Combined Effects of Biological Control Formulations, Cultivars, and Fungicides on Preharvest Colonization and Aflatoxin Contamination of Peanuts by *Aspergillus* Species

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ABSTRACT

A 3-yr field study was conducted to determine the effect of biological control formulations of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus*, peanut cultivars, and fungicides on preharvest aflatoxin contamination of peanuts. Formulation treatments consisted of (a) no biocontrol treatment, (b) the fungi cultured on rice via solid-state fermentation, (c) conidia of the fungi coated onto the surface of rice, and (d) conidia coated onto the surface of wheat (year one) or hulled barley (years two and three). Experiments consisted of factorial combinations of the four formulation treatments, two peanut cultivars (Florunner or Georgia Green), and two fungicide treatments (chlorothalonil or combinations of chlorothalonil and tebuconazole). Florunner and Georgia Green peanuts were each planted in 32 individual plots consisting of six rows 15.2 m in length. Biological control formulations, consisting of a mixture of nontoxigenic strains of *A. flavus* (NRRL 21882) and *A. parasiticus* (NRRL 21369), were applied to the same plots in each of the 3 yr at a rate of 56 kg/ha. Foliar applications of fungicides were made as recommended for control of leaf spot, with one treatment being full-season applications of chlorothalonil, and the other being two applications of chlorothalonil followed by four applications of tebuconazole and remaining applications of chlorothalonil. Only in year two of the study was late-season drought sufficient to produce preharvest aflatoxin contamination. All biocontrol formulation treatments produced significant reductions in aflatoxin compared with untreated controls, averaging 81%. There was also a significant cultivar effect on aflatoxin with Georgia Green averaging 119 µg/kg compared with 402 µg/kg for Florunner. No differences were observed between the two fungicide treatments, and there was no interaction among the three factors. Analysis of soil for populations of *A. flavus* and *A. parasiticus* throughout the study showed that all formulations, except the conidia-coated wheat in the first year, were effective in delivering competitive levels of the nontoxigenic strains. In the third year, which did not result in significant aflatoxin contamination, analysis of peanuts for fungal colonization showed no significant differences among biocontrol treatments (including control) for total amounts of *A. flavus* and *A. parasiticus* in peanuts. However, the incidence of toxigenic isolates in peanuts was significantly reduced by all three biocontrol formulations.

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Key Words: *Arachis hypogaea* L., groundnut, *Aspergillus flavus*, *Aspergillus parasiticus*, biocontrol, competitive exclusion.

Aflatoxins are potent hepatotoxic, carcinogenic metabolites produced by *Aspergillus flavus* Link, *A. parasiticus* Speare, and *A. nomius* Kurtzman *et al.* (Diener *et al.*, 1987; Kurtzman *et al.*, 1987; CAST, 1989). *Aspergillus flavus* and *A. parasiticus* invade various agricultural commodities during maturation in the field or after harvest and contaminate them with aflatoxins. Corn, peanuts, cottonseed, and various tree nuts are particularly susceptible to preharvest aflatoxin contamination when environmental conditions during crop maturation are characterized by high temperatures and moisture stress and when insect injury is prevalent (Hill *et al.*, 1985; Cole *et al.*, 1989; Cotty and Lee, 1989; Payne, 1992). Because of the toxicity and carcinogenicity of aflatoxins, contaminated commodities destined for human or animal consumption pose a serious health hazard and are, therefore, closely monitored and regulated (van Egmond, 1995). Apart from its effect on health, aflatoxin also impacts agricultural economies through the loss of produce and the time and costs involved in monitoring and decontamination efforts (Shane, 1994; Lamb and Sternitzke, 2001).

One strategy that has been developed for reducing preharvest aflatoxin contamination of crops is biological control, in which nontoxigenic strains of *A. flavus* and/or *A. parasiticus* are applied to soil and competitively exclude native toxigenic strains. Application of non-aflatoxigenic strains of *A. parasiticus* to peanut plots reduced aflatoxin contamination of peanuts in 3 successive yr (Dorner *et al.*, 1992). Aflatoxin contamination was reduced by 74.3 to 99.9% in studies demonstrating that higher application rates of inocula containing nontoxigenic strains of *A. flavus* and *A. parasiticus* produced greater reductions in contamination (Dorner *et al.*, 1998). Treatment in the field also was found to have a carry-over effect of reducing aflatoxin contamination of peanuts during storage (Dorner and Cole, 2002). Reduction in aflatoxin contamination of cottonseed was achieved by applying a non-aflatoxigenic strain of *A. flavus* to soil around developing cotton plants (Cotty, 1994). Inoculation of corn plots resulted in aflatoxin reductions in harvested corn of 66 to 87% (Dorner *et al.*, 1999).

