Journal of General Microbiology (1985), **131**, 487–494. *Printed in Great Britain*

Circumstantial Evidence for Phytoalexin Involvement in the Resistance of Peanuts to *Aspergillus flavus*

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(*Received 11 October 1984*)

Three stilbene phytoalexins, elicited by slicing and incubating imbibed peanut kernels under aerobic conditions, inhibited spore germination and hyphal extension of *Aspergillus flavus* with ED_{50} values in the range 4·9–12·8 μg ml^{-1}. Phytoalexin yield was dependent on cultivar, conditions and duration of incubation after slicing, and crop history. The yield of phytoalexin from ten cultivars studied, after slicing and incubating at 25 °C for 24 h, ranged from 28 to 935 μg per g fresh weight and was negatively correlated with dry kernel colonization by *A. flavus* [$r = -0·868$ when plotted as ln (phytoalexin concn) against ln (percentage peanut colonization)]. When the incubation period was extended to 96 h there was no such correlation. Reduced phytoalexin yields were obtained when sliced kernels of one cultivar studied were incubated in water or at 37 °C, and no phytoalexin was obtained when the slices were incubated under nitrogen gas or frozen before aerobic incubation. Drought stress during pod development in four cultivars studied reduced phytoalexin yields of sliced kernels incubated at 25 °C for 24 h by 17–65% compared with non-stressed controls.

INTRODUCTION

Strains of *Aspergillus flavus* Link and the closely related species *A. parasiticus* Speare produce a group of secondary metabolites termed aflatoxins. Most work has been done with aflatoxin B_1, which is the most poisonous (Wogan & Pong, 1970). This compound, in addition to its toxicity to a wide range of animals, is also teratogenic and carcinogenic (Heathcote & Hibbert, 1978*b*; Wogan, 1966). These studies suggest that aflatoxin contaminated food may be a health hazard to man. Indeed, a positive association between aflatoxin ingestion and liver cancer in man has been found in many population studies in tropical regions of Africa, India, South-East Asia and the Philippines (Heathcote & Hibbert, 1978*b*). Aflatoxin contamination of food has also been associated with kwashiorkor (Hendrickse *et al.*, 1982).

Aflatoxins can occur in many food materials but peanut meal from tropical countries is one of the commodities most frequently contaminated (Hiscocks, 1965). Originally it was thought that invasion of peanuts and aflatoxin prouction occurred mainly in storage, but it is now known that infection may occur before or during harvest (Ashworth & Langley, 1964; Williams & McDonald, 1983).

Kernels of different cultivars of peanuts vary widely in their susceptibility to invasion by *A. flavus* when inoculated with a spore suspension, and this has been correlated with their susceptibility to *A. flavus* invasion in the field (Zambettakis, 1983). Variation in susceptibility to *A. flavus* invasion is also influenced by both pre- and post-harvest environmental factors (Mixon, 1981). These include the type of plant residue in the soil (Griffin & Garren, 1976), the

Abbreviation: ED_{50}, effective dose (50%).

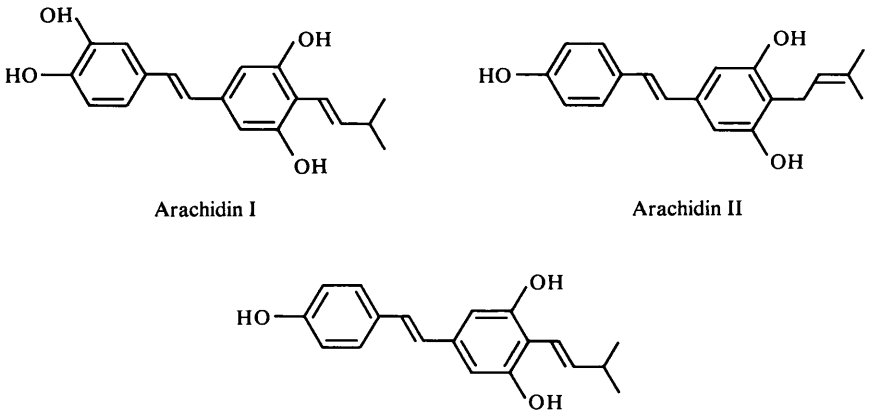

Fig. 1. Chemical structures of three peanut kernel phytoalexins.

