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Aflatoxin Production in Six Peanut (*Arachis hypogaea* L.) Genotypes Infected with *Aspergillus flavus* and *Aspergillus parasiticus*, Isolated from Peanut Production Areas of Cordoba, Argentina

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Aflatoxin contamination is one of the main factors affecting peanut seed quality. One of the strategies to decrease the risk of peanut aflatoxin contamination is the use of genotypes with resistance to *Aspergillus* infection. This laboratory study reports the resistance to *Aspergillus* infection and aflatoxin contamination of six peanut genotypes inoculated with 21 *Aspergillus* isolates obtained from the peanut production region of Cordoba, Argentina. The resistance was investigated in the seed coat and cotyledons of three resistant genotypes (J11, PI 337394, and PI 337409) and three breeding lines (Manfredi 68, Colorado Irradiado, and Florman INTA) developed at the Instituto Nacional de Tecnologia Agropecuaria (INTA), Manfredi Experimental Station, Cordoba, Argentina. Resistance to fungal colonization and aflatoxin contamination was found to be associated with seed coat integrity in the PI 337394, PI 337409, and J11 genotypes, whereas the INTA breeding lines such as Colorado Irradiado showed a moderate resistance and the Manfredi 68 and Florman INTA genotypes the least resistance. Furthermore, another type of resistance associated with cotyledons was found only in the PI 337394 genotype.

KEYWORDS: Aflatoxins; peanut; *Arachis hypogaea*; *Aspergillus* colonization

INTRODUCTION

Aflatoxin contamination is one of the main factors affecting peanut seed quality. Aflatoxins are toxic secondary metabolites produced by *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare, which are potent carcinogens for animals and in humans have been linked to liver cancer (*1*, *2*). Aflatoxin contamination can occur either before or after the crop is harvested. Preharvest contamination is actually the major economic problem in the peanut industry (*3*). One of the strategies to decrease the risk of peanut aflatoxin contamination is the use of genotypes with resistance to *Aspergillus* infection. The resistance to *Aspergillus* infection in the peanut fruit can be localized in the shell, seed coat, and cotyledons (*4*−*6*).

During the second half of the 20th century, peanut improvement programs were developed, based on the selection of genotypes according to the resistance to *Aspergillus* infection and aflatoxin contamination. Studies performed to detect resistant genotypes can be made with field assays and laboratory assays in which the levels of *Aspergillus* colonization and aflatoxin production on the peanut kernels are measured (*7*, *8*). In field assays, natural contamination and artificial contamination produced by direct inoculation of *Aspergillus* spores are evaluated. These field assays are very representative, but their disadvantages are the annual variability influenced by the seasonal weather conditions and the capability of performing only one study each year. The laboratory assay allows a larger number of studies to be carried out under controlled conditions and a lower variation of aflatoxin level than in the field assays.

The objective of this study was to evaluate in a laboratory assay the resistance to *Aspergillus* infection and aflatoxin contamination in six peanut genotypes inoculated with 21 *Aspergillus* isolates obtained from the peanut production region of Cordoba, Argentina. It was also proposed to investigate the seed coat and cotyledon participation in the seed resistance of these genotypes. The genotypes selected for this study were three lines (J11, PI 337394, and PI 337409) characterized as resistant to *Aspergillus* infection (*8*−*10*) and three breeding lines (Manfredi 68, Colorado Irradiado, and Florman INTA) with unknown susceptibility, developed at the Instituto Nacional de Tecnologia Agropecuaria (INTA), Manfredi Experimental Station, Cordoba, Argentina.

MATERIALS AND METHODS

Source of Seed. The genotypes used in this study were (a) Junagah 11 (J11), a commercial cultivar from India; (b) PI 337394 and PI

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337409, genotypes that were discovered in northeastern Argentina and central Paraguay and introduced in the germplasm collection of the U.S. Department of Agriculture; (c) Florman INTA, obtained by a selection of Florunner over 9 years; (d) Colorado Irradiado INTA, obtained by a mutation carried out by irradiation with X-rays in Colorado INTA; and (e) Manfredi 68 INTA, a cross between Maní negro 4 (Corrientes, Argentina) and Fla. 249-40-B3 (United States). The seeds samples of all of the tested genotypes were provided by Manfredi Experimental Station, INTA, and obtained from the same growing season. The genotypes were planted in a two-row plot 10 m long, with an interrow distance of 0.70 m at 1 seed/20 cm linear row, in a completely randomized block design with two replicates. In turn, to eliminate all weeds, preplant (Imazetapir 100 cm^3 of ai/ha) and postemergence (Cletodin 175 cm^3 of ai/ha) herbicides were used. Leaf spot was avoided using contact fungicide (Mancozeb 1 kg of ai/ha). Two complementary irrigations (50 mm) were carried out in February and March to avoid drought stress. Each genotype was manually harvested at its optimum maturity and threshed. Harvested pods, naturally dried to 5.5% moisture, were hand sorted to remove and discard visibly damaged pods and stored in bags in a seed chamber at 7 ± 3 °C until use. Pods were hand-shelled before the experiments were performed.

Natural infection was evaluated by using the "blotter test" according to ISTA rules (*11*). Two hundred seeds of each genotype were used in four replicates. The percentage of colonized seed was calculates; a 5% maximum level of natural contamination was considered to be acceptable according to the findings of Mehan and MacDonald (*12*).