Many of the field biocontrol studies utilized inoculum that was produced by solid-state fermentation of the fungi on rice (Dorner *et al.*, 1998) or wheat (Cotty, 1994; Bock and Cotty, 1999). Other formulation techniques tested for production of inocula include starch encapsulation (McGuire and Shasha, 1992), encapsulation in alginate pellets (Daigle and Cotty, 1995), and extrusion of wheat gluten, kaolin, and conidia to produce pesta (Connick *et al.*, 1991; Daigle *et al.*, 1997). Tests comparing colonized rice, pesta, and corn-flour granules for biocontrol showed that all were effective in establishing nontoxigenic strains in soil and reducing aflatoxin contamination of peanuts (Dorner *et al.*, 2003). Because those formulation processes are time-consuming and laborious, or use relatively expensive raw materials, a simple spore-coating process was developed whereby spores of the nontoxigenic strain are trapped on the surface of a small grain (Cole and Dorner, 2001).

This study had three objectives. The primary objective was to compare spore-coated formulations of nontoxigenic strains of *A. flavus* and *A. parasiticus* with a proven formulation produced by solid-state fermentation in establishing a dominant population of nontoxigenic strains in soil and potentially reducing aflatoxin contamination. A secondary objective was to compare the susceptibility to aflatoxin contamination between cultivars Florunner and Georgia Green. Much of the data on preharvest aflatoxin contamination of peanuts was gathered during the 1980s and 1990s with Florunner, but Georgia Green has become the predominant southeastern U.S. cultivar in recent years. The final objective was to determine if inclusion of the fungicide tebuconazole, which is used in leaf-spot control spray programs, would have an effect on aflatoxin contamination. Tebuconazole is a sterol dimethylation-inhibiting fungicide that controls leaf spots [caused by *Cercospora arachidicola* Hori and *Cercosporidium personatum* (Berk. et Curt.)] in peanut (Brenneman and Murphy, 1991) as well the soil-borne pathogens *Sclorotium rolfsii* (Sacc.) and *Rhizoctonia solani* Kuhn (Brenneman *et al.*, 1991). The control of these diseases by tebuconazole results in healthier plants that produce higher peanut yields (Brenneman *et al.*, 1991). Theoretically, tebuconazole treatment might reduce aflatoxin contamination if healthier plants better withstand the stresses of drought associated with preharvest aflatoxin contamination. This paper reports the results of a 3-yr factorial experiment designed to meet those objectives.

Materials and Methods

Fungal Strains. Strains of *Aspergillus* used for biological control in this study were a nontoxigenic strain of *A. flavus* (NRRL 21882), originally isolated from natural infection of a peanut, and a UV-induced, orange-brown color mutant of *A. parasiticus* (NRRL 21369). Each strain lacks the ability to produce aflatoxins, cyclopiazonic acid, and known biosynthetic precursors of aflatoxin (Dorner *et al.*, 1998). Cultures were maintained on Czapek agar slants at 5 C.

Formulations. Each nontoxigenic strain was used in the production of three formulations. First, rice was colonized by solid-state fermentation as previously described by Dorner *et al.* (1998). After fermentation, the rice was dried to a moisture content ≤ 7% and stored at 5 C. Second, long-grain rice was coated with conidia (Cole and Dorner, 2001), which was purchased from a private company (Higuchi Matsunosuke Shoten, Osaka, Japan) that specializes in production of conidia of *Aspergillus* species for use in koji fermentations. Rice was coated by suspending conidia in soybean oil (10^8 conidia/mL of oil) and spraying the suspension (350 mL) onto the surface of the rice (22.7 kg) as it was tumbling in a 100-L capacity cement mixer. Diatomaceous earth (2.5% by weight) was added to absorb oil to make the formulation free-flowing. This resulted in an approximate concentration of 1.5×10^6 conidia/g of grain. Third, spore-coated wheat (year one) and spore-coated hulled barley (years two and three) were prepared in the same way as the spore-coated rice.

Experimental Design. Sixty-four individual peanut plots were established in a 6.2-ha field located approximately 10 miles west of Dawson, GA, and except for research plots, the field was not under cultivation (fallow). Plots were separated by 9 m end-to-end and by 3.7 m side-to-side. Four replicates of factorial combinations of cultivar, leaf spot control, and biocontrol formulations were completely randomized across the field. The area around and between plots was routinely harrowed to minimize organic debris that could support fungal colonization. Plots contained six rows of peanuts 0.9 m apart and 15.2 m in length, and were planted at a rate of one seed every 5 cm. Half of the plots were planted to cv. Florunner and half to Georgia Green. All peanuts were grown according to cultural practices recommended by the Georgia Coop. Ext. Serv. (Peanut Production Field Guide, 1997). Half of the plots of each cultivar received a full-season spray schedule of chlorothalonil at 1.26 kg/ha. The other half received two sprays of chlorothalonil followed by four treatments with tebuconazole (0.23 kg/ha), with subsequent treatments consisting of chlorothalonil as necessary, depending on the length of the growing season. Spraying began approximately 25 d after planting (DAP) each year and followed a 10 to 14 d schedule as recommended based on weather conditions. Equal weights of the separately prepared formulations of *A. flavus* and *A. parasiticus* were mixed in a cement mixer prior to application to soil. Each mixed formulation was applied at a rate of 56 kg/ha. Each plot was planted with the same cultivar and received the same spray and biocontrol formulation treatments every year. Differences in planting and harvest dates and in sampling and

processing procedures are described below for each year.

