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Increased Susceptibility and Reduced Phytoalexin Accumulation in Drought-Stressed Peanut Kernels Challenged with *Aspergillus flavus*

HILARY R. WOTTON and RICHARD N. STRANGE*

Department of Botany and Microbiology, University College London, London WC1E 6BT, United Kingdom

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Three genotypes of peanut (*Arachis hypogaea* L.), with ICG numbers 221, 1104, and 1326, were grown in three replicate plots and drought stressed during the last 58 days before harvest by withholding irrigation water. Within each plot there were eight levels of stress ranging from 1.1 to 25.9 cm of water. Kernels harvested from the plots were hydrated to 20% moisture and challenged with *Aspergillus flavus*. Fungal colonization, aflatoxin content, and phytoalexin accumulation were measured. Fungal colonization of non-drought-stressed kernels virtually ceased by 3 days after inoculation, when the phytoalexin concentration exceeded 50 μg/g (fresh weight) of kernels, but the aflatoxin concentration continued to rise exponentially for an additional day. When fungal colonization, aflatoxin production, and phytoalexin accumulation were measured 3 days after drought-stressed material was challenged, the following relationships were apparent. Fungal colonization was inversely related to water supply (*r* varied from −0.848 to −0.904, according to genotype), as was aflatoxin production (*r* varied from −0.876 to −0.912, according to genotype); the phytoalexin concentration was correlated with water supply when this exceeded 11 cm (*r* varied from 0.696 to 0.917, according to genotype). The results are discussed in terms of the critical role played by drought stress in predisposing peanuts to infection by *A. flavus* and the role of the impaired phytoalexin response in mediating this increased susceptibility.

Earlier studies reporting the predisposition of plants to disease by drought stress were summarized by Cook and Papendick (9) and Schoeneweiss (25–27). Perennial woody species are particularly well represented in these and later studies (2–4, 28, 30), but there are reports of increased disease severity in annual crop plants that have been drought stressed as well. These include infections of sorghum and cotton by *Macrophomina phaseoli* (9), wheat by *Fusarium culmorum* (8), safflower by *Phytophthora cryptogaea* (10, 15, 18), and soybeans by *Phomopsis* sp. (11). Particular attention has been paid to the enhanced levels of invasion of drought-stressed peanuts by *Aspergillus flavus* (5–7, 16, 19, 20, 23, 24). Many strains of this fungus, as well as of *Aspergillus parasiticus*, are capable of synthesizing a group of powerful mycotoxins known as aflatoxins. In addition to their toxicity, these compounds are reported to be carcinogenic, mutagenic, and teratogenic (13). They have also been implicated in hepatitis (17) and kwashiorkor (14). Control of aflatoxin contamination of peanuts is therefore a highly desirable objective. One approach is to select genotypes of the plant that are resistant to the fungus. Zambettakis (32) has shown that kernels harvested from different peanut cultivars vary widely in their resistance to *A. flavus* when inoculated with a spore suspension and that this variation correlates with resistance to the fungus in the field. Recently, we confirmed variation in resistance among peanut cultivars and showed that resistance is correlated with a potential for rapid accumulation of stilbene phytoalexins on wounding (31). We also showed that phytoalexin accumulation in response to wounding was decreased if the plant had had a history of drought stress (31). We now report the results of two experiments with peanut kernels inoculated with spores of *A. flavus* in which fungal development, aflatoxin production, and phytoalexin accumulation were measured. The first was a time course study in which kernels from plants that had not been drought stressed were used. The second experiment involved kernels from three plant genotypes that had been drought stressed to various degrees during the last 58 days before harvest. In this experiment measurements were made 3 days after inoculation.

* Corresponding author.

MATERIALS AND METHODS

Peanut kernels. Kernels in their pods were kindly supplied by R. C. N. Rao and J. H. Williams of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru P O, Andhra Pradesh 502 324, India. They came from plants that were grown under uniform irrigation conditions until 72 days after sowing; they were then subjected to various degrees of water stress with a line source sprinkler system (12) until harvest 58 days later. The sprinkler system delivered various amounts of water to the plants within the plots according to their distance from the line, resulting in eight intensities of drought stress. The entire experiment was replicated three times. Kernels were dispatched by air to Britain and stored at 4°C until use.