***Aspergillus* Isolates.** A total of 21 isolates of *A. flavus* and *A. parasiticus* obtained from the peanut seeds of plants developed in the production region of the Province of Cordoba, Argentina, were selected according to infective and toxigenic efficiency of an *Aspergillus* populations study. The fungi were isolated from peanut hull and kernel and identified and classified by Dr. Vaamonde (*13*).

Seed Treatment. The resistance study of the seed coat was performed by selecting mature seeds with undamaged testas (whole seeds). The resistance study in cotyledons was carried out by slicing the kernels into halves (sliced seeds). The seeds selected for the assays were surface sterilized by immersion in 0.5% sodium hypochlorite solution for 3 min (*14*), rinsed four times in sterile water, and hydrated to reach a 20% w/w moisture content (*12*).

Seed Infection. Conidial suspensions were produced from a 10-day-old culture grown in potato dextrose agar slant. Conidia were suspended in 0.05% v/v Tween 20, and spore concentrations were determined by a hematocytometer. The conidial suspensions were diluted to obtain 1×10^6 spores/mL in 0.05% Tween 20.

Twenty grams of both whole and sliced peanut seed was placed in a 200 mL sterile flask and inoculated with 1 mL of conidial suspension. The flasks were shaken to get a homogeneous spore distribution and incubated at 30 °C for 8 days (whole seeds) or 16 days (sliced seeds). The sliced seeds were infected with 21 *Aspergillus* isolates, and the whole seeds were infected with 9 of 21 isolates chosen in a random sample selection. The infection experiment of the sliced seeds was conducted once, whereas the whole seeds experiment was conducted twice.

Measurement of Fungal Colonization. Fungal colonization in whole seeds was determined by visual inspection using a scale from 1 to 5 (1, 0−20%; 2, 21−40%; 3, 41−60%; 4, 61−80%; 5, 81−100% of the kernel surface covered by conidiophores bearing conidia) (*15*).

Fungal colonization on sliced seeds was determined by ergosterol analysis (*16*) due to abundant *Aspergillus* growth. For this assay, 7 of 21 isolates that infected the genotypes were chosen in a random sample selection and used for ergosterol analysis. Briefly, the samples were ground and a weighed portion (20−40 mg) was placed in 17 mL culture tubes and saponified with 2 mL of methanol and 0.5 mL of 2 M sodium hydroxide using microwaves. Then, the ergosterol was extracted with 3×3 mL of pentane and evaporated, and the residue was dissolved in methanol. The ergosterol was separated and measured by HPLC using a Hewlett-Packard chromatograph with a Hypersil ODS column (125×4 mm i.d., 5 μm, Hewlett-Packard). The ergosterol was eluted with acetonitrile/methanol (80:20, v/v) and detected with an Uvis 200 UV detector (Konik, Barcelona, Spain) at 282 nm. The identification and quantification were carried out by comparison with the retention times and areas of the ergosterol standards (Fluka Chemie Gmbh, Buchs, Switzerland). A daily calibration curve was carried out with ergosterol solutions with a concentration range between 0.1 and 20 μg/mL. Each sample was analyzed in duplicate. Confirmation of ergosterol in the sample was obtained by UV spectra determinations in a photodiode array spectrophotometer, Multispec-1501 (Shimadzu), and co-injection.

Aflatoxin Analysis. The aflatoxins were extracted from infected seeds (20 g each) with 100 mL of 60% (v/v) methanol in water and 4 g of NaCl (extraction solvent) in a tissue homogenizer (Sorvall Omni-Mixer) for 3 min. The homogenized samples were centrifuged at 3000*g* for 5 min at 4 °C, and the supernatant was filtered through a Whatman no. 1 filter and a Vicam microfiber filter.

The cleaning up of the extract for HPLC was carried out with an Aflatest P immunoaffinity column (55×5 mm i.d., Vicam, Watertown, MA) using the manufacturer's procedure. The sample solution was passed through the columm with a flow of 1 drop per second. The aflatoxins were eluted with acetonitrile (HPLC grade), and the eluate was evaporated to dryness on a rotary evaporator, derivatized by adding 50 μL of trifluoroacetic acid (Fluka Chemie Gmbh) and stirring with a vortex mixer for 30 s, and evaporated again. The residue was dissolved in 200 μL of injection solution (acetonitrile/water, 90:10, v/v). Derivatized samples were subsequently injected (20 μL) into the HPLC system. The chromatography was carried out with a Hewlett-Packard 1100 chromatograph equipped with a fluorescence detector (excitation = 360 nm, emission = 440 nm), HP ChemStation Software integrator and a 250×4.6 mm i.d., 5 μm, Hypersil ODS column, using the AOAC−IUPAC method (*17*) modified to separate and detect the aflatoxins in peanuts. The mobile phase consisted of acetonitrile/methanol/water (25:25:50, v/v), and the flow rate was of 0.8 mL/min. The identification and quantification were carried out by comparison with the retention times and areas of the aflatoxin B_1, B_2, G_1, and G_2 standards (Sigma-Aldrich, St. Louis, MO). A daily calibration curve was carried out with aflatoxin solutions with a concentration range between 10 and 150 ng/mL for B_1 and G_1 aflatoxins and between 5 and 25 ng/mL for B_2 and G_2 aflatoxins. Each sample was analyzed in duplicate. Confirmation of aflatoxin in the sample was obtained by co-injection with the standards.

