

Improved Screening Techniques for *In-vitro* Seed Colonization and Pre-harvest Seed Infection by *Aspergillus flavus* in Groundnut

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Abstract

Pre-harvest infection of groundnut seeds by toxigenic strains of *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare is a major cause for aflatoxin contamination. The objective of this study was to refine screening techniques for in-vitro seed colonization and pre-harvest seed infection that could be effectively used in resistance breeding programs. A highly aggressive and toxigenic strain of *A. flavus*, Af 11-4, was used to improve the screening techniques for in-vitro seed colonization and pre-harvest seed infection. The in-vitro seed colonization technique involved the use of sodium hypochlorite (NaOCl - clorax 20%) instead of mercuric chloride (HgCl₂ 0.1%) or ethanol (ethyl alcohol 70%) as seed surface disinfectant and multiwell dishes to incubate single inoculated seeds that facilitated selection of seeds resistant to *A. flavus* colonization. The pre-harvest seed infection screening technique involved development of *A. flavus* infested beds and imposing soil moisture stress (54-63% water deficit) during the last 48 days of the crop growth. Both in-vitro seed colonization and field screening methods were successfully used to screen a number of groundnut genotypes with consistent results.

Keywords: *Aspergillus flavus*, in-vitro seed colonization, pre-harvest seed infection, groundnut

Introduction

Aflatoxin contamination of groundnut (*Arachis hypogaea* L.) by *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare is an important health problem for humans, cattle and poultry, and an economic issue for farmers and traders (Cotty *et al.*, 1994). Of the four naturally occurring aflatoxins (B₁, B₂, G₁ and G₂), B₁ is most common and most toxic (Cullen and Newberne, 1994). *A. flavus* also produces cyclopiozonic acid (CPA), which is toxic to animals and humans (Urano *et al.*, 1992).

Pre-harvest infection of groundnut kernels is a major cause for aflatoxin contamination in the semi-arid tropics environment, which contributes more than 60% of the world groundnut production (Anderson *et al.*, 1995). This is influenced by abiotic factors, such as soil moisture stress and temperature during pod maturation (Dorner *et al.*, 1989), by biotic factors, such as presence of toxigenic strains of *A. flavus* and *A. parasiticus*, (Horn *et al.*, 1994), soil insects that can damage pods (Lynch *et al.*, 1991), and by agronomic management practices, such as application of gypsum, irrigation, harvesting time and drying method (Bhat *et al.*, 1992). Seeds from pods with damaged shells are more frequently contaminated with *A. flavus* than those from intact pods.

Considering the complex nature of the problem, no single control measure is likely to be effective. Among the several

control measures that could be strategically integrated, host plant resistance is a major component. Efforts on breeding for resistance to preharvest aflatoxin contamination in groundnut have not met with desired success mainly due to the lack of reliable screening techniques and thus nonavailability of sources with high level of stable resistance. Several screening methods have been used (Holbrook *et al.*, 2000) to determine preharvest seed infection, but no method has provided reliable and consistent results. The objective of this study was to refine screening techniques for in-vitro seed colonization and pre-harvest seed infection that could be effectively used in resistance breeding programs.

Materials and methods

Screening strains of *Aspergillus flavus* for aggressiveness

Isolates of *A. flavus* (48) obtained from seeds of different groundnut cultivars and their rhizosphere soils from ICRISAT-Patancheru and Anantapur were screened for their growth rate and aflatoxin production potential, and three most aggressive isolates were selected. A number of single-spore isolates were obtained from these three isolates and further selection was made for rapid seed colonization, profuse sporulation, and high aflatoxin production. From

these. a highly aggressive and toxigenic strain of *A. flavus* (referred to as *Af 11-4* throughout) was identified and used in all the experiments reported here.

