Evaluation of Field Inoculation Techniques for Screening Peanut Genotypes for Reaction to Preharvest *A. flavus* Group Infection and Aflatoxin Contamination[1]

M. E. Will[2], C. C. Holbrook[3]*, and D. M. Wilson[4]

ABSTRACT

Efforts are being made to screen peanut (*Arachis hypogaea* L.) genotypes for resistance to invasion of seed by the *Aspergillus flavus* (Link ex Fries) group fungi and subsequent contamination by aflatoxins. Field inoculation studies are needed to develop reliable screening techniques that insure infection of susceptible peanut seed by the *A. flavus* group fungi and minimize the number of escapes. The objectives of this study were to evaluate the effects of *A. parasiticus* (Speare) inoculum formulation and timing of application on shell and seed colonization and aflatoxin contamination. Soil population densities of *A. flavus* and plant survival were also measured. Plots were inoculated using various rates either at-plant or at mid-bloom of an organic-matrix infested with *A. parasiticus* NRRL 2999, and/or using an aqueous conidial suspension of the fungus. Mid-bloom inoculation produced an increase of *A. flavus* group population densities in the soil. There were no statistical differences between treatments in aflatoxin content, or colonization of shells or seed. At-plant inoculation was associated with reduced plant emergence or survival. Inoculation at mid-bloom with the organic-matrix resulted in peanuts that ranked high in aflatoxin content. There were no correlations between aflatoxin content, colonization of seed or shells, and population densities of *A. flavus* in soil. Inoculation at mid-bloom with the organic-matrix, resulting in greater soil population densities and relatively higher aflatoxin levels, was an effective method to use in screening for resistance to invasion by *A. flavus* group fungi and contamination by aflatoxins.

Key Words: Peanut, aflatoxin, *Arachis hypogaea*, *Aspergillus flavus*, *Aspergillus parasiticus*, inoculation techniques.

Contamination of peanuts (*Arachis hypogaea* L.) with aflatoxins, toxic secondary metabolites of *Aspergillus flavus* (Link ex Fries) and *A. parasiticus* (Spear), is a worldwide problem. Peanut genotypes with some resistance to invasion by *A. flavus* have been reported (Bartz *et al.*, 1978; Kushalappa *et al.*, 1979; Mehan *et al.*, 1981; Mixon and Rogers, 1973; Tavasolian, 1977; Zambettakis *et al.*, 1977, 1981). These genotypes were identified by screening germplasm using *in vitro* colonization by *A. flavus* of rehydrated sound mature kernels. This *in vitro* screening method has produced inconsistent results when compared to a natural field situation. Davidson *et al.* (1983) and Blankenship *et al.* (1985) did not observe significant levels of preharvest aflatoxin resistance in field studies using genotypes which had been reported as resistant from *in vitro* screening. Kisyombe *et al.* (1985) observed significant field resistance in only one of 14 *in vitro* resistant selections.

To begin the process of developing peanut cultivars resistant to *A. flavus*, a source of high resistance is necessary. The development of reliable field inoculation techniques will aid in the identification of sources of resistance. A more complete understanding of the environmental factors controlling activities of the aspergilli fungi has allowed development of experimental field conditions under which these fungi thrive and toxin production is favored.