1998 Growing Season and Sample Processing. Peanuts were planted on 8 May and pre-weighed formulations were sprinkled over each row by hand (78 g/row) on 10 July (63 DAP). Peanuts were mechanically dug on 7 Oct. (152 DAP) and dried in the windrow for 5 d. Digging occurred later than would usually be expected for these cultivars because lack of early-season rainfall delayed plant development and pod maturation. Each windrow (2 rows × 15.2 m) was harvested with a Lilliston 1500 combine with a bagging attachment and peanuts were collected in 35/45 plain cabbage bags (Cady Industries Consolidated, Pearson, GA). Bags were transported to the Natl. Peanut Res. Lab., placed in a conventional peanut drying wagon, and dried to a moisture content of approximately 10%.

Peanuts from the middle windrow of each plot were cleaned with a specially designed farmers' stock precleaner that removes foreign material and separates loose-shelled kernels (LSK) from pods. Pods were shelled with a "model 4 sample sheller" designed to produce the same shelling characteristics as equipment used in a typical commercial shelling plant (Davidson *et al.*, 1981). Kernels were combined into two groups based on the definition of edible and inedible shelled stock grades (Official Trade Rules, 2000). Edible kernels included jumbo, medium, number 1, and sound split size categories after removal of visibly damaged kernels. Inedible kernels included the oil stock size category, LSK, and visibly damaged kernels removed from the edible category. The edible and inedible fractions were analyzed for aflatoxins separately.

1999 Growing Season and Sample Processing. Peanuts were again planted on 8 May and inoculated as in 1998 on 23 July (76 DAP), except that the coated wheat formulation was replaced with coated hulled barley. Peanuts were dug on 22 Sept. (137 DAP) and harvested the next day as peanuts were relatively dry as a result of prolonged drought stress. The remainder of sample handling and processing was the same as for 1998 except that damaged kernels removed from the edible categories were analyzed for aflatoxins separately.

2000 Growing Season and Sample Processing. Peanuts were planted on 29 April and inoculated on 21 July (83 DAP). Mechanical inoculation of biocontrol formulations was done with a microband granular applicator (Canaan Industries, Inc., Dothan, AL) calibrated separately for rice and barley to deliver 56 kg/ha in a band over the row. Peanuts were dug on 27 Sept. (151 DAP) and harvested 2 d later.

A change in sample processing protocol was made in 2000 so that the degree of fungal colonization of peanuts could be assessed in addition to aflatoxin quantitation. After drying to a moisture content of approximately 10%, all peanuts from each plot were combined and shelled with a Penco Pearman moisture sheller (Pearman Manufacturing, Chula, GA). Each shelled kernel lot was randomly sampled, and the sample (20 kg) was combined with 20 kg of water and homogenized in a 60 L Hobart vertical cutter mill (VCM) to produce a peanut-water slurry. Separate subsamples of the slurry (200 g) were analyzed for aflatoxins and dilution plated to quantify the colony-forming units of *A. flavus* and *A. parasiticus* in peanuts (Dorner, 2002). The incidence of toxigenic strains of *A. flavus* in peanuts was determined by transferring conidia from 10 randomly selected colonies per sample to 4-mL vials containing 1 mL of liquid medium containing 150 g of sucrose, 20 g of yeast extract, 10 g of soytone, and 1 L of distilled water (pH 6.0). Cultures were incubated in the dark for 7 d at 30 C and analyzed for aflatoxins and cyclopiazonic acid by thin-layer chromatography and high performance liquid chromatography (HPLC) (Horn *et al.*, 1996).

Fungal Soil Populations. Soil samples from each plot were taken immediately after planting and immediately prior to digging each year to determine the density and composition of *A. flavus* and *A. parasiticus* populations. From each plot, six subsamples of soil (approx. 100 cm^3 each) were removed with a sterile trowel from the top 5 cm in the peanut pod zone. Subsamples were combined in a paper bag and allowed to dry for 2 to 3 wk at 25 to 30 C. Soil was thoroughly mixed, sieved through a no. 20 standard testing sieve (0.833-mm opening), and stored at 5 C. The soil dilution plating technique of Horn and Dorner (1998) was used to enumerate colonies of *A. flavus* and *A. parasiticus*. The applied color mutant of *A. parasiticus* was readily distinguishable from wild-type strains. All wild-type colonies of *A. parasiticus* were presumed to be toxigenic based on the rarity of natural occurrence of nontoxigenic *A. parasiticus* (Horn *et al.*, 1996; Tran-Dinh *et al.*, 1999). The incidence of toxigenic and nontoxigenic strains of *A. flavus* was determined as described above for peanut-water slurries.

