Effects of Diniconazole on *Aspergillus* Populations and Aflatoxin Formation in Peanut Under Irrigated and Nonirrigated Conditions

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ABSTRACT

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Florunner peanuts (*Arachis hypogaea*) planted in irrigated and nonirrigated plots were either not treated or sprayed four times with diniconazole at 0.07, 0.14, and 0.28 kg/ha in 1988 and 1989. Paired plots in the nontreated and 0.28 kg/ha treatments were infested with a conidial suspension of *Aspergillus parasiticus* (a member of the *Aspergillus flavus* group) or left noninfested. Populations of the *A. flavus* group and the *Aspergillus niger* group were monitored in the soil throughout the season as well as in the shells and seed after harvest. Treatment with diniconazole had no effect ($P \leq 0.05$) on populations of the *A. flavus* group in the soil or shells, and reduced populations in seed only in 1989. The fungicide had no effect on soil populations of *A. niger* but reduced isolation frequencies from both shells and seed. Artificial infestation with *A. parasiticus* significantly increased *A. flavus*-group soil populations but had little effect on isolation frequencies from shells or seeds. Irrigation decreased isolation frequencies of both *A. flavus* and *A. niger* from shells and seed, but irrigation had no effect on soil populations of *A. niger* and variable effects on *A. flavus* populations. Aflatoxins were detected only in 1989. Mean concentrations were 11 and 2 ppb for the nonirrigated and irrigated plots, respectively. Neither fungicide treatment nor infestation with *A. parasiticus* affected aflatoxin levels. Aflatoxin concentrations were significantly correlated ($P = 0.0001$) with *A. flavus*-group populations in both shells and seed ($r = 0.70$ and 0.57, respectively).

Additional keyword: mycotoxins

Aflatoxins are toxic secondary metabolites produced by *Aspergillus flavus* Link:Fr. and *Aspergillus parasiticus* Speare as they grow parasitically on various substrates. These fungi are in the *A. flavus* group (19), a term that will henceforth be used for both fungi because they were not differentiated in this study. These fungi commonly colonize peanut (*Arachis hypogaea* L.) pods, and the aflatoxins formed are of grave concern to the peanut industry. Fungi in the *A. flavus* group are distributed throughout the world, and aflatoxins have been found in peanuts grown in all major peanut-producing countries (11). Another species that also is widely distributed is *Aspergillus niger* Tiegh. Although it does not produce aflatoxin, it is a pathogen of peanut and is antagonistic to *A. flavus* (17,18).

Environmental parameters, particularly moisture, have proven to be significant factors affecting the degree of colonization of peanut by *Aspergillus* species and the resulting contamination by aflatoxin. Pettit et al (18) reported decreased aflatoxin contamination and reduced susceptibility to invasion by *A. flavus* and *A. parasiticus* in irrigated versus nonirrigated peanuts. Wilson and Stansell (24) indicated that late-season drought was the most critical period for aflatoxin formation and suggested that other environmental parameters were important as well. A 1973 study in North Carolina linked aflatoxin production with hot, dry soils, which in turn were associated with high levels of both *A. flavus* and lesser cornstalk borer (10). Sanders et al (20) found that damage such as insect injury increases aflatoxin levels. They also looked at soil temperature and drought, and documented differences in pod maturity that influenced aflatoxin levels. Much of this work was summarized by Cole et al (8), who indicated that drought stress seemed to be required for aflatoxin production, despite up to 80% invasion by *A. flavus* and *A. parasiticus*.

Various techniques other than water management that have been tried to reduce aflatoxin levels in peanut have met with limited success (11). One approach was the use of fungicides either directly or indirectly toxic to *Aspergillus* species. Badii and Moss (2) found that tridemorph, fenpropimorph, and fenarimol all decreased growth of *A. parasiticus* but actually increased aflatoxin production. Conversely, Latif et al (16) discovered that pyrazolines and pyrazoles did not affect mycelial growth of *A. flavus* but did inhibit aflatoxin production. In 1965, Jackson (14) found that some fungicides had activity against *Aspergillus* species in vitro and speculated they could be used to treat pods in storage. Bell and Doupnik (4) screened a variety of chemicals including polyvalent metal compounds, oxidants, enzyme inhibitors, and fungicides for that purpose, and later tested some of the more promising compounds in the field (5). Although the results were encouraging, this approach has not been extensively pursued.