Soil moisture, and temperature to some extent, can be controlled by employing shelters to induce drought stress for the appropriate period of time and thereby enhance infection of peanut pods by the *A. flavus* group. Inoculation of the peanut plant or soil in which it is growing has been used to increase the natural *A. flavus* group population densities and enhance the potential for contact between these fungi and peanut pods (Azaizeh *et al.*, 1989; Kisyombe *et al.*, 1985; Wilson and Stansell, 1983; Wilson *et al.*, 1989). The most common means of delivery of *A. flavus* inoculum is an aqueous suspension of conidia (D. M. Wilson, unpubl. data). Aqueous suspensions of *A. flavus* and/or *A. parasiticus* conidia are either mixed into the surface of the soil (Azaizeh *et al.*, 1989) or sprinkled directly on the plant (Wilson and Stansell, 1983; Wilson *et al.*, 1989). Cracked, sterilized corn, an organic-matrix infested with, and acting as the substrate for, the fungus has also been used as a delivery system (Kisyombe *et al.*, 1985; Wilson *et al.*, 1989). Mehan *et al.* (1988) observed an increase in seed infection and aflatoxin contamination from using a labor intensive method of inoculating developing pegs and pods with an aqueous suspension of *A. flavus*. However, no attempts have been made to assess the effects of large-scale inoculation techniques on soil population levels and seed infection by *A. flavus* group fungi or aflatoxin contamination.

A consistently successful and reproducible field inoculation technique is essential to the development of efficient field screening procedures for resistance to preharvest infection and aflatoxin contamination. The objectives of this research were to evaluate the effects of *A. parasiticus* inoculum formulations and timing of application in the field on shell and seed colonization and aflatoxin contamination. *Aspergillus flavus* group population densities in the soil and plant survival were also measured.

Materials and Methods

Field Experiment. Field experiments were conducted at the Coastal Plain Experiment Station on Tifton loamy sand soil (fine, loamy, siliceous, thermic Plinthic Kandiudult). In 1990, Pronto peanuts were planted 25 May and harvested 31 Aug., and Florunner peanuts were planted 15 June and harvested 9 Oct. In 1991, Florunner peanuts were planted 17 May and harvested 16 Oct. Double row plots (0.9 x 1.8 m) were seeded at four to six seeds/30 cm linear row. The experiment was designed as a completely randomized block with 10 treatment replications in each planting. Organic matrix treatments were roasted cracked corn (25% moisture content) inoculated with a spore suspension of *A. parasiticus* NRRL 2999 and incubated at 25 C for 3 d. Aqueous spore suspension treatments received approximately 500 mL of a suspension of conidia (approx. 1×10^6 conidia/mL) of *A. parasiticus* NRRL 2999 in tap water.

[1]Cooperative investigation of the USDA-ARS and Univ. of Georgia, College of Agriculture.

[2]Formerly Post-doctoral Res. Assoc., Dept. of Plant Pathology, Univ. of Georgia, Tifton, GA 31793 (present address: 648 Robertsville Rd., Oak Ridge, TN 37830).

[3]Res. Geneticist, USDA-ARS, Coastal Plain Exp. Station, Tifton, GA 31793.

[4]Prof., Dept. of Plant Pathology, Univ. of Georgia, Tifton, GA 31793.

*Corresponding author.

At-plant organic-matrix treatments were applied immediately after planting and lightly raked into the soil surface within 10 cm of either side of the seed furrow. Mid-bloom treatments were applied 18 July and 8 Aug. to both cultivars in 1990, and 18 July 1991 to Florunner. Mid-bloom organic-matrix treatments were sprinkled by hand directly on the foliage, then gently dislodged to the soil surface under the canopy. Mid-bloom aqueous spore suspension treatments were applied by sprinkling the suspension from a garden-watering can onto and through the foliage. Treatments per plot were (a) at-plant 57 g infested organic-matrix, (b) at-plant 113 g infested organic-matrix, (c) at-plant 227 g infested organic-matrix, (d) at-plant 113 g infested organic-matrix plus mid-bloom 113 g infested organic-matrix, (e) at-plant 113 g infested organic-matrix plus mid-bloom 500 mL aqueous spore suspension, (f) mid-bloom 57 g infested organic-matrix, (g) mid-bloom 113 g infested organic-matrix, (h) mid-bloom 227 g infested organic-matrix, (i) mid-bloom 500 mL aqueous spore suspension, and (j) uninoculated control. Rain-out shelters were used to induce drought stress during the final 35 to 40 d of growth. Stand counts were made immediately prior to harvest. Pods were harvested by hand in 1990 and mechanically in 1991. Harvested pods were dried to 7% moisture using forced air at 32 C. Visibly damaged pods were removed and discarded. Each peanut sample was split into two subsamples; 150 pods were used for evaluation of shell and seed colonization, and the remainder (approx. 100 pods) were used for aflatoxin analysis.