***A. flavus*.** An isolate of *A. flavus*, Imperial Mycological Institute no. 91019b-iii, was obtained from the Tropical Development and Research Institute, London. The fungus was maintained on malt-peptone agar in the dark at 25°C. Spore suspensions were prepared by growing the fungus on agar (Czapek Dox) in the dark at 30°C. Cultures that were 8 to 10 days old were flooded with sterile distilled water (SDW) and agitated with a bent glass rod. The resulting spore suspension was filtered through four thicknesses of sterile cheesecloth, washed twice in SDW in a centrifuge, and made up in SDW to a concentration of 4×10^6 ml^{-1} with a hemocytometer.

Inoculation with *A. flavus*. Triplicate samples (ca. 10 g

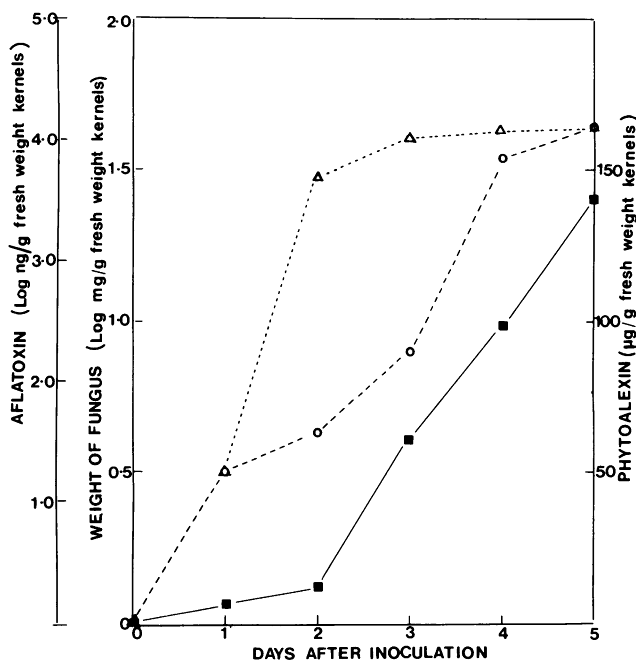

FIG. 1. Time course study of colonization of peanut kernels (ICG 221) by *A. flavus*, aflatoxin production, and phytoalexin accumulation. Symbols: △, fungus; ○, aflatoxin; ■, phytoalexin.

each, when shelled) of peanut kernels were surface sterilized in sodium hypochlorite (0.5%) for 2 min and washed twice with SDW. They were soaked in SDW for 15 min to allow them to reach a moisture level of 20% and placed in sterile petri dishes. Washed spore suspension (1 ml) was added to the petri dishes, and the kernels were rolled in the suspension. The petri dishes were incubated over water in semirigid plastic boxes with well-fitting lids at 25°C in the dark.

Measurement of fungal colonization. Samples of inoculated and uninoculated, surface-sterilized kernels (10 g) were homogenized in 80% aqueous acetone (40 ml) and filtered. Part of the residue (0.5 g [dry weight]) of each sample was assayed for chitin by the procedure described by Ride and Drysdale (21). Triplicate samples of surface-sterilized, uninoculated peanuts (10 g) were also assayed to serve as controls, and the mean absorbance values were subtracted from those obtained for inoculated samples.

Measurement of the chitin content of *A. flavus*. The fungus was grown on malt-peptone agar at 30°C. Plugs (diameter, 8 mm) of a 3- to 4-day old culture were transferred to liquid medium (Czapek Dox) containing yeast extract (0.7%) and were incubated for 4 days at 30°C without shaking. The mycelium was collected on muslin and washed in distilled water before it was homogenized in acetone (30 ml) for 30 s. Three portions of the suspension (0.9, 0.6, 0.3 ml) were transferred to glass centrifuge tubes, centrifuged (1,500 × g, 5 min), and assayed for chitin (21).

