Susceptibility of Pods of Different Peanut Genotypes to Aspergillus flavus Group Fungi

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ABSTRACT

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Pods and seeds from 17 peanut genotypes were tested for resistance to colonization by *Aspergillus parasiticus* after each of two successive growing seasons. In each year's tests, pods of three genotypes remained completely free from colonies of the test fungus, whereas all the pods of two genotypes in the first year and of three genotypes in the second year had at least one colony. The percentage of inoculated pods with surface colonies of *A. parasiticus* was not directly proportional to that of inoculated seeds; rather, they were inversely correlated (r = −0.5 and −0.6 in tests 1 and 2, respectively). Thus, peanuts selected for resistance to *A. parasiticus* through the standard inoculated seed assay would usually produce pods that are more susceptible to colonization than would genotypes producing more susceptible seed. The shell of the intact pod seemed to provide an effective barrier to *A. parasiticus*. The quantity of aflatoxin B_1 in seeds of four genotypes was correlated (r = 0.89) with the percentage of pods with surface colonies at 21 days after inoculation. The latter values also were correlated (r = 0.89 and 0.94) with the percentage of seeds that had been penetrated and those with surface colonies, respectively. Resistance of pods to *A. parasiticus*, however, may not be a true plant resistance, since genotypes that were relatively resistant one year were susceptible the next, and vice versa. Colonies arising from natural inoculations occurring before the pods were artificially inoculated accounted for only three of the seven significant changes in the percentage of pods with colonies between the first and second tests.

Additional key words: Arachis hypogaea L., groundnut, mycotoxins.

Seeds of certain peanut (*Arachis hypogaea* L.) genotypes were reported to be resistant to colonization by *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare based on inoculations of hand-harvested, hand-shelled seed (14). Resistance was reduced or eliminated by practices that damaged the testa of the seed. Machine harvest (stripping the pods from the plants) and machine shelling decreased resistance (15), and abrading the testa with Carborundum or pricking it with pins eliminated resistance (11,12). Seeds without testae had no resistance (2). Resistant seeds seemed to have greater surface wax accumulations (13), more compact cells and a greater number of fibers in the testa along with smaller hila (17), and a greater concentration of tannins (4).

All these reports involved studies on seed shelled by hand and established that resistance to *A. flavus* in peanut seed was a function of the testa, which acts as a barrier to movement of the fungus into the seed. In a normal commercial operation, farmers sell unshelled peanuts to processors, and the peanut lots are inspected for visible *A. flavus* in the seed before the sale. The processor shells the seed and either processes them immediately or stores them under carefully controlled environmental conditions to to prevent rancidity and mold. During harvest, when the peanut is most susceptible to the *A. flavus* group, the environment cannot be controlled (6). If the resistant testa is to provide an effective barrier to the *A. flavus* group, it must do so before the seed is shelled.

The following is a report of tests on the effect of the shell on the susceptibility of pods of different peanut genotypes to *A. flavus* group fungi.

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MATERIALS AND METHODS

Standard agronomic procedures were used to produce and harvest pods from 17 peanut genotypes (breeding lines and cultivars) for two successive years—1974 and 1975. The peanuts were dug at optimum maturity with a digger-shaker-inverter, dried (cured) in a stackpole 4–6 wk, and mechanically picked. The harvested pods were freed of debris and stored in paper bags at room temperature (20–25 C).

Inocula were prepared from 10 to 15 day old Czapek's dox agar cultures of *A. parasiticus* (culture No. 2999) obtained from the Northern Regional Research Laboratory, SEA, USDA. Conidia were removed from the surface of the cultures with a transfer loop and deposited into an aqueous solution of 0.02% polyoxyethylene (20) sorbitan monolaurate (Tween 20). Spore concentrations were adjusted to 5×10^6 conidia per milliliter.