Seed surface disinfectants for *in-vitro* colonization by *A. flavus*

Three commonly used seed surface disinfectants; sodium hypochlorite (Clorax 5.25% - the Clorox company, Oakland, CA 9412, USA), mercuric chloride (0.1% - S.D. Fine Chemicals Ltd., Boisar 401 506, Maharashtra, India), and ethanol (ethyl alcohol - Shymlakhs Internationals, New Bond Street, London, UK) were used to compare their effects on seed colonization by *A. flavus*. Dry seeds (30g) of cv. J 11 were surface sterilized by soaking them in 25 ml of clorax (20% of the commercial grade), mercuric chloride (0.1%), or ethanol (70%) for 3 min followed by three washings with distilled sterilized water (dsw), 25 ml each at 23-25° C. This process enhanced the seed moisture content from the initial 7% to above 20% (measured by Portable multigrain moisture tester, Dicky-john Corp. Auburn, USA), essential for seed colonization by *A. flavus*.

The above treated seeds were inoculated by spraying spore suspension (1×10^6 spores mL⁻¹) of *Af 11-4* (2.5 ml suspension for 24 seeds of normal genotypes or 12 seeds of large-seeded confectionary genotypes) in a sterilized beaker. The beaker was shaken gently to ensure uniform distribution of inoculum on the seed surface. The inoculated seeds were placed individually in a multiwell (12 or 24 wells) dish and incubated in a moist chamber with 100% RH (plastic trays lined with wet cotton wool and blotting paper and covered with a lid lined with wet blotting paper) at 28 °C in dark. Data on seed colonization were recorded seven days after incubation. The experiment was repeated twice.

Evaluation of germplasm and breeding lines

Sixteen promising breeding lines and germplasm accessions including two checks were evaluated. From each line, 48 visibly healthy, mature and intact seeds were surface sterilized. Half of the seeds (24) were wounded by pinpricking uniformly with a sterile needle (six pricks up to 2-mm deep in a normal seed and 10 pricks in a large seed), to allow easy invasion by *A. flavus* spores and hyphae. The remaining 24 seeds were not wounded and kept as healthy. The wounded and healthy seeds were placed separately in sterilized beakers and spray inoculated with *Af 11-4* and incubated using multiple dishes as described above.

Individual seeds in the multiwell dish were scored for colonization severity using a modified progressive scale of 1 to 4 where, 1 = <5% seed surface colonized with scanty

mycelial growth and no sporulation; 2 = 5-25% seed surface colonized with good mycelial growth and scanty sporulation; 3 = 26-50% seed surface colonized with good mycelial growth and good sporulation; and 4 > 50-100% seed colonized with heavy sporulation. Mean colonization severity was calculated for each line. Seeds that showed resistance to seed colonization (seed coat resistance in unwounded seeds) and seed infection (cotyledon resistance in wounded seeds) could be selected for advancing generation and further testing. The experiment was repeated once.

Field screening technique

Inoculation. Inoculum of *Af 11-4*, growing on autoclaved pearl millet grain, was used to inoculate field soil. Inoculum from 200g pearl millet grains (1×10^7 spores g⁻¹ seed) was mixed with 1 kg of finely powdered field soil in a plastic bucket (5 l capacity). This inoculum (about 25 g) was applied uniformly into the seed furrows (4 rows, 4m long) and covered with a thin layer of soil before sowing the groundnut seeds. In another experiment, the *Af* inoculum was applied (as mentioned above) at flowering of groundnut lines by opening a narrow furrow beside the row. Care was taken in order to ensure that roots were not damaged while opening the furrow.

