Visual Estimation of Aflatoxin Production in Peanut with *Aspergillus* Norsolorinic Acid Mutants[1]

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ABSTRACT

Peanut seed and pods are susceptible to contamination by aflatoxin (AF), a carcinogenic mycotoxin produced by *Aspergillus flavus* Links Fr. and *A. parasiticus* Speare. Efforts to evaluate peanut lines for resistance to AF contamination have been impeded by limitations to the methodologies available for AF detection. AF cannot be seen by visible light and its detection involves grinding seed tissue in organic solvents, separation of phases, and detection by ELISA, high performance liquid chromatography (HPLC) or thin layer chromatography. These methodologies are time-consuming, expensive, labor-intensive, and are uninformative in defining the tissues of the peanut seed and pod that are most frequently contaminated with AF. *Aspergillus* AF mutants which accumulate norsolorinic acid (NOR), an orange-pigmented AF pathway intermediate, provide an easy and convenient mean to detect AF contamination. A visual rating scheme for NOR contamination of peanut seed was developed that correlated favorably to HPLC detection of both NOR and AF (r = 0.96 and 0.95, respectively). When screening the 38 plant progenies that comprise Tamspan 90 (a spanish cultivar), NOR was first seen in the intercotyledonary cavity and the interfacial surface of cotyledons and testae in seeds examined from infected pods. Immature pods were often heavily contaminated with NOR. Six of the 38 lines accumulated low levels of NOR in two laboratory tests. Additional studies are needed to determine if these results are predictive of aflatoxin contamination under field conditions.

Key Words: *Aspergillus parasiticus*, *Arachis hypogaea* L., Tamspan 90, resistance, groundnut, NOR mutant.

Peanut, *Arachis hypogaea* L., though recognized as an oilseed crop in most countries of the world, is used for food wherever it is grown. However, when the crop is grown under certain stress environments or stored improperly, aflatoxin (AF) contamination in *Aspergillus flavus* Link Fr. and *A. parasiticus* Speare infected seed

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can be a threat to human and animal health (7, 8, 12, 13). Prevention of field infection by these fungi and/or the restriction of AF contamination by the fungi in infected seed is an international priority need. Host resistance to the organisms, or to production of the carcinogenic compound in infected peanuts, could provide a comprehensive and cost-effective solution to the AF problem.

A major deterrent to the breeding of cultivars resistant to *Aspergillus* spp. infection and AF production is the lack of a rapid and reliable screening technique. Current immunological and chromographic assays used to detect AF are time-consuming, expensive, seed destructive, and poorly suited to the evaluation of large numbers of entries as required in strong breeding programs. Neither is the ultra-violet light inspection used by the U.S. peanut industry well suited for screening. *Aspergillus* strains which produce visual evidence of infection concomitant with AF production might be a useful tool in selection for resistance.

Infection of peanut by *A. parasiticus* (15) and *A. flavus* (22) mutants in which the AF synthesis pathway is partially blocked, results in a highly visible orange-red color as a result of an accumulation of norsolorinic acid (NOR), the first stable intermediate in the AF pathway. Mutant NOR strains produce NOR and AF concurrently (5). The brightly pigmented, highly visible NOR is much easier to detect than AF (4) and is useful in AF studies. Keller and coworkers (14) used *A. flavus* and *A. parasiticus* NOR mutants successfully to visualize portions of corn kernels where AF accumulates. The management of NOR mutants is relatively simple, requires little special skill or training, and the cost is economical; all of which are favorable components of a useful screening technique.

