A Phytoalexin and Aflatoxin Producing Peanut Seed Culture System

S.M. Basha*[1], R.J. Cole[2] and S.K. Pancholy[1]

ABSTRACT

An *in vitro* seed culture system was established to grow peanut seed of different maturities *viz.* white, yellow, orange, brown and black, using a modified Murashige and Skoog medium. Under this system peanut seed of yellow, orange, brown and black maturity categories grew to maturity as measured by increase in their size and germinability. *In vitro* cultured seeds produced significant amounts of phytoalexins and were contaminated with aflatoxins following their inoculation with *Aspergillus* spp. while the noninoculated sterile controls did not produce any phytoalexins. Exposure of seed cultures to water stress using various concentrations of mannitol (0 to 1 M) and polyethylene glycol 8000 (0-30% w/v) caused a significant decrease in their phytoalexin producing ability, and enhanced fungal growth compared to the nonstressed controls. The seeds that were stressed with mannitol and subsequently inoculated with *A. flavus* and *A. parasiticus* showed a significant increase in the aflatoxin contamination of stressed seed compared to the unstressed control. This would indicate that *in vitro* grown seeds responded to water stress similar to the field grown peanuts by loosing their ability to produce phytoalexins and increased susceptibility to aflatoxin contamination. Hence, this system has a potential application in evaluating peanut genotypes for aflatoxin resistance under water stress.

Key Words: Aflatoxins, *Aspergillus flavus*, *in vitro* culture, mannitol, peanut, phytoalexins, polyethylene glycol.

Preharvest aflatoxin contamination is a major economic problem of the peanut industry and does not occur when peanuts are supplied with adequate moisture during the growing season; however, a late season drought can lead to aflatoxin contamination (3,5,9,11,14,15,19). Further, invasion by *Aspergillus* spp. and aflatoxin contamination in peanuts subjected to water-stress usually occurs first and to a greater degree in small, immature peanuts (16). It was suggested (4) that in immature peanuts some seed resistance mechanisms inhibiting growth and aflatoxin production by the fungi break down in response to water and temperature stresses. This resistance mechanism has been reported as the production of phytoalexins, the antibiotic secondary metabolites produced by plants in response to injury and invasion by certain pathogens (1,10). Wotton and Strange (21) indicated that resistance of peanut seeds to invasion by *A. flavus* was correlated with their capacity to synthesize phytoalexins as an early response to wounding, and conditions that promoted invasion of peanuts by *A. flavus* inhibited phytoalexin production. Dorner *et al.* (7) demonstrated that as peanut seed water content decreased during drought and temperature stresses, the capacity of seeds to produce phytoalexins decreased, resulting in fungal activity and aflatoxin contamination. Therefore, they state that as long as peanut seeds had the capacity for phytoalexin production (high kernel moisture), significant amounts of aflatoxin did not form.

Although loss of phytoalexin producing capacity following stress will enhance *Aspergillus* spp. invasion, subsequent aflatoxin production by the invading pathogen may be due to drought-induced compositional changes in the host plant. Since colonization or invasion of peanuts by *A. flavus* does not always result in aflatoxin contamination, it is believed that altered seed composition may play a significant role in triggering aflatoxin biosynthesis by the invading fungi. Hence, it is essential to understand the interrelationship of phytoalexin production, seed composition and aflatoxin biosynthesis. However, it is difficult to control environmental factors under field conditions to determine the effects of these factors on susceptibility of peanut seed to aflatoxin contamination. Furthermore, screening of germplasm to detect aflatoxin resistant lines also requires a reliable system in which aflatoxin contamination can be induced consistently.

In this paper, we report establishment of a phytoalexin and aflatoxin producing peanut seed/fungus co-culture system which can be used in studying the relationship between seed composition and aflatoxin biosynthesis. In addition, this system appears to be useful in screening peanut germplasm to identify peanut lines less prone to aflatoxin contamination under water stress.