Aflatoxin Analyses. In 1998 and 1999, edible and inedible kernel fractions were separately ground in a VCM for 7 min to produce a homogeneous paste (Dorner and Cole, 1993). Sample sizes ranged from a few hundred grams for the inedible category to as much as 11 kg for edibles. Damaged kernels, which were analyzed as a separate component in 1999, were directly extracted without VCM grinding and subsampling (aflatoxins in 2000 crop year peanuts were determined from the peanut-water slurry also used to quantify *A. flavus* and *A. parasiticus* colonization of peanuts). VCMs in increasing size included: Cuisinart Pro Custom 11 Food Processor (East Windsor, NJ); Robot Coupe RSI6Y-1 (Robot Coupe USA, Inc., Ridgeland, MS); Stephan UM-12 (Stephan Machinery Corp., Dayton, OH); and Hobart VCM 60 (Hobart Manufacturing Co., Troy, OH). VCM usage was based on sample size to produce the most homogeneous peanut paste possible. A 200-g subsample of ground peanut paste was analyzed for aflatoxins by HPLC

(Dorner and Cole, 1988) with certain modifications. The HPLC system consisted of a Waters 3.9 × 150 mm Nova-PAK C_{18} column with a mobile phase of water/methanol/butanol (700/355/12; v/v/v). Instead of using postcolumn iodination to enhance fluorescence of aflatoxins B_1 and G_1, postcolumn derivatization was achieved with a photochemical reactor (Joshua, 1993) placed between the column and a Shimadzu Model RF551 fluorescence detector with excitation and emission wavelengths of 365 and 440 nm, respectively. Injection solvent consisted of methanol/water (62/38; v/v) with 0.1% acetic acid. Aflatoxin standards were prepared from crystals according to AOAC method 970.44 (Official Methods of Analysis, 1995), and aflatoxin determinations were not corrected for recovery.

Statistics. Aflatoxin concentrations were log transformed as necessary to normalize distributions. Yield and aflatoxin data were analyzed by three-way ANOVA, and fungal soil population and peanut colonization data were analyzed by one-way ANOVA using SigmaStat for Windows Version 3.10 (Systat Software, Inc., Richmond, CA). Multiple comparisons were made with the Holm-Sidak method.

Results

Growing Season Conditions. The 3 yr of this study were characterized by different weather conditions, as evidenced by rainfall during the growing seasons (Fig. 1). In 1998, only 4.6 cm of rain fell during the first 60 DAP. However, peanuts received abundant rainfall for the remainder of that season (42.2 cm). There was abundant early rainfall during 1999 (22.4 cm in the first 60 d), but only 10.7 cm fell during the last 78 d with no rain in the last 27 d, which subjected plants to severe drought stress. Peanuts in the 2000 growing season received timely rainfall throughout the year.

Peanut Yields. Treatment with different biological control formulations had no significant effect on yield, but there were significant ($P < 0.001$) cultivar effects in 1998 and 1999 and a significant ($P = 0.021$) fungicide effect in 1998 (Table 1). Yields for Florunner and Georgia Green were 5074 and 5394 kg/ha, respectively, in 1998 and 2716 and 3151 kg/ha, respectively, in 1999. Inclusion of tebuconazole in the spray program increased yield from 5150 to 5318 kg/ha in 1998. There was no significant interaction of factors with regard to yield. The highest overall yield occurred during the 1998 growing season, which was the first time peanuts had been planted on this land in several years. Yields were greatly reduced in 1999, primarily as a result of the severe drought stress during the pod set and maturation period. Yield rebounded in 2000 (overall mean = 4245 kg/ha), but there was much more, but not significant, plot-to-plot variability. Tomato spotted wilt virus (TSWV) incidence was very low in 2000, and this may have resulted in a higher-than-expected yield for Florunner compared with the relatively TSWV-resistant Georgia Green.