Several reports of partial resistance to these carcinogenic producing fungi in peanut are in the literature (19, 20, 21, 24). Among the germplasm lines reported as partially resistant is TxAG-5 (Tx798736) which traces to a F_4 selection from Toalson × UF 73-4022 (also reported as UF 73513) (29). Tamspan 90 which also has resistance to several other important soilborne fungi, was selected from germplasm line TxAG-5 (30). The 38 $F_{4:8}$ progenies composited to form Tamspan 90 were selected phenotypically on the basis of pod size, pod shape, and shelling percentage. Pettit and coworkers (23) found that seed of the cultivar Toalson withstood windrow curing without significant increase in *Aspergillus* infestation. Azaizeh and coworkers (2) reported that TxAG-5 had lower mean fungal infestation levels and lower AF levels than 17 other genotypes included in field tests. In 1990, Szerszen and Pettit (31) reported an isozyme band in response to challenge by *Aspergillus* that was unique to TxAG-5, even on seed produced under drought stress. They hypothesized that the band might be a defense-related protein. Although Tamspan 90 is maintained as a bulk population, the families have been maintained separately. *Aspergillus* spp. reaction was not a selection criterion for either TxAG-5 or Tamspan 90. If the 38 Tamspan 90 component lines differ genetically in resistance to AF production when challenged by the toxin-producing fungi, a higher level of resistance might result from altering the composition of the cultivar. The objectives of this study were to (a) develop a repeatable procedure for the inoculation and infection of peanut seed by *A. parasiticus* NOR mutants for comparative assessments of NOR (and AF) contamination, (b) devise a visual rating system that would discriminate among peanuts with varied AF concentrations, (c) employ visual classification of relative norsolorinic acid content in screening peanut lines for varied content (as might occur in screening lines segregating for resistance) and confirm the differences among lines by high performance liquid chromatography (HLPC), and (d) ascertain whether AF production would differ among detached pods and shelled seed of the 38 component lines of Tamspan 90 challenged in the laboratory with the *Aspergillus* NOR mutant.

Materials and Methods

All fungi were maintained at -80 C as glycerol stocks. Isolates used for the studies were *A. parasiticus* American Type Culture Collection (ATCC) 98106 (*A. parasiticus* SK1 in Keller *et. al.* (14), and *A. parasiticus* Southern Regional Research Center (SRRC) 162. Both isolates accumulate NOR and AF concomitantly. *Aspergillus parasiticus* was grown at 30 C on potato dextrose agar (PDA) for conidia production. Conidia were harvested in Tween 20 (0.1%) water and the dilution adjusted to the desired concentration for seed inoculations. Pod inoculum was prepared by growing *A. parasiticus* ATCC 98106 and *A. parasiticus* SRRC 162 on rice seeds (D.M. Wilson, pers. commun., 1993). Both *A. parasiticus* isolates ATCC 98106 and SRRC 162 were used and applied randomly. Suspensions of conidia in Tween 20 water were poured onto moist, sterile rice seed and packaged in plastic containers with cotton plugged openings for gas exchange. The inoculated seed were stored at room temperature for incubation and rotated daily until infection among the seed appeared uniform.

Pod Inoculations

Pods of the 38 F_8 lines composited to form Tamspan 90 and four spanish market-type check cultivars were increased on a field site where peaches had been cropped for several years previously and evaluated for relative fungal growth and AF production in the laboratory. The four checks included two partially AF field-resistant spanish-type cultivars—SN55-437, a widely grown drought-tolerant cultivar in West Africa (6, 31) and Junagah-11 (J-11), a commercial cultivar in India (18, 20, 31)—and two susceptible U.S. spanish-type cultivars, Pronto (3, 31) and Starr (27, 31).

Twenty intact, full-sized, visibly mature, two-seed pods each with 2-4 cm of peg attached were randomly handpicked. The pods were forced air oven-dried at 32 C until the average moisture content of the seed was between 15 and 22%. The moisture content was estimated by timing, sampling, and measurement of seed moisture (moist weight - dry weight/dry weight) of similar samples of Tamspan 90 harvested 2-3 d previously from the same location and dried under the same temperature setting in the same drying oven. Periodic subsamples of the test materials were collected and rapid dried to a constant weight to affirm the moisture content was as predicted on the basis of the previously dried Tamspan 90 samples. The pods were surface-sterilized by a series of baths — distilled

water (2×, 30 sec each), 70% ethanol (1×, 3 min), 20% sodium hypochlorite (1×, 3 min), and distilled-sterile water (2×, 30 sec each)—and placed on lids suspended above glycerin solution within sterile, 473 mL (1 pt), wide-mouth jars. Five mg of infested rice was sprinkled over the 20 peanut pods. Containers were incubated at 28.5 C and relative humidity in the jars was maintained at about 95% with a 24% glycerin solution (10). After 2 wk the containers were moved into a freezer at -70 C where they remained until visual readings were made. Treatments (lines) were replicated four times.

