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Relationship between Fungal Growth and Aflatoxin Production in Varieties of Maize and Groundnut

E. Priyadarshini* and P. G. Tulpule

Available methods to quantitate fungal growth in infected grains are far from satisfactory. A chemical method to estimate fungal growth in infected plant tissue has been successfully used to estimate accurately the somatic amount of *Aspergillus parasiticus* on infected grains. Varieties of maize and of groundnuts were studied with respect to their capacity to promote fungal growth and aflatoxin production. No direct correlation was observed between fungal growth and aflatoxin production by the fungus among varieties of maize and groundnut, suggesting that genotypes support different amounts of fungal growth and also different amounts of aflatoxin production per unit growth of the fungus.

Contamination of food grains by some storage fungi such as *Aspergillus flavus* and *Aspergillus parasiticus* is known to occur very widely. During their growth, these fungi produce a group of toxic metabolites collectively known as aflatoxins which are potent hepatotoxins and carcinogens. Consumption of such contaminated foodstuffs have been shown to be hazardous to a variety of animals including the monkey (Butler, 1974; Gopalan et al., 1972; Tilak, 1975) and more recently to man (Krishnamachari et al., 1975a,b).

Considerable efforts have therefore been directed toward preventing aflatoxin contamination of food grains. Among new approaches to the problem has been an attempt to identify and develop varieties which are resistant to aflatoxin production. Earlier work reported from this Institute (Rao and Tulpule, 1967; Nagarajan and Bhat, 1972, 1973) and from elsewhere (Mixon and Rogers, 1973; LaPrade, 1973) have shown that varietal differences do occur among genotypes of maize and groundnut with respect to their capacity to support production of aflatoxins by *A. flavus* and *A. parasiticus*. It has also been observed that under experimental conditions, aflatoxin production appreciably increased in some common food grains following their exposure to ^{60}CO irradiation (Priyadarshini and Tulpule, 1976). The basis for these differences in aflatoxin production, however, is not clear. It may be due to differences in fungal growth, to differences in the amount of toxin produced per unit growth of the fungus, or to both. Methods available so far to quantitate fungal growth in natural substrates are far from satisfactory. Recently, Ride and Drysdale (1972) have successfully developed a chemical method for quantitating the growth of *Fusarium* strains in infected leaflets of the tomato plant, using chitin as a biochemical marker. Chitin, a β, 1,4 linked linear polymer of 2-*N*-acetyl-D-glucosamine, cannot be detected in higher plants but forms a major constituent of most fungi and green algae. Chitin cannot be estimated directly, but Ride and Drysdale (1972) have suggested an alkali treatment which partially depolymerizes and deacetylates chitin to produce chitosan units, which can then be assayed for glucosamine by the modified method of Tsuji et al. (Ride and Drysdale, 1972).

Using this method, wherein glucosamine values can be used to determine fungal growth, studies were undertaken to quantitate the growth of *A. parasiticus* and to determine the correlation between fungal growth on the one hand and the amount of aflatoxin produced on the other.

EXPERIMENTAL SECTION

Aspergillus parasiticus NRRL 2999 strain was maintained on Potato Dextrose Agar slants. To determine the conversion factor (i.e., translational unit of glucosamine content into fungal growth) an in vitro glucosamine estimation of the fungus grown on a standard synthetic medium was done. Spores from a 4-day-old culture were inoculated into sterilized flasks containing 50 mL of standard synthetic medium (Adye and Mateles, 1964) and incubated at 27 °C for 7 days. At the end of the incubation period, the well-formed fungal mycelial mat was thoroughly washed with distilled water and homogenized to a known volume in a mechanical homogenizer. The dry weight of the fungal mycelial mat was determined by transferring 3-mL aliquots of the homogenate into preweighed aluminium foil boats and drying in an oven at 115 °C to constant weight. Suitable aliquots from the same homogenate were processed and assayed for glucosamine as described by Ride and Drysdale (1972), with a few minor variations. The initial alkali hydrolysis was run for 2 h at 130 °C. All centrifugations were run for 20 min at 2000*g*. These variations were introduced to ensure complete hydrolysis and better separation of the particles. To determine varietal differences, 10-g lots of 13 varieties of maize and six varieties of groundnut in duplicates were infected in the laboratory with a uniform spore suspension