Estimation of *Af*-population. Soil samples were collected on September 9, 1999 (before *Af*-application) and on 30 Nov 1999 (after *Af*-application) from the field (0.36 ha). The field (60m × 60m) was divided into 144 quadrates (5m × 5m each) and a total of 83 samples (50g each) from the cross-sections of subplots, at 5 cm depth, were collected. The samples were stored in brown paper bags at 4 °C until processed. Soil samples were dried in a low temperature dry air oven at 35°C for four days or in shade (25-30 °C) for six days. The soil samples were made into fine powder and screened through a 20-mesh sieve to get rid of plant debris. From each sample 10g soil was suspended in 90 ml dsw and diluted serially up to 1×10^{-4} for plating. Using a micropipette, 0.5ml soil suspension from each of two dilutions (1×10^{-3} and 1×10^{-4}) was placed on to the AFPA medium (Pitt *et al.*, 1983) and spread uniformly by sterilized glass spreader. For each soil dilution, at least two plates were used, thus there were at least four plates for each sample. The plates were incubated at 28°C in dark for four days. The pigmented (bright orange yellow) colonies of *A. flavus* on AFPA, when viewed underside of the plate, were counted and colony-forming unit (cfu) per gram of soil were determined. The plates were incubated further for a day to confirm the typical *Af 11-4* colonies by sporulation. The cfu data were subjected to the log₁₀ transformation before analysis.

Field experiments

Two field experiments using two cultivars, JL 24 susceptible and J 11 resistant (Mehan, 1989) were conducted at ICRISAT Patancheru during the postrainy season (November 1999-April 2000), and rainy season (July-November, 2000). The treatments included *A. flavus* inoculum application at sowing and at flowering, and a control without inoculum. The experiments were laid out in a randomized complete block design with four replications. The plot size was 4 rows 4 m long with rows spaced at 75 cm and plants within a row at 20 cm on ridges. An additional control was maintained about 500 m from the experimental field where the two cultivars were grown under normal conditions without *Af* inoculum. This was done to reduce the chance of dry spores of *A. flavus* contaminating the control plots.

Imposing soil moisture stress

During the 1999-2000 postrainy season, plots were irrigated to field capacity at 10-day intervals up to 80 days after sowing, using sprinklers. Soil moisture stress (near-wilting situation) was created for the remaining 48 days of the crop (Mar 02-Apr 18, 2000) by manipulating the duration of irrigation. The exact amount of water applied at each irrigation was measured using catch cans placed perpendicular to the sprinkler line in the center of each plot in two of the four replications. A total of eight irrigations were provided during March 2 to April 18, with duration of each irrigation reduced from 180 min in non-stressed plots to 60 min in stressed plots and the total amount of water reduced from 50 mm in non-stressed plots to 21 mm in the stressed plots. Uninoculated, non-stressed groundnut plots were maintained 12 m away from the experimental plots. This was done to reduce inter-plot interference, particularly with irrigation. In the rainy season experiment (Jul-Nov 2000), because of frequent rains, only three irrigations were applied during the last 48 days (12 Oct-28 Nov) of the crop at the rate of 19 mm water at each irrigation. The soil moisture stress was expressed in terms of water deficit (Mehan et al., 1988), which was calculated thus: $Y = [(X_1 - X_2) / X_1] \times 100$

Where Y is the percent water deficit, X_1 is cumulative evaporation measured during drought treatment, and X_2 is cumulative amount of water applied during the drought treatment. To determine soil moisture, the soil samples were collected from 0-10 cm layer at 48 h after each irrigation and just before the next irrigation from each plot, using an auger. The samples were oven dried at 105 °C for 48 h and the soil moisture content was measured using the following formula: Soil moisture (%) = $\{(Fresh\ wt\ of\ soil - Dry\ wt\ of\ soil) / Dry\ wt\ of\ soil\} \times 100$

Table 1. Soil and irrigation parameters of two field experiments during the post-rainy (Nov 1999 - Apr 2000) and the rainy season (July - Nov 2000) at ICRISAT, Patancheru

Parameter	Expt-1 ^a		Expt-2 ^a	
	(post-rainy season)		(rainy season)	
	Non-stressed	Stressed	Non-stressed	Stressed
Number of irrigation	8	8	3	3
Duration of each irrigation (min)	180	60	120	50
Amount of water (mm)	50	21	45	18
Total irrigation water (mm)	400	168	360	54
Water deficit (%)	-	63.8	-	54.5
Soil moisture (%) 48h after irrigation	5.4	3.5	5.3	2.8
Soil moisture (%) before next irrigation	3.4	1.8	3.8	1.7
Soil temperature (°C) at 0830h	22.8	24.5	20.5	22.9
Soil temperature (°C) at 1430h	30.0	35.2	24.8	29.6