After spray with pure hexane to rinse spores from pod surfaces, seed were carefully removed from the pods by hand under biosafety hoods. Each seed was cut perpendicular to the suture into approximate halves and visually classified for seed color at the cut surface on an individual seed basis. Sample readings of seed from the pod inoculations were made by counting the number of seeds assigned to each of four NOR concentration classes (Fig.1):

1. 0 = no visible pigmentation on the cut surface.
2. L = low concentration (orange pigmentation covering less than 25% of the cut surface).
3. M = medium concentration (orange pigmentation covering 25-50% of the cut surface).
4. H = high concentration (orange pigmentation covering more than 50% of the cut surface).

Fig. 1. Visual scale for extent of orange pigmentation because of NOR in peanut seeds. From left to right: high, medium, low, and no color classes, respectively.

The number of seed in each class was recorded and multiplied (weighted) by factors of 0.01, 0.25, 0.50, and 1.0 for classes 1 through 4, respectively. Each sample score was the mean of the seed scores within each jar.

Although this classification method seemed practical for general screening, the classes were too broad and few in number for close examination of the correlation of visual rating and amount of AF as measured by HPLC. To more clearly define the association of visible color changes with NOR and AF concentrations, a supplementary study was made using a more detailed classification of seed color followed by quantitation for NOR and AF by HPLC. Seed were assigned to classes visibly on a scale of 0 (no coloration) to 9 with assignments for classes 1 through 6 as the proportion of the seed surface that was colored increased. Seed surfaces completely orange in color were assigned classes 7 through 9 with the highest values for the deepest orange coloration. Weighting factors were assigned to each class to facilitate numerical analysis with the HPLC measured concentrations. Weights assigned consisted of a factor of 0.01 for class 0, and increased at equal increments of 0.11 for classes 1 through 9, respectively.

The test was conducted with inoculated and incubated seed of five randomly chosen Tamspan 90 lines and Pronto which were visually rated and then analyzed for AF and NOR in accordance with published procedures. In preparation for HPLC analyses, each sample was weighed and then macerated using a mortar and pestle with 35 mL of acetone:water (75:25 v:v) (28). Following centrifugation for 10 min at 1500 rpm, the supernatant was washed with 3 mL of hexane and extracted with 2 mL of chloroform three times. The extracts were evaporated and redissolved in 1 mL of chloroform, and divided in half for AF and NOR analyses. AF was analyzed by HPLC using the methodology described by Shepherd *et al.* (26). For NOR analysis, the sample in chloroform was evaporated and redissolved in 2 mL of methanol and analyzed by HPLC following methodology described by McCormick and coworkers (17). The column was a 10 mm × 25 cm ODS 10-µm Waters reversed-phase column operated at 1 mL/min. The solvent system utilized was binary with 90% of solvent A (methanol:tetrahydrofuran, 1:1) and 10% of solvent B (0.1 M of acetic acid). Peaks were detected at 313 nm, and the retention time was 4.8 min. Three extraction variations were assessed—(a) extraction of multiple seed (pooled from a class or subclass) in 35-mL extraction solvent (acetone-deionized water (75:25 v:v), (b) extraction of multiple seed in an amount of solvent proportional to the number of seed in the sample at 35 mL/seed, and (c) extraction of one seed in 35 mL of solvent. Extractions were performed separately for all classes and subclasses. Visual NOR color scores were compared with HPLC measured AF and NOR concentrations.

Seed Inoculations

Seed of the 38 composite lines of Tamspan 90 and the four checks (as described in the pod inoculation study) were increased and evaluated for fungal growth and AF production in the laboratory. Seed of three developmental stages were used for seed inoculation studies: fresh immature (FI), fresh mature (FM), and dry mature (DM). Seed were classified as immature and mature on the basis of size, testae appearance, and internal pericarp color (11). FI and FM seed were produced in the greenhouse. Seed tested as FI were similar to those in groups 2-4 of Williams and Drexler (32). Dry, mature-appearing, field-grown stages 5 and 6 seed (32), were forced-air dried at 32 C, and stored 30 d. Two, five-seed sample replicates of each entry were surface-sterilized, inoculated, and incubated. Seed in pods visibly punctured or damaged were excluded from the tests.

Seed were surface-sterilized as described for the pod inoculation studies. Seed were split into cotyledonary halves and one cotyledon of each was placed with the facial surface up in covered sterile petri dishes on a moistened filter paper. Five cotyledons per container were inoculated with 2.5 mL of 6×10^6 conidia/mL suspension of *A. parasiticus* ATCC 98106. The other cotyledon, with germ intact, was stored for propagation. Surface-sterilized seed inoculated with water only were included as checks. Petri dishes containing the seed were placed in the dark at 28.5 C for 3 d for incubation. One hundred percent relative humidity was maintained by adding 450 µL of Tween 20 water every

6 hr. The third day following inoculation, cotyledons were rated visually for extent of NOR pigmentation (i.e., orange color).