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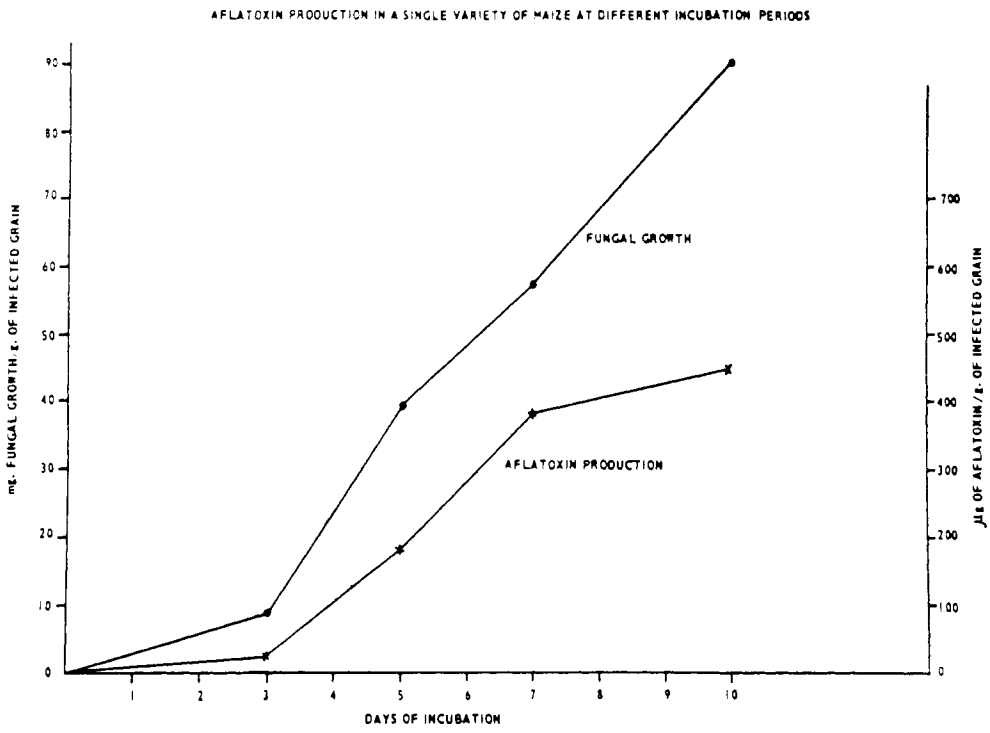

Figure 1.

of *A. parasiticus* and incubated at 27 °C for 7 days. The material was then dried in an oven at 60 °C and powdered. Aliquots (~100 mg) in duplicate were taken for the processing and estimation of glucosamine (Ride and Drysdale, 1972), and the rest of the material was used for extraction and estimation of aflatoxin by the multimycotoxin method of Stoloff et al. (1971). Quantitation of aflatoxin on TLC plates was done using a Photo-volt densitometer. Ten-gram lots of uninfected grains were also processed similarly. To determine the relationship between fungal growth and toxin production at different periods of incubation, 10-g lots of a single variety of maize in duplicate were infected with *A. parasiticus* and measurements made on days 3, 5, 7, and 10 of incubation at 27 °C. In all the experiments, the grains were sterilized by autoclaving at 15 lb for 15 min and then inoculated with 1 mL of a uniform spore suspension (approximately 8×10^5 spores/mL) of a 4-day-old culture of *A. parasiticus* NRRL 2999.

RESULTS

In six replicates, the glucosamine content (expressed as microgram/milligram of dry weight of the fungus) of mycelia of *A. parasiticus* grown on standard synthetic media varied within a narrow range of 370–390 μg/mg of dry weight of the fungus with a mean value of 380 μg/mg of dry weight of the fungus.

The relationship between the growth of *A. parasiticus* and aflatoxin production in a single variety of maize is shown in Figure 1. It is clearly seen that a linear and parallel correlation exists between growth of the fungus and aflatoxin production, up to 7 days of incubation, after which toxin production tends to fall off from linearity although the fungus continues to grow up to 10 days. The correlation between fungal growth and aflatoxin production at different incubation periods are shown in Table I. There exists a significant correlation between aflatoxin production and fungal growth with incubation time.

The results of the relationship between fungal growth and toxin production in different varieties of maize and groundnut for a given incubation time are presented in Tables II and III, respectively. They clearly indicate the presence of wide variations between varieties of both maize and groundnut with respect to not only the growth of the fungus as calculated from the values of glucosamine but also the quantity of aflatoxin produced. In all varieties, a correlation similar to that observed in a single variety (Figure 1) may be expected between aflatoxin production and fungal growth. However, the absolute values vary

Table I. Mean Values of Fungal Growth (in Terms of Glucosamine) and Aflatoxin Production at Different Incubation Periods with Level of Significance[a]

