Aflatoxin Production by *Aspergillus flavus* and *A. parasiticus* on Visibly Sound Rehydrated Peanut, Corn and Soybean Seed[1]

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ABSTRACT

Peanut, corn and soybean seed were inoculated with 14 isolates of *Aspergillus flavus* Link and *A. parasiticus* Speare. The seeds were hand sorted to remove all visibly damaged seeds and were fumigated under vacuum (-95.25 k Pa Hg) with 2.2% cyano (methylmercuri) guanidine at 37 C for 48-96 hours. All fumigated seed had a minimum of 95% germination and a maximum of 5% residual contamination by fungi and bacteria. Corn and peanut samples (100 g/flask) were rehydrated to 28% moisture and inoculated with all isolates; soybean samples (100 g/flask) were rehydrated to 28% moisture and inoculated with four *A. flavus* and two *A. parasiticus* isolates. Samples were incubated for 10 days at 30 C and analyzed for aflatoxins. *Aspergillus parasiticus* isolates produced aflatoxin B_1, B_2, G_1 and G_2 while *A. flavus* isolates produced aflatoxin B_1 and B_2. Mean B_1 production for 12 isolates was 34 mg/kg in peanut seed and 3.6 mg/kg in corn seed. Two *A. flavus* isolates produced 3.8 to 5.4 mg/kg B_1 in peanut seed, and 2.2 mg/kg in corn seed. Overall, the mean B_1 production was about 10 times higher on peanut seed than on corn seed. However, more G_1 was produced on soybean seed than B_1. The isolate and the substrate are apparent limiting factors in aflatoxin production. Peanut seed accumulated more aflatoxin than corn or soybean seed when inoculated with the same isolates and incubated under similar conditions.

Key Words: *Aspergillus flavus, Aspergillus parasiticus*, aflatoxins, peanut, corn and soybean seed.

The production of aflatoxins on peanut, corn and soybean seed has been studied extensively (7,8,9,10,12,13). However, most of these investigators did not compare crops or fungal isolates in their experiments. Diener and Davis (3) have carried out the most extensive studies of aflatoxin formation in freshly dug and stored whole peanuts.

Joffe (6) found that almost 90% of 1626 isolates collected from peanut seed and peanut fields over 5 years produced aflatoxin B and that 8.4% of the isolates produced aflatoxins B and G. Diener and Davis (2) found 86% of their isolates produced toxins. Shroeder and Boller (11) reported that toxins were produced by 96% of the isolates from peanut, 79% from cotton, 49% from sorghum and 35% from rice seeds. Hesseltine et al. (4) tested 67 *Aspergillus flavus* Link group isolates for the production of aflatoxin B_1, G_1, and M_1. Eleven of 14 *Aspergillus parasiticus* Speare isolates produced B_1, G_1 and M_1, two of the other three strains produced low levels of B_1 and G_1 and one strain produced low levels of B_1. A group of five isolates taxonomically intermediate between *A. flavus* and *A. parasiticus* produced B_1, G_1 and M_1. The group of toxin producing isolates identified as *A. flavus* produced B_1 and M_1. Hesseltine et al. (4) concluded that *A. parasiticus* regularly produces B_1 and G_1 and that *A. flavus* produces B_1 and only rarely G_1 in low levels. It should be noted, though, that the differentiation of species between *A. flavus* and *A. parasiticus* is sometimes quite difficult.

Priyadarshini and Tulpule (9) found that *A. parasiticus* growth and aflatoxin production was not correlated among corn and peanut genotypes, consequently different plant genotypes support different amounts of fungal growth and aflatoxin production. Nagarajan and Bhat (8) reported differences in *A. flavus* group isolates and peanut cultivars in aflatoxin production. The relative differences between isolates were consistent with gentoype but different genotypes supported different amounts of aflatoxin.

The objectives of these experiments were to find if isolates of the *A. flavus* group (*A. parasiticus* and *A. flavus*) known to be toxin producers have differences in invasive potential in rehydrated sound, viable corn, peanut and soybean seed and if aflatoxin production is dependent more on the isolate or the crop.

Materials and Methods

The corn, peanut and soybean seeds used for these experiments were hand sorted to remove all visibly damaged seeds and fumigated to reduce microfloral contamination (1). Corn and soybean seeds were rinsed with 0.53% (w/v) NaOCl three times and dried for 40 minutes at 38 C. The seeds were placed in the upper chamber of a vacuum dessicator over an open petri dish containing 15 mL of 2.2% cyano (methylmercuri) guanidine. The dessicator was evacuated until the liquid began to boil (-95.25 k Pa Hg) and then the vacuum port was closed. Corn seeds were fumigated under vacuum for 72 hours at 37 C and soybean seeds were fumigated at 37 C for 96 hours. Peanut seeds were treated in the same manner except no NaOCl rinse was used and the seeds were fumigated at 37 C for 48 hours. The temperature and time exposures were determined to maximize kill of seedborne microflora and minimize toxicity to the seeds from organic Hg vapor. All seeds were aseptically removed and stored in sterile containers at 7 C until use.