Visual evaluation of disease intensity in cotyledons was made on a scale of 1 to 5 (1 = no fungal growth and no NOR pigmentation, 5 = heavy fungal growth and strong pigmentation). For analyses, the number of seed in each class was weighted for infection intensity with progressive values of one for class 1 through five for class 5, added, and divided by the total number of seed in the sample. The average seed score of each sample was used in all statistical analyses. Data obtained on NOR visual ratings and both AF and NOR concentrations were analyzed using simple correlation and 2-way analyses of variance.

Results and Discussion

Pod Inoculations (PI)

The weighted visual scores for the seed of the 38 Tamspan 90 lines and four checks ranged from 0.52 to 0.17 (Table 1). Lines 24 and 12 showed the highest and lines 15 and 34 the lowest percentage of NOR pigmentation. The highest score was three times the lowest, but the differences among means were not significant at $P \leq 0.05$.

Table 1. Weighted visual scores of orange (NOR) color in seed following pod inoculation of checks and Tamspan 90 lines.

Entry	Seed from mature full size fresh pods[a]
Pronto	0.45 n.s.
Starr	0.44
55-437	0.38
J-11	0.36
Tamspan 90 lines range:	
Max.	0.52
Min.	0.17

[a]The number of seed in each class was recorded and multiplied by a factor of 1.0, 0.50, 0.25, and 0.01 for high medium, little, and no pigmentation, respectively. n.s. = not significant.

Color in the inoculated and incubated mature shells varied from none to an extensive splotchy orange color. Damaged shell tissue surrounding minute previously unnoticed penetration sites (e.g., by piercing mouth insects) became intensely orange. Immature pods, mistakenly chosen as mature based on external features, consistently developed intense orange pigmentation.

In general, pods with heavy external fungal growth had seed with high NOR production. However, a few exceptions occurred where little seed coloration was found in pods with heavy fungal growth and vice versa. Similar occasional associations of *Aspergillus* spp. mycelial growth and AF formation have been reported previously (9, 25).

The orange color in the seed was most prominent in the intercotyledonary cavity (lumen) and the interfacial surface of the cotyledons and testae. In many samples, the facial surfaces were deeply orange-stained with the color diffusing from those surfaces into interior portions of the cotyledons. In other samples, coloration of the facial tissues was very light orange or not apparent, indicative of little or nor NOR production. Much care was required with such seed to differentiate the very light NOR pigmentation from the interior tan surface of the testae.

Seed Inoculations

It was observed during preliminary tests, that cotyledons with germinating embryos inhibited fungal growth and NOR production in petri dishes with water-agar media. In the absence of germination, however, it was usual that fungal growth and infection in the two cotyledons of the same seed, with or without the germ, followed a similar, mirror-image pattern. Thus, embryos were subsequently removed from all cotyledons tested to avoid germination that might alter or inhibit AF production. Cotyledons with the germ intact could be used for reproduction and conservation of the genotype. Keller *et al.* (14) reported similar inhibition of *Aspergillus* growth and aflatoxin production by germinating corn embryos.

Fresh Immature Seed (FI). Scores for the four checks differed only by 0.9 points. 55-437 had less NOR than Pronto and J-11 but others differences among parents were not different statistically ($P \leq 0.05$) (Table 2). The range in the weighted scores of the Tamspan 90 lines was much greater with a range from 5.00 to 0.19. Twelve lines were not different from 55-437, the check with the least NOR coloration.

Fresh Mature Seed (FM). Very little fungal growth or NOR production was observed within any of the lines and four checks (Table 2). Similar results were found in a separate experiment where mature freshly harvested (undried) seed did not support fungal growth (16). The reason(s) for lack of fungal invasion at this stage are unknown as simultaneous inoculation of dried, stored seed were highly infected.

Table 2. Weighted visual scores of orange (NOR) color in seed following pod inoculation of checks and Tamspan 90 lines.