Measurement of aflatoxins. The 80% acetone filtrate (see above) was made up to 50 ml with the same solvent, and 12.5 ml was reserved for phytoalexin analysis. The remainder (37.5 ml) was assayed for aflatoxins by the method described by Romer (22). Aflatoxin concentrations were measured by comparison with a standard preparation on thin-layer chromatographic plates. Samples and standards (1 to 5 µl) were spotted on precoated thin-layer chromatographic plates (Kieselgel 'G') (thickness, 200 µm) and developed in a solvent consisting of ether-methanol-water (94:4.5:1.5; vol/vol/vol). Plates were dried in a fume cupboard and viewed under a UV lamp (λ_{max} = 365 nm). Concentrations of the aflatoxins were estimated by matching the intensity of fluorescence of samples with that of standards.

Measurement of phytoalexins. Samples (12.5 ml) from the 80% acetone filtrate were evaporated on a film evaporator, and the remaining aqueous phase was partitioned against petroleum ether (boiling point, 40 to 60°C; 2 times, 5 ml) and against ethyl acetate (3 times, 5 ml). The ethyl acetate fractions were combined, dried over anhydrous sodium sulfate, and evaporated to 0.75 ml. The stilbene phytoalexins were estimated by analytical high-pressure liquid chromatography, as described previously (1).

RESULTS

Fungal growth on non-drought-stressed peanut kernels was almost logarithmic for 2 days after inoculation; but very little growth occurred on further incubation, and by day 3, when the phytoalexin concentration had reached >50 µg/g (fresh weight) of kernels growth had essentially ceased (Fig. 1). In contrast, aflatoxin concentrations continued to increase almost logarithmically for 4 days before the rate of increase declined. At 5 days the value recorded was 12,600 ng/g of fresh weight (Fig. 1). During the course of the experiment the amount of aflatoxins accumulated per milligram of fungus rose from about 10 ng at 2 days after inoculation to 1,087 ng at 5 days. Aflatoxins B_1 and G_1 accumulated in approximately equal proportions and together accounted for >98% of the total.

Water deprivation during the last 58 days before harvest had a pronounced effect on the resistance of kernels when inoculated with *A. flavus*. In all three genotypes there was an inverse linear relationship between fungal colonization 3 days after inoculation and the cumulative water supplied. ICG 221 supported consistently greater growth of the fungus at all levels of water compared with the other two genotypes, which had levels that were similar to each other (Fig. 2).

Aflatoxin accumulation was also inversely and linearly related to the cumulative water supply in all three genotypes. The highest concentrations of aflatoxins was found in ICG 221 and the least was found in ICG 1326. ICG 1104 had an intermediate concentration (Fig. 3). Mean values for produc-

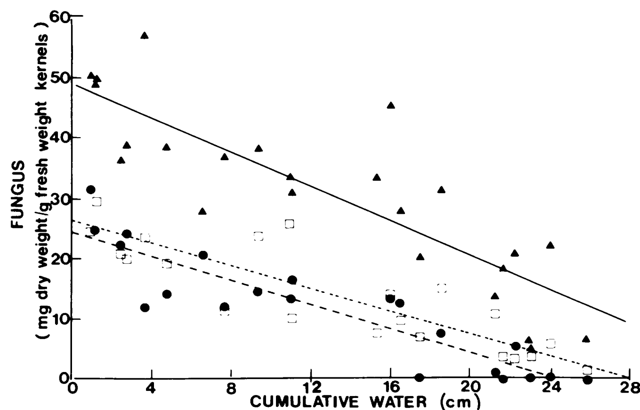

FIG. 2. Colonization of peanut kernels from three genotypes by *A. flavus* 3 days after inoculation relative to cumulative water supply during the last 58 days before harvest. Symbols: ▲, ICG 221 (r = −0.847); □, ICG 1104 (r = −0.862); ●, ICG 1326 (r = −0.904).

tion of aflatoxins per milligram of fungus did not vary significantly among genotypes. These values (nanograms ± standard deviation) were 252 ± 91, 302 ± 87, and 254 ± 96 for ICG 221, ICG 1104, and ICG 1326, respectively.