Seven aflatoxin producing isolates of *A. parasiticus* and five aflatoxin producing isolates of *A. flavus* were obtained from the USDA-NRRL collection at Peoria, Illinois for these experiments. Four *A. parasiticus* and two *A. flavus* strains were isolated originally from peanut seed, three *A. parasiticus* and three *A. flavus* strains were isolated originally from corn, and two *A. flavus* strains were isolated from soybean seed in Georgia by D. K. Bell. A fresh spore suspension of each isolate was prepared to contain ca 10^6 conidia/mL and used to inoculate the rehydrated seed. Four replicates of inoculated flasks containing 100 g of seed/flask/isolate were used in all subsequent tests.

One hundred g of corn, peanut or soybean seeds were each placed in cotton plugged, sterile 250 mL erlenmeyer flasks and were rehydrated to 28% moisture content by adding sterile water and equilibrating 12 hours at 4 C. Four flasks of each type of rehydrated seed were then inoculated with 0.25 mL of the spore suspension from each isolate in the case of corn and peanuts and with six isolates in the case of soybeans. The inoculated seeds were incubated for 10 days at 30 C. After 10 days, the samples were observed for fungal growth, sporulation and then were extracted for aflatoxin determinations. Aflatoxin analyses and confirmations were performed using AOAC official methods (5); for corn and peanut seed the CB method was used and for soybean seed the rapid modification method was used. These methods are suitable for the B and G aflatoxins, but cannot be used for the M aflatoxins.

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Therefore, M aflatoxin data were not collected in these experiments. Aflatoxins B_1 and G_1 were confirmed using both a H_2SO_4 spray and the TFA derivative (5).

Results and Discussion

These experiments were designed to determine major differences between isolate infectivity and subsequent aflatoxin production when sound, viable, rehydrated corn, peanut and soybean seeds were inoculated with different *A. flavus* and *A. parasiticus* isolates and incubated for 10 days at 30 C. The germination and residual microflora data in the seed lots before rehydration are listed (Table 1). All seed had 5.0% or less residual contamination by fungi and bacteria after fumigation. Many published studies on growth of *A. flavus* group isolates have been with cracked seed or autoclaved seed when comparing growth and aflatoxin production. We were interested in studying infectivity of toxic species of the *A. flavus* group beginning with sound, viable, whole grain.

All 14 isolates infected rehydrated peanut and soybean seed, and fungi were growing and sporulating heavily on all seed surfaces after 10 days. In corn, however, there was a paucity of visible mycelium and sporulation occurred on less than 10% of the seeds. There was little apparent damage, except the darkened germ, in the corn seed which had no visible sporulation on the surface. When these seeds from each isolate were hand sorted and surface disinfested for 5 minutes with 0.53% aqueous NaOCl, plated on malt-salt agar and incubated for 7 days at 28 C, colonies of *A. flavus* group were observed growing from over 85% of the corn seed indicating good infectivity by all of the *A. flavus* and *A. parasiticus* isolates.

All *A. flavus* and *A. parasiticus* isolates produced aflatoxins in the corn, peanut and soybean seed (Table 2). The *A. parasiticus* isolates produced aflatoxins B_1, B_2, G_1 and G_2 whereas the *A. flavus* isolates produced aflatoxins B_1 and B_2.

Table 1. Germination and residual microflora of corn, peanut and soybean seed after vacuum fumigation with 2.2% cyano(methylmercuri) guanidine.

Seed	Germination (%)	Residual microflora	(%)
Corn	98.1	*Fusarium* sp.	2.0
		Bacteria	0.1
		Total	2.1
Peanuts	94.3	*Aspergillus niger*	0.5
		Fusarium sp.	0.3
		Mucor haemalis	0.1
		Penicillium sp.	0.1
		Rhizopus arrhizus	0.3
		Trichoderma sp.	0.1
		Bacteria	3.3
		Total	4.7
Soybeans	98.3	*Phomopsis sojae*	1.3
		Bacteria	3.7
		Total	5.0

Overall, peanut seed accumulated almost 10 times more aflatoxin B_1 than corn, with mean B_1 production in soybean seed being the lowest but not significantly different than corn (Table 3). With the exception of isolates A22657 and A22551, the average aflatoxin B_1 production was relatively high for *A. flavus* and *A. parasiticus* in peanut seed. In both corn and peanut seed aflatoxin G_1 production was more variable but equal to or higher than B_1 in all *A. parasiticus* isolates except A20341. For the two *A. parasiticus* tested in soybean seed, aflatoxin

Table 2. Aflatoxins found in inoculated seed.