Entry	Fresh immature seed[a]	Dry mature seed[a]
Pronto	1.40	2.75
Starr	0.80	2.41
55-437	0.50	2.58
J-11	1.30	3.58
Tamspan 90 lines range:		
Max.	5.00	4.08
Min.	0.19	1.00
LSD (0.05)	0.64	1.14

[a]The number of seed in each class x progressive weighting values of one for class 1 to 5 for class 5, added, and divided by the total number of seed in the sample.

Dried Mature Seed (DM). The range in NOR scores was wide but less than in the FI test (Table 2). NOR production on two Tamspan 90 lines (5 and 26) was less ($P \leq 0.05$) than in all checks. Among the check varieties, Starr and 55-437 ranked lowest in NOR production and Starr was different ($P \leq 0.05$) from J-11. The J-11 seed available for this comparison were small and of marginal quality which might have been a factor in these results.

Reactions of the fungus on seed of the same entry were variable, both among and within petri dishes. Fungal growth and NOR production on FM seed differed greatly from that on DM stored seed. Variation within petri dishes was possibly a result of physiological age as well as other factors. The correlation between the visual ratings of color intensity and quantity of aflatoxin as determined by HPLC analysis on seven Tamspan 90 lines was significant ($r = 0.50$, $P \leq 0.05$).

We were unable to obtain data on the association of these laboratory data and aflatoxin production under field conditions. If the fungal infection and AF production in cotyledons, or in detached, newly harvested, partially dried pods related informatively to fungal infection and AF production in nature, the technique might be very useful in preliminary screening for AF resistance. Our attempts to investigate the relationship between the laboratory and field reactions were unsuccessful because of scarcely detectable AF development in samples collected from three field tests at different times and locations which were inoculated artificially with the fungus.

The results from the immature seed of the Tamspan 90 lines did not correlate well with the results from the dried mature seed or the intact pod inoculations. Results from the latter two methods were similar, perhaps because the seed in both tests were mature. The main differences in seed test conditions between the DM and pod tests were the enclosure of seed in intact shells during inoculation, and the difference in humidity chambers. Six lines (11, 13, 25, 26, 28, and 37) were among the 50% of the Tamspan 90 lines with the least color in both the DM and PI methods.

In both the DM and pod procedures, the coefficients of correlation for visual ratings, NOR, and AF on multiple-seed extractions of the five randomly chosen T-90 lines and Pronto were significant ($P \leq 0.05$) (Table 3).

Table 3. Coefficients of correlation for weighted visual color score and amount of AF and NOR in sample (multiple seed) as measured by HPLC.

Correlation coefficient	r values	
	Sample[a] basis	Entry[b] basis
Visual[c] vs. AF	0.64**	0.81**
Visual vs. NOR	0.48**	0.59**
AF vs. NOR	0.64**	0.73**

[a]48 samples.

[b]24 entries, two replications.

[c]Visual: Average weighted visual score based on a four-class scale: high, medium, low, and no orange (NOR) color.

**Significant at P = 0.001.

The "r" values were higher when calculated on an entry mean basis than on a sample basis. Nevertheless, the coefficients were lower than we expected based on preliminary studies using smaller samples of seed. Although NOR is reported as insoluble in hexane, the concentration of NOR from multiple seed samples in the standard 35 mL of hexane was hypothesized to be affecting the results. To test our hypothesis, simultaneous analyses were made on 12 single and five multiple-seed samples from three lines with results as shown in Tables 4 and 5. The associations of NOR and AF and the visual score

Table 4. Effect of sample size and amount of solvent on quantification of NOR and AF by HLPC in Table 4. Effect of sample size and amount of solvent on quantification of NOR and AF by HLPC in selected Tamspan 90 lines.

Line no.	Seed	Color class[a]	Solvent	Visual score[b]	Total NOR	Total AF
	no.		mL		ppb × 1000	
10	1	9	35	1.00	653.7	29.5
10	1	8	35	0.89	453.9	17.1
10	1	7	35	0.78	403.2	16.8
10	1	6	35	0.67	453.8	13.4
10	1	5	35	0.56	211.0	9.3
10	1	4	35	0.44	137.5	9.2
10	1	3	35	0.33	153.5	6.9
10	1	2	35	0.22	46.6	0.5
10	1	1	35	0.11	38.9	1.7
10	1	0	35	0.01	0.1	0.0
10	1	0	35	0.01	0.0	0.0
10	1	0	35	0.01	0.0	0.0
10	5	H	35	5.00	43.4	15.5
10	6	M	35	3.00	135.8	10.3
10	10	L	35	2.50	4.5	22.9
12	9	H	160	9.00	577.7	36.3
13	12	H	160	12.00	515.7	64.2

[a]Classes: 0 (no coloration) through 9 (uniformly bright orange) assigned on basis of proportion and intensity of color in surface of cut cotyledons:

Weighted (No. seed per color class × factor)/total no. seed per sample where factor assignments are 0 = 0.01, and classes 1 through 9 increased incrementally to a maximum of 1.00 for class 9.