To evaluate *A. flavus* group population densities, soil samples were taken 14 d before harvest to a 5-cm depth within 10 cm of the seed furrow. Five subsamples from each row were composited for each replicate.

Analyses. Dried pod samples were shelled using a Peerless peanut sheller. Kernels were ground in a household food processor and aflatoxin contamination was determined using the Vicam immunoaffinity column method (Trucksess, 1991). Results were recorded as total aflatoxin (B_1 + B_2 + G_1 + G_2) in parts per billion (ng/g) of peanut seed.

Pods of the 150-pod subsample were surface sterilized in 10% Clorox (0.525% NaOCl) for 3 min and rinsed in tap water. After shelling by hand, 50 seed and 50 half shells were placed in Petri dishes on malt salt medium (20 g malt extract and 100 g/L salt). Colonization by *A. flavus* group fungi was visually determined after 7 d incubation at 30 C. Results were recorded as the percentage of shells and seed colonized by *A. flavus* group fungi.

Soil samples were air-dried, sieved (2 mm), and a 5-g subsample was mixed with 100 mL 0.38% w/v water agar. A 1-mL aliquot of a 1:20 or 1:100 dilution was plated on M3S1B, a medium selective for *A. flavus* - *A. niger* group fungi (Griffin and Garren, 1974). Plates were incubated for 5 d at 30 C before counts of *A. flavus* group colonies were recorded. Results were recorded as colony forming units of *A. flavus* group fungi per gram soil.

Data were ranked and analyzed using the GLM procedure (SAS Institute, Inc., 1975) and the Duncan-Waller K-Ratio T-Test (Waller and Duncan, 1969). All differences discussed are significant at $P \leq 0.05$ unless indicated otherwise.

Results and Discussion

Application timing of fungal inoculation affected the number of Florunner plants remaining in the row at harvest in 1990 (Table 1). All five treatments involving inoculation at planting resulted in numerically lower stand counts in comparison to the five treatments which did not involve at-plant inoculation timing. Two at-plant inoculation treatments resulted in stand counts which were significantly lower than the stand counts observed in the treatments which did not involve at-plant inoculation timing. The presence of the *A. flavus* group fungi, or other microorganisms using the organic-matrix as a substrate (e.g., *Fusarium*, *Pythium*), may have inhibited seedling emergence. No differences were seen among treatments in the Pronto cultivar in 1990 (Table 2) and no stand counts were recorded for Florunner in 1991. This indicates that there were important differences in soil physical and/or biological conditions at the time of seedling emergence.

Because of the spatial variability inherent with soil-borne fungi, artificial inoculation techniques can be very useful in eliminating escapes, resulting in more reliable measurements of plant response. Data in Tables 1-3 indicate that soil population densities of *A. flavus* group fungi were higher in plots treated with the organic-matrix formulation at mid-bloom than those in at-plant treatments or the controls. The lack of differences among the levels of

Table 1. Number of plants per plot, population densities of *A. flavus* group fungi in soil, concentration of aflatoxin, and percentage of seed and shell colonization by *A. flavus* in Florunner peanut, 1990.

Treatment[a]		Plants	*A. flavus*		Colonization[b]	
At-plant	Mid-bloom	per plot[b]	group[b]	Aflatoxin[b]	Seed	Shell
g		No.	cfu/g soil	ppb	%	
57	—	12 c	766 e	3256 NS	20 NS	45 NS
113	—	14 bc	1090 e	1400	50	69
227	—	14 bc	1852 cd	4716	64	94
113	113	14 bc	5322 a	2535	23	49
113	Aqueous	13 c	1913 c	2800	29	55
—	57	21 a	5236 a	3557	36	65
—	113	20 ab	6204 a	3265	39	54
—	227	19 ab	6208 a	1830	35	77
—	Aqueous	19 ab	3546 b	3222	41	56
Control	—	19 ab	1228 de	1697	22	44

[a]Inoculation treatment = infested organic-matrix; aqueous = 500 mL spore suspension.