		ng/g of indicated aflatoxin[a]											
NRRL		Corn				Peanut				Soybean			
Isolate No.	Source	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
Aspergillus parasiticus													
2999	peanuts	3500b	337bc	3125c	375a	25,000bc	6000cd	25,000c	3675c	2998b	166b	12076a	1099a
3240	peanuts	2750b	337bc	5000bc	750a	40,000a	8250b	40,000abc	8250a				
3145	peanuts	2000b	300bc	8750a	787a	30,000bc	3562e	41,000abc	4500bc				
A14330	peanuts	3750b	339bc	4375bc	712a	32,500abc	8250b	35,000bc	4650bc				
A22437	corn(silk)	3500b	412bc	4250bc	525a	37,500ab	10500a	50,000ab	8250a				
A20267	corn	3750b	337bc	6250ab	625a	35,000ab	8250b	55,000a	6300ab	1499bc	140b	10494a	550b
A20341	corn	6125a	712ab	2250c	375a	40,000a	9000ab	8,750d	150d				
Aspergillus flavus													
3357	peanuts	3500b	356bc	ND[c]	ND	40,000a	9000ab	ND	ND				
A11608	peanuts	3333b	325bc	ND	ND	37,500ab	4050de	ND	ND	6829a	500a	ND	ND
5520	corn	3665b	425bc	ND	ND	37,500ab	8250b	ND	ND	475c	19c	ND	ND
A22657	corn	2250b	131c	ND	ND	5,375d	157f	ND	ND				
A22551	corn	2250b	112c	ND	ND	3,812d	168f	ND	ND				
SB-1[b]	soybeans	4007b	919a	ND	ND	30,000bc	6913bc	ND	ND	546c	39c	ND	ND
AP-2[b]	soybeans	4162b	120c	ND	ND	32,000abc	3006e	ND	ND	766bc	40c	ND	ND

[a] Aflatoxins (ng/g) are average values from four 100 g replications. Means within columns bordered by the same letter are not significantly different at P=0.01 by Duncan's multiple range test.

[b] SB-1 and AP-2 *A. flavus* isolates were isolated from soybeans in Georgia.

[c] ND, indicated aflatoxin not detected.

G_1 production was much higher than B_1. Aflatoxin B_2 and G_2 production were both isolate and substrate dependent.

Table 3. Mean aflatoxin production in peanut, corn and soybean seed by all toxin producing isolates.

	ng/g of indicated aflatoxin[a]			
	B_1	B_2	G_1	G_2
Peanut	28413 a	5690 a	36393 a	5110 a
Corn	3236 b	344 b	4857 c	600 b
Soybean	1874 b	129 b	11285 b	824 b

[a]Means within columns bordered by the same letters are not significantly different at P=0.01 by Duncan's multiple range test. R-square values overall were .958 (B_1), .951 (B_2), .899 (G_1) and .909 (G_2).

These data indicate that there are not major differences between the infectivity of toxic *A. flavus* group isolates when tested with corn, peanut and soybean seed under our conditions. But there are important differences in aflatoxin production that should be considered in experimental design. Peanuts supported more aflatoxin production than corn and soybeans. Aflatoxin production on corn and soybeans was not signficiantly different for B_1, B_2 and G_2, however, more G_1 was produced on soybean than corn (Table 3). In field experiments it may be better to analyze for all four aflatoxins when collecting data but the use of total ($B_1 + B_2 + G_1 + G_2$) aflatoxins may be less meaningful than B_1 data when the results are statistically analyzed because B_1 data alone may have less variation than total aflatoxins.

The use of *A. parasiticus* rather than *A. flavus* as inoculum in experiments appears to be quite logical since there is little difference in infectivity between the two species and data on aflatoxin B_1, B_2, G_1 and G_2 can be collected. If field data were statistically analyzed using only G_1 or $G_1 + G_2$ results, this would effectively ignore background B_1 clutter from *A. flavus* since *A. flavus* rarely produces aflatoxin G_1 and G_2. There would of course still be some G_1 and G_2 background, but less than 10% of natural *A. flavus* group isolates produce G_1 and G_2 (6) and high levels of G_1 and G_2 are rarely seen in field or storage experiments. Unpublished identifications and analyses conducted in Georgia over the last 5 years at the Mycotoxin Analysis Research Center confirm Joffe's (6) conclusion that *A. flavus* and aflatoxin B_1 are most often found in uninoculated situations.

The conclusions from these experiments are as follows: (1) There is little difference between *A. flavus* and *A. parasiticus* in infectivity of sound rehydrated corn, peanut and soybean seed. (2) Aflatoxin production is dependent on both the fungal isolate and on the substrate inoculated. (3) The use of *A. parasiticus* as inoculum for field and storage experiments followed by statistical analyses of G_1 or $G_1 + G_2$ data may be more useful than the current practice of *A. flavus* inoculation followed by statistical analyses of B_1 or total aflatoxin data.

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