Materials and Methods

Seed Material. Peanut (*Arachis hypogaea* L. cv. Florunner) plants were grown in the field at the Florida A&M Univ., Tallahassee, FL, following recommended cultural practices. Plants were harvested 80 to 130 d after planting, and pods were washed and separated into different maturity categories following the method of Williams and Drexler (18).

Seed Culture. After classification, the pods were surface sterilized with 2% (v/v) sodium hypochlorite (NaOCl) for 15 min. The pods were rinsed with sterile water, split open and the seeds were collected with seed coats intact. The seeds were then surface sterilized with 0.5% NaOCl containing 0.01% Tween 80 for 5 min. The seeds were rinsed thrice with sterile distilled water and cultured in 20 mL of sterile basal medium at 25 C on rotary shakers with 16/8 hr light (average of 100 mE/$m^{-2}s^{-1}$ from cool white fluorescent lamps)/dark cycle. The basal medium consisted of Murashige and Skoog (12) salts, B_5 vitamins (8), 3% sucrose, 2% glutamine and pH adjusted to 5.8 prior to autoclaving. Glutamine was filter sterilized and added after autoclaving (13,17). Growth of the seed was monitored by measuring increase in size, weight, and germination.

Water Stress Imposition. The seed cultures of various maturities were established by culturing seeds under nonstress conditions for 7 to 10 d. The seeds were then aseptically transferred to the basal medium containing various concentrations of mannitol (0 to 1 M) and polyethylene glycol 8000 (PEG) (0 to 30%, w/v) and incubated for 10 d under the conditions described above.

Culture Inoculation. Following the stress treatment, seeds were harvested and inoculated with a spore suspension of *A. flavus* and *A. parasiticus* and incubated in the dark at 25 C. The fungal inoculant had approximately 5 x 10^6 spores/mL from 3-wk-old cultures grown on Czapek's agar. The controls used in phytoalexin study were not inoculated with *Aspergillus* spp. and kept sterile until harvest to prevent phytoalexin induction. After 5 d of incubation, the seeds were harvested, freeze-dried, ground to a powder, and stored at -20 C for chemical analysis.

Phytoalexin Extraction and Analysis. Phytoalexins were extracted (1) and analyzed on HPLC (2,7). Briefly, the peanut meal (2 g) was extracted with ethanol, filtered, and dried. The dried material was taken

[1]Division of Agric. Sciences, Florida A&M Univ., Tallahassee, FL 32307.

[2]USDA-ARS, National Peanut Research Laboratory, Dawson, GA 31742.

*Corresponding author.

up in benzene and clarified by passing through a Silica Sep-pak (Waters, Milford, MA) cartridge. Phytoalexins were fractionated on a silica column with cyclohexane:methyl-t-butyl ether:methanol (79:20:1, v/v/v) as mobile phase and UV detection at 335 nm.

Aflatoxin Analysis. Aflatoxin extraction and HPLC analysis was carried out essentially as described by Dorner and Cole (6). Briefly, peanut meals were extracted with methanol:water (80:20, v/v), filtered and subjected to mini-column clean-up. An aliquot of this sample was dried and aflatoxins in the samples were detected by HPLC. The HPLC system consisted of Radial-Pak silica gel column and solvent system of water-saturated chloroform supplemented with 0.6% methanol. All of the above experiments were conducted in four replications and the results are consistently reproducible.

Results and Discussion

Establishing the Seed Cultures. Peanut seed of white, yellow, orange, brown, and black maturity categories were cultured on a Murashige and Skoog medium as modified by Obendorf *et al.* (13) containing glutamine as the nitrogen source and sucrose as the carbon source. Initially, attempts were made to culture the white seed of different sizes (8-10 mg). However, seeds smaller than 10 mg failed to grow in the culture even after prolonged incubation. Seeds larger than 12 mg showed some growth. After 1 wk in culture, the skins of large (> 15 mg) white seed cracked and cotyledons protruded out of the seed coats (Fig. 1A). In addition, the radicle swelled and cotyledons split open with increasing incubation time (10-20 d). Significant growth was seen only with very large (30-40 mg) white seeds, while the smaller (10-20 mg) white seeds showed inconsistent growth pattern.