Incubation period in days		Mg of fungal dry weight/g of infected grain	Aflatoxin production in μg/g of infected grain
3		8.58	20.6
5		39.60	179.0
7		57.30	381.0
10		85.55	447.0
CD[d] at 5%		19.81	134.79
at 1%		30.01	223.54
Replicates			
I		50.43	244.10
II		43.79	269.70
I + II		47.76	256.90
Analysis of variance table:		*F* ratio:	
Source	(d.f)[d]		
Replicates	(1)	0.62	0.48
Incubation period	(3)	32.05[c]	32.08[b]
Error	(3)	1.00	1.00
Total	(7)		

[a] When differences between replicates were not significant, the replicate sum of squares were mixed with error sum of squares and was used when testing the significance of sum of squares due to Incubation period. (2) Those marked with asterisks are only significant. (3) The coefficient of correlation: between incubation period and fungal growth = 0.9913 ($P < 0.01$); between incubation period and aflatoxin production = 0.9594 ($P < 0.05$); between aflatoxin production and fungal growth = 0.9706 ($P < 0.05$). [b] $P < 0.01$. [c] $P < 0.001$. [d] CD, confidence difference; d.f, degrees of freedom.

Table II. Fungal Growth (in Terms of Glucosamine) and Aflatoxin Production in 13 Varieties of Maize[c]

Sl. no.	Variety of maize	Mg of fungal dry weight/g of infected grain*	Aflatoxin production	
			μg/g of grain	μg/mg of fungal dry weight
1.	EH-400675	22.5	278	12.4
2.	Comp. H-3	23.6	505	21.4
3.	Syn B-21	25.1	562	22.4
4.	Comp. B-VI	26.6	440	16.5
5.	Syn. B-19	28.1	455	16.2
6.	EH-401075	28.6	360	12.6
7.	DHM-101	34.7	480	13.8
8.	Syn. B-23	38.1	406	10.7
9.	Ganga-5	43.4	495	11.4
10.	EH-400175	47.2	356	7.5
11.	EH-400575	49.2	340	6.9
12.	Vijay comp.	50.1	585	11.7
13.	Shakti	68.5	510	7.45
*CD at 5% between duplicates			1.1083	
CD at 5% between varieties			2.9320	
Analysis of variance table: F ratio				
Source (d.f)				
Duplicates (1)		0.05[a]		
Varieties (12)		218.25[b]		
Error (12)		1.00		
Total (25)				

[a] Not significant. [b] $P < 0.001$ (significant). [c] Correlation coefficient (r) between fungal growth and aflatoxin production among varieties = 0.19 N.S.

Table III. Mean Values of Fungal Growth (in Terms of Glucosamine) and Aflatoxin Production in Six Varieties of Groundnut[c]

Sl. no.[d]	Variety of groundnut	Mg of fungal dry weight/g of infected grain[a]	Aflatoxin production[b]	
			μg/g of grain	μg/mg of fungal dry weight
1.	J_{11} Khargoan	25.4	206.5	8.13
2.	Karad 4-11	28.8	146.4	5.08
3.	J_{11} Ranchi	29.2	183.0	6.27
4.	99-5	29.4	57.8	1.97
5.	Kopergoan-1	32.6	126.4	3.88
6.	NG-268	33.2	115.2	3.47

Analysis of variance and F ratio test was done. [a] Glucosamine values between varieties are not significant. [b] Significant differences ($P < 0.001$) were seen in aflatoxin production among varieties. [c] Correlation coefficient (r) between fungal growth and aflatoxin production among varieties is not significant. [d] Sl, serial number.

considerably from one variety to another at a given point of incubation period. The results thus show that the amount of toxin produced per unit growth of the fungus differed considerably from one genotype to another. For example, in two varieties of maize, namely Vijay Comp. and EG-400575, fungal growth was essentially similar (50.1 and 49.2 mg, respectively) and yet the amount of aflatoxin produced by the latter was only about 60% of that produced by the former (585 and 340 μg, respectively). Similarly, in the six genotypes of groundnut examined, fungal growth varied within a narrow range of 25.4–32.2 μg/g while the amount of aflatoxin produced showed wide variation with a range of 57.8–206.5 μg/g.

A soft endosperm variety of maize like "Shakti" was found to support higher fungal growth (68.5 mg/g) than did a hard endosperm variety like Comp. H-3 (23.6 mg/g); but the aflatoxin production was not different (510 and 505 μg/g, respectively).