Whole undamaged pods were inoculated following a procedure patterned after that described by Mixon and Rogers (14) for peanut seed. Pods and seeds were moistened with distilled water to 25 and 20%, respectively, (w/w) water on an oven dry weight basis (72 hr at 90 C) and contained in petri plates. Inoculum was added to each plate (1.5 ml for pods and 0.5 ml for seeds), and the plate was gently agitated to distribute the conidia. The plates were placed in polyethylene bags, then held at 25 C. The incubation period—7, 14, or 21 days—depended on the test.

Colonization of pods or seeds. The inoculated pods or seeds were examined for sporulating colonies that appeared similar to those of the test fungus (16) after 7 days' incubation. At least one colony on the surface of a pod or seed was considered evidence that the entire unit had been colonized.

Invasion of the seed within a pod. After 14 days' incubation, the percentage of pods with colonies was recorded. The pods were then

immersed in 1.5% NaClO for 5 min, rinsed twice in water, and shelled by hand. The seeds were immersed in 1.5% NaClO for 5 min, rinsed twice in distilled water, and plated on malt-salt-agar (20 g of malt extract, 75 g of NaCl, and 15 g of agar to 1 L with distilled water). Development of fungi resembling the *A. flavus* group on or around the seeds after 5 days' incubation was considered positive evidence that the seed had been invaded.

Colonization of aflatoxin B_1 in seeds within pods. After 21 days' incubation, the percentage of pods with surface colonies of the *A. flavus* group was recorded, the pods were shelled, and the percentage of seeds with colonies of the *A. flavus* group was recorded. Aflatoxin B_1 in seeds within inoculated pods was estimated using the method of the Tropical Products Institute (8). Inoculated pods were incubated for 21 days, then dried at 50 ± 2 C for 24 hr. The seeds from two plates with pods were bulked, mixed with Filter-Cel (a form of Celite, or diatomaceous silica), and milled. A portion of the ground product was extracted with hexane in a Soxhlet apparatus for 4 hr. The resulting fat-free material was dried at 105 C for 30 min. A portion was removed, weighed, and extracted with chloroform in an Erlenmeyer flask for 30 min. The chloroform phase was decanted and filtered through Whatman 2 paper. Three 10-ml portions of each extract were chromatographed on Adsorbosil-1 thin-layer plates (Applied Science Lab., Inc., State College, PA 16801). The plates were developed in diethyl ether, allowed to dry, and developed with a 19:1 chloroform-methanol solution (v/v). The intensity of the fluorescent spots corresponding in location to that of the B_1 standard was measured with a densitometer.

Surface disinfestation of pods before inoculation. Fungi other than those of the *A. flavus* group were observed growing on the surface of inoculated and control pods in both test years. To eliminate fungi arising from airborne conidia, pods of 12 genotypes were immersed in 0.5% NaClO for 5 min before being inoculated. This treatment has been reported to disinfect the surface of peanut pods (7). Because the hypochlorite pretreatment reduced the apparent resistance of some of the pods, it was also used in the test on the relationship between colonization of the pods and seeds within pods and aflatoxin B_1 in the seeds.

Even though pure cultures of *A. parasiticus* were used for inoculations, colonies of this fungus as well as other members of the *A. flavus* group undoubtedly developed from natural infections that began before inoculation. Thus, we have used the terms "colonies of the test fungus" and "colonies of the *A. flavus* group."

The statistical analyses used were the one-way analysis of variance test, Duncan's new multiple range test, and the *t* test of the significance of various correlation coefficients (r).

RESULTS

Comparison of resistances of seeds and of pods. The results of assay of hand-shelled seeds and whole pods for resistance to colonization by *A. parasiticus* in 1974 and 1975 are presented in Table 1. In general, the pods seemed more resistant to colonization than did the seeds. Although pods of four genotypes in 1974 and three in 1975 were quite susceptible (> 85% with colonies), those of eight genotypes in 1974 and 10 in 1975 were free or nearly so (< 5%) of colonies. None of the seeds, on the other hand, were as susceptible as the pods or free or nearly free of colonies in either year. The relationship between the percentage of hand-shelled seeds and whole pods with colonies was inverse (r = −0.54* and −0.62* in 1974 and 1975, respectively).