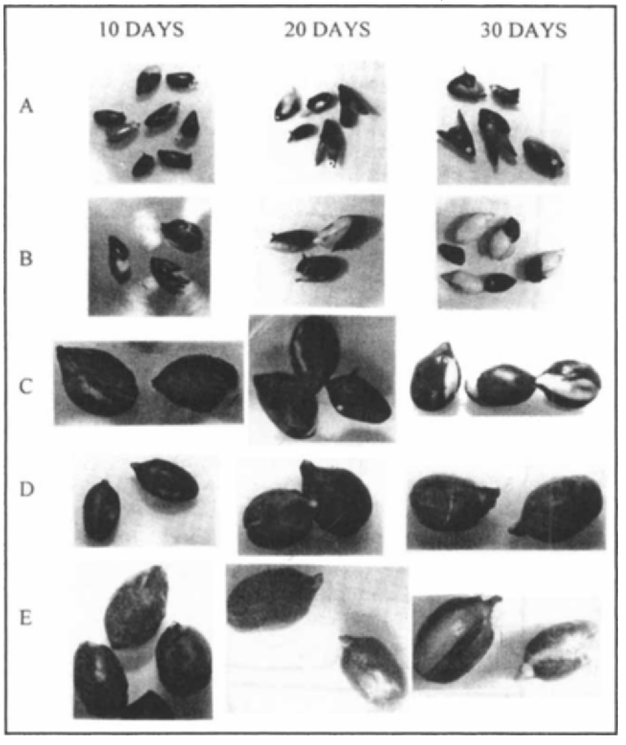

Fig. 1. Growth pattern of peanut seed of different maturities cultured *in vitro*. After harvest the pods were classified into different maturities and cultured in a modified Murashige and Skoog media for 10, 20 and 30 d. Seed maturity categories: A= White, B= Yellow, C= Orange, D= Brown and E= Black.

This would suggest that seeds must attain a minimum maturity in order for them to grow independently off of the plant. Similar, observations were made with soybean seed cultures in which seeds weighing less than 50 mg showed little growth (13).

Seeds of the yellow maturity category were more responsive to culture. The yellow seed category showed rapid growth relative to increased seed size and seed coat rupture (Fig. 1B) as compared to the white maturity category. The seed coats of the orange maturity category cracked, but the cotyledons remained within the seed coats (Fig. 1C). In contrast, the skins of brown and black maturity categories did not show any cracks and remained intact throughout the culture period (Fig. 1D and 1E). These observations suggest that during culture of the immature seeds (white, yellow) the skins either failed to grow or did not grow enough to accommodate the cotyledon growth. Furthermore, absence of seed coat cracking in the brown and black categories indicated that the cotyledons of seeds in these categories had attained complete development and hence, did not expand like those of the white and yellow categories. The seed cultures derived from the yellow category germinated after about 6-8 wk in culture, while the orange, brown and black seeds attained full maturity during culture, and germinated sooner than the yellow category.

Phytoalexin and Aflatoxin Production. Phytoalexin and aflatoxin producing potential of the *in vitro* cultured seeds was determined both by mechanically damaging the seed by slicing (2,7) and inoculating the seeds with *A. flavus* and *A. parasiticus*. Figure 2 shows colonization of seed cultures (white and yellow stages) by the fungi following their inoculation with *A. flavus* spores. Similar infestation levels were observed with other maturity categories and *A. parasiticus* (not shown). The fungi readily colonized *in vitro* cultured seed and sporulated within 5-10 d. Analysis of the infested seed for phytoalexins showed that *in vitro* cultured seed produced significant amounts of phytoalexins following wounding by slicing (Fig. 3b) and also after inoculation with *A.* spp. (Fig. 3c). The control seed, which was kept sterile and not diced, did not produce phytoalexins (Fig. 3a). The phytoalexin profiles of the cultured seed was similar to that of the field grown seed (2), indicating that both responded similarly to fungal invasion or wounding. In addition to phytoalexins, the cultured seed supported production of large amounts of aflatoxins (13,240 and 958,640 units of B_1 and B_2, respectively, per mg of meal) following their infestation with *A. flavus*. These data show that cultured seeds do posses the ability to produce phytoalexins as well as being contaminated with aflatoxins and hence, may be suitable as substitutes for field grown seeds in studies involving measuring phytoalexin and aflatoxin responses of peanut seed.

