Rapid Screening Method for Resistance to Aflatoxin Production in Peanut[1]

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ABSTRACT

A simple, economical, and rapid method to detect aflatoxin in peanut is described in this paper. The extract from 1 g of peanut is added to 1 mL of $NH_4H_2PO_4$-$HgCl_2$ solution containing 0.005 g agar in a tube. The concentration of aflatoxin can be visually estimated by the tube fluorescence which is directly correlated with the concentration of aflatoxin on a simple linear regression ($r=0.999$, $P<0.01$). The lowest aflatoxin amount that could be detected was 0.02 μg/g by a spectroflurophotometer and was 0.04 μg/g by visual observation under UV light. There was no significant difference between the amounts of aflatoxin estimated by the tube fluorescence method and by TLC ($P>0.10$) in an experiment containing 10 samples. This method should be applicable for fast identification of peanut genotypes of single plants with resistance to aflatoxin production.

Key Words: Peanut, aflatoxin, resistance, screening, method.

Following recognition of the peanut (*Arachis hypogaea* L.) aflatoxin problem in 1960, there has been much research into control and prevention of aflatoxin contamination in peanut by *Aspergillus flavus* Link:Fr. and *Aspergillus parasiticus* Speare. Of several control strategies for aflatoxin contamination in peanut, breeding for resistance is considered as a sound, long-term and effective approach (12, 15).

In 1967 Rao *et al.* (14) and Kulkarni *et al.* (5) each reported a resistant variety of peanut to aflatoxin contamination. Since then, at least two kinds of resistance have been discovered in peanut, ie. resistance to invasion by *A. flavus* and resistance to aflatoxin production even though invasion occurs (8, 9, 10, 11, 12, 13, 15). About thirty genotypes were reported to have resistance to seed invasion by *A. flavus* (11), and only two genotypes were reported to have resistance to aflatoxin production (9).

A laboratory inoculation method was developed by Mixon and Rogers (12) for screening peanut genotypes for resistance to *A. flavus* invasion of rehydrated, mature, sound seeds. It is simple and inexpensive and it has been adopted by many researchers. Mehan and McDonald (7) developed a laboratory method to screen peanut for resistance to aflatoxin production in which the assay of aflatoxin was by minicolumn or thin-layer chromatography (TLC) methods. By this method they tested 502 peanut genotypes and found two resistant genotypes, U4-7-5 and VRR245 (9, 10, 17).

In the screening procedure to identify genotypes resistant to aflatoxin production, peanut seeds inoculated by aflatoxigenic strains of *A. flavus* must be tested for aflatoxin content. Breeders require screening vast numbers of peanut genotypes rapidly and economically. Such screening differs from programs which survey agricultural commodities, mixed feeds, and raw or processed foods to quantify aflatoxin concentrations. The breeders' interest is in how to rapidly identify resistant genotypes rather than quantify the amount of aflatoxin per se in each genotype. In this regard, currently available screening methods including minicolumn and TLC are unsuitable because of expensive, time-consuming or laborious procedures (17). In addition, these methods are also unsuitable to screen single plants because too many seeds (20-50 g) are needed to test. Thus it is difficult by these methods to identify resistant genotypes in the F_2 generation from crosses between resistant and adapted genotypes, and also to study the genetics of the resistance.

To rapidly identify aflatoxigenic isolates of *A. flavus*, several screening methods for aflatoxin production have been devised which rely on a blue fluorescence in agar medium under ultraviolet (UV) light (2, 3, 6). Cotty (1) described a somewhat similar method but used a quantitative measure of agar medium fluorescence in a tube by a scanning densitometer to estimate aflatoxin production of isolates of *A. flavus*. This method is fast and simple. However, as with other agar media mentioned above, the ingredients are complex to support the growth of *A. flavus*. The author also did not show how the method was used for estimating aflatoxin in peanut.

This paper describes a simple, rapid and inexpensive method, the tube fluorescence (TF) method, somewhat similar to Cotty's method (1), for large-scale screening of peanut genotypes for resistance to aflatoxin production.

Materials and Methods

Equipment and Reagents– The equipment used included a small grinder, high speed shaker, water bath and UV-lamp with 365 nm. The reagents used included CH_3OH, $CHCl_3$, $NH_4H_2PO_4$, $HgCl_2$, and agar powder. A solution of $NH_4H_2PO_4$-$HgCl_2$ consisted of 8.69×10^{-2} M $NH_4H_2PO_4$, 5×10^{-4} M $HgCl_2$, and distilled water.

Inoculation procedure-In an experiment containing 10 samples, about 20 g of peanut seeds of variety 5105 were inoculated according to the method described by Mehan *et al.* (7). Intact mature seeds were surface sterilized for three min in a 0.1% aqueous solution of mercuric chloride, rinsed in five changes of sterile water and hydrated to 20% moisture. The seed were then placed in a plate and their testae were scarified with a sterile needle. They were then surface inoculated with one mL of conidial suspension (4×10^6 conidial/mL) of an aflatoxin B_1-producing strain of *A. flavus* (AFP81-1). After incubation at 25 C for 3-10 days (3 days for samples A and B, 5 days for samples F and G, 7 days for samples C, E and J. 10 days for samples D, H and I) the seeds were tested for aflatoxin production by TF and TLC, respectively.