other mycoflora present (Joffe, 1969), the crop rotation practices (Pettit & Taber, 1968), the age of kernels and the condition of the plants (McDonald & Harkness, 1967). Invasion of the kernels is more rapid if the pods are damaged, for example by insects (Minton & Jackson, 1967), by pod rotting fungi (Ashworth & Langley, 1964), or by machinery during harvest (Dickens & Khalsa, 1967). Severe drought stress during the last 4–6 weeks of the growing season increases the incidence of invasion of peanut kernels by *A. flavus* (Pettit *et al.*, 1971) as does storage at high temperatures and relative humidities (Heathcote & Hibbert, 1978*a*).

The nature of the greater resistance to *A. flavus* of some peanut genotypes compared with others is unknown, but has been associated with certain characteristics of the testa. These include wax accumulation on the surface (Zambettakis & Bockelee-Morvan, 1976), pigmentation (Mixon 1981), thinness and tightness of fit (Glueck *et al.*, 1977; Waliyar & Abadie, 1978), low permeability to aqueous stains (La Prade & Bartz, 1972) and low concentrations of certain amino acids (Amaya *et al.*, 1980).

Recently, three stilbene phytoalexins were isolated in this laboratory from peanut kernels exposed to their native microflora; two of the compounds were novel and one had been previously described (Aguamah *et al.*, 1981). These compounds have been assigned the trivial names arachidin I, II and III (Fig. 1). The relationship between the accumulation of these compounds in response to wounding and the resistance to colonization by *A. flavus* is the subject of this paper.

METHODS

Peanut kernels. Kernels of peanut (*Arachis hypogaea*) in their pods were kindly supplied by Dr R. C. N. Rao and Dr J. H. Williams of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Pantancheru PO, Andhra Pradesh 502324, India. Four cultivars were grown under drought stress conditions and were compared with the same cultivars grown under non-stress conditions. Details of the irrigation regime are given in Table 1. Seed was treated with fungicides Captan and Thiram (3 g per kg seed) and sown at ICRISAT on 27 November 1982 in 12 m rows. The inter-row spacing was 30 cm and the spacing between plants in the rows 10 cm. The crop was protected against pests and diseases until maturity.

A. flavus. An isolate of the fungus IMI no. 91019biii was obtained from the Tropical Development Research Institute, 56 Gray's Inn Road, London, UK. It was grown on malt/peptone agar in the dark at 25 °C.

Phytoalexins. Peanut kernels were shelled, sorted into 10 g batches and surface sterilized by immersion in H_2O_2 (30%, w/v) for 30 min. They were rinsed three times in sterile distilled water and allowed to imbibe sterile distilled water for 16 h at 25 °C. Samples were cut into transverse sections 2 mm thick with a sterile multi-blade knife before incubating in Petri dishes (90 mm diam.) at 25 °C or (for one experiment) 37 °C in the dark. Routinely, the Petri dishes were opened under sterile conditions every 24 h and the contents stirred to ensure adequate aeration. In some experiments the batches of sliced kernels were incubated with 0, 1, 2, 3, 4 or 5 ml sterile distilled water while in others the samples were gassed with N_2.

Table 1. *Irrigation regime in drought stressed and non-stressed plots*

Four cultivars of peanut, NcAc 17094, EC 76446, Robut 33 and TMV_2, were planted on 27 November 1982 at ICRISAT, Andhra Pradesh, India.