Effect of Water Stress. Suitability of the seed culture system to measure peanut seed response to *water stress*, and its affect on phytoalexin and aflatoxin production was determined by imposing water stress on cultures followed by inoculation with *A. flavus* and *A. parasiticus*. Figure 4a shows the phytoalexin profile of the control and water stressed seeds. Unstressed seed produced four (A,B,C,D) phytoalexin components following fungal infestation.

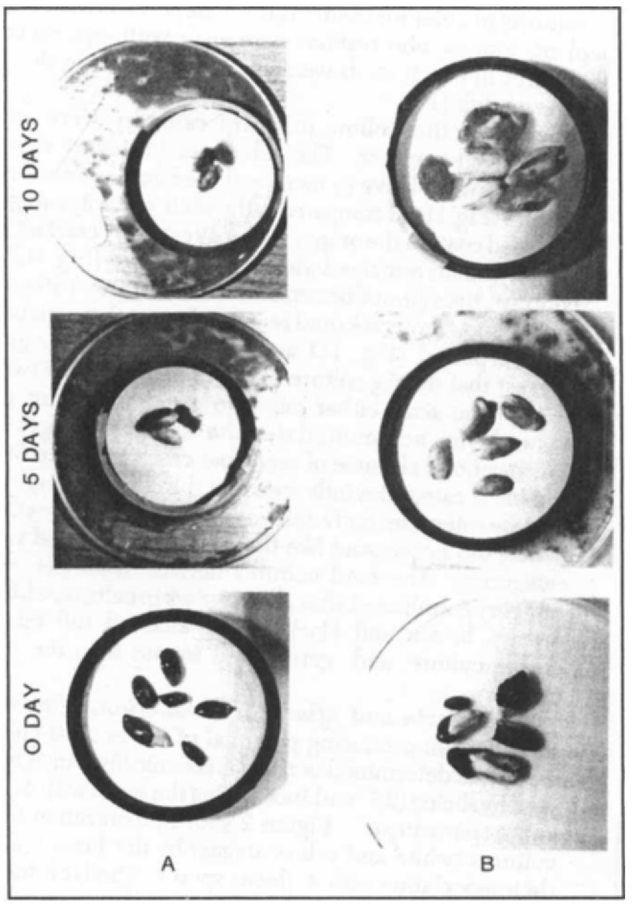

Fig. 2. Colonization of *in vitro* cultured seed by *A. flavus*. Peanut seed of different maturities were cultured in a modified Murashige and Skoog media for 29-30 d, inoculated with *A. flavus* and incubated at 25 C for 5 to 10 d. Seed maturity categories: A= White, B= Yellow.

However, imposition of water stress greatly decreased the phytoalexin producing ability of these seed. In stressed seed, the decrease was more rapid in phytoalexin components C and D as compared to components A and B. As the mannitol concentration increased (0 to 1 M), phytoalexin producing ability of the seed decreased (Fig. 4). This would indicate that water stress decreased the ability of the cultured seed to produce phytoalexins. Maximum phytoalexin inhibition occured with mannitol concentrations above 0.25 M. Like the mannitol, PEG also inhibited phytoalexin production in the cultured seed (Fig. 4). Unlike mannitol, inhibition by PEG was inconsistent. This may be attributed to the different osmotic effects of mannitol and PEG and/or to the toxicity of PEG. In addition, it was observed that the water stressed seeds were more heavily colonized by the fungi than the unstressed controls (not shown), indicating that water stress enhanced *Aspergillus* growth.