Reference standards–Pure aflatoxin B_1 (AFB_1) (SIGMA Chemical Company) was added to tubes (15x100 mm) at concentrations of 0.00, 0.02, 0.04, 0.08. 0.12, 0.16, 0.24, 0.32, 0.40, 0.48, 0.56, 0.64, 0.72, 0.80 and 0.88 μg, each being replicated three times. The solvent of Benzene-Acetonitrile (49:1, V/V) in the tube was evaporated to dryness at 90 C in a water bath, and one ml of $NH_4H_2PO_4$-$HgCl_2$ solution was added with 0.005 g of agar powder. After the agar was dissolved at 90 C, the tube was shaken and then kept still until the agar solution solidified. The tube fluorescence was determined by a spectrofluorophotometer (RF-540: Shimadzu Scientific Instruments. Inc., Japan) with 366.6 nm excitation wavelength. The fluorescence intensity (443.7 nm) was recorded on a linear scale of 0 to 100. Then a linear regression analysis was carried out for the correlation between AFB_1 amounts in the tubes and the tube fluorescences.

Assay by TF– The previously inoculated seeds were surface sterilized with 70% aqueous solution of alcohol. After the alcohol volatilized, the seeds were dried at 90 C for one hour and then ground. One g of each sample powder with two replicates was mixed with 10mL of methanol-

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water solution (55:45, V/V) in a 20x160 mm tube for 5-10 sec at high speed, followed by incubation for 20 min. One mL of supernatant liquid was extracted with 2 mL of chloroform in a 15x100 mm tube for several sec at high speed. After the layers separated, the upper layer was removed by an air pump. The chloroform was evaporated to dryness at 90 C in water bath and one ml of $NH_4H_2PO_4$-$HgCl_2$ solution was added with 0.005 g of agar powder. After agar in the tube was dissolved at 90 C in water bath, the tube was shaken and then kept still until the agar solution solidified. The tube fluorescence was visually determined under UV light by comparison with the intensities of standard references and then aflatoxin amount was estimated by the regression equation. If the tube fluorescence of sample was too intense to match the highest standard, the agar solution had to be diluted.

To test recovery of the toxin by this method two samples of variety 5105 were spiked with pure AFB_1 at concentrations of 0.4 and 0.8 μg/g, each being replicated five times. For spiking, a measured volume of AFB_1 standard solution was added to a tube and evaporated to dryness before the ground sample was added in.

Assay by TLC–To compare the tube fluorescence determination with aflatoxin concentration, aflatoxin level of the same samples (A-J) were measured by TLC, each being replicated two times. One g of sample powder was extracted with 10 mL of methanol-water solution (55:45, V/V) and 2 mL of petroleum ether for 30 min at 300 rpm. One mL of methanol-water filtrate was extracted with 2 mL of chloroform for several sec at high speed. After the layers separated, the upper layer was removed, 2 mL of water was mixed and removed again. The chloroform extract was dried with one g of anhydrous sodium sulfate. The one mL of the chloroform extract was evaporated to dryness at 90 C in a water bath. The residue was then dissolved in one ml of benzene-acetonitrile (98:2), V/V) for TLC using silica gel G coated (250-μm-thick) plates and developing them in chloroform-acetone (92:8, V/V) (16).

Data obtained from TF and TLC were analyzed by t test of significance using Paired-sample test.

Results and Discussion

A highly significant direct linear relationship occurred between the tube fluorescence of the reference standards and the amounts of AFB1 in the tubes (Fig. 1). No significant difference was observed among replicates (P>0.05). The lowest AFB_1 concentration that could be determined was 0.02 μg/g although the difference of tube fluorescence between 0.00 μg/g and 0.02 μg/g was not significant (Table 1.) The lowest AFB_1 concentration that could be visually detected under UV light was 0.04 or 0.08 μg/g.

By this tube fluorescence (TF) method, one naturally contaminated sample and two samples spiked with AFB_1 were analyzed. The rates of recovery of AFB_1 in spiked samples were 78% and 82% respectively (Table 2).

In the experiment containing 10 samples, the concentrations indicated by TF method were slightly less than those by TLC but there were no significant difference

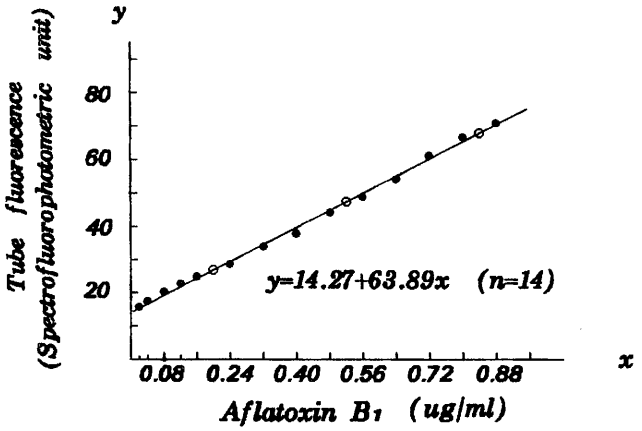

Fig. 1. A linear relationship between aflatoxin B_1 concentration and tube fluorescence (r^2=0.997, P<0.01, n=14).