Days after planting	Water applied to plots (mm)	
	Dry treatment	Wet treatment
3	Field capacity	Field capacity
11	10	–
16	10	10
27	–	10
47	–	10
60	–	10
69	–	10
71, 72	70	–
81	–	Field capacity
89	–	Field capacity
97	–	Field capacity
105	–	Field capacity
106	60	–
112	60 + 11·5*	Field capacity + 11·5*
119	–	Field capacity
126	–	Field capacity
133	50	–
134	–	Field capacity

* Water fell as rain.

The phytoalexins were extracted and quantified by HPLC, essentially as previously described (Aguamah *et al.*, 1981). Larger samples of the phytoalexins, for antifungal assays, were also prepared by HPLC (Aguamah *et al.*, 1981).

Antifungal assays. Spores of *A. flavus* from malt/peptone agar Petri plates (1–2 weeks old) were harvested in sterile distilled water (10 ml) by agitation with a sterile glass rod. The resulting suspension was filtered through four thicknesses of sterile muslin and centrifuged for 15 min at 700 *g*. The pelleted spores were resuspended in sterile distilled water and washed twice more in the centrifuge. They were finally suspended in sterile distilled water (10^5 spores ml^{-1}).

The three peanut phytoalexins were dissolved in 95% (v/v) ethanol, and duplicates of a dilution series were distributed to the wells of a microtest plate (Flow Laboratories). After removal of the alcohol by evaporation in a current of air, Vogel's medium (Vogel, 1956) containing 2% (w/v) sucrose and 1·5% (w/v) agar (0·1 ml) was added to each well. The final concentrations of the phytoalexins were 100·00, 36·10, 10·00, 3·61, 1·00 and 0·00 μg ml^{-1}.

Spore suspension (4 μl) was placed on the agar in each well and the plate incubated in the dark at 25 °C. After incubation for 12 h a drop of cotton blue/lactophenol was added to prevent any further growth and to stain the fungus. Germination was assessed for 100 spores from each well, those spores with the germ tube longer than the diameter of the spore being scored as germinated. The percentage inhibition caused by the phytoalexins was calculated using the formula $[(C - T)/C] \times 100$, where C = mean percentage germination in controls, and T = mean percentage germination in tests.

The effect of the phytoalexins on germ tube extension was also assessed. Cellophane discs (5 mm diam.) were boiled for 2 min in water to remove plasticizers, dried, and sterilized at 121 °C in the autoclave for 15 min before being placed on Vogel's medium, containing 2% sucrose and 1·5% agar (20 ml), in a Petri dish (9 cm diam.). Spore suspension (2 μl) was placed on each disc and incubated for 15 h. A drop of cotton blue/lactophenol was placed on a sample of the discs and the length of 25 germ tubes per disc measured under a microscope using the × 40 objective and a graticule eye piece. A microtest plate was set up as described above for the spore germination tests (0·1 ml agar per well together with a dilution series of the phytoalexins). One cellophane disc with sporelings was placed on the agar in each well, and the microtest plate incubated for a further 15 h. A drop of cotton blue/lactophenol was placed in each well, and 25 germ tubes from each well were measured. The average length was calculated and converted into percentage inhibition using the formula $[(L_C - L_F)/(L_C - L_O)] \times 100$, where L_C = the final length of the germ tubes incubated without phytoalexins, L_O = the length of the germ tubes at the time of phytoalexin addition, and L_F = the final length of the germ tubes incubated with phytoalexins.

ED_{50} values were calculated from the regression of percentage inhibition on $\log_{10}$(concentration of phytoalexins).