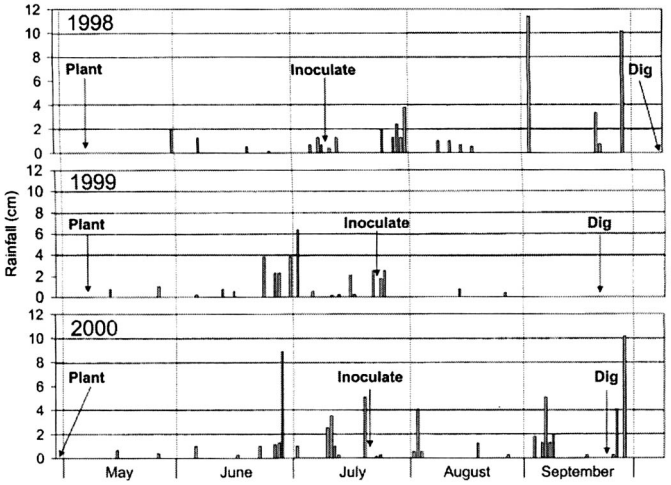

Fig. 1. Rainfall accumulations during the 1998-2000 growing seasons with times of planting, inoculation, and digging indicated.

Effect of Biocontrol Formulations on Populations of A. flavus in Soil. Prior to initial application of biocontrol formulations, there were no significant differences among plots for total *A. flavus* and *A. parasiticus*, with populations ranging from 1650 to 2505 CFU/g soil (Fig. 2). More than 90% of the isolates recovered were *A. flavus*, and were assumed to be toxigenic, although toxigenicity was not determined (Fig. 3). Applications of the coated and fermented rice formulations resulted in significantly ($P < 0.05$) higher total *A. flavus* and *A. parasiticus* populations at harvest in 1998 compared with the control and coated wheat treatments (Fig. 2). The coated wheat treatment reduced the proportion of toxigenic *A. flavus* isolates compared with the control (Fig. 3), but it was apparent that the coated wheat formulation was not as effective in displacing toxigenic strains as the other two formulations. Therefore, the coated wheat formulation was replaced by the coated hulled barley formulation for experiments in 1999 and 2000.

By planting time in 1999, total populations of *A. flavus* and *A. parasiticus* had fallen considerably in the coated and fermented rice treatments (Fig. 2). The percentage of toxigenic isolates also increased in those treatments compared with harvest of 1998 (Fig. 3). By harvest of 1999, total populations were very high in all treated plots, and the fermented rice treatment resulted in a significantly

Table 1. Three-way ANOVA results for main effects of cultivar, fungicide, and biocontrol formulation on peanut yields in 1998, 1999, and 2000.

	Cultivar		Fungicide		Formulation	
Year	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
1998	20.7	< 0.001	5.7	0.021	1.3	0.286
1999	13.5	< 0.001	0.1	0.903	0.7	0.567
2000	2.5	0.121	1.6	0.208	0.2	0.879

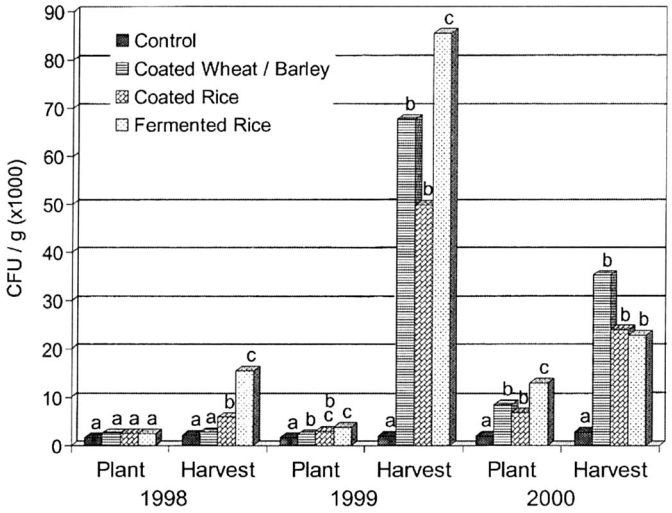

Fig. 2. Population densities of all strains (wild-type and applied) of *A. flavus* and *A. parasiticus* in soils treated with different formulations of nontoxigenic strains. Soils were sampled immediately after planting and immediately prior to harvest in 1998-2000. Bars within a specific time (plant or harvest) for each year with different letters are significantly different ($P < 0.05$). Data shown for planting in 1998 are grouped according to formulation treatment even though those treatments had not yet been applied.