Statistical Analysis. Principal component analysis (PCA) and biplot graphics were carried out as an exploratory tool to analyze the relationship between genotypes and isolations. The presence of isolate−genotype interaction is interpreted as the consistent lack of the differences among genotypes across the *Aspergillus* isolates. As the sliced seed infection was made once, the comparison of aflatoxin contamination and fungal colonization (in sliced seeds) among genotypes was performed with the Friedman test followed by multiple-comparison tests (*18*). The isolates that were found to be interacting with some genotypes in the PCA were not considered in the comparison of the genotypes by the Friedman test.

Fungal colonization data in whole seeds were analyzed using a proportional odds model (*19*), a specific method for ordinal variables that assumes that there exists an underlying continuous variable that generated the five categories. It models the probability that the response is below a category c, $c = 1, ..., 4$. In this case, a model of principal effects of genotypes and isolations was adjusted; that is, if y_{ijk} is the response of the kth replication ($k = 1, 2$) in the combination of the ith genotype and the jth isolation, then

$$\text{logit}[P(y_{ijk} \leq c)] = \theta_c + \alpha_i + \beta_j$$

where θ is the "cutpoint" for category c, α_i is the effect of the ith genotype, and β_j is the effect of the jth isolation. The parametrization used considers both the 5th isolate and the PI 337394 genotype as baseline levels. Therefore, $\exp(\alpha_i)$ is the ratio of the odds of being below a certain category instead of being above that category between the ith and PI 337394 genotypes. If this value is significantly greater than unity, the odds are higher for the ith genotype than for the PI 337394 genotype; if it is equal to unity, both genotypes have similar resistance to isolates, and if it is lower than unity the odds are higher for the PI 337394 genotype than for the ith genotype.

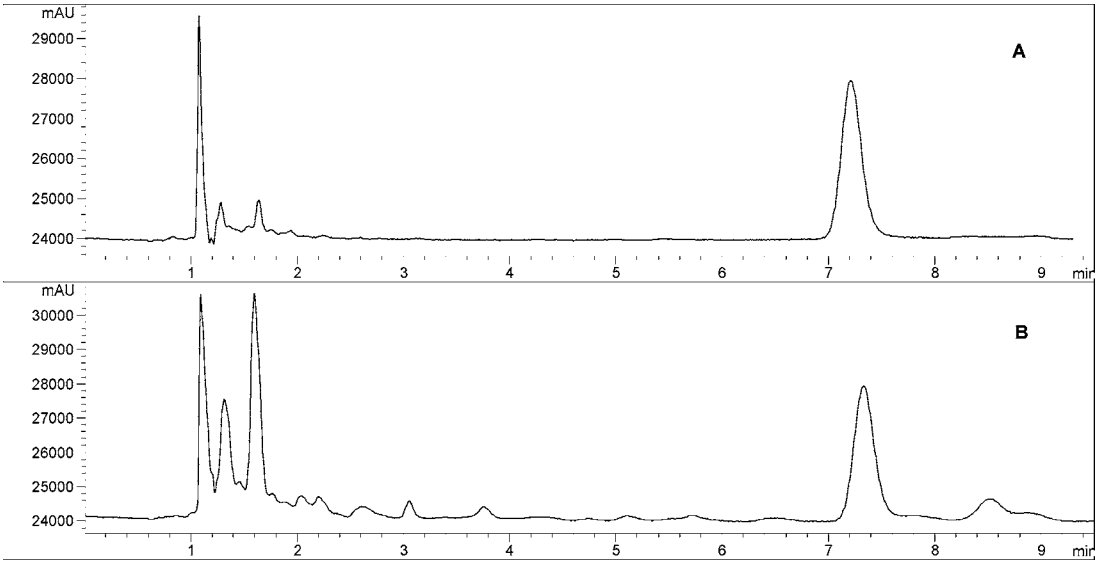

Figure 1. Chromatograms of ergosterol analysis by HPLC: (**A**) chromatogram of ergosterol standard; (**B**) chromatogram of peanut seed infected with *Aspergillus*. The retention time of ergosterol peak was 7.3 min.

Table 1. Fungal Colonization Modes on Whole Peanut Seeds from Six Genotypes Inoculated with Four *A. flavus* and Three *A. parasiticus* Isolates

isolate[a]	Florman INTA[b]	Manfredi 68	Colorado Irradiado	J11	PI 337409	PI 337394
6 (AF)	4[c]	5	5	1	2	1
	5	4	1	4	1	1
7 (AF)	5	1	2	2	1	1
	5	1	5	1	1	1
11 (AF)	4	3	3	1	1	2
	4	3	2	1	1	1
14 (AF)	4	3	1	1	1	1
	5	5	3	1	1	1
15 (AP)	5	4	2	1	1	1
	5	5	3	1	1	1
17 (AP)	4	5	1	1	1	1
	5	1	2	1	1	1
21 (AP)	4	5	3	1	1	1
	4	5	1	1	1	1

[a] *A. flavus* (AF) and *A. parasiticus* (AP) isolates. [b] Peanut genotypes. [c] Mode of fungal colonization rating from 1 to 5 (1, 0–20%; 2, 21–40%; 3, 41–60%; 4, 61–80%; 5, 81–100% of the kernel surface covered by conidiophores bearing conidia).

Contrasts for specific comparisons among genotype levels were also estimated.

RESULTS

Fungal Colonization Comparison among Different Genotypes. The result of the natural infection study by blotter test in the genotypes seeds was lower than 2%, which was considered to be satisfactory.

In the whole seed assay, the fungal colonization modes for each replicate are presented in **Table 1**. The model of principal effects of genotypes and isolations was suitable (deviance = 115.05, df = 199, $p = 1$); therefore, we can assume that interaction terms are not significant. PI 337409 and J11 genotypes were not significantly different from the PI 337394 genotype ($p = 0.975$ and 0.493, respectively). Moreover, the hypothesis of no differences among these three genotypes was not rejected ($p = 0.693$). This group was characterized by a low percentage of fungal colonization.