Another approach was the application of a fungicide to the plants while they were still growing. Some field studies have shown that fungicides did not reduce aflatoxin levels when applied to peanut foliage (6) or to soil (18). However, Gangawane and Reddy (12) postulated that chemical management in the field might be possible.

The introduction of new fungicides continues to offer new prospects for chemical control. One of the most promising classes of fungicides for peanut disease control is the ergosterol biosynthesis inhibitors (EBIs). Diniconazole is an EBI that has shown excellent activity in vitro and in the field against several major peanut pathogens (9). The sensitivity of *Aspergillus* species to this fungicide has not been reported. Diniconazole also is a plant growth regulator and produces decreased shoot growth and thicker leaves when applied to peanut plants (15). These structural changes could make the plants more drought tolerant and thus less susceptible

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to invasion by *Aspergillus* species. Alternatively, a reduced canopy provides less ground coverage, which could mean a greater loss of soil moisture.

The objectives of this study were to evaluate 1) the in vitro sensitivity of *A. parasiticus* to diniconazole and chlorothalonil, the fungicide currently used on virtually 100% of the peanuts grown in Georgia for foliar disease control, and 2) the effects of diniconazole on aflatoxin production and populations of *Aspergillus* species in peanut shells, kernels, and field soils under irrigated versus nonirrigated conditions.

MATERIALS AND METHODS

In vitro sensitivity. The following fungicides were utilized: chlorothalonil (Bravo 720) and diniconazole (Spotless, 25 WP). Based on preliminary studies, concentration ranges of 0.1–0.5 μg/ml and 1.0–100 μg/ml were used for chlorothalonil and diniconazole, respectively. Appropriate fungicide suspensions were prepared in sterile distilled water and pipetted into flasks containing sterile water agar (1.5%) cooled to approximately 55 C. The medium was stirred to ensure uniform mixing and dispensed into 60-mm-diameter petri dishes.

A single *A. parasiticus* isolate (NRRL 2999) was evaluated. The inoculum consisted of conidia from cultures on potato-dextrose agar (PDA) suspended in sterile deionized water containing two drops of Tween 80 per 100 ml of water. A Vortex-Genie test tube mixer was used to achieve a uniform suspension of approximately 7×10^5 conidia per milliliter. A sterile Pasteur pipette was used to deliver a single drop of inoculum (approximately 0.031 ml) to the center of fungicide-amended plates that had been under a transfer hood at room temperature to ensure that the agar surface was dry. All treatments were replicated six times, and plates were incubated with a 12-hr photoperiod and a 27/24 C day/night temperature regime. Colony diameters were measured after 8 days of incubation, and percent inhibition was calculated as a percentage of the growth of the fungus on a nonamended medium. Growth inhibition was plotted as a function of fungicide concentration, and linear regression was used to determine concentrations giving 50% reduction of growth (ED_{50} values). Because of the wide range of concentrations, $\log_{10}$ values were used for the diniconazole treatments. The test was repeated, and the results shown represent the mean of the two tests.

Field studies. Tests were conducted in 1988 and 1989 in a field of Tifton loamy sand (fine-loamy, siliceous, thermic Plinthic Paleudults), pH 6.1, with a history of tobacco production but fallow in 1986 and 1987. Florunner peanut (112 kg/ha) was planted 24 May 1988 and 12 May 1989. Soil was deep turned with a moldboard plow and disked before each crop, and standard management practices were followed for weed, disease, and insect control. All plots were sprayed with chlorothalonil (1.23 kg a.i./ha) every 2 wk to eliminate foliar diseases as a confounding factor. Peanuts were inverted 20 October 1988 and 3 October 1989. Plots were harvested mechanically on 26 October 1988 and 13 October 1989. Peanuts were dried to approximately 10% moisture prior to storage at room temperature.

The experiment was a fractional factorial with a split-split-plot design utilizing whole plots consisting of eight beds (14.4 m wide by 12.2 m long) of irrigated or nonirrigated peanuts. Water (2.54 cm) was applied weekly via solid set sprinklers to irrigated plots unless there was an equivalent amount of rainfall that week. Nine and seven irrigation events were used in 1988 and 1989, respectively. Rainfall by month was 5.3 cm in June, 7.9 in July, 6.4 in August, and 24.6 in September 1988; and 28.2 cm in June, 14.5 in July, 7.4 in August, and 1.4 in September 1989.