[b]Values within a column followed by the same letter are not different ($P \leq 0.05$) according to Waller-Duncan multiple-range test; NS = nonsignificant ($P \geq 0.05$).

Table 2. Number of plants per plot, population densities of *A. flavus* group fungi in soil, concentration of aflatoxin, and percentage of seed and shell colonization by *A. flavus* in Pronto peanut, 1990.

Treatment[a]		Plants	*A. flavus*		Colonization[b]	
At-plant	Mid-bloom	per plot[b]	group[b]	Aflatoxin[b]	Seed	Shell
g		No.	cfu/g soil	ppb	%	
57	—	37 NS	973 de	1561 NS	18 NS	49 NS
113	—	35	908 e	1012	7	59
227	—	36	905 e	2894	14	59
113	113	34	4976 ab	1098	27	73
113	Aqueous	30	2522 c	772	6	42
—	57	32	6295 a	1760	43	85
—	113	33	5018 ab	1998	32	67
—	227	36	5867 a	1129	30	54
—	Aqueous	37	4130 b	212	19	42
Control	—	36	1521 cd	2572	18	45

[a]Inoculation treatment = infested organic-matrix; aqueous = 500 mL spore suspension.

[b]Values within a column followed by the same letter are not different ($P \leq 0.05$) according to Waller-Duncan multiple-range test; NS = nonsignificant ($P \geq 0.05$).

organic-matrix inoculum applied at mid-bloom demonstrated that mid-bloom inoculum at the lowest level (57 g/row) was the most efficient treatment for increasing *A. flavus* group population densities in the soil using this inoculation method. Mid-bloom aqueous spore inoculum treatments applied alone also consistently increased *A. flavus* group population densities in the soil over controls, but not as much as mid-bloom organic-matrix inoculum. The choice between infested organic-matrix and spore suspension inocula may be made on the basis of technical and handling considerations. An aqueous spore suspension involves the least complex laboratory preparation and requires less material to be handled. The organic-matrix inoculum, however, may be prepared in large quantities, frozen for short-term storage (up to 6 mo), shipped in bulk to other research sites, and applied at various rates in the field.

There were no differences in aflatoxin concentration in peanut seed or percentage colonization of shells or seed in the three crops (Tables 1-3). However, mid-bloom organic-matrix treatments at 57 and 113 g/row consistently produced seed that ranked high in aflatoxin concentration over the three crops. This trend is compatible with the higher soil population densities generated by mid-bloom organic-matrix inoculation.

Differences existed within treatments between crops for all response measures. In general, where differences occurred the values for aflatoxin concentration, percentage of colonization of seed and shells by *A. flavus* fungi, and soil population density of fungi were higher in 1990 than in 1991. This was probably due in part to low soil moisture and high soil temperatures between May and mid-August 1990 which were conducive to growth of *Aspergillus* fungi and aflatoxin production. These factors were important in field screening experiments in North Carolina (Kisyombe *et al.*, 1985) and field trials with Florunner in Georgia (Cole *et al.*, 1985; Hill *et al.*, 1983). Duration of soil moisture stress influenced the degree of *A. flavus* invasion and aflatoxin contamination in peanut (Azaizeh *et al.*, 1989; Mehan *et al.*, 1988; Sanders *et al.*, 1985). The differences between the Florunner and Pronto cultivars in 1990 may be due to pre-drought-stress rains inhibiting late season infection and aflatoxin production by *A. flavus*.

Table 3. Population densities of *A. flavus* group fungi in soil, concentration of aflatoxin, and percentage of seed and shell colonization by *A. flavus* in Florunner peanut, 1991.