Neither the average percentage of pods with colonies nor that of seeds with colonies in 1974 was correlated with corresponding data in 1975. The pods of genotypes 19 and 18 were quite susceptible (> 85% with colonies) in 1974 but resistant (< 5%) in 1975. The reverse occurred with genotype 7. The results from tests of pods and seeds in 1974 were paired with those in 1975 for individual genotypes. Seven and four such pairings were significantly different for pods and seeds, respectively, at $P = 0.05$ by the one-way analysis of variance test.

The uninoculated (control) pods in both 1974 and 1975 possessed colonies of various fungi. In 1974, fungi of the *A. flavus* group appeared in 80, 80, 75, 20, and 20% of the pods of genotypes 18, 19, 17, 21, and 20, respectively. In 1975, none of the pods of any of the genotypes had naturally occurring colonies of the *A. flavus* group. The presence of the *A. flavus* group in pods of genotypes 18, 19, and 21 in 1974 and their absence in 1975 may explain the significant shift in susceptibility noted for pods of those genotypes between the 1974 and 1975 tests.

The shell as a barrier in peanut pods to A. flavus group fungi. Because in 1974 and 1975 several genotypes produced pods that appeared resistant to colonization by the *A. flavus* group, the relationship between the percentage of pods with *A. flavus* group colonies and the percentage of invaded seeds within the pods was investigated in 12 genotypes. Penetration through the testae of seeds within pods was highly correlated (r = 0.89***) with colonization of the pods (Table 2). Invaded seeds, however, were found in pods that appeared free of the *A. flavus* group and, conversely, seeds free of the test fungus were found in colonized pods. Treatment of pods with NaClO before inoculation led to significantly ($P = 0.05$) increased susceptibility for pods of genotypes 2, 13, 14, 15, and 21, but the percentage of testae penetrated was increased for only genotype 19. The percentage of inoculated seeds colonized in the earlier test and that of testae penetrated in this test were not correlated.

Pods of four genotypes were used to compare colonization of pods, colonization of seeds within the pods, and content of aflatoxin B_1 in seeds within the pods (Table 3). Colonization of pods was highly correlated with aflatoxin B_1 in seeds (r = 0.89**) and with the percentage of seeds with colonies (r = 0.90**). In addition, the latter two results were correlated (r = 0.94**). The results for pods with colonies and the effect of the NaClO treatment in this test were remarkably similar to earlier results for the four genotypes presented in Tables 1 and 2, even though the incubation period was increased from 7 and 14 days, respectively, to 21 days. The data for respective pods with colonies were highly correlated (r = 0.98***), and the NaClO treatment significantly ($P = 0.05$) increased the percentage of pods with colonies for genotypes 14 and 21 but not for genotypes 16 and 19 compared with the control samples. This increase in pods with colonies, however, was not reflected by a significant increase in seeds with colonies, although seeds of genotype 14 had more aflatoxin B_1 than did those from

TABLE 1. Percentage of peanut seeds and pods of 17 genotypes grown in 1974 and 1975 with colonies of the *Aspergillus flavus* group after inoculation[x] with conidia of *A. parasiticus* and incubation for 7 days at 25 C

		1974		1975	
	Genotype[y]	Seeds	Pods	Seeds	Pods
19	74-4045	13 f[z]	88 b	25 abc	2 d
16	74-4021	19 ef	88 b	26 abc	100 a
14	74-4005	24 def	11 de	19 bcd	40 b
9	74-114	27 def	3 c	37 ab	2 d
13	74-4001	30 cdef	0 e	14 cd	34 c
20	74-4055	30 cdef	4 e	40 ab	7 d
18	74-4039	34 bcde	100 a	33 abc	0 d
17	Altika	35 bcde	100 a	10 d	100 a
7	74-112	41 abcd	0 e	23 bcd	100 a
15	74-4009	42 abcd	23 cd	22 bcd	56 b
24	Tamnut	43 abcd	16 e	30 abc	2 d
22	Starr	44 abcd	23 c	26 abc	2 d
21	Tifspan	45 abcd	23 c	31 abc	2 d
6	74-111	49 abc	0 e	26 abc	3 d
2	74-104	54 ab	0 e	23 bcd	0 d
26	74-515	64 a	2 e	47 a	3 d
1	74-102	64 a	3 e	29 abc	0 d

[x] 20 g of dried pods or 15 g of dried seeds were moistened to 25 and 20%, respectively, and exposed to 4×10^5 or 2×10^5 conidia of *A. parasiticus* (NRRL, No. 2999) per gram of pods and seeds, respectively, in petri plates.