Table 5. Coefficients of correlation for weighted visual scores of NOR, ppb of AF and NOR in multiple and single seed samples.

Correlation coefficient	Multiple[a] seed	Single[b] seed
Visual vs. AF HPLC	0.87**	0.95**
Visual vs. NOR HPLC	0.45	0.96**
AF HPLC vs. NOR HPLC	0.72**	0.97**

[a]Extractions from up to 12 seed in 35 mL solvent.

[b]Extraction of one seed in 35 mL solvent acetone-deionized water (75:25 v:v).

**Significant at P = 0.01.

with amounts of NOR and AF were much better in the single seed than in the multiple-seed analyses. In several comparisons, equal amounts or more AF and NOR were detected in single than in multiple-seed samples of the same NOR color category (Table 4). To confirm those findings, 48 additional single-seed samples from lines 10 and 25 were analyzed visually and with the HPLC. The relationship among these observations was strong (Tables 6 and 7). This verified that the quantitation of NOR and AF in the multiple-seed samples was less precise than in the single-seed samples, and indicated that NOR is somewhat soluble in hexane. The linear regression of visual scores on the quantity NOR among the 48 single seed samples was highly significant (P = 0.0001) with an r^2 = 0.87 (Fig. 2). An even closer relationship of NOR content and visual score was revealed by regression analysis using a quadratic model (r^2 = 0.94). The confounding effect factor is suspected to be seed size; the visual score underestimated the quantity of NOR in large, heavily pigmented seed. These results confirmed the similarity of results from visual and chromatographic methods of analysis on single-seed samples.

The purpose for extracting from multiple-seed samples was to evaluate the average toxin production by class within an experimental unit (jar). The seed within a jar were separated into color groups and analyzed. Since the number of seed in the different color groups within a jar varied, the number of seed per analysis varied. With limited solvent, multiple seed samples and high NOR contamination, demarcation of the extraction layers (peanut oil, hexane, and organic) was obscured by dark coloration. The oil layer acquired a distinct orange color, indicating a possible loss of NOR into the oil, and the intensity of the yellow color in the hexane layer increased proportionately with the intensity of orange in the seed. This suggested that some NOR was being discarded with the hexane. With solvent increased proportionate to the sample size, it was much easier to differentiate the oil layer, larger quantities of AF and NOR were detected, and the color of the extracted NOR more closely resembled the actual orange-red of pure NOR (results not shown). It became evident that for good purification the quantity of solvent should be proportional to the size of the seed sample.

The results indicate that (a) visual rating of NOR contamination of peanut reflect relative concentrations of AF contamination, (b) the extraction procedure which produced the most accurate HPLC quantitation of both NOR and AF was the single seed extraction, but reasonable estimation can be obtained for multiple seed evaluation by proportional adjustment in quantity of solvent, (c) there was no obvious advantage to either

Table 6. Relative NOR and AF in individual seed of one Tamspan 90 line as measured by HPLC and as estimated by weighted visual score.

Line	Class[a]	Visual score[b]	NOR	AF
			ppb × 1000	ppb × 1000
10	9	1.00	584.4	38.5
10	8	0.89	392.6	18.6
10	7	0.78	393.6	23.3
10	6	0.67	359.8	16.2
10	5	0.56	195.7	8.7
10	4	0.44	192.4	6.6
10	3	0.33	89.0	6.0
10	2	0.22	33.2	2.0
10	1	0.11	26.5	2.5
10	0	0.01	0.0	0.0
10	0	0.01	0.0	0.0
10	0	0.01	0.0	0.0

[a]Classes: 0 (no coloration) through 9 (uniformly bright orange) assigned on basis of proportion and intensity of color in surface of cut cotyledons:

Weighted (No. seed per color class × factor)/total no. seed per sample where factor assignments are 0 = 0.01, and classes 1 through 9 increased incrementally to a maximum of 1.00 for class 9.