Treatment[a]		*A. flavus*		Colonization[b]	
At-plant	Mid-bloom	group[b]	Aflatoxin[b]	Seed	Shell
g		cfu/g soil	ppb	%	
57	—	947 cd	1066 NS	20 NS	16 NS
113	—	233 d	2503	17	21
227	—	1460 bc	841	9	9
113	113	3300 a	1535	10	22
113	Aqueous	2160 abc	537	8	16
—	57	1920 abc	1335	13	12
—	113	3000 a	2153	17	23
—	227	2633 ab	244	19	13
—	Aqueous	2087 abc	1232	18	23
Control	—	340 d	509	14	16

[a]Inoculation treatment = infested organic-matrix; aqueous = 500 mL spore suspension.

[b]Values within a column followed by the same letter are not different ($P \leq 0.05$) according to Waller-Duncan multiple-range test; NS = nonsignificant ($P \geq 0.05$).

There were no significant correlations between aflatoxin concentration, percentage colonization of seed and shells, and soil population densities of *A. flavus*. These results indicate that the presence of the fungi in the soil or peanut shells or seed is not a useful indicator of aflatoxin production and supports the results reported by Kisyombe *et al.* (1985) and Azaizeh *et al.* (1989). Mehan *et al.* (1986), however, reported a correlation between seed infection and aflatoxin content of late-harvested peanuts.

Our results indicate the need for a better understanding of the relationships among soil *A. flavus* group population dynamics, peanut invasion, and aflatoxin production. It is evident that soil population densities of *A. flavus* can be increased by field inoculation, perhaps to a point detrimental to seed or seedlings. Soil population densities of *A. flavus* group were relatively high under all treatments, including the uninoculated. This indicates there may have been sufficient native populations of *Aspergillus* in these fields for maximal peanut pod invasion. The need remains, however, for a reliable field technique that insures the infection of peanut pods by the *A. flavus* group fungi in order to minimize the number escapes. We feel that mid-bloom organic-matrix inoculation, resulting in greater soil population densities and high aflatoxin levels, is an effective method to be used in screening germplasm for resistance to invasion by *A. flavus* group fungi and contamination by aflatoxins, especially at sites with low native population densities of *Aspergillus*.

Acknowledgment

This work was supported in part by a grant from the National Peanut Foundation.