Subplots were either not treated or sprayed four times with diniconazole (0.28, 0.14, and 0.07 kg a.i./ha). Treatments were applied with a CO_2-pressurized backpack sprayer with three D2-13 nozzles per row delivering 124 L of spray per hectare at 345 kPa. Diniconazole was applied on a 21-day schedule initiated in the eighth week after planting. Subplots were single beds (12.2 m × 1.8 m) with two rows per bed on a 0.91-m spacing for the 0.07 and 0.14 kg/ha treatments, and double beds (12.2 × 3.6 m) for the nontreated and 0.28 kg/ha treatments. A 6.1-m fallow alley separated plots, and the outside bed of each whole plot served as a border.

Paired beds treated with either the highest rate of diniconazole (0.28 kg a.i./ha) or left untreated were infested with a conidial suspension of aflatoxin-producing *A. parasiticus* (NRRL 2999) or left noninfested. Infestation by a similar method has been demonstrated to increase the isolation frequencies of *A. flavus*-group fungi from peanut kernels and to increase aflatoxin production (17). The fungal inoculum was grown in 2.8-L Fernbach flasks containing 750 g of soil, 50 g of corn grits, and 250 ml of H_2O that had been autoclaved for 30 min on two consecutive days. Plugs of inoculum from cultures growing on PDA were used to inoculate flasks, which were then incubated at room temperature for 10 days. Flasks were filled twice with water containing 10 ml of biodegradable detergent (Fisher FL70), mixed, and the contents filtered through cheesecloth into an 18.9-L bucket. The bucket was filled with water, and 400 ml of this suspension was further diluted to 7.6 L. This volume was distributed with a sprinkler-type watering can over the peanut foliage in a single plot (2 rows × 12.2 m) approximately 50 days after planting.

Soil samples were collected four times during the season (60, 90, and 120 days after planting, and just prior to digging) from nontreated plots and from those receiving the highest rate of diniconazole (sub-subplots). Soil probes were used to collect five cores (5.1 cm deep) per row from an area 30 cm wide for a total of 10 samples per plot. These were mixed thoroughly, and a 5-g sample was suspended in 100 ml of 0.2% water agar. Aliquots (1 ml) of this suspension were pipetted onto each of five petri plates (60 cm diameter) containing M3S1B medium (13). The suspension was spread evenly over the agar surface, and the plates were incubated at 30 C. Colonies were counted 5–7 days later.

A random sample of 100 pods from each sub-subplot was surface disinfested in 0.52% NaOCl for 5 min, drained, and hand shelled. Two hundred half shells and seed from each plot were plated on malt salt agar (100 g NaCl, 20 g malt extract, 20 g agar, 1 L H_2O), a semiselective medium for *Aspergillus* species. Plates were incubated 7 days at 30 C before counting visible colonies of the *A. flavus* group.

Another 2-kg sample of peanuts from each subplot was mechanically shelled and the seeds chopped to obtain a uniform mixture. Aflatoxins were determined in 50-g subsamples. The samples were extracted and purified according to the method of Thean et al (22). The extracts thus obtained were analyzed by high-performance liquid chromatography with postcolumn iodine derivatization as described by Beaver et al (3).

Data were subjected to analyses of variance and Fisher's protected LSD ($P \leq 0.05$) utilized to separate means (21). Yearly data were analyzed separately. Aflatoxin data were transformed ($\log_{10} \times +1$) before analysis, a procedure commonly used for data that cover a wide range of values.

RESULTS AND DISCUSSION

In vitro sensitivity. Chlorothalonil was extremely efficacious against *A. parasiticus*. Conidia did not germinate on media amended with only 0.5 μg/ml of chlorothalonil (Fig. 1A). The sensitivity range to chlorothalonil was narrow, with 0.1 μg/ml providing only 26% growth inhibition. Diniconazole did not completely prevent spore germination, even at 100 μg/ml (Fig. 1B). However, the limitations of spore germination as a parameter for evaluation of EBIs are such that this method may underestimate their efficacy (7). Still, the differences in subsequent mycelial growth indicate that *A. flavus* is more sensitive to chlorothalonil than to diniconazole.