Table 1 shows the effect of varying concentrations of mannitol and PEG on the total phytoalexin and aflatoxin contents of the *A. flavus* infested seed cultures. Aflatoxin analysis of the *A. flavus* inoculated seed cultures showed higher aflatoxin levels in stressed seed compared to the unstressed seed. Seeds stressed with 0.125 to 0.5 M mannitol were contaminated with relatively higher (1016 to 1552 x 10^3 ppb) levels of aflatoxins (B_1 and B_2) than the controls. Interestingly, seed cultures stressed with mannitol concentrations above 0.5 M contained relatively lower (853 x 10^3 ppb) amounts of aflatoxins than the seed cultures stressed with mannitol concentrations below 0.5 M. Seed cultures grown in high vs. low concentrations of mannitol

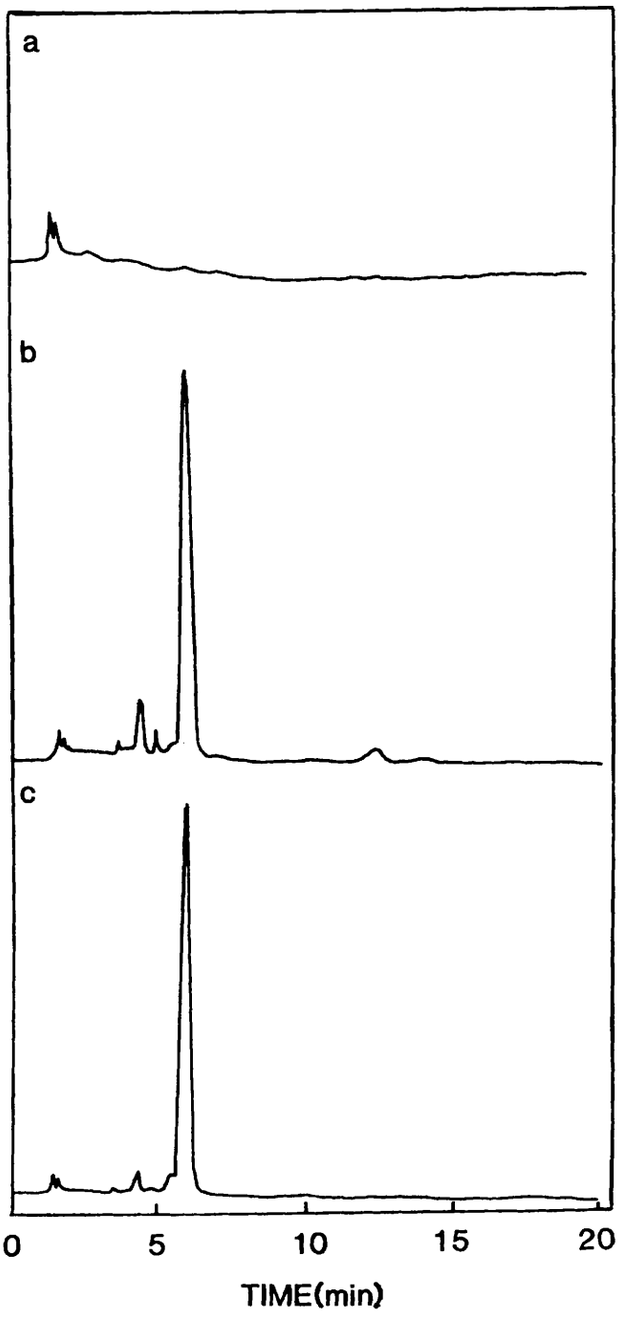

Fig. 3. Phytoalexin producing ability of *in vitro* cultured peanut seed. A=Uninjured sterile control, B=injured by slicing and C=infected with *A. flavus*. Phytoalexins were extracted from the above seed and resolved on a silica gel column using HPLC.