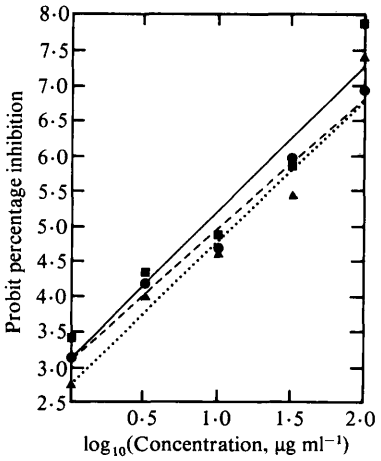

Fig. 2

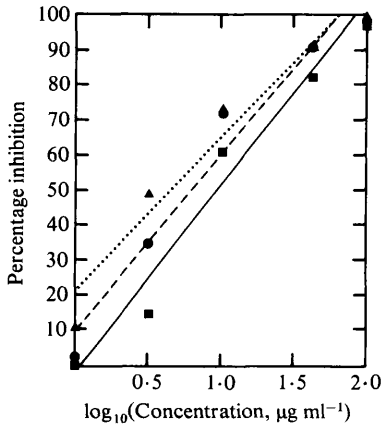

Fig. 3

Fig. 2. Relationship between probit percentage inhibition of spore germination and the logarithm of phytoalexin concentration. (Combined results from three experiments, maximum SD < 1·0 probit unit.) ▲·····▲, Arachidin I; ●---●, arachidin II; ■——■, arachidin III. The ED_{50} values for these compounds were 12·8, 12·7 and 8·9 μg ml^{-1} respectively.

Fig. 3. Relationship between percentage inhibition of hyphal extension and the logarithm of phytoalexin concentration. (Combined results from three experiments, maximum SD < 12%.) ▲·····▲, Arachidin I; ●---●, arachidin II; ■——■, arachidin III. The ED_{50} values for these compounds were 4·9, 6·8 and 9·9 μg ml^{-1} respectively.

RESULTS

Inhibition of A. flavus by the peanut phytoalexins

Spore germination and hyphal extension of *A. flavus* were inhibited by all three peanut phytoalexins, as compared with controls incubated without the compounds. Both probit percentage inhibition of germination and percentage inhibition of hyphal extension were proportional to the logarithm of phytoalexin concentration in the range 1–100 μg ml^{-1}. The ED_{50} values for spore germination were 12·8, 12·7 and 8·9 μg ml^{-1} for arachidin I, II and III respectively (Fig. 2). The ED_{50} values for hyphal extension were 4·9, 6·8 and 9·7 μg ml^{-1} for arachidin I, II and III respectively (Fig. 3).

Phytoalexin accumulation in peanut kernels after wounding

Phytoalexins accumulated within 24 h of wounding peanut kernels and reached maximum concentrations in 96–120 h, after which they began to decline (Fig. 4). The three cultivars chosen differed in their speed of response. Cultivar TMV_2 produced the least phytoalexin at 24 h (177 μg per g fresh wt of kernels) but outstripped the other cultivars at 96 h (3817 compared with 1028 and 1647 μg per g fresh wt). When phytoalexin concentration in ten cultivars was measured 24 h after wounding and compared with their reported susceptibility to 'dry seed' colonization by *A. flavus* (Mehan *et al.*, 1981), a negative correlation was found between the two factors, particularly when the data were expressed as natural logarithms ($r = -0{\cdot}868$) (Fig. 5). No such relationship held between phytoalexin concentrations accumulated 96 h after wounding and dry seed susceptibility to colonization (Table 2).

Incubation of sliced kernels of cultivar TMV_2 at 37 °C rather than 25 °C slowed phytoalexin accumulation (Fig. 6). The addition of varying amounts of sterile distilled water to sliced samples of the cultivar in Petri dishes also decreased phytoalexin accumulation, suggesting that aerobic conditions might be important (Fig. 7). Gassing samples of TMV_2 with N_2 virtually abolished phytoalexin accumulation (after an incubation period of 96 h the mean phytoalexin