There were strong differences between PI 337394 and Manfredi 68 and Florman INTA genotypes. Precisely, the estimated odds of being below a certain category instead of being above that category for Manfredi 68 and Florman INTA were 0.004 ($p < 0.001$) and 0.002 ($p < 0.001$) times lower than for PI 337394, respectively. Manfredi 68 and Florman INTA were the most affected genotypes, and there were not significant differences between them ($p = 0.212$).

Table 2. Ergosterol Levels (Micrograms per Gram) on Sliced Peanut Seeds from Six Genotypes Inoculated with Four *A. flavus* and Three *A. parasiticus* Isolates

isolate[a]	Florman INTA[b]	Manfredi 68	Colorado Irradiado	J11	PI 337409	PI 337394
7 (AF)	1548	2955	1701	860	1782	766
8 (AF)	3049	3410	4835	6024	7885	1374
11 (AF)	6660	7228	5347	5545	5314	3769
13 (AF)	5521	4631	2891	4310	4101	1993
15 (AP)	3480	4945	2997	4236	2815	3760
16 (AP)	258	1160	2214	532	430	4792
17 (AP)	5834	5488	5535	5250	5478	3660

[a] *A. flavus* (AF) and *A. parasiticus* (AP) isolates. [b] Peanut genotypes.

Colorado Irradiado genotype was significantly different from Manfredi 68 ($p = 0.035$), Florman INTA ($p = 0.002$), and PI 337394 ($p < 0.001$), showing moderate fungal growth. For instance, the odds of being below a certain category instead of being above that category for Colorado Irradiado were 0.016 times lower than for PI 337394 and 8 times higher than for Florman INTA genotype.

The ergosterol chromatograms of HPLC analysis of a standard and sample are shown in **Figure 1**. The ergosterol concentrations in sliced seeds of the peanut genotypes infected with seven *A. flavus* and *A. parasiticus* isolates are shown in **Table 2**. The RSD% of the ergosterol determination in the duplicates was between 2 and 14%, with a mean of 8%. The PCAprincipal component analysis of the sliced seeds assay is shown in a biplot graphic (**Figure 2**) that includes vectors that represent the genotypes and the objects that represent the isolates. The isolates close to the graphic center have little interaction with the genotypes, and those that are distant from the center or origin and in the same direction with some genotype have positive interaction with each other. The biplot graphic made up of the two first principal components (PC1 and PC2) accounted for 88% of the total data variation (**Figure 2**). The differences

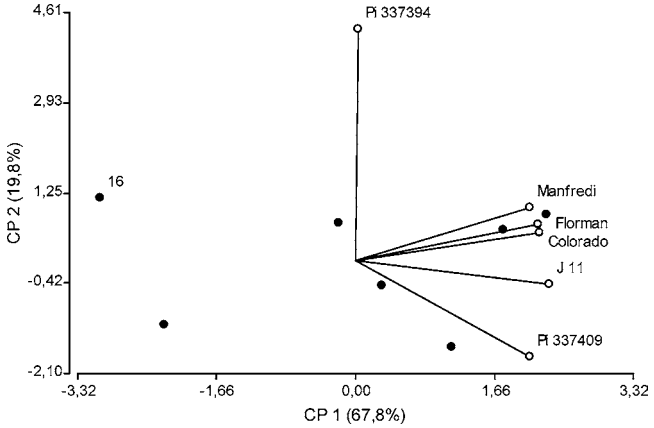

Figure 2. Biplot of the first two principal components based on the fungal colonization in sliced seeds by seven *Aspergillus* isolates. Vectors represent the genotypes, and full circles represent the *Aspergillus* isolates.

among genotypes were consistent across most of the isolates except for the 16th isolate, which was positively associated with a greater fungal colonization in the PI 337394 genotype.

Significant differences were obtained by the Friedman test ($p = 0.024$) among the genotypes. These differences were observed between the PI 337394 genotype (with the lowest ergosterol amount) and J11, Florman INTA, and Manfredi 68 (with the highest ergosterol amount). The Colorado Irradiado and PI 337409 genotypes did not show significant differences with the remainding genotypes.

Aflatoxin Contamination Comparison among Different Peanut Genotypes. Aflatoxin separation by HPLC is shown in **Figure 3**. Total aflatoxin production and the percentage of aflatoxin composition produced on whole seeds of the peanut genotype are shown in **Tables 3** and **4**, respectively. The PCA of aflatoxin production in six peanut genotypes by nine *Aspergillus* isolates in the whole seeds assay is represented in the biplot graphic made up from the first and second principal components (PC1 and PC2) (**Figure 4**). It accounted for 78% of the total data variation. Positive interactions were detected among the 7th isolate and J11−Florman INTA, the 11th isolate and PI 337394−Colorado Irradiado, and the 21st isolate and Manfredi 68−PI 337409 genotypes, respectively.

The Friedman test detected significant differences in the mean aflatoxin contamination among genotypes ($p = 0.01$). The multiple-comparison procedure showed two different clusters; the first cluster consisted of PI 337409, J11, and PI 337394 genotypes, with the lowest aflatoxin contamination, and the second cluster consisted of Colorado Irradiado, Manfredi 68, and Florman INTA, with the highest aflatoxin contamination.

