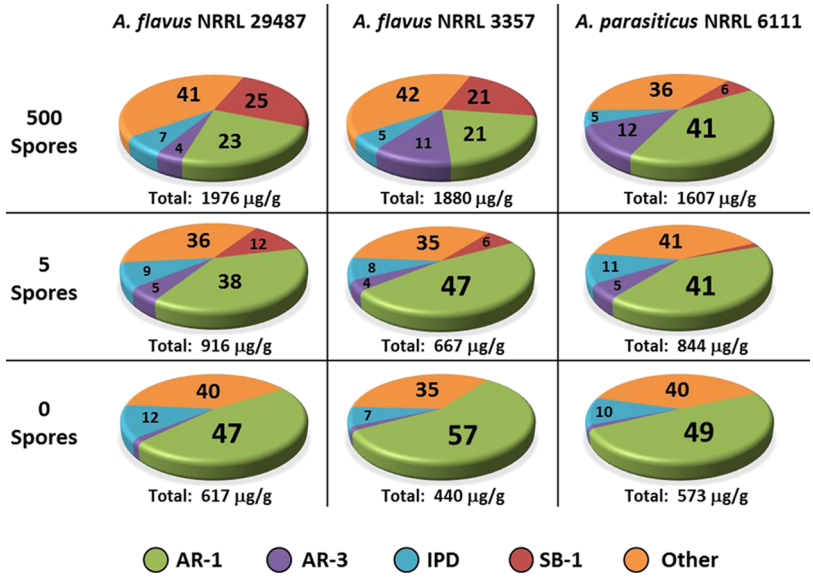

Figure 8. Phytoalexin composition in wounded seeds incubated for 24 h prior to application of different numbers of fungal spores. Abbreviations: AR-1, *trans*-arachidin-1; AR-3, *trans*-arachidin-3; IPD, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; other, all other stilbenoids. Each pie graph presents data from eight experiments; statistical analysis is provided in Table 2.

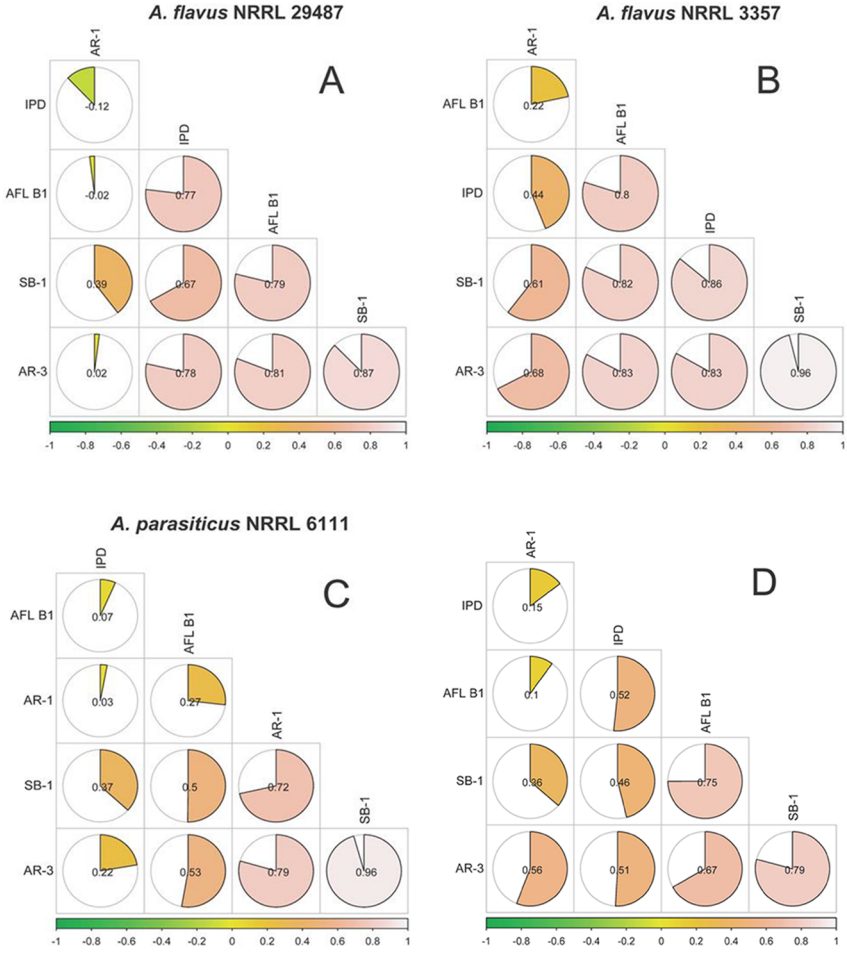

Figure 9. Correlograms demonstrate the correlation of a series of data obtained in the course of peanut−fungus interaction: plate A, with *A. flavus* NRRL 29487; plate B, with *A. flavus* NRRL 3357; plate C, with *A. parasiticus* NRRL 6111; plate D, shows combined data from the experiments with the three fungi. Abbreviations: AR-1, *trans*-arachidin-1; AR-3, *trans*-arachidin-3; IPD, *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene; AFL B1, aflatoxin B_1.