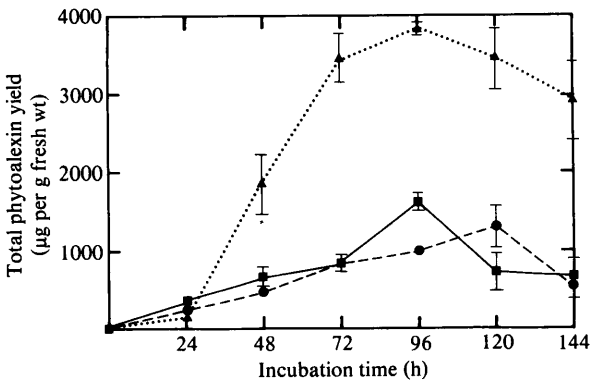

Fig. 4

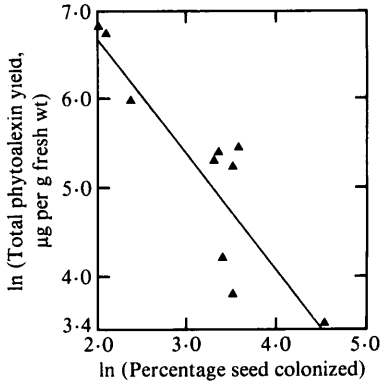

Fig. 5

Fig. 4. Phytoalexin accumulation in relation to the incubation time after wounding by slicing in three cultivars of peanut: TMV_2 (▲·····▲), Tamnut 74 (●---●) and Florunner (■——■). The bars represent ±1 SD for three replicates.

Fig. 5. Relationship between the natural logarithms of phytoalexin yield 24 h after wounding by slicing and percentage colonized kernels when inoculated with a spore suspension of *A. flavus* ($r = -0{\cdot}868$) for ten cultivars of peanut (see Table 2). The data on percentage colonization are from Mehan *et al.* (1981).

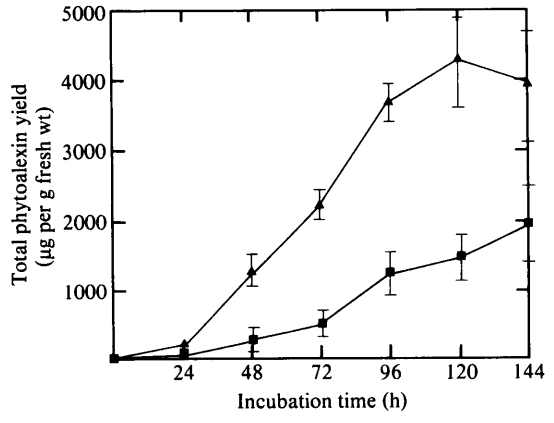

Fig. 6

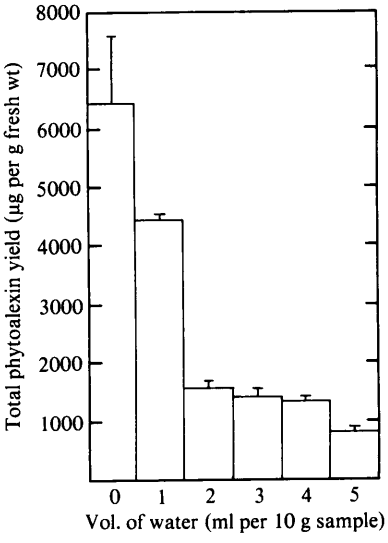

Fig. 7

Fig. 6. Effect of incubation, after wounding by slicing, at two different temperatures, 25 °C (▲) and 37 °C (■), on phytoalexin yield in cultivar TMV_2. The bars represent ±1 SD for three replicates.

Fig. 7. Effect of the addition of water to sliced kernels of cultivar TMV_2 on phytoalexin yield after incubation for 96 h. The bars represent ±1 SD for three replicates.

level, ± SD, was 103 ± 50 μg per g fresh wt compared with 4743 ± 1090 μg per g fresh wt for controls). Killing the samples of TMV_2 by alternate freezing and thawing before incubation prevented any phytoalexin accumulation.

Kernels from drought stressed plants of four cultivars studied accumulated less phytoalexin by 17–65% than kernels from non-stressed plants (Fig. 8).