Total aflatoxin production and the percentage of aflatoxin composition produced on sliced seeds of the peanut genotypes are shown in **Tables 5** and **6**, respectively. The RSD% of the aflatoxin determination in the duplicates was between 3.8 and 16%, with a mean of 10%. The PCA of the sliced seeds assay is represented by a biplot graphic made up from the two first principal components (PC1 and PC2) and accounted for 87% of the total data variation (**Figure 5**). The differences of aflatoxin

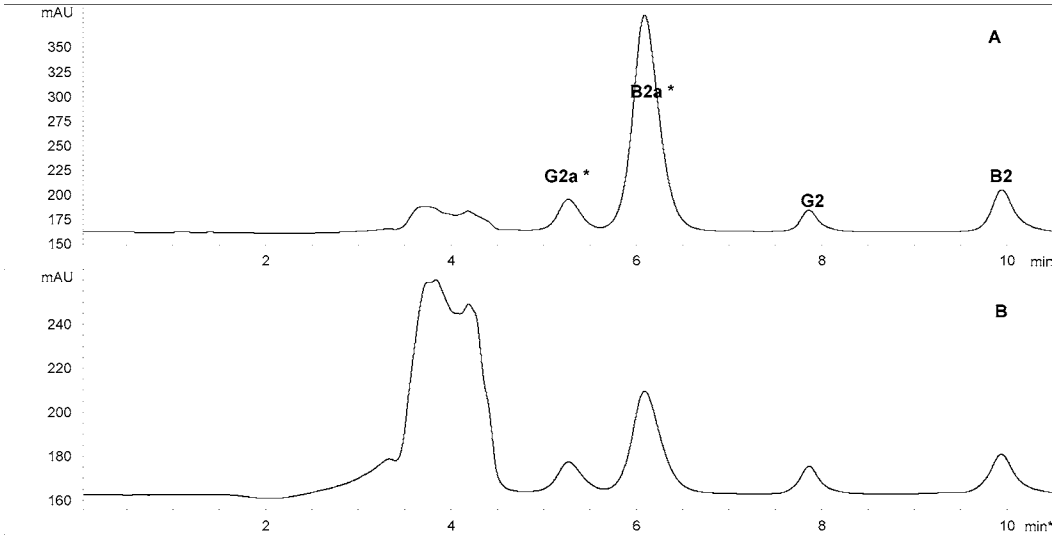

Figure 3. Chromatograms of aflatoxin determination by HPLC: (**A**) chromatogram of aflatoxins standards; (**B**) chromatogram of peanut seed infected with *A. parasiticus*. G2a, derivatized aflatoxin G_1; B2a, derivatized aflatoxin B_1.

Table 3. Total Aflatoxin Levels (Nanograms per Gram) on Whole Seed from Six Peanut Genotypes Inoculated with Five *A. flavus* and Four *A. parasiticus* Isolates

	Florman INTA[a]		Manfredi 68		Colorado Irradiado		J11		PI 337409		PI 337394	
isolate[b]	mean[c]	RSD%[d]	mean	RSD%	mean	RSD%	mean	RSD%	mean	RSD%	mean	RSD%
5 (AF)	1226	76.1	203	11.4	3500	71.8	6	5.2	698	85.2	8	54.4
6 (AF)	6105	21.4	6896	13.4	3662	42.1	4893	89.5	1021	68.5	3958	89.0
7 (AF)	39609	88.2	1232	68.9	1381	60.1	29877	98.0	43	97.3	36	80.4
11 (AF)	3841	55.5	6063	64.0	5943	84.1	3149	81.4	2368	72.3	41337	90.3
14 (AF)	4114	82.1	3179	46.0	4788	62.1	3601	58.8	76	37.5	516	53.5
15 (AP)	5127	9.4	321	95.4	5506	52.0	79	97.0	115	85.2	44	37.9
17 (AP)	3469	69.1	9836	61.0	3406	10.9	103	13.5	874	90.4	9991	61.9
18 (AP)	44885	32.8	660	76.5	2958	70.6	6	73.1	11	32.8	30	0.4
21 (AP)	24727	32.6	26602	75.6	4899	42.5	3999	9.6	4154	63.6	17282	72.1

[a] Peanut genotypes. [b] *A. flavus* (AF) and *A. parasiticus* (AP) isolates. [c] Mean of two replications. [d] Relative standard deviation.

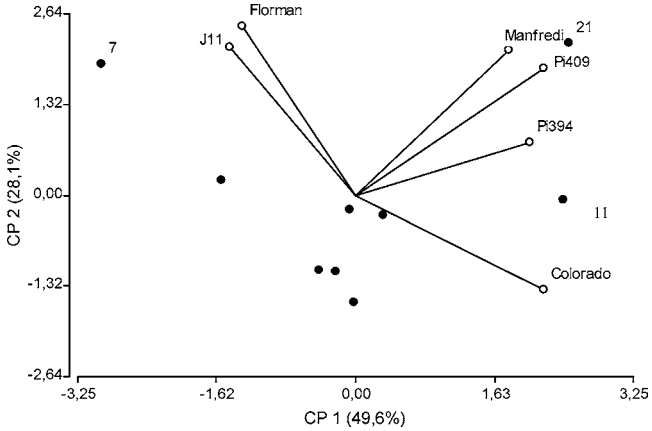

Figure 4. Biplot of the first two principal components based on the aflatoxin production in whole seeds by nine *Aspergillus* isolates. Vectors represent the genotypes, and full circles represent the *Aspergillus* isolates.