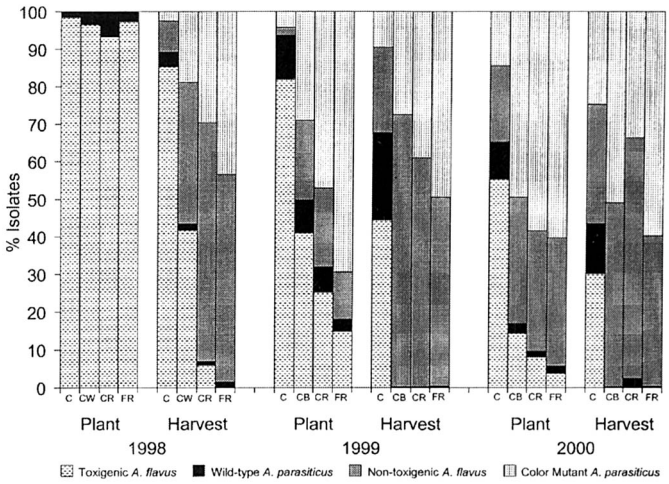

Fig. 3. Percentage of toxigenic and nontoxigenic isolates of *A. flavus* and *A. parasiticus* from soil samples taken from plots treated with various biocontrol formulations during the 1998-2000 growing seasons. Toxigenicity of *A. flavus* isolates was determined by analysis of representative colonies from dilution plates. Wild-type *A. parasiticus* (presumed to be toxigenic) was readily distinguishable from the nontoxigenic *A. parasiticus* color mutant on dilution plates. Abbreviations for treatments: C = control; CW = coated wheat; CR = coated rice; FR = fermented rice; CB = coated barley.

higher population than the other two treatments (Fig. 2). The incidence of toxigenic strains was less than 1% in all three treatments compared with a toxigenic strain incidence of 67.7% in control soils (Fig. 3). Populations again fell dramatically during the winter of 1999-2000. At planting in 2000, soil from the two coated formulation treatments still had significantly lower *Aspergillus* populations than the fermented rice-treated soil (Fig. 2). Although these overall populations had decreased, the proportion of toxigenic strains increased during the winter (Fig. 3).

The increase in total populations during the growing season of 2000 was not as dramatic as in 1999, and no significant differences were seen among the three formulation treatments at harvest (Fig. 2). Toxigenic strains at harvest of 2000 accounted for less than 1% of the total population in the coated hulled barley and fermented rice treatments and 2.3% of the total population in the coated rice treatment (Fig. 3). In control plots, 43.4% of the isolates were toxigenic compared with 89.3% at harvest in 1998.

Aflatoxin Contamination. Minimal aflatoxin contamination occurred in 1998 with no treatment exceeding 1 μg/kg. No significant differences were found with regard to formulations, cultivars, or fungicide treatments. Aflatoxins were not detected in the vast majority of samples. The highest concentrations among the 64 edible and inedible samples were 2.8 and 33.7 μg/kg, respectively, both from plots not treated with biological control.

The severe late-season drought in 1999 (Fig. 1) resulted in high concentrations of aflatoxins in peanuts (Table 2). Significant differences were associated with cultivars and formulation treatments, but not with the two fungicide treatments. There were no significant interactions among any of the main study variables (cultivars, fungicides, and formulations). Georgia Green peanuts of all kernel categories contained significantly less aflatoxin than Florunner. All biocontrol formulations produced significant reductions in aflatoxin compared with peanuts grown in untreated soil. The average reduction in aflatoxin in all biocontrol-treated peanuts was 81% compared with untreated controls.

In 2000, shelled kernels from each sample (20 kg) were homogenized together in a 60 L VCM to produce a peanut-water slurry that could be used both for aflatoxin and

Table 2. Mean aflatoxin concentrations (μg/kg) in various kernel categories of peanuts of two cultivars treated with two fungicides and four biocontrol formulations in 1999.

		Kernel category[a]			
Factor		Total[b]	Edible	Inedible	Damaged
Cultivar	Florunner	402 a	238 a	929 a	41,625 a
	Georgia Green	119 b	69 b	279 b	21,429 b
Fungicide	Chlorothalonil	199 a	111 a	528 a	30,234 a
	Chlorothalonil + Tebuconazole	322 a	196 a	681 a	32,821 a
Formulation	Control	668 a	403 a	1703 a	70,322 a
	Fermented rice	90 b	43 b	181 b	18,252 b
	Coated rice	154 b	78 b	300 b	19,346 b
	Coated barley	130 b	89 b	234 b	17,189 b

[a]For each factor, means within a column followed by a different letter are significantly different ($P < 0.05$).

[b]Aflatoxin concentrations for total kernels are the weighted averages of the edible, inedible, and damaged kernels.

fungal analyses. Therefore, aflatoxin was not determined on separate kernel components as in previous years. Similar to 1998, very little aflatoxin contamination occurred in 2000, and no significant differences were associated with cultivars, formulations, or fungicide treatments. However, the incidence of toxigenic strains of *A. flavus* and *A. parasiticus* colonizing peanuts was significantly ($P < 0.05$) reduced by all three formulations (Table 3). This displacement of toxigenic strains by nontoxigenic strains in treated peanuts did not result in an increase in the overall colonization of peanuts by *A. flavus* and *A. parasiticus* (Table 3), even though soil populations were significantly higher where formulations were applied (Fig. 2). There were no significant cultivar or fungicide effects on colonization.