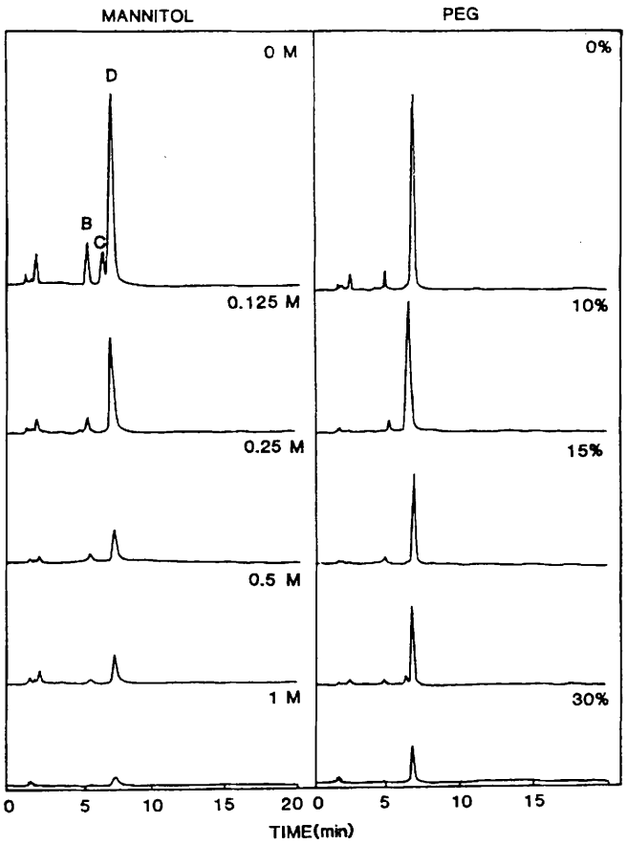

Fig. 4. Effect of imposition of water-stress on the phytoalexin producing ability of the peanut seed. Seeds were cultured *in vitro* for 10 d and then stressed by transferring into a media containing various concentrations of mannitol (0 to 1 M) and polyethyleneglycol 8000 (0-30%). After 10 d, the seeds were inoculated with *A. flavus* and incubated at 25 C for 5 d. Phytoalexins were extracted and analyzed by HPLC.

had no visible difference in mycelial growth. Hence, relatively lower aflatoxin contamination in these seed suggest that severe stress had little effect on fungal growth but did reduce aflatoxin production. Likewise, the PEG-treated cultures showed higher aflatoxin contamination at concentrations between 10 and 15% whereas treatments with PEG concentrations above 15% produced relatively lower levels of aflatoxins. These data suggest that water stress adversely affected phytoalexin producing ability of the seed, but enhanced aflatoxin production but only to a certain point, above which aflatoxin production decreased. This observation is consistent with the previous findings (7,21). Moderate stress enhances while severe stress decreases aflatoxin production possibly because of enzyme inhibition in the aflatoxin biosynthetic pathway. Unlike the aflatoxins, phytoalexin content of the seed decreased with increasing levels of mannitol and PEG.

Overall, the data suggested that the culture system used to grow peanut seed of various maturities supported *in vitro* seed growth, and the seeds responded to *A. flavus* and *parasiticus* infestation by producing phytoalexins and contaminated with aflatoxins. In addition, it was also possible to impose water stress in the cultures, inhibit

Table 1. Effect of water stress on phytoalexin and aflatoxin producing ability of peanut seed cultures.

Osmoticum	Total phytoalexins	Aflatoxins		
		B_1	B_2	Total
	μ volt-sec x 10^3	ppb x 10^3		
Mannitol (M)				
0.125	6498	1016	10	1026
0.250	4060	1552	22	1575
0.500	2094	1243	16	1260
1.000	853	853	1	855
Polyethyleneglycol 8000 (%)				
10	3905	892	7	900
15	2792	1263	14	1278
20	3027	56	1	57
30	3043	14	0.5	14
Control	9240	239	3	242

phytoalexin production, and obtain consistent aflatoxin production in the stressed seed. Hence, the *in vitro* seed culture system appears to be a reliable method for determining genetic variation for aflatoxin resistance as well as relationships between seed composition and aflatoxin biosynthesis under water stress.