DISCUSSION

Several methods to quantitate fungal growth have been used by different workers (Laprade, 1973; Zambettakis, 1975). The visual colonization method can only identify and estimate qualitatively fungal growth as colonies on the infected grains. The chemical method used in this study, based mainly on the method developed by Ride and Drysdale (1972), accurately determines fungal growth in infected maize and groundnut; the least amount of glucosamine determined by this method is 0.25 μg/mL. Naturally contaminated grains would generally be expected to have lower glucosamine values than grains artificially infected under laboratory conditions. In view of the high sensitivity of the method, it can also be used for quantifying fungal infestation under natural conditions, in terms of glucosamine content. The glucosamine values obtained from mycelia grown on standard synthetic medium (expressed as microgram/milligram of dry weight of the fungus; known as the conversion factor) are of special significance since they are used to translate glucosamine contents of infected tissue in terms of fungal growth. Each fungal strain has its own conversion factor since chitin concentration varies from one fungal strain to another. The glucosamine values obtained among replicates show good agreement with coefficient of variation ±5%. This method, being easy and quick, can be easily extended to study several other infected grains, including under natural conditions.

The observations on varieties of maize and groundnut made here confirm earlier findings from our own laboratory as well as from other laboratories, that varietal differences do occur among genotypes with respect to aflatoxin production. The reasons for these variations are not

known. Data presented here indicate that there are variations in fungal growth when they are grown on different varieties of maize and groundnut. They also show that a soft endosperm variety (Shakti) supports more fungal growth than did a hard variety (Comp. H-3). This may be due to the fact that a soft endosperm variety permits easier penetration and proliferation of the fungus in the grain. The amount of toxin production, however, among these varieties is not consistent with fungal growth, suggesting that increases or decreases in growth of the fungus do not run parallel to increases or decreases in aflatoxin production. It would thus appear that differences in the amount of toxin produced by the fungus, on different varieties of the same food grain are independent of quantitative differences in growth, but related to qualitative changes characteristic of the genotype. This may be due to the presence of varying amounts of stimulatory and inhibitory factors in the genotype. Further studies are in progress to explore such possibilities.

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A Chemical Assay for Saxitoxin. Improvements and Modifications

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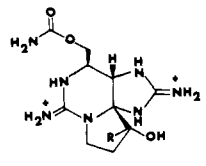
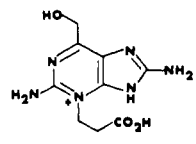
Saxitoxin (**1a**), the paralytic shellfish poison, can be oxidized to a purine (**2**) the concentration of which may be determined by ultraviolet absorbance or fluorescence. This is the basis of a sensitive chemical assay for saxitoxin. Several improvements and modifications are presented, as well as a procedure to check the functioning of each separate operation in the assay. The constant which relates saxitoxin concentration to ultraviolet absorbance after oxidation has been remeasured using pure saxitoxin.

Recently we presented a chemical assay procedure for saxitoxin (**1a**), the paralytic shellfish poison, based on oxidation to a fluorescent purine (**2**) (Bates and Rapoport, 1975). This chemical assay is superior to the previously used mouse bioassay in many respects, and its implementation is being considered for routine analysis of West Coast shellfish samples. The purpose of the present paper is to clarify several procedural details and indicate certain improvements and modifications in the chemical assay which increase its accuracy and reproducibility. Using several samples of purified saxitoxin, we have remeasured the constant which relates saxitoxin concentration to the UV absorbance after oxidation. We also describe a procedure to check the functioning of each separate operation of the assay.



1a, R=OH Saxitoxin
b, R=OEt



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EXPERIMENTAL SECTION

Materials and Equipment. The concentration of reagent grade hydrogen peroxide (~30%) was determined by titration with potassium permanganate (Welcher, 1963) and a 10% solution was prepared by dilution. When stored at 5 °C, it is stable for months. For routine work, hydrogen peroxide concentrations between 9 and 11% are satisfactory, introducing less than 1% deviation in the amount of **2** produced. Glassware and solvents must be kept free of dust and metallic particles capable of decomposing the hydrogen peroxide. Decomposition will lead to decreased and irreproducible oxidation of saxitoxin.

A stock solution of saxitoxin dihydrochloride monohydrate in water (50–100 μg/mL) is prepared and kept refrigerated in a glass container fitted with a rubber septum to prevent evaporation. Evaporation can be significant when ground glass stoppered flasks are used. After its concentration has been determined, a dilute solution of saxitoxin (~5 μg/mL) is accurately prepared from the original solution and stored in the same way. Volumes of less than 1 mL of either saxitoxin solution must be measured with microliter pipets inserted through the septa. Microliter syringes are totally unsatisfactory as they absorb saxitoxin on their ground glass surfaces.

All solutions should be checked periodically for contamination by fluorescent materials.

Prepare Bio-Rex 70 ion-exchange resin, 50–100 mesh

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