[y] Florida number and cultivar or Florida peanut breeding line.

[z] Each value represents the average of three replicates. Values within each column not followed by the same letter were different at $P = 0.05$ by Duncan's new multiple range test.

untreated pods. Finally, the data for colonized seeds (Table 3) were highly correlated (r = 0.95***) with the data for invaded seeds (Table 2) for genotypes 14, 16, 19, and 21. Slightly more seeds were invaded than were colonized.

DISCUSSION

This study was originally designed to relate the resistance of hand-shelled seed to *A. flavus* group fungi, as defined by Mixon and Rogers (14,15), with resistance to development of that fungus in seeds within inoculated pods. However, when pods of 17 genotypes were inoculated in tests in 1974 and 1975, obvious differences occurred in the development of *A. flavus* group fungi on the surfaces of the pods. The percentage of pods of the genotypes with colonies was inversely rather than directly correlated with the percentage of inoculated seeds with colonies. Later, the percentage of pods with colonies was found to correlate with the percentage of seeds within pods that had been invaded and colonized. Thus, resistant seed would be exposed to a greater inoculum load if the enclosing shell were moistened and inoculated than would more susceptible seed.

The absence of colonies on the pod surface of some genotypes appeared to be a highly variable type of resistance. Pods of some genotypes were completely free of colonies of the *A. flavus* group in both 1974 and 1975, whereas 100% of pods of other genotypes had colonies. Ranking of the 17 genotypes for resistance in 1974, however, differed from that in 1975. Significant ($P = 0.05$) changes were noted in the percentage of pods with colonies for seven of the 17 genotypes between 1974 and 1975. Naturally occurring *A. flavus* group fungi present in the shell in 1974 and absent in 1975 accounted for only three of the seven changes. The magnitude of the shifts ranged up to nearly the entire scale of resistance used in these tests (10–100% colonization). The variability of the resistance of pods to colonization between the test years resembles that of the resistance of seeds that has appeared extremely variable not only between years but also between harvest dates and field locations in a single year (2,3).

Although extremely variable between the 1974 and 1975 tests, pod resistance was more effective than seed resistance in preventing the *A. flavus* group fungi from invading seeds within intact pods. However, pod resistance may be too variable to be readily used in breeding programs and may not be a property of the plant. In each year's tests, colonies of many different fungi were observed on the surface of uninoculated as well as inoculated pods. The *A. flavus* group colonies that developed as a consequence of inoculations were generally scattered unless the pods had been dried immediately after being dug (9; J. A. Bartz, *unpublished*). In the latter study, the entire surface of every shell was covered with colonies of the *A. flavus* group.

Many workers have reported the existence of fungi in the shell as well as in the testa of the seed of cured peanuts (4–7,10,18), and some have suggested that some of the microflora found may antagonize the *A. flavus* group (4–6). Thus, we conclude that the resistance in pods to the *A. flavus* group we have described was caused by antagonistic microflora that were in the shell before inoculation. The preinoculation sodium hypochlorite soak we used was expected to affect the microflora in the shell and did, in fact, reduce the resistance of pods of some genotypes (Tables 2 and 3). This supports our conclusion. Complete breakdown of pod resistance after the hypochlorite treatment did not occur and was not expected, since the shell was not sterilized.

Implicit in our conclusion concerning pod resistance is the concept that antagonistic microflora are responsible for a varietal resistance. This concept is supported by the literature. Atkinson et al (1) reported differences in the bacterial populations in the rhizosphere of wheat genotypes that were susceptible and resistant to common root rot of wheat. Jackson (7) reported differences in the fungal communities in shells of three peanut cultivars grown in a randomized complete block design. Thus, the peanut genotype itself can influence the identities of the organisms that colonize its pods.