Kernels from plants that had received >11 cm of water accumulated phytoalexins in response to infection by *A. flavus*, and the amounts were approximately linearly related to the amount of water applied to the plots (Fig. 4). In contrast, little phytoalexin (<10 μg/g [fresh weight] of kernels accumulated in any cultivar that received 11 cm of water or less, although fungal colonization was still inversely related to water supply (Fig. 4). To investigate these data further, fungal colonization of plants that had received 0.9 to 2.7 cm of water was compared with that of plants that had received 7.6 to 11.0 cm (Table 1). Analysis of variance showed that these values were significantly different, suggesting that some mechanism other than the inability to accumulate phytoalexin is responsible for the increased susceptibility of the plants that received the lesser amount of water. The analysis also showed that ICG 221 was significantly more susceptible than the other two cultivars, the susceptibilities of which did not differ significantly from each other.

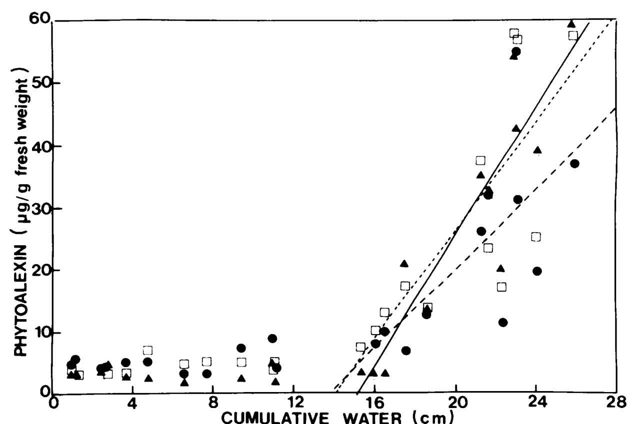

FIG. 4. Phytoalexin accumulation in peanut kernels from three genotypes 3 d after inoculation with *A. flavus* relative to cumulative water supply during the last 58 d before harvest. Symbols and correlation coefficients for data points where water supply exceeded 11 cm: ▲, ICG 221 (r = 0.917); □, ICG 1104 (r = 0.787); ●, ICG 1326 (r = 0.696).

DISCUSSION

We previously provided circumstantial evidence for the involvement of phytoalexins in the resistance of peanuts to *A. flavus* (31). Results of these studies showed inter alia that three stilbene phytoalexins from the plant inhibited hyphal extension in vitro by 50% at concentrations of 4.9 to 9.9 μg/ml and that drought-stressed kernels had a reduced capacity to synthesize phytoalexins following wounding. Data reported here show that phytoalexins are also elicited by *A. flavus*. In a time course experiment with non-drought-stressed kernels, the phytoalexin concentration only reached 12 μg/g (fresh weight) 2 days after inoculation (Fig. 1). This slow accumulation probably reflects the low level of challenge provided by spores that have only just germinated and have had little time to penetrate host tissues, as well as the general sluggishness of phytoalexin accumulation, a consequence of their de novo synthesis. Thereafter, accumulation was rapid and by day 3 a sufficient amount of phytoalexin was present, on the basis of in vitro studies (31), to explain the virtual cessation of growth of the fungus. In contrast, aflatoxin levels continued to rise exponentially until day 4. These data suggest that, in common with many other secondary metabolites of fungi, aflatoxin synthesis may be promoted by conditions that limit fungal growth (29). By day 5 the rate of increase of aflatoxin accumulation was no longer exponential, possibly reflecting the limited amount of fungus that was present.