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(Figure 6A–C; Table 1). The same pattern was also observed for the first aflatoxin precursor, norsolorinic acid (**6**) (Figure 6D). The highest levels and frequency of aflatoxin production were observed when seeds were inoculated with fungal spores immediately after wounding them. In contrast, after appreciable incubation time, particularly after 72 h, aflatoxin and norsolorinic acid production was minimal or was completely suppressed (Figure 6A–D, Table 1, and Table A of the Supporting Information). Table A shows that in some experiments norsolorinic acid was produced by the *A. parasiticus* NRRL 6111 strain, while no aflatoxin production was detected in the same samples. We note, however, that the limited number of experiments does not allow one to make sound conclusions on the selective suppression of aflatoxin vs norsolorinic acid biosynthesis under the experimental conditions used.

Combined data, regardless of the fungal strains used, number of spores applied, and time of incubation before inoculation, demonstrate that of all the seeds that showed fungal growth, 57.7% were aflatoxin-free after 72 h of incubation (Table 1). Statistical comparison of mean values for aflatoxin B_1 is given in Table 2. The most significant difference in aflatoxin levels in the *A. flavus* NRRL 29487 and *A. flavus* NRRL 3357 was observed between 0 (no incubation time before spore inoculation) and longer incubation times, 24, 48, and 72 h (Table 2). This fact suggests the influence of peanut stilbenoids on the mechanism of plant–fungus interaction.

Total phytoalexin production by wounded peanut seeds at different incubation times and spore concentrations in response to fungal challenge is shown in Figure 7A–C and Table 2. With all three fungal isolates, a significant difference was most noticeable between the controls (no fungal spores) and the experimental seeds (Table 2). In contrast, in most of the experiments, there was no significant difference between total phytoalexin concentrations within the experimental time and the number of spores applied (Table 2). These data allow one to conclude that regardless of the toxigenic potential of the fungi tested and the concentrations of their spores that contact the seed wound, as well as the incubation time, overall phytoalexin production stays within insignificantly different limits (Table 2). However, there was a significant difference in the composition of individual stilbenoids (Figure 8 and Figure B of the Supporting Information). For example, after 24 h of incubation prior to spore application, arachidin-1 (**1**) was the predominant stibenoid in the extracts of individual control seeds, while SB-1 (**4**) appeared only in the presence of growing fungi or fungal spores, and the ratio of **1** in the matrix was decreased in favor of **4** from the controls to seeds with higher number of spores, although application of only five fungal spores was sufficient to cause the formation of SB-1 (**4**). Orange sectors in the pie charts (Figure 8 and Figure B of the Supporting Information) represent the sum of other than listed (Figure 1) stilbenoids (Material and Methods).

The interrelationship between all experimental data is shown in Figure 9; the correlograms demonstrate the simultaneous change in value of all the variables obtained in the course of peanut–fungus interaction. The analysis of data correlation from all experiments with individual fungal isolates (Figure 9A–C) as well as combined data correlation (Figure 9D) allows one to conclude, for example, that there was a strong positive correlation between aflatoxin B_1, arachidin-3, IPD, and SB-1 in the experiments with *A. flavus* NRRL 29487 and *A. flavus* NRRL 3357. On the other hand, aflatoxin B_1 practically did not correlate with the major stilbenoid arachidin-1 (**1**) in all the experiments with *A. flavus* NRRL 29487 and moderately correlated with in the experiments with *A. flavus* NRRL 3357 and *A. parasiticus* NRRL 6111 (Figure 9A–C). Of particular interest is a very high correlation level between arachidin-3 (**2**) and SB-1 (**4**) in all experiments with all fungal isolates. Such a correlation may be attributed to a probable conversion of stilbenoid **2** into **4** through **1** within the time frame of the experiments, as earlier suggested.[32] In agreement with our previous research,[26] production of aflatoxin B_1 in some experiments did not necessarily positively correlate with the degree of fungal colonization of wounded seeds as apparent from Table A of the Supporting Information.

The present research demonstrated that in the course of peanut–fungus interaction, in the majority of experiments, aflatoxin production was suppressed in all fungi tested when lower concentrations of spores were applied to wounded seeds; with all fungi tested, higher concentrations of aflatoxin B_1 (**5**) were produced with a higher concentration of spores and shorter incubation times before spore application; production patterns of aflatoxin B_2 and the primary aflatoxin precursor, norsolorinic acid (**6**), were very similar to that of aflatoxin B_1; of all experimental seeds that showed fungal growth, 57.7% were aflatoxin-free after 72 h of incubation; overall production of stilbenoids was not significantly different between experiments with different fungi tested; concentrations of stilbenoids in wounded peanuts increased with higher concentrations of spores contacting them; compared to the control, the composition of stilbenoids changed significantly upon interaction with fungal structures; phytoalexin profiles and production changed with incubation time before fungal application and the number of spores applied; formation of SB-1 (**4**) was observed only in the presence of fungal spores or actively growing fungi; arachidin-1 (**1**) was the predominant phytoalexin in all the experiments with the exception of the experiment with the most aggressive fungus, *A. flavus* NRRL 29487, 500 spores used, and zero and 24 h of incubation before spore application.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b01969.

Figures showing the production of aflatoxin B_2 by *Aspergillus* species and the phytoalexin composition in wounded seeds after the application of fungal spores (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DAD, diode array detector; HPLC, high-performance liquid chromatography; UPLC, ultra-performance liquid chromatography; MS, mass spectrometer, mass spectrometry, mass spectra; APCI-MS, atmospheric pressure chemical ionization mass spectrometry, mass spectrum

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