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Literature Cited

1. Aguamah, G.E., P. Langcake, D.P. LeWorth, J.A. Page, R.J. Pryce, and R.N. Strange. 1981. Two novel stilbene phytoalexins from *Arachis hypogaea*. Phytochemistry 20:1381-1388.
2. Basha, S.M., M.N. Musingo, B. Mohanty, J.W. Dorner, and R.J. Cole. 1990. Factors affecting phytoalexin production in peanut seed. J. Plant Physiol. 136:143-148.
3. Blankenship, P.D., R.J. Cole, T.H. Sanders, and R.A. Hill. 1984. Effect of geocarposphere temperature on pre-harvest colonization of drought-stressed peanuts by *Aspergillus flavus* and subsequent aflatoxin contamination. Mycopathologia 85:69-74.
4. Cole, R.J., T.H. Sanders, R.A. Hill, and P.D. Blankenship. 1985. Mean geocarposphere temperatures that induce pre-harvest aflatoxin contamination of peanuts under drought-stress. Mycopathologia 91-41-46.
5. Dickens, J.W., J.B. Satterwhite, and R.E. Sneed. 1973. Aflatoxin contaminated peanuts produced on North Carolina farms in 1968. J. Amer. Peanut Res. Educ. Assoc. 5:48-55.
6. Dorner, J.W., and R.J. Cole. 1988. Rapid determination of aflatoxins in raw peanuts by liquid chromatography with post-column iodination and modified minicolumn clean up. J. Assoc. Off. Anal. Chem. 71:43:47.
7. Dorner, J.W., R.J. Cole, T.H. Sanders, and P.D. Blankenship. 1989. The interrelationship of kernel water activity, soil temperature, maturity and phytoalexin production in pre-harvest aflatoxin contamination of drought-stressed peanuts. Mycopathologia 105:117-128.
8. Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. Plant Physiol. 5:372-375.
9. Hill, R.A., P.D. Blankenship, R.J. Cole, and T.H. Sanders. 1983. Effect of soil moisture and temperature on preharvest invasion of peanuts by the *Aspergillus flavus* group and subsequent aflatoxin development. Appl. Environ. Microbiol. 45:628-633.
10. Keen, N.T., and J.L. Ingham. 1976. New stilbene phytoalexins from American cultivars of *Arachis hypogaea*. Phytochemistry 15:1794-1795.

11. McDonald, D., and C. Harkness. 1967. Aflatoxin in groundnut crop at harvest in Northern Nigeria. Trop. Sci. 9:148-161.
12. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
13. Obendorf, R.L., E.E. Timppo, M.C. Byrne, T.V. Toai, G.T. Rytko, F.C. Hsu, and B. G. Anderson. 1984. Soybean seed growth and maturation *in vitro* without pods. Ann. Bot. 53:853-863.
14. Pettit, R.E., R.A. Taber, H.W. Schrooder, and A.L. Harrison. 1971. Influence of fungicides and irrigation practice on aflatoxin in peanuts before digging. Appl. Microbiol. 22:629-634.
15. Porter, D.M., and K.H. Garren. 1968. An analysis of endogeocarpic microflora of peanuts in Virginia. Trop. Sci. 10:100-106.
16. Sanders, T.H., R.A. Hill, R.J. Cole, and P.D. Blankenship. 1981. Effect of drought on the occurrence of *Aspergillus flavus* in maturing peanuts. J. Amer. Oil Chem. Soc. 58:966-970.
17. Thompson, F.J., J.T. Madison, and A-M. E. Muenster. 1977. *In vitro* culture of immature cotyledons of soybean (*Glycine max* L. Merr.). Ann. Bot. 41:29-39.
18. William, E.J., and J.S. Drexler. 1981. A nondestructive method for determining peanut pod maturity. Peanut Sci. 8:131-141.
19. Wilson, D.M., and J.R. Stansell. 1983. Effect of irrigation regimes on aflatoxin contamination of peanut pods. Peanut Sci. 10:54-56.
20. Wooten, H.R., and R.N. Strange. 1985. Circumstantial evidence for phytoalexin involvement in the resistance of peanuts to *Aspergillus flavus*. J. Gen. Microbiol. 131:487-494.
21. Wooten, H.R., and R.N. Strange. 1987. Increased susceptibility and reduced phytoalexin accumulation in drought-stressed peanut kernels challenged with *Aspergillus flavus*. Appl. Environ. Microbiol. 53:270-273.

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