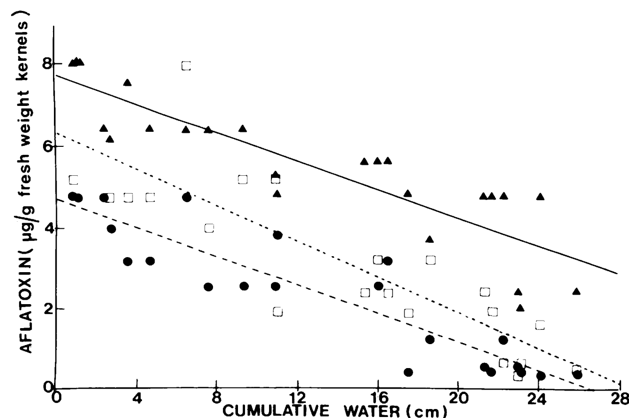

FIG. 3. Aflatoxin accumulation in peanut kernels from three genotypes 3 days after inoculation with *A. flavus* relative to cumulative water supply during the last 58 days before harvest. Symbols: ▲, ICG 221 (r = −0.876); □, ICG 1104 (r = −0.855); ●, ICG 1326 (r = −0.912).

Reduced water availability during the last 58 days of the growing season dramatically affected the susceptibility of peanut kernels to subsequent challenge by *A. flavus*. In all three genotypes both fungal colonization and aflatoxin accumulation were linearly and inversely related to water supply when measured 3 days after inoculation (Fig. 2 and 3). Other investigators, as described above, also have found that drought stress predisposes peanuts to infection by *A. flavus*, and in some cases this has been related to increased concentrations of aflatoxin (6, 23).

Conversely, significant phytoalexin accumulation only occurred in plants that received more than 11 cm water. Increases in water supply above this amount resulted in approximately linear increases in phytoalexin accumulation (Fig. 4). No plateau was reached, suggesting that even at the highest level of watering plants were not expressing their full capacity to synthesize phytoalexins. These data raise the question of the importance of phytoalexin accumulation in resistance. In the experiments described here, 10-g samples of peanut kernels were used, and no attempt was made to

TABLE 1. Colonization by *A. flavus* of peanut kernels from plants that received two ranges of drought stress

Plant genotype	Amt[a] of fungus in plants receiving the following levels of cumulative water[b]:	
	0.9–2.7	7.6–11.0
ICG 221	46.24 ± 5.72	35.01 ± 2.83
ICG 1104	23.62 ± 3.70	17.84 ± 7.07
ICG 1326	25.63 ± 3.39	14.10 ± 1.73

[a] Values are milligrams of fungus (dry weight) g (fresh weight) of kernel^{-1} ± standard deviation.

[b] Level of cumulative water (in centimeters) received by plants during the last 58 days before harvest.

measure localized phytoalexin accumulation. Thus, challenged tissue was effectively diluted by tissue that may not have been in contact or even close to the parasite. Consequently, the most susceptible genotype ICG 221 appears to accumulate the most phytoalexin probably because more tissue was invaded than in the other two genotypes. These genotypes, in contrast, accumulated far more phytoalexin per milligram of fungus. For example, taking values for plants that had received 20 cm or more of cumulative water, the most susceptible genotype ICG 221 accumulated only 2.95 μg of phytoalexin per mg of fungus, whereas the comparable values for ICG 1104 and ICG 1326 were 9.19 and 31.99 μg/mg, respectively. In ICG 1326 the fungus was detectable in only two of seven plants receiving 20 cm or more of water. It is highly probable, therefore, that in these two cultivars concentrations of phytoalexin that were sufficient to inhibit the fungus were attained by the time of sampling.

In conclusion, the data presented in this report show that phytoalexins are synthesized by peanut kernels in response to inoculation with spores of *A. flavus* and that their accumulation to inhibitory concentrations may provide sufficient reason for the observed cessation of fungal growth in the time course study. The importance of the phytoalexin response in determining resistance to *A. flavus* is underscored by the data for drought-stressed material. Here, the phytoalexin response was impaired and there was a corresponding increase in susceptibility. These data, together with those published previously (31), encourage the view that selection of genotypes with a rapid and efficient phytoalexin response might provide a means of controlling *A. flavus*. Attention, however, will have to be paid to the possibility of enhanced aflatoxin production by the organism when inhibited by phytoalexins.

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