Field studies. The effects of irrigation on populations of the *A. flavus* group in soil were variable. In 1988, June, July,

and August were relatively dry; and irrigation resulted in significantly increased *A. flavus*-group populations for the first two sampling dates (Fig. 2A). Because of higher than average rainfall in the early part of the 1989 season, populations in the soil were similar in the irrigated and nonirrigated plots at 56 and 90 days after planting (DAP). Mid to late season was much drier, and nonirrigated plots had higher *A. flavus*-group populations than did irrigated plots (Fig. 2A). Fungi of the *A. flavus* group were consistently isolated more frequently from shells and kernels in nonirrigated than in irrigated plots (Table 1).

Populations of *A. niger* in soil were not affected by irrigation in either year of the study, with no significant differences found at any sampling date (Fig. 3A). The general trend both years was one of a gradual population increase over time, with populations being generally higher in 1989 than in 1988. Isolation frequencies of *A. niger* were usually higher from shells and kernels in nonirrigated than in irrigated plots (Table 2).

Four applications of diniconazole at the highest rate (0.28 kg/ha) did not alter populations of the *A. flavus* group in the soil compared to populations in soil receiving no fungicide (Fig. 2B). Populations in 1989 declined at 90 DAP before rising sharply to about 250–300 propagules per gram of soil at harvest. Diniconazole had little effect on isolation frequencies of *A. flavus* from shells and kernels (Table 1).

Diniconazole also had little effect on *A. niger* populations in the soil, although the 1989 harvest sample indicated a significant ($P \leq 0.05$) decrease in population where the fungicide was used (Fig. 3B). Populations of *A. niger* in the soil increased slowly during the growing season in both 1988 and 1989. Diniconazole applications resulted in lower isolation frequencies of *A. niger* from shells in

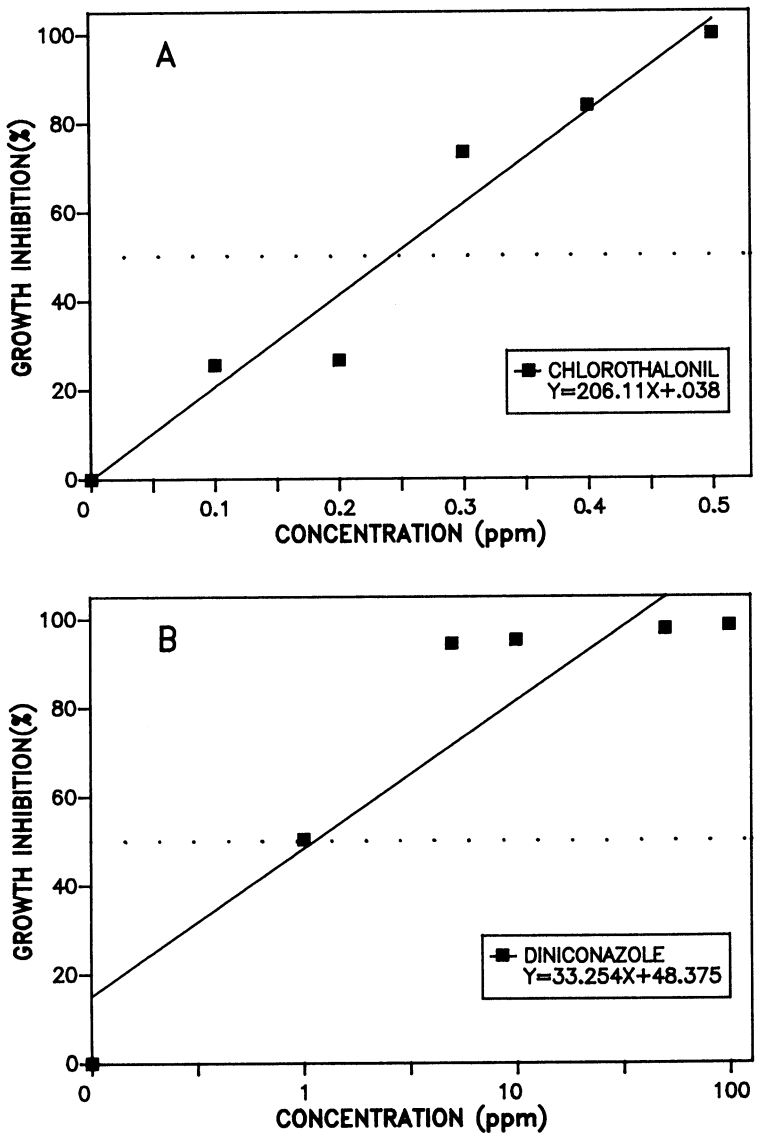

Fig. 1. In vitro sensitivity of *Aspergillus parasiticus* to **(A)** chlorothalonil and **(B)** diniconazole ($\log_{10}$ scale).