The responsible microflora seem to be those that colonize the pod during the curing process. The community present at the beginning of and during the curing process may not be the same as that present at the end of the process. Thus, additional information is needed before the usefulness of pod resistance to the farmer can be predicted. Diener and Davis (6) stated that if curing is interrupted, as by rain, peanuts are quite vulnerable to attack by *A. flavus*. A useful resistance must protect peanuts during the entire curing period.

Finally, we neither proved nor disproved the usefulness of seed resistance, because the shell influenced the amount of inoculum reaching the surface of the seed. With one genotype (Table 3), seed colonization and aflatoxin B_1 content apparently were greater because inoculum reaching the seed was increased after increased colonization of the pod. However, greater concealed damage to the testae due to machine harvesting (15) could have influenced the extent of seed resistance.

TABLE 2. Percentage of peanut pods of 12 genotypes with surface colonies and seeds within pods invaded[x] by *Aspergillus flavus* group fungi after immersion in 0.5% NaClO solution, inoculation,[y] and incubation for 14 days at 25 C

		Control		Treated	
	Genotype	Pods	Seeds	Pods	Seeds
1	74-102	0 d[z]	0 e	35 cd	12 c
2	74-104	0 d	2 de	19 c	12 bc
21	Tifspan	2 d	6 cde	14 cd	13 c
19	74-4045	2 d	9 bcde	7 cd	0 d
9	74-114	4 d	8 bc	7 cd	0 d
6	74-111	3 d	0 e	5 d	0 d
26	74-515	3 d	0 e	7 cd	3 d
13	74-4001	34 c	9 bcde	64 b	19 bc
14	74-4005	40 c	8 cde	92 a	27 b
15	74-4009	56 b	21 ab	79 b	23 bc
16	74-4021	100 a	29 ab	100 a	51 a
7	74-112	100 a	40 a	100 a	53 a

[x]After 14 days' incubation, seeds were removed, surfaces were disinfected, testae were removed, and cotyledons were incubated in malt-salt-agar plates for 5 days at 25 C. Sporulating colonies of the *A. flavus* group were positive for invasion.

[y]Pods contained 25% moisture at the beginning of incubation and were exposed to 2×10^5 conidia of *A. parasiticus* (NRRL, No. 2999) per gram of pods.

[z]Each value represents the average of three replicates. Values within each column not followed by the same letter were different at $P = 0.05$ by Duncan's new multiple range test.

TABLE 3. Percentage[s] of peanut pods and seeds within pods with surface colonies of the *Aspergillus flavus* group and aflatoxin B_1 content[t] of seeds after immersion of intact pods of four breeding lines in NaClO solution, inoculation,[u] and incubation for 21 days at 25 C

	Control[v]			Treated[w]		
Line no.	Pod[x]	Seed[x]	B_1 (ppb)[y]	Pod	Seed	B_1 (ppb)
19	4 c[z]	4 b	120 c	8 c	3 c	260 b
21	9 c	7 b	650 b	17 c	14 bc	830 b
14	43 b	10 b	420 bc	86 b	19 b	1,320 a
16	85 a	26 a	1,330 a	100 a	31 a	1,370 a

[s]Average of three 20-gm samples.

[t]Seeds from two replicates were bulked, dried for 24 hr at 50 C, ground, and extracted with hexane for 4 hr. Fat-free residue was dried and extracted with $CHCl_3$, and extract was chromatographed on Adsorbosil-1 thin-layer plates against pure aflatoxin B_1.

[u]Pods at 25% moisture were exposed to 4×10^5 conidia of *A. parasiticus* (NRRL, No. 2999) per gram of pods in petri plates.

[v]Pods immersed in distilled water.

[w]Pods immersed in 0.5% NaClO for 1 min.

[x]Average of three samples.

[y]Average of three samples of one extract.

[z]Values within each column not followed by the same letter were different at $P = 0.05$ by Duncan's new multiple range test.

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