^a Each value is the mean of two replications

Soil temperatures in the pod zone were recorded by thermocouples placed at 5 cm depth in two of the four replications (reps 1 and 3) of each treatment. The data were recorded daily at 0830 and 1400 h both in stressed and non-stressed plots. The details of irrigation, amount of water, soil moisture, soil temperatures are provided in Table 1. The soil water deficits of 63.8% in experiment 1 and 54.5% in experiment 2 were created in the stressed plots.

Assay for Seed infection

Pods were shelled after drying for 2-3 days in shade or sun, and from each plot 100 matured and apparently healthy seeds, and 100 immature, shriveled seeds were selected. Seeds were surface disinfected by soaking in clorax (20%) for 3 min and washed with two changes of dsw, 2-3 min each. Ten seeds were placed in each plate of Czapeck's Dox Agar (CDA: Sucrose 30g, NaNO₃ 3g, KH₂PO₄ 0.3g, K₂HPO₄ 0.7g, MgSO₄ 7H₂O 0.5g, KCl 0.5g, NaCl 10g, Bacto agar 20g, (Hi Media, Mumbai, India), amended with chloramphenicol 0.2g, rose Bengal 0.3g, streptomycin 1g, dsw 1L; chloramphenicol, streptomycin and rose Bengal added after autoclaving, pH 6.5), and incubated at 28°C in dark for four days. Seeds were scored visually for *A. flavus* growth on them. Percentage of seeds showing *A. flavus* infection was determined for each plot.

Data were analyzed using analysis of variance with GENSTAT program to determine the level of significance.

Results and discussion

Identification of an aggressive and toxigenic strain of *A. flavus*

Eight of the 48 *A. flavus* isolates were more aggressive for rapid seed colonization and higher aflatoxin production than the others. Further evaluation led to the identification of a single-spore isolate, *Af 11-4*, which was highly aggressive and toxigenic (85-100% *in vitro* seed colonization and 210,102 mg kg⁻¹ aflatoxin B1). In addition, *Af 11-4* produced numerous white sclerotia within 3-4 days on CDA that later turned brown. This unique morphological trait was used as a phenotypic marker for identification of *Af 11-4*. This isolate was used in all the experiments reported here.

Pre- and post-harvest colonization of groundnut seeds by *A. flavus* group of fungi and subsequent aflatoxin production is common. However, all colonized seeds do not contain aflatoxin, for only 40-60% of the *A. flavus* strains are toxigenic. A strain of *A. flavus*, which is virulent and highly toxigenic, could be used for evaluation of groundnut genotypes for genetic resistance to aflatoxin contamination. The unique morphological traits of fast growth and rapid sclerotial formation facilitated the monitoring of the strain in soil and on seed. However, a better marker, such as resistance to a fungicide or DNA-based markers would be desirable for studying soil population dynamics of the strain.

Effect of seed surface disinfectants.

Of the three seed surface disinfectants used, ethanol-treated seeds recorded 94% seed colonization and 3.7 colonization severity compared with 86% colonization and 3.4 colonization severity in clorax treatment, and 33% colonization and 1.7 colonization severity in mercuric chloride treatment (Table 2). Both ethanol and clorax treatments induced profuse sporulation on the seed surface, while mercuric chloride induced only mycelial growth and scanty sporulation, and water control showed severe contamination with other fungi. The mercuric chloride, which is highly toxic and expensive than the other treatments, also induced seed germination (88%) in plates (data not presented) thus rendering seeds unfit for selection and planting.

Clorax proved most effective in this study because it washed the least amount of phenols from the seed coat and favored better colonization of seeds by *Af 11-4*. Higher seed colonization of ethanol-treated seeds seems to have occurred due to washing of excessive amount of phenols from the seed coat (unpublished data). Phenolics (phytoalexins) are well known antifungal compounds that impart resistance against fungal invasion (Wootton and Strange, 1987).