Discussion

This study corroborates previous studies demonstrating biological control of aflatoxin contamination in peanuts by competitive exclusion (Dorner *et al.*, 1992, 1998; Dorner and Cole, 2002). The primary objective of the current study was to determine if a biocontrol formulation produced by coating a small grain with conidia of nontoxigenic strains was as effective as a formulation produced by solid state fermentation. Results showed that both the coated rice and coated hulled barley formulations were as effective as fermented rice in reducing aflatoxin contamination. In the first 2 yr of the study, the fermented rice formulation resulted in the highest soil populations of *A. flavus* and *A. parasiticus*, the great majority of which were the added nontoxigenic strains. Nevertheless, all formulations had similar effects on the levels of aflatoxin observed. Although significant aflatoxin contamination did not occur in 2000, quantitative analysis of *A. flavus* and *A. parasiticus* colonization of peanuts showed that significant displacement of toxigenic strains by nontoxigenic strains occurred with all formulation treatments without increasing overall colonization of peanuts. The results on total colonization of peanuts by *A. flavus* and *A. parasiticus* corroborates earlier work, which showed that treating soil with competitive strains of *A. flavus* and *A. parasiticus* did not result in increased colonization of peanuts by *A. flavus* and *A. parasiticus* (Dorner and Cole, 2002). That study also showed that the displacement of toxigenic strains by nontoxigenic strains that occurs in the field prevents significant aflatoxin contamination when conditions during storage facilitate *A. flavus* growth.

Table 3. *Aspergillus flavus* and *A. parasiticus* colonization (CFU/g of peanuts) and incidence of toxigenic strains in peanuts grown in soil treated with different biocontrol formulations in 2000.

Formulation	CFU/g[a]	% Toxigenic[b]
Control	8466	60.0 a
Fermented rice	4288	11.2 b
Coated rice	6350	19.1 b
Coated barley	4680	14.5 b

[a]Values are the means of 16 determinations among which there were no significant differences.

[b]Means in a column followed by a different letter are significantly different ($P < 0.05$).

The spore-coated formulation offers several advantages compared with the formulation produced by solid-state fermentation. First, the fermentation process requires sterilization of the substrate prior to inoculation with the nontoxigenic *A. flavus*. This is not necessary with the coating process because spores are applied at a concentration ($\geq 10^5$ CFU/g of grain) that ensures rapid growth and sporulation in the field. Second, the fermentation process requires a 24 hr incubation period for each batch fermented, regardless of batch size (Bock and Cotty, 1999). However, seed coating is a "flow-through" process in which 6 to 8 t of formulation can be produced per hour with modern seed-coating equipment. Third, fermentation requires that the fermented product be dried at temperatures not exceeding 58 C for approximately 48 hr. Slow drying is necessary to prevent killing the fungus with high temperature. The spore-coated formulation, on the other hand, does not require any drying because water is never added in the process. These advantages mean that much more biocontrol formulation can be produced in a given amount of time with the coating process, and the production is much more economical because of the savings in time and costs associated with sterilization and drying. If aflatoxin biocontrol technology is ever to be implemented commercially, the coating process provides the means for the economical, large-scale production that would be necessary.

The second objective of the study was to compare aflatoxin contamination in cultivars Florunner and Georgia Green. Results demonstrated a significant cultivar difference in the only year of significant contamination (1999). Georgia Green had significantly less aflatoxin than Florunner in all kernel components, particularly in peanuts not treated with nontoxigenic strains. This may be related, at least partially, to greater resistance of Georgia Green to TSWV (Culbreath *et al.*, 2000). TSWV infection causes plants to develop stress earlier in a drought than do non-infected plants, and longer stress duration increases aflatoxin concentrations (Sanders *et al.*, 1985; Dorner *et al.*, 1989). Although virus pressure was not quantified in this study, visual observation suggests that Florunner plants were much more affected by the virus than were Georgia Green plants in 1999. Other unidentified factors could also play a role in the lower aflatoxin concentrations associated with Georgia Green. The cultivar difference seen in this study highlights the need to identify other genotypes that offer even greater resistance to contamination.

No differences in aflatoxin contamination were associated with the inclusion of tebuconazole in the leaf spot control program. The only significant effect associated with the tebuconazole treatments was the yield increase in 1998. Although yields were not increased consistently in this study, that effect has been well documented in earlier work (Brenneman and Murphy, 1991; Brenneman *et al.*, 1991).