Table 1. Effects of irrigation and diniconazole on *Aspergillus flavus* isolations from peanut shells and kernels

	Isolation frequency[a] (%)					
Source	**Irrigated**	**Nonirr.**	**Difference[b]**	**Diniconazole[c]**	**Nontrt.**	**Difference**
Shells 1988	1.85	3.45	*	2.65	2.65	NS
Shells 1989	2.19	6.19	*	2.51	5.09	NS
Kernels 1988	0.30	1.03	*	0.90	0.43	NS
Kernels 1989	1.50	5.15	*	1.80	4.00	*

[a]Based on isolations from 200 shells or kernels per plot for each of five replications.
[b]Significant (*) or nonsignificant (NS) differences between paired means as determined by ANOVA ($P \leq 0.05$).
[c]0.28 kg/ha Applied four times.

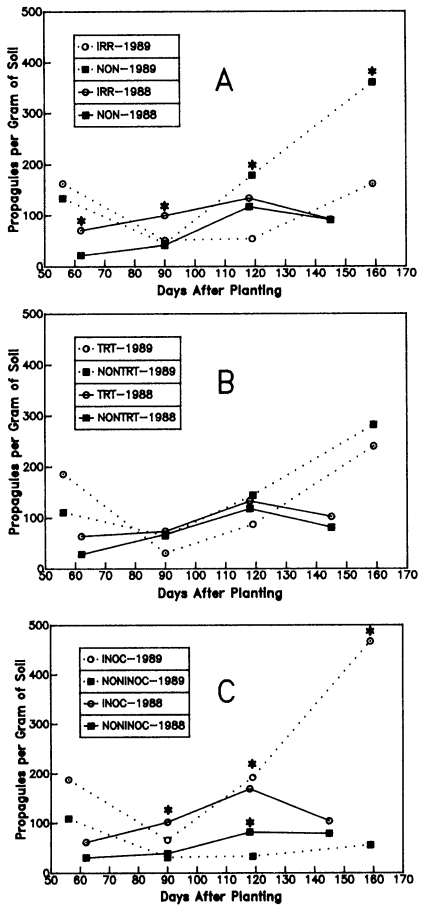

Fig. 2. Populations of *Aspergillus flavus*-group fungi in peanut field soil in **(A)** irrigated (irr) versus nonirrigated (non) plots, **(B)** diniconazole treated (trt) versus nontreated (nontrt) plots, and **(C)** artificially infested (inoc) versus noninfested (noninoc) plots in 1988 and 1989. * = Significant difference ($P < 0.05$) between treatments within years.

both years of the study and from kernels in 1989 only (Table 2).

Infestation of plots with *A. parasiticus* conidia did raise populations of that fungus in the soil. This increase was significant at 90 and 120 DAP in 1988, and at 120 and 160 DAP in 1989 (Fig. 2C). In 1989, the infested plots contained almost 500 propagules per gram of soil compared to less than 100 in the noninfested plots.

Aflatoxin was not detected in any kernels sampled in 1988. In 1989, low levels were found in irrigated peanuts, and concentrations as high as 14.4 μg/kg were detected in kernels from nonirrigated plots (Fig. 4). Irrigation was the only factor that significantly affected aflatoxin concentrations. The concentrations were significantly correlated ($P = 0.0001$) with isolation frequencies of *A. flavus* from both shells ($r = 0.71$) and kernels ($r = 0.58$), but not with *A. flavus* populations in the soil.