Table 7. Coefficients of correlation for visual NOR pigmentation, HPLC quantifitation of AF and NOR for one-seed samples[a].

Correlation coefficient	Sample basis[b]	Entry basis[c]
Visual vs. AF	0.83**	0.88**
Visual vs. NOR	0.95**	0.95**
AF vs. NOR	0.86**	0.97**

[a]Mean of two replications of each of two Tamspan 90 lines, 10 and 25.

[b]Values calculated on 48 samples.

[c]Values calculated on 24 entries.

**Significant at P ≤ 0.01.

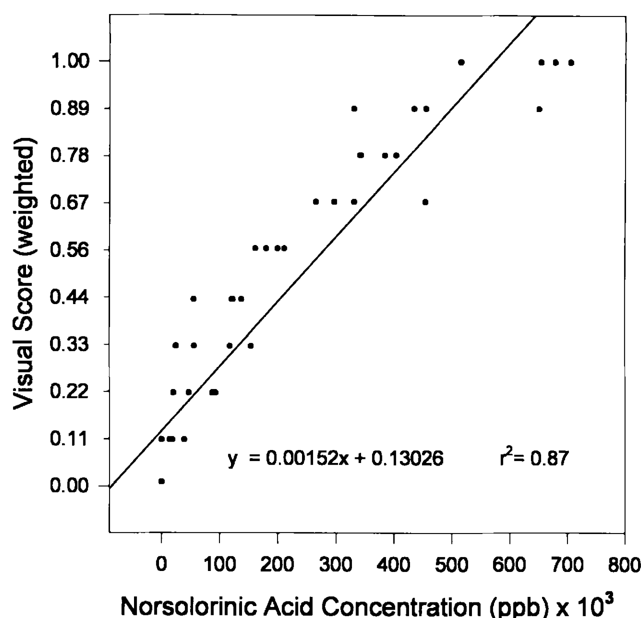

Fig. 2. Regression of weighted visual score on norsolorinic acid (ppb) in 48 seed of two Tamspan 90 component lines.

inoculation procedure with regard to NOR rating although the age of the seed did appear to be a factor, and (d) the pod inoculation method potentially affords a means to understand the route of entry and progressive infection of peanut seed by *Aspergillus* as well as showing which tissues are most likely to be contaminated with AF.

Our results indicate that comparative evaluations of seed lots for AF content following infection by NOR *A. parasiticus* mutants can be made visually, rapidly, and economically. Such characteristics are needed in procedures applicable to plant breeding. The test under laboratory conditions produced uniformity and repeatability which has been extremely difficult to achieve under field conditions for this trait.

We do not know whether the difference in AF production among the Tamspan 90 entries ($P \leq 0.05$), if repeatable, would be sufficient to be of importance if the field and laboratory response are correlated. False hopes for useful AF resistance have been generated through previous research (21, 33). Perhaps the need for host plant resistance as a control for AF contamination has induced researchers to grasp for differences which, as suggested by Anderson *et al.* (1) were too small or nonexistent. Caution should be exercised in hastening toward conclusions without appropriate confirmations.

If results from these procedures and field results are adequately correlated, the utilization of NOR mutants as a screening technique could be used to complement more accurate quantitation methods. It could be very helpful in breeding for *Aspergillus*/AF resistance to have a basis for discarding large numbers of lines with low potential for resistance without the need to conduct expensive and time consuming analyses such as HPLC or ELISA. This study suggests that visual classification of seed following infection with NOR mutants might offer that opportunity. In practice, the visual evaluation could be restricted to the four main categories (i.e., high, medium, low and no NOR concentrations).

The strong orange color that was observed in immature shells could be of importance to the feed industry. Perhaps fungal growth and AF production is aided by nutrients present in immature pods, or defense mechanisms are not active in these pods. It would be useful to conduct a more in-depth study on the use of *Aspergillus* NOR mutants in evaluating AF contamination of shells with regard to the use of shells in the animal feed industry.

Our evidence was insufficient to conclude whether the variability in reaction for *Aspergillus* growth and AF production among the Tamspan 90 lines was a result of genetic resistance or sampling error. However, because of the spread in color scores observed we are not comfortable in concluding that differences among the lines did not exist. The potential for some lines (11, 13, 25, 26, 28, and 37) to have useful resistance to the molds and to toxin production should be explored. However, a critical question persists as to the predictive ability of the laboratory results relative to that which might occur under field conditions.

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