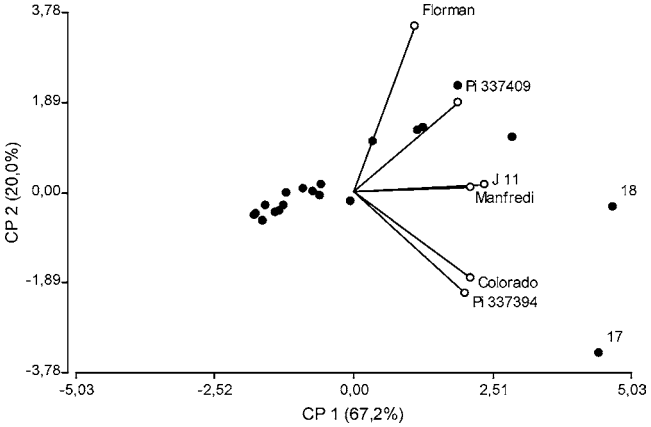

Figure 5. Biplot of the first two principal components based on the aflatoxin production in sliced seeds by 21 *Aspergillus* isolates. Vectors represent the genotypes, and full circles represent the Aspergillus isolates.

Table 4. Percentage of Individual Aflatoxin Produced on Whole Peanut Seed by *A. flavus* and *A. parasiticus* Isolates

	aflatoxin B_1		aflatoxin B_2		aflatoxin G_1		aflatoxin G_2	
isolate[a]	mean[b]	SD[c]	mean	SD	mean	SD	mean	SD
5 (AF)	93.1	1.1	6.9	1.1	ND[d]		ND	
6 (AF)	95.2	2.6	4.8	2.6	ND		ND	
7 (AF)	91.4	4.5	8.6	4.5	ND		ND	
11 (AF)	93.8	2.3	6.2	2.3	ND		ND	
14 (AF)	99.1	1	0.9	1	ND		ND	
15 (AP)	77.8	4.1	10.8	2.8	11.1	7.3	0.3	0.3
17 (AP)	65.7	6.9	2	1	28.7	4.2	3.5	1.8
18 (AP)	67.6	24	1.8	4.6	30.2	20	0.4	0.2
21 (AP)	66.9	6.6	2.7	1.1	27.3	5.1	2.9	2.3

[a] *A. flavus* (AF) and *A. parasiticus* (AP) isolates. [b] Mean of individual aflatoxin percentage produced on six peanut genotypes. [c] Standard deviation. [d] Not detectable.

Table 5. Total Aflatoxin Levels (Nanograms per Gram) on Sliced Seeds from Six Peanut Genotypes Inoculated with 14 *A. flavus* and 7 *A. parasiticus* Isolates

isolate[a]	Florman INTA[b]	Manfredi 68	Colorado Irradiado	J11	PI 337409	PI 337394
1 (AF)	2440	516	1160	1410	745	389
2 (AF)	633	331	991	360	256	125
3 (AF)	2211	2734	4900	1208	3346	519
4 (AF)	4787	1664	4133	1301	7611	2108
5 (AF)	327	152	275	110	390	123
6 (AF)	169	217	105	417	229	120
7 (AF)	1027	860	3315	622	2985	1274
8 (AF)	14126	6785	6389	12130	8681	1676
9 (AF)	3118	5709	14402	7183	9355	2210
10 (AF)	5852	3304	9210	4333	7203	949
11 (AF)	27826	13727	19196	12657	13954	3409
12 (AF)	19300	6000	19479	14938	12183	2955
13 (AF)	6160	3573	6036	4226	4025	1887
14 (AF)	560	335	5249	294	16	281
15 (AP)	18250	11900	11800	9550	12697	4538
16 (AP)	1952	15800	73257	22597	7593	22860
17 (AP)	5832	38587	34168	29033	20930	10138
18 (AP)	4749	2072	416	2027	3252	1462
19 (AP)	7224	4191	3675	6533	1544	3489
20 (AP)	12060	9330	24666	18767	31489	7331
21 (AP)	1134	1275	5073	1899	2981	392

[a] *A. flavus* (AF) and *A. parasiticus* (AP) isolates. [b] Peanut genotypes.

contamination among genotypes were consistent in most of the isolates with the exception of the 17th and 18th isolates that were positively associated with a higher aflatoxin production

Table 6. Percentage of Individual Aflatoxin Produced on Sliced Peanut Seeds by *A. flavus* and *A. parasiticus* Isolates

	aflatoxin B_1		aflatoxin B_2		aflatoxin G_1		aflatoxin G_2	
isolate[a]	mean[b]	SD[c]	mean	SD	mean	SD	mean	SD
1 (AF)	95.0	2.7	5.0	2.7	ND[d]		ND	
2 (AF)	95.1	2.8	4.9	2.8	ND		ND	
3 (AF)	99.0	1.3	1.0	1.3	ND		ND	
4 (AF)	99.1	1.0	0.9	1.0	ND		ND	
5 (AF)	91.8	5.1	8.2	5.1	ND		ND	
6 (AF)	98.0	3.0	2.0	3.0	ND		ND	
7 (AF)	91.5	2.6	8.5	2.6	ND		ND	
8 (AF)	94.8	0.7	5.2	0.7	ND		ND	
9 (AF)	96.4	2.9	3.7	2.9	ND		ND	
10 (AF)	99.1	0.9	0.9	0.9	ND		ND	
11 (AF)	92.2	1.5	7.8	1.5	ND		ND	
12 (AF)	92.2	1.9	7.8	1.9	ND		ND	
13 (AF)	95.4	0.7	4.6	0.7	ND		ND	
14 (AF)	99.1	0.3	0.9	0.3	ND		ND	
15 (AP)	45.5	2.3	1.6	0.1	51.5	2.3	1.4	0.1
16 (AP)	56.3	4.8	4.9	0.9	37.0	4.1	1.8	0.5
17 (AP)	54.0	3.7	4.5	0.9	38.9	3.7	2.6	0.7
18 (AP)	37.9	7.0	3.0	0.5	54.7	6.9	4.4	1.1
19 (AP)	73.7	5.8	1.0	0.9	24.8	7.9	0.5	0.8
20 (AP)	44.9	2.7	8.5	2.6	41.0	4.9	5.6	1.1
21 (AP)	60.1	9.4	0.9	1.1	37.9	8.8	1.1	2.0