Soil populations of *A. flavus* and *A. niger* were generally high, at times over 500 propagules per gram of soil. This was much higher than reported by Griffin and Garren (13) in Virginia peanut field soils. Inoculation with *A. flavus* conidia did result in higher populations of the fungus in soil; however, the inoculation procedure did not alter isolation frequencies from kernels as it did in a previous study (17). Mehan et al (17) and Pettit et al (18) also found that aflatoxin levels were correlated with isolation frequencies from kernels. This was verified by our study only in 1989. Apparently this correlation does not occur under all environmental conditions (5).

A. niger has been reported to be antagonistic to *A. flavus* (1,11). The ratio of *A. flavus* to *A. niger* has been shown to be significant in predicting aflatoxin levels, and irrigation can lower that ratio (23). This was consistent with our results only in 1989. However, shells and kernels from irrigated plots usually had lower isolation frequencies of both *A. niger* and *A. flavus* than did those from nonirrigated plots (Tables 1 and 2). Analysis of population data indicates that irrigation reduced the *A. flavus*-to-*A. niger* ratio in shells from 0.54 to 0.16 in 1988 and from 0.69 to 0.39 in 1989. A similar reduction occurred with populations from kernels, where irrigation reduced the ratio from 0.36 to 0.24 in 1988 and from 1.32 to 0.74 in 1989. Apparently, irrigation reduced populations of *A. flavus* in shells and kernels more than it did populations of *A. niger*. In the soil, irrigation had no effect on populations of *A. niger* and variable effects on populations of *A. flavus*.

Diniconazole was only moderately toxic to *A. parasiticus* in vitro, but the fungus was sensitive at a level which could be significant in the field, especially since the fungicide is systemic (15). Movement is generally apoplastic, but residues can be found in shells at harvest after foliar applications. This fact, along with the possible reduction in drought stress experienced by diniconazole-treated plants, led us to postulate that this chemical could effectively reduce aflatoxin contamination of peanuts. Unfortunately, aflatoxin was not significantly affected by applications of diniconazole. Because *A. flavus*-group fungi are very sensitive to chlorothalonil, the effects of diniconazole may have been confounded by the foliar applications of chlorothalonil. Since chlorothalonil is an integral component of peanut disease management, these results indicate no added benefits of diniconazole in reducing infection by *A. flavus*-group fungi