Table 1. Tube fluorescence (TF) and aflatoxin B_1 (AFB_1) concentration in standard reference tubes.

standard reference	AFB_1 Concentration (μg/g)	Tube fluorescence *
1	0.88	71.01 ± 2.57 [a]
2	0.80	66.83 ± 1.27 [b]
3	0.72	61.24 ± 1.32 [c]
4	0.64	54.19 ± 0.09 [d]
5	0.56	49.12 ± 1.50 [e]
6	0.48	44.37 ± 0.35 [f]
7	0.40	37.94 ± 0.36 [g]
8	0.32	34.05 ± 1.68 [h]
9	0.24	28.77 ± 0.14 [i]
10	0.16	24.95 ± 1.64 [j]
11	0.12	22.78 ± 0.69 [k]
12	0.08	20.20 ± 0.75 [l]
13	0.04	17.45 ± 0.53 [m]
14	0.02	15.71 ± 0.17 [mn]
Control	0.00	13.72 ± 1.57 [n]

* Spectrofluorophotometric units on a linear scale of 0 to 100. Tube fluorescence values were inverted to $\sin^{-1}\sqrt{p}$. Values not followed by the same letter are significantly different (P<0.01) by analysis of variance. The linear mean square and error mean square values were 1116.76 and 1.57, respectively. Means were separated (P=0.05) by Duncan's multiple range test.

Table 2. AFB_1 estimation on spiked peanut samples by tube fluorescence (TF) method.

Sample	Amt of AFB_1 added (μg)*	Amt of AFB_1 estimated (μg/g)**	Rate of recovery (%)
Control (unspiked)		0.53±0.137	
A	0.4	0.86±0.136	82.0
B	0.8	1.15±0.175	78.0

* Pure aflatoxin B1 added to a ground peanut sample.
** Means of 5 replicates, each consisting of a 1 g sample.

between them (P>0.10, n=10) (Table 3.). In addition, it was observed in this experiment that the finer the seeds were ground, the less difference there was between the two methods (as the samples D to H and J).

The results of this study indicate that the TF method could be accepted for estimating aflatoxin in peanut. The TF procedure saves time and materials required for large-scale screening of peanut genotypes for resistance to aflatoxin production. Most peanut genotypes support aflatoxin production of *A. flavus*, and the yields of aflatoxin are generally above 20 μg/g seed (9, 11). In this situation, the TF method is very conveniently used for isolating a resistant genotype in which aflatoxin yield is below 20 μg/g seed or 10 μg/g seed from large numbers of peanut genotypes because all of the sensitive genotypes can be easily eliminated by their very strong tube fluorescence intensities. Of course, aflatoxin can be more accurately determined by a spectrofluorphotometer or a scanning densitometer as described by Cotty (1) if necessary.

Only AFB_1 was used in this paper because we only have this kind of toxin. Though the fluorescence color of AFG_1

Table 3. Comparison of TF and TLC methods of aflatoxin estimation using peanut samples artificially inoculated with *Aspergillus flavus*.

Sample	TF method		Aflatoxin by TLC (μg/g)
	Tube fluorescence[a]	Amt. of aflatoxin estimated (μg/g)[b]	
A	29.62	2.40	2.00
B	31.95	2.77	3.60
C[c]	43.39	4.56	5.71
D[c]	47.24	5.16	5.05
E[c]	47.24	5.16	5.60
F	47.24	5.16	6.00
G	49.14	5.46	5.60
H[c]	49.14	5.46	6.11
I[c]	61.21	7.35	6.67
J[d]	61.21	7.35	7.98

[a]: The value of tube fluorescence was given by visual comparison with those of reference standards and inverted to $\sin^{-1}\sqrt{p}$.

[b]: The amount of aflatoxin was estimated by the equation $x=(y-14.27)/63.89$ ($r^2=0.997$, $P<0.01$, $n=14$).

[c,d,e]: The extractions of these samples were diluted 42 times for "c", 21 times for "d" and 2 times for "e", respectively, so the amounts of aflatoxin estimated were 1/42 for "c", 1/21 for "d" and 1/2 for "e" of the totals of aflatoxin.

and G_2 is yellow green, all aflatoxins will contribute to the tube fluorescence intensity as is the situation in the minicolumn method (4, 16).

The TF procedure also saves peanut seeds required for assaying aflatoxin besides saving time and materials. It should be especially useful in identifing the resistance genotypes of single plants in the F_2 generation and studying the genetics of resistance to aflatoxin production.

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