[a] *A. flavus* (AF) and *A. parasiticus* (AP) isolates. [b] Mean of individual aflatoxin percentage produced on six peanut genotypes. [c] Standard deviation. [d] Not detectable.

in the Colorado Irradiado−PI 337394 and J11−Manfredi 68 genotypes, respectively.

The Friedman test showed significant differences in the averages of aflatoxin contamination among the genotypes ($p < 0.0001$). The multiple-comparison test showed PI 337394 to be significantly different from the other genotypes with the lowest aflatoxin mean contamination, followed by Manfredi 68. Colorado Irradiado and Florman formed the cluster with the highest aflatoxin contamination.

DISCUSSION AND CONCLUSIONS

Aspergillus infection in peanuts is conditioned by three principal characteristics: the aggressiveness of the fungus, peanut genotype susceptibility, and environmental conditions of moisture and temperature (*20*). The invasive and toxigenic capacities of the fungus were the two measurements of its aggressiveness. Aflatoxin production of *A. flavus* and *A. parasiticus* isolates on peanut was from 100 to 70000 ppb

(**Tables 3** and **5**). The broad range of aflatoxin production shows the toxigenic differences among the isolates, with *A. parasiticus* being the most toxigenic.

The seed infection resistance was studied with or without the presence of integument in order to obtain information about integument and cotyledon as components of seed resistance. In the mean comparison study of fungal colonization and aflatoxin contamination among the genotypes in the whole seeds assay, three clusters with significant differences were described. The first cluster consisted of the PI 337394, PI 337409, and J11 genotypes and was classified as a resistant one; the second cluster consisted of the Colorado Irradiado genotype as moderately resistant, and the third cluster consisted of Florman INTA and Manfredi 68 genotypes as the least resistant. The integument or seed coat presence in the seeds was demonstrated to be a barrier to *Aspergillus* entry and seed colonization. Seed coat elimination largely increased the fungal colonization and aflatoxin contamination irrespective of genotype and isolate.

The resistance of PI 337394, PI 337409, and J11 genotypes to aflatoxin contamination and *Aspergillus* colonization shown in the whole seeds assay has been reported by others (*8*, *10*, *21*, *22*). In those studies an *Aspergillus* reference strain with a known aggressiveness was used to evaluate the resistance. However, the resistance of these genotypes when they are infected with several *A. flavus* and *A. parasiticus* strains has not been described.

In the sliced seeds assay, the cotyledon exposure allowed the fungi to reach the necessary nutrients to colonize the seed. Despite the high aflatoxin contamination and fungal colonization, it was possible to detect significant differences among the genotypes ($p < 0.05$). PI 337394 was the only genotype with a significantly lower aflatoxin contamination and fungal infection than the others. It showed the highest resistance to aflatoxin contamination and fungal colonization in the cotyledons that have yet to be described. Manfredi 68 was the second lowest aflatoxin-contaminated genotype but had the highest fungal colonization. In contrast, the resistance of the PI 337409 and J11 genotypes observed in the whole seeds assay disappeared with the elimination of seed coat integrity. The aflatoxin contamination resistance in the cotyledons might be associated with lower aflatoxin production as a consequence of fungal growth inhibition (PI 337394) or aflatoxin synthesis inhibition (Manfredi 68).

The differences of fungal colonization and aflatoxins contamination among genotypes were consistent in most of the isolates. However, there were six isolates that showed interactions with the genotypes. Those interactions were related to a higher aflatoxin contamination (7th and 18th versus J11; 11th versus PI 337394; 17th versus Colorado Irradiado; 21st versus Manfredi 68) and fungal colonization (16th versus PI 337394) on the specific genotypes. Because there were interactions among specific isolates and resistant genotypes, the 7th isolate (*A. flavus*) and the 11th, 16th, and 18th isolates (*A. parasiticus*) might be characterized as aggressive isolates.

The results of laboratory assay and field assay carried out in more than one country have shown discrepancies, where some genotypes (including the resistant genotypes used in this research) showed resistance in laboratory assays but susceptibility in field assays. The discrepancies observed in these studies might be related to differences in the methodology used in field assays. Seed coat resistance is operative only in seeds with intact testa, and its effectiveness in the field assay is reduced by mechanical operations causing pod and seed damage or by faulty curing, drying, and storage conditions. In addition, differences in the composition of microflora soils, weather conditions, and *Aspergillus* strain aggressiveness that colonize the soil are variables of field assays that could justify the discrepancies. With regard to the aggressiveness of a strain, it is important to emphasize the interaction between local *Aspergillus* strains and resistant genotypes that might be one of the causes of the reduced resistance under field assays.