Literature Cited

1. Azaizeh, H.A., R.E. Pettit, O.D. Smith, and R.A. Taber. 1989. Reaction of peanut genotypes under drought stress to *Aspergillus flavus* and *A. parasiticus*. Peanut Sci. 16:109-113.
2. Bartz, J.A., A.J. Norden, J.C. La Prade, and T.J. Demuynk. 1978. Seed tolerance in peanuts (*Arachis hypogaea* L.) to numbers of *Aspergillus flavus* group of fungi. Peanut Sci. 5:53-56.
3. Blankenship, P.D., R.J. Cole, and T.H. Sanders. 1985. Comparative susceptibility of four experimental peanut lines and the cultivar Florunner to preharvest aflatoxin. Peanut Sci. 12:70-72.
4. Cole, R.J., T.H. Sanders, R.A. Hill, and P.D. Blankenship. 1985. Mean geocarposphere temperatures that induce preharvest aflatoxin contamination of peanuts under drought stress. Mycopathologia 91:41-46.
5. Davidson, J.I., R.A. Hill, R.J. Cole, A.C. Mixon, and R.J. Henning. 1983. Field performance of two peanut cultivars relative to aflatoxin contamination. Peanut Sci. 10:43-47.
6. Griffin, G.J., and K.H. Garren. 1974. Populations levels of *Aspergillus flavus* and the *A. niger* group in Virginia peanut field soils. Phytopathology 64:322-325.
7. Hill, R.A., P.D. Blankenship, R.J. Cole, and T.H. Sanders. 1983. Effects of soil moisture and temperature on preharvest invasion of peanuts by the *Aspergillus flavus* group and subsequent aflatoxin development. Appl. Environ. Microbiol. 46:628-633.
8. Kisyombe, C.T., M.K. Beute, and G.A. Payne. 1985. Field evaluation of peanut genotypes for resistance to infection by *Aspergillus parasiticus*. Peanut Sci. 12:12-17.
9. Kushalappa, A.C., J.A. Bartz, and A.J. Norden. 1979. Susceptibility of pods of different peanut genotypes to *Aspergillus flavus* group fungi. Phytopathology 69:159-162.
10. Mehan, V.K., D. McDonald, S.N. Nigam, and B. Lalitha. 1981. Groundnut cultivars with seed resistance to invasion by *Aspergillus flavus*. Oléagineux 36:501-505.
11. Mehan, V. K., D. McDonald, and N. Ramakrishna. 1988. Effect of adding inoculum of *Aspergillus flavus* to pod-zone soil on seed infection and aflatoxin contamination of peanut genotypes, Oléagineux 43:21-28.
12. Mehan, V.K., D. McDonald, N. Ramakrishna, and J.H. Williams. 1986. Effects of genotype and date of harvest on infection of peanut seed by *Aspergillus flavus* and subsequent contamination with aflatoxin. Peanut Sci. 13:46-50.
13. Mehan, V.K., R.C. Nageswara Rao, D. McDonald, and J.H. Williams. 1988. Management of drought stress to improve field screening of peanuts for resistance to *Aspergillus flavus*. Phytopathology 78:659-663.
14. Mixon, A.C., and K.M. Rogers. 1973. Peanut accessions resistant to seed infection by *Aspergillus flavus*. Agron. J. 65:560-562.
15. Sanders, T.H., R.J. Cole, P.D. Blankenship, and R.A. Hill. 1985. Relationship of environmental stress duration to *Aspergillus flavus* invasion and aflatoxin production in preharvest peanuts. Peanut Sci. 12:90-93.
16. SAS Institute. 1985. SAS User's Guide: Statistics, Version 5. SAS Institute, Cary, NC.
17. Tavasolian, B. 1977. Native Iranian peanut resistance to seed infection by *Aspergillus flavus*. J. Agric. Food Chem. 25:1422-1423.
18. Trucksess, M.W., M.E. Stack, S. Nesheim, S.W. Page, R.H. Albert, T.T.J. Hansen, and K.F. Donahue. 1991. Immunoaffinity column coupled with solution fluorometry or liquid chromatography postcolumn derivitization for determination of aflatoxins in corn, peanuts and peanut butter: Collaborative study. J. Assoc. Off. Anal. Chem. 74: 81-88.
19. Waller, R.A., and D.B. Duncan. 1969. A Bayes rule for the symmetric multiple comparison problem. J. Amer. Stat. Assoc. 64:1484-1499.
20. Wilson, D.M., and J.R. Stansell. 1983. Effect of irrigation regimes on aflatoxin contamination of peanut pods. Peanut Sci. 10:54-56.
21. Wilson, D.M., M.E. Walker, and G.J. Gascho. 1989. Some effects of mineral nutrition on aflatoxin contamination of corn and peanuts, pp. 137-151. *In* A.W. Engelhard (ed.), Soilborne Plant Pathogens: Management of Diseases with Macro- and Micronutrients. APS Press, St. Paul, MN.
22. Zambettakis, Ch., A. Bockelee-Morvan, F. Waliyar, and J. Rossion. 1977. Varietal differences in groundnut sensitivity to contamination by *Aspergillus flavus* in the field and in artificial conditions. Oléagineux 32:377-385.
23. Zambettakis, Ch., F. Waliyar, A. Bockelee-Morvan, and O. dePins. 1981. Results of four years of research on resistance of groundnut varieties to *Aspergillus flavus*. Oléagineux 36:377-385.

Accepted September 30, 1994