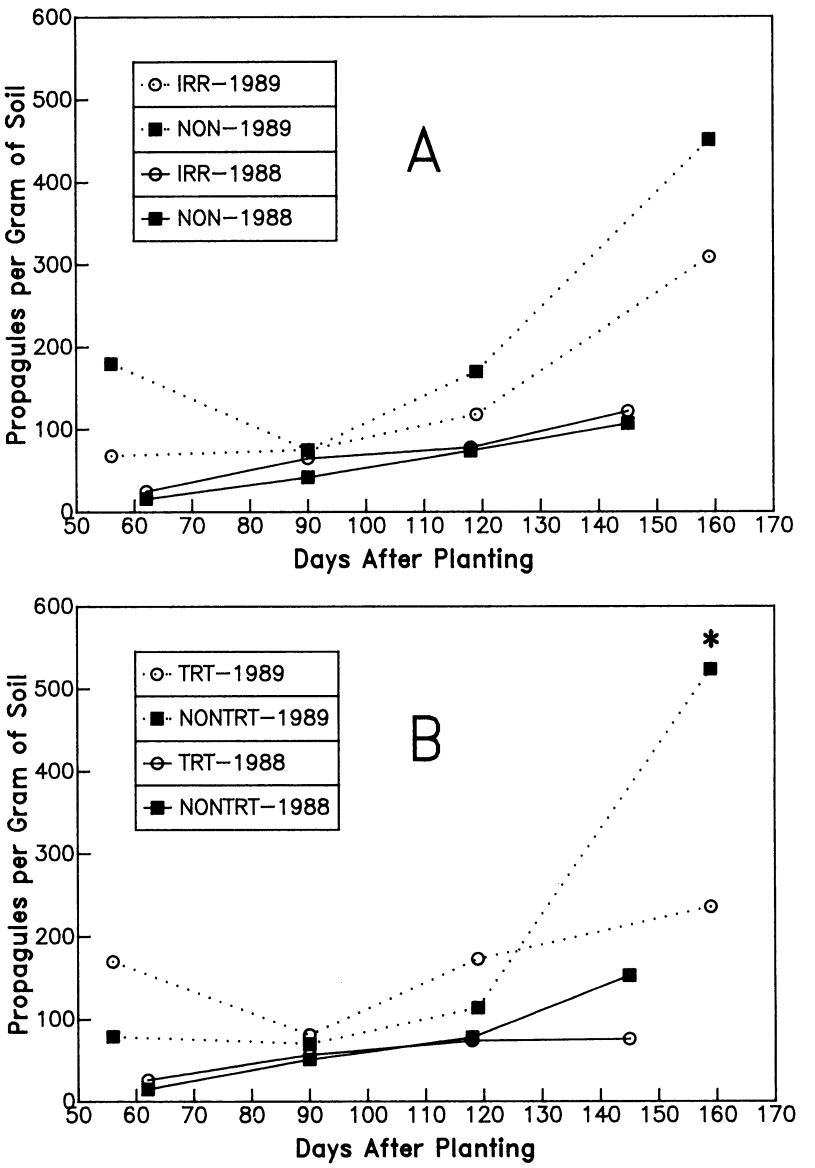

Fig. 3. Effects of **(A)** irrigation and **(B)** diniconazole treatments on populations of *Aspergillus niger* in peanut field soil in 1988 and 1989. * = Significant difference ($P < 0.05$) between treatments within years.

Table 2. Effects of irrigation and diniconazole on *Aspergillus niger* isolations from peanut shells and kernels

	Isolation frequency[a] (%)					
Source	**Irrigated**	**Nonirr.**	**Difference[b]**	**Diniconazole[c]**	**Nontrt.**	**Difference**
Shells 1988	11.15	6.43	*	7.00	10.58	*
Shells 1989	5.67	8.91	*	4.69	9.14	*
Kernels 1988	1.25	2.85	*	1.73	2.38	NS
Kernels 1989	2.03	3.90	*	2.10	3.19	*

[a]Based on isolations from 200 shells or kernels per plot for each of five replications.
[b]Significant (*) or nonsignificant (NS) differences between paired means as determined by ANOVA ($P \leq 0.05$).
[c]0.28 kg/ha Applied four times.

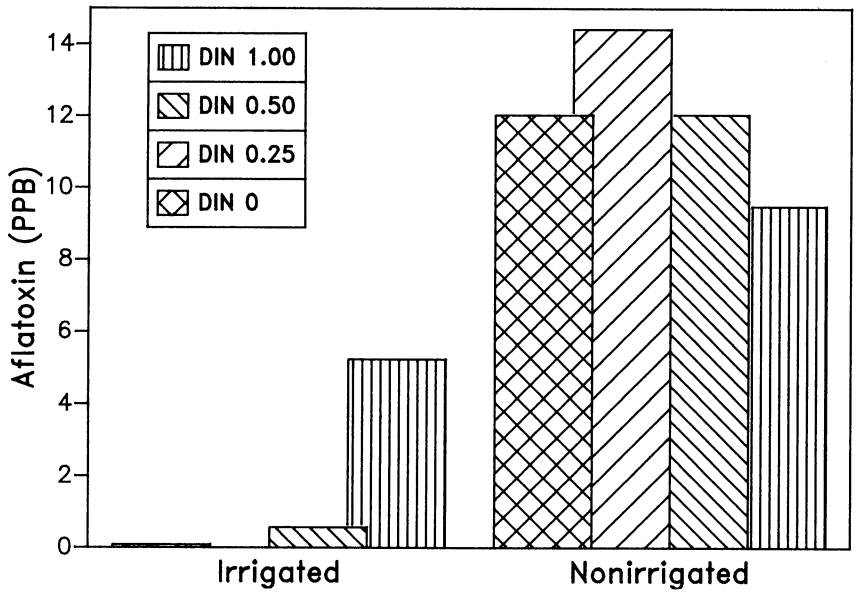

Fig. 4. Effects of diniconazole (DIN) treatment on aflatoxin production in irrigated or nonirrigated peanuts, 1989. (DIN 1.00, DIN 0.50, DIN 0.25, and DIN 0 refer to four applications of diniconazole each, at 0.28, 0.14, 0.07, and 0 kg a.i./ha, respectively.)

or aflatoxin contamination of kernels. It is possible that other new fungicides, even other EBIs, will prove to be more effective; but diniconazole, even at this relatively high use rate, is not as effective as irrigation in reducing aflatoxin contamination in peanuts.

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