Table 2. Effect of surface disinfectants on *in-vitro* seed colonization by *Aspergillus flavus* (strain *Af 11-4*) in groundnut

Treatment	Seed colonization ^a		Remarks
	Severity	(%)	
Mercuric chloride (0.1%)	1.7	33 (35) ^b	Profuse mycelial growth, no sporulation, seed germination
Ethanol (70%)	3.7	94 (78)	Profuse sporulation, no seed germination
Clorox (20%)	3.4	86 (69)	Profuse sporulation, no seed germination
Water-control	3.6	95 (81)	Heavy contamination with other fungi
Untreated-control	3.4	91(75)	Heavy contamination with other fungi
LSD (P<0.05)	0.49	16.4 (13.1)	

^aMean of four replications, with 24 seeds/ replication.

^bArc sin transformed values in parenthesis

Table 3. *In vitro* seed colonization and seed infection in groundnut genotypes by *Aspergillus flavus* (strain *Af 11-4*) in two experimental runs

Genotype	Colonization severity ^a	
	I	II
ICGV 87110	2.9	2.7
ICGV 88145	3.7	3.3
ICGV 89104	1.9	2.0
ICGV 91278	3.9	3.8
ICGV 91279	3.4	3.5
ICGV 91283	3.6	3.6
ICGV 91284	2.8	2.5
ICGV 91315	3.1	3.2
ICG 1448 (U 4-7-5)	1.7	1.5
ICG 4601 (VAR 27)	2.3	2.0
ICG 4749 (PI 337394F)	1.1	1.2
ICG 4750 (PI 337409)	1.0	1.3
ICG 7101 (VRR 245)	2.6	2.4
ICG 7633 (UF 71513)	1.0	1.1
J 11 (Control)	3.3	3.2
JL 24 (Control)	2.0	2.2
Mean	2.5	2.5
SE(m) ±	0.24	0.23

^aBased on 24 healthy, unwounded seeds inoculated with *Af 11-4* and incubated for 7 days at 25° C and >95% RH

In-vitro seed colonization

Of the 16 groundnut lines evaluated for *in-vitro* seed colonization, the mean severity ranged from 1.0 in ICG 7633) to 3.9 in ICGV 91278 (Table 3), and several individual resistant seeds were selected for growing and evaluating the next generation seeds. Known resistance/ tolerance of several

genotypes (ICG 4749, ICG 4750, ICG 7633, and ICG 1448) to seed colonization by *A. flavus* was confirmed. A very high correlation was found among colonization severities of genotypes in the two experimental runs. However, the two control cultivars J 11 and JL 24 recorded severity scores of 3.3 and 2.0, respectively. All the pin-wounded and inoculated seeds were highly susceptible (score 4). The results, thus indicated the existence of resistance at the testa level but lack of it at the cotyledon level.

Incubation of inoculated seeds in the multiwell dish was an improvement over the earlier methods where glass petridishes and glass vials were used. In petridishes, seeds tend to clog together, which makes scoring of individual seeds difficult. The use of vial caps was better than the petridish but these were not convenient for handling as only one seed could be placed in a vial cap. The multiwell dish contained 24 seeds, facilitated easy handling and scoring of seed colonization, and selection of individual resistant seeds. Thus, the seed coat resistance/tolerance to *A. flavus* colonization in several genotypes, including ICG 1448, ICG 4749, ICG 4750, ICG 7633, and ICGV 89104 was confirmed. Mixon and Rogers (1973) first recognized the role of resistance to seed colonization by *A. flavus* as an effective means of preventing aflatoxin contamination, and they identified two Valencia-type genotypes, PI 337394F (ICG 4749) and PI 337409 (ICG 4750) that showed resistance. Similar findings of resistance in ICGV 89104 and ICGV 88145 to *A. flavus* seed infection were reported by Rao *et al.*, (1995). Other researchers (Mehan, 1989) also reported several groundnut genotypes having resistance to seed colonization. A progressive grading of 1 to 4 to account for the severity of seed colonization was evolved, which allowed easy grouping of seeds that were completely free from those that were heavily colonized, and facilitated selection of individual seeds. This process

would be helpful in understanding genetic variability within a genotype by screening seeds of different filial generations.