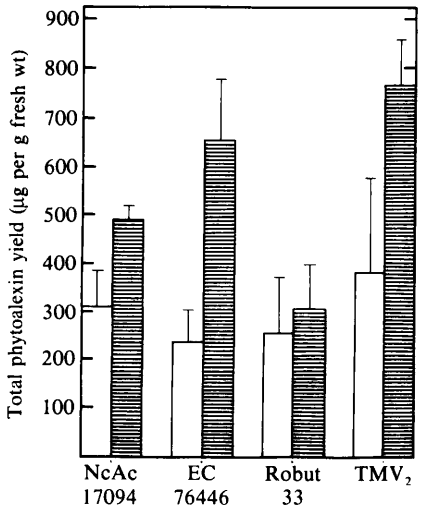

Fig. 8. Effect of drought stress on the capability of kernels to synthesize phytoalexins in four cultivars of peanut. Unhatched, dry treatment; hatched, wet treatment. Samples were incubated for 24 h after wounding by slicing. The bars represent ±1 SD for four replicates.

Table 2. *Phytoalexin accumulation in peanut kernels 96 h after wounding by slicing*

Cultivar (ICG no.)	Percentage of seeds colonized when inoculated with *A. flavus**	Total phytoalexin† [μg (g fresh wt)$^{-1}$]
4749	7·2	2981 ± 431
4750	8·0	2421 ± 376
1326	10·3	1940 ± 348
3836	27·3	1195 ± 55
TMV_2	28·4	2325 ± 269
4747	29·7	1109 ± 203
Florunner	32·8	1059 ± 56
801	32·8	1921 ± 474
2716	33·7	1020 ± 32
7867	94·1	2343 ± 62

* Data from Mehan *et al.* (1981).
† Means of three replicates, ± SD.

DISCUSSION

All three peanut phytoalexins inhibited both germination and hyphal extension of *A. flavus* in the μg ml^{-1} range. Since peanut kernels are capable of synthesizing these compounds in the low mg g^{-1} range (Aguamah *et al.*, 1981), an investigation was begun into why *A. flavus* colonizes peanut kernels. There were differences between various cultivars in the initial rate of accumulation of phytoalexins in response to slicing and these differences correlated with their dry seed resistance to colonization by *A. flavus*. It seems possible, therefore, that resistance to *A. flavus* is related to the time of phytoalexin synthesis, and not to the final amounts that accumulate after prolonged incubation. This is in line with several reports documenting the role of phytoalexins in resistance. For example, Rossall & Mansfield (1978) demonstrated, in experiments on the infection of *Vicia faba* leaves by *Botrytis* spp., that phytoalexin accumulation occurred at the right time to explain the inhibition of fungal growth during resistant reactions. Also Bailey *et al.* (1980) showed that in the invasion of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*, inhibition of hyphal growth occurred shortly after phytoalexins began to accumulate. Yoshikawa

et al. (1978) have demonstrated that, in the challenge of soybean by *Phytophthora megasperma* var. *sojae*, glyceollin accumulated at a greater rate in resistant rather than in susceptible tissue.

In this study, conditions which promoted the invasion of peanuts by *A. flavus* also inhibited phytoalexin production. Thus kernels from drought stressed plants, which are more susceptible to *A. flavus* than kernels from non-drought stressed plants (Wogan & Pong, 1970), produced less phytoalexin in response to wounding by slicing. Also, sliced kernels incubated at 37 °C produced less phytoalexin than sliced kernels incubated at 25 °C. This latter finding may be of particular significance in the context of the work of Sanders *et al.* (1981) and Hill *et al.* (1983), who found that peanuts were more susceptible to *A. flavus* in soils at high rather than low temperatures. They also suggested that under conditions of drought stress the kernels were more susceptible to *A. flavus* invasion because the leaf canopy was reduced and, therefore, the temperature of the soil below the plants was higher.

Our results indicate that resistance of peanut kernels to invasion by *A. flavus* is correlated with their capacity to synthesize phytoalexins as an early response to wounding. Work is now in progress to determine whether the two phenomena are connected in a cause and effect relationship.

We are grateful for the receipt of a grant by one of us (H. R. W.) from the Tropical Development and Research Institute.

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