In conclusion, three lines of peanut genotypes (PI 337394, PI 337409, and J11) showed a resistance to fungal colonization and aflatoxin contamination associated with seed coat integrity, whereas the INTA breeding line Colorado Irradiado showed a moderate resistance and Manfredi 68 and Florman INTA genotypes showed the least resistance. A partial resistance to *Aspergillus* colonization and aflatoxin contamination associated with cotyledons was observed only in PI 337394 genotype.

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LITERATURE CITED

(1) Ueno, Y. Toxicology and biochemistry of mycotoxins. In *Toxicology, Biochemistry and Pathology of Mycotoxins*; Uraguchi, K., Ed.; Kodansha Press: Tokyo, Japan, 1978; pp 645−832.

(2) Groopman, J. D.; Cain, L. G.; Kensler, T. W. Aflatoxin exposure in human populations: measurements and relationship to cancer. *Crit. Rev. Toxicol.* **1988**, *19*, 113−145.

(3) Keenan, J. I.; Savage, G. P. Mycotoxins in groundnuts, with special reference to aflatoxin. In *Groundnut Crop: A Scientific Basis for Improvement*; Smartt, J. S., Ed.; Chapman and Hall: London, U.K., 1994; pp 509−541.

(4) Zambetakkis, C.; Waliyar, F.; Bockelee-Morvan, F.; dePins O. Results of four years of research on resistance of groundnut varieties to *Aspergillus flavus*. *Oleagineux* **1981**, *36*, 377−385.

(5) Sanders, T. H.; Mixon, A. C. Effects of peanut tannins on percent seed colonization and in vitro growth by *Aspergillus parasiticus*. *Mycopathologia* **1978**, *66*, 169−173.

(6) Mehan, V. K.; McDonald, D.; Ramakrishna, N. Varietal resistance in peanut to aflatoxin production. *Peanut Sci.* **1986**, *13*, 7−10.

(7) Will, M. E.; Holbrook, C. C.; Wilson, S. M. Evaluation of field inoculation techniques for screening peanut genotypes for reaction to preharvest *A. flavus* group infection and aflatoxin contamination. *Peanut Sci*. **1994**, *21*, 122−125.

(8) Mehan, V. K.; McDonald, D.; Nigam, S. N.; Lalitha, B. Groundnut cultivars with seed resistant to invasion by *Aspergillus flavus*. *Oleagineux* **1981**, *30*, 501−507.

(9) Mehan, V. K.; McDonald, D.; Rajagopalan, K. Resistance of peanut genotypes to seed infection by *Aspergillus flavus* in field trials in India. *Peanut Sci.* **1987**, *14*, 17−21.

(10) Kisyombe, C. T.; Beute, M. K.; Payne, G. A. Field evaluation of peanut genotypes for resistance to infection by *Aspergillus parasiticus*. *Peanut Sci.* **1985**, *12*, 12−17.

(11) ISTA. International Seed Testing Association Rule Book. *Seed Sci. Technol.* **1985**, *13*, 299−355.

(12) Mehan, V. K.; McDonald, D. Screening for resistance to *Aspergillus flavus* invasion and aflatoxin production in groundnuts. In *Groundnut Improvement Program*; ICRISAT: Patancheru, India, 1980; Occasional Paper 2, pp 1−15.

(13) Vaamonde, G.; Degrossi, C.; Comerio, R.; Fernández Pinto, V. *Aspergillus flavus* y *A. parasiticus* en maní cultivado en la provincia de Córdoba (Argentina): Carácteristicas diferenciales y capacidad aflatoxicogenica. *Bol. Soc. Argent. Bot.* **1995**, *30*, 191−198.

(14) Fajardo, E. J.; Waniska, R. D.; Cuero, R. G.; Pettit, R. E. Phenolic compounds en peanut seeds: enhanced elicitation by chitosan and effects on growth and aflatoxin B1 production by *Aspergillus flavus*. *Food Biotechnol.* **1995**, *9*, 59−78.

(15) Tubajika, K. M.; Damann, K. E. Sources of resistance to aflatoxin production in maize. *J. Agric. Food Chem.* **2001**, *49*, 2652−2656.
(16) Young, J. C. Microwave-assisted extraction of the fungal metabolite ergosterol and total fatty acids. *J. Agric. Food Chem.* **1995**, *43*, 2904−2910.
(17) AOAC. *Official Methods of Analysis of AOAC Intenational*; Patricia, C., Ed.; AOAC: Gaithersburg, MD, 1999; Natural Toxins Method 970.44.
(18) Conover, W. *Practical Nonparametric Statistics*, 2nd ed.; Wiley: London, U.K., 1980; pp 335−402.
(19) McCullagh, P. Regression models for ordinal data (with discussion). *J. R. Stat. Soc., Ser. B* **1980**, *42*, 109−142.
(20) Middleton, K. J.; Pande, S.; Sharma, S. B.; Smith, D. H. Diseases. In *Groundnut Crop: A Scientific Basis for Improvement*; Smartt, J. S., Ed.; Chapman and Hall: London, U.K., 1994; pp 336−378.
(21) Mixon, A. C.; Rogers, K. M. Peanut accessions resistant to seed infection by *Aspergillus flavus*. *Agron. J.* **1973**, *65*, 560−562.
(22) Pettit, R. E.; Azaizeh, H. A.; Taber, R. A. Screening groundnut cultivars for resistance to *Aspergillus flavus*, *Aspergillus parasiticus*, and aflatoxin contamination. In *Aflatoxin Contamination of Groundnut: Proceedings of the International Workshop*; ICRISAT: Patancheru, India, 1989; pp 291−303.

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