Field screening

Seed infection. In experiment 1, seed infection in two treatments (*Af* at sowing and *Af* at flowering) by *Af* 11-4 ranged from 82% to 87% in mature seeds and 70% to 88% in immature seeds of J 11 and JL 24 (Table 4). There were no significant differences in infection levels between the two cultivars and between two levels of maturity. In control 1 (plots in the same field) the seed infection ranged from 72% to 77%, which was not significantly different from the above two treatments. In control 2 (plots 500 m from the experimental field), seed infection ranged from 1% to 4%, which was significantly different from the above treatments. In experiment 2, seed infection ranged from 50% to 79% in mature seeds and 29% to 59% in immature seeds in the two cultivars (Table 4). There were significant differences between seed infection in immature JL 24 (29%) and other treatments. In control 1, the infection level ranged from 39% to 82%, again the lowest infection being in immature seeds of JL 24. In control 2, the seed infection ranged from 2% to 4% with no significant difference between mature and immature seeds and between cultivars.

Population density of *A. flavus* in the field. The mean of *Af*-population from 83 soil samples before *Af* 11-4 application in the field was $44 \times 10^3 \text{ g}^{-1}$ soil compared with $35.6 \times 10^3 \text{ g}^{-1}$ soil collected from in-between the rows of plants after *Af* 11-4 applications. The latter population was predominantly of *Af* 11-4, which was applied at sowing while the former population had none to very few colonies that resembled *Af* 11-4. In experiment 1, the *Af* 11-4 population at 40, 80, and 120 days varied in different

Table 4. Pre-harvest seed infection in groundnut at different times in two field experiments during the post-rainy season (Dec 1999 - Apr 2000) and the rainy season (July - Nov 2000) at ICRISAT, Patancheru

	Seed infection (%) ^a by <i>A. flavus</i> in							
	Experiment 1 (post rainy season)				Experiment 2 (rainy season)			
	Mature seed		Immature seed		Mature seed		Immature seed	
	J 11	JL 24	J 11	JL 24	J 11	JL 24	J 11	JL 24
Af at sowing	82	82	70	84	67	50	59	29
Af at flowering	83	87	81	88	79	70	50	57
Control-1 ^b	72	77	73	72	82	79	68	39
Control-2 ^c	4	1	4	4	4	3	3	2
LSD (P<0.05)	19.4		14.8		17.6		27.7	

^a Mean of four replications

^b Control-1 = No *Af* application in plots in the same experimental field

^c Control-2 = No *Af* application in plots 500 m away from the experimental field

Table 5. Population density of the introduced *Aspergillus flavus* (Af 11-4) in two field experiments during the post-rainy season (Dec1999-Apr 2000) and the rainy season (Jul-Nov 2000), ICRISAT, Patancheru

	Af population (\log_{10} cfu) ^a at different days after sowing											
	Experiment 1 (post rainy season)						Experiment 2 (rainy season)					
	40d		80d		120d		40d		80d		120d	
	J 11	JL 24	J 11	JL 24	J 11	JL 24	J 11	JL 24	J 11	JL 24	J 11	JL 24
Af at sowing	4.5	4.4	3.6	3.6	3.4	3.5	4.3	4.4	4.7	4.6	4.6	4.4
Af at flowering	4.4	4.6	3.3	3.6	4.2	3.9	4.3	4.3	4.7	4.7	4.7	4.5
Control-1 ^b	4.6	4.0	3.7	3.4	3.7	3.4	4.3	4.4	4.6	4.6	4.6	4.6
Control-2 ^c	0	0	0	0	0	0	0	0	0	0	0	0
LSD (P<0.05)	0.45		0.34		0.50		0.16		0.13		0.21	

^a Mean of four replications. ^b Control-1 = No Af application in plots in the same experimental field. ^c Control-2 = No Af application in plots 500 m away from the experimental field

treatments. At 40 days it was in the range of 4.0 - 4.6 \log_{10} cfu g^{-1} soil; at 80 days from 3.3 to 3.6; and at 120 days, from 3.4 to 4.2 \log_{10} cfu g^{-1} soil (Table 5). There was no significant difference in populations in between treatments and control 1 plots. However, in control 2 plots no Af colonies resembling Af 11-4 was detected. In experiment 2, the population density trend remained similar to that of experiment 1, and there were no differences in Af populations when it was applied either at sowing or at flowering (Table 5).

In the two field screenings the population density of Af 11-4 remained stable, when the inoculum was applied each time of screening. It remains to be examined the fluctuations in its population density as influenced by several biotic and abiotic factors. For effective screening of groundnut genotypes the current population of \log_{10} 3.5-4.5 cfu g^{-1} soil appears adequate to induce over 80% seed infection in susceptible genotypes under favorable conditions. Lack of significant differences between seed infection levels and between Af populations among treatments and controls 1 plots could be due to rapid spread of Af inoculum between plots.

Soil moisture stress during the pod-filling to pod-maturing stage seems to enhance invasion by *A. flavus* and subsequent aflatoxin production in the kernels (Wilson and Stansell, 1983). In experiment 1, with 63.8% water deficit, 3.5 - 1.8% soil moisture and soil temperature range of 24.5 - 35.2 °C, the seed infection was 70 - 88% compared with those in experiment 2, where water deficit was 54.5%, soil moisture 2.8 - 1.7% and soil temperature range of 22.9 - 29.6 °C with seed infection of 29-82% (Tables 1, 4). Influence of soil moisture stress and the resultant elevated soil temperature are known to favor seed infection by *A. flavus* (Mehan *et al.*, 1988) although drought-tolerant genotypes have not been found to be resistant to seed infection by

A. flavus. Recently, Holbrook *et al.*, (2000) found that drought tolerant genotypes had less preharvest aflatoxin contamination than those of drought intolerant genotypes. By regulating frequency and duration of irrigation during the postrainy season experiment, soil moisture stress equivalent to water deficit of 63.8% was created that proved more effective in enhancing seed infection than those at 54.5% water deficit during the rainy season. However, it was easier to create water deficit during the postrainy season than during the rainy season. A significant, positive, linear relationship between water deficit and seed infection by *A. flavus* has been reported (Mehan *et al.*, 1988). These results clearly show that soil water deficit in the range of 63 to 65% could be useful for effective screening of groundnut genotypes.

Large variations have been recorded for seed colonization and seed infection among different genotypes during the same season or between seasons. Seeds from the postrainy season irrigated crops had higher colonization than those from rainy season crops (Mehan *et al.*, 1983), although it is highly dependent on the drying and curing process. Fluctuation in soil moisture during pod development and rapid drying during hot dry conditions of harvesting weaken and damage the seed testa resulting in easy fungal invasion (Glueck *et al.*, 1977; Mehan *et al.*, 1983). Large genetic variability exists in groundnut cultivars for resistance to *A. flavus* colonization and infection and this could easily be detected with improved screening methods. However, because of the basic nature of asynchronous flowering in groundnut, within and between plant variation for physiological seed maturity occurs and that further adds to the genetic variation.

The correlation between resistances to *in-vitro* seed colonization and seed infection has not been consistent (Davidson *et al.*, 1983; Waliyar and Bockelee-Morvan,

1989). This could be due to independent resistance genes for seed colonization and seed infection, and other unknown factors. Large variability seems to exist for tolerance to seed colonization, seed invasion, and aflatoxin production. The use of these refined screening techniques will allow for this variation to be exploited in breeding for resistant varieties.

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