Application of biocontrol formulations each year was timed to coincide with rain events (Fig. 1) after peanut canopy had fully developed in order to stimulate rapid fungal growth and sporulation on the substrates. Moisture uptake by the inoculum is necessary to germinate conidia, and the abundant moisture provided by rainfall (either before or after application) provides an optimum environment for rapid sporulation. Inoculation can usually be timed to coincide with rainfall during the month of July in Georgia, though not necessarily in every year. Having a well-developed canopy at the time of inoculation also offers several advantages. First, the relative humidity underneath the canopy, particularly in the early morning hours, is very high, and this may provide adequate moisture for fungal growth in the absence of rainfall. Second, the canopy provides protection from elements such as solar radiation, which promotes drying of the inoculum, and heavy rainfall, which increases the potential for inoculum runoff (Horn *et al.*, 2001). Third, inocula are less visible to birds, which may be attracted to them as a food source, than they would be if applied early in the growing season. In addition, the cooler temperatures associated with an early-season inoculation are not as conducive to *A. flavus* growth as the warmer temperatures of July.

Data indicated a slow but steady increase in the proportion of nontoxigenic strains in control plots over time. At harvest in 1998, toxigenic strains comprised 89.3% of the total population, and this decreased to 67.7% at harvest in 1999 and 43.4% at harvest in 2000. Although attempts were made to isolate plots, unavoidable movement of equipment, wind, and insects through the plots partially compromised the true nature of the *A. flavus*/*A. parasiticus* composition of non-treated soils. Differences in aflatoxin contamination that were observed may underestimate the differences that might have been realized if the natural *A. flavus* and *A. parasiticus* composition of control plots had been maintained.

Conclusions

Aflatoxin biological control formulations prepared by the simple, economical, and rapid technique of coating small grain with conidia of a nontoxigenic strain proved to be as effective as the formulation produced by solid-state fermentation. This technique greatly simplifies the process of producing formulations in which fungi are the active ingredient. The savings in time and money offered by this technique compared with solid-state fermentation make commercialization of this biocontrol technology much more plausible. In the only year in which significant aflatoxin contamination of peanuts occurred (1999), the Georgia Green cultivar was found to be significantly less contaminated than Florunner. Although Florunner is no longer in widespread production, the differences in contamination associated with the two cultivars suggest that even greater genotypic resistance to aflatoxin can be found. Although treatment with tebuconazole did not have an effect on aflatoxin contamination, studies with other chemicals that might enhance peanut plant health should be considered as part of an overall strategy for reducing contamination. A combination of several strategies, such as biological control, more resistant cultivars, and enhanced plant health through disease control, offers the best chance for achieving maximum reduction in aflatoxin contamination of peanuts.

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Literature Cited

Bock, C., and P.J. Cotty. 1999. Wheat seed colonized with atoxigenic *Aspergillus flavus*: Characterization and production of a biopesticide for aflatoxin control. Biocontr. Sci. Technol. 9:529-543.

Brenneman, T.B., and A.P. Murphy. 1991. Activity of tebuconazole on *Cercosporidium personatum*, a foliar pathogen of peanut. Plant Dis. 75:699-703.

Brenneman, T.B., A.P. Murphy, and A.S. Csinos. 1991. Activity of tebuconazole on *Sclerotium rolfsii* and *Rhizoctonia solani*, two soilborne pathogens of peanut. Plant Dis. 75:744-747.

CAST. 1989. Mycotoxins: Economic and Health Risks. Council for Agric. Sci. and Technol., Ames, IA.

Cole, R.J., and J.W. Dorner. 2001. Biological control formulations containing spores of nontoxigenic strains of fungi for toxin control of food crops. U.S. Patent 6,306,386.

Cole, R.J., T.H. Sanders, J.W. Dorner, and P.D. Blankenship. 1989. Environmental conditions required to induce preharvest aflatoxin contamination of groundnuts: Summary of six years' research, pp. 279-287. *In* S.D. Hall (ed.) Aflatoxin Contamination of Groundnut: Proc. of the Intl. Workshop, 6-9 Oct. 1987, ICRISAT, Patancheru, A.P. 502 324, India.

Connick, W.J., Jr., C.D. Boyette, and J.R. McAlpine. 1991. Formulation of mycoherbicides using a pasta-like process. Biol. Control 1:281-287.

Cotty, P.J. 1994. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. Phytopathol. 84:1270-1277.

Cotty, P.J., and L.S. Lee. 1989. Aflatoxin contamination of cottonseed: Comparison of pink bollworm damaged and undamaged bolls. Trop. Sci. 29:273-277.

Culbreath, A.K., J.W. Todd, D.W. Gorbet, S.L. Brown, J. Baldwin, H.R. Pappu, and F.M. Shokes. 2000. Reaction of peanut cultivars to spotted wilt. Peanut Sci. 27:35-39.

Daigle, D.J., W.J. Connick, Jr., C.D. Boyette, M.P. Lovisa, K.S. Williams, and M. Watson. 1997. Twin-screw extrusion of 'Pesta'-encapsulated biocontrol agents. World J. Microbiol. Biotechnol. 13:671-676.

Daigle, D.J., and P.J. Cotty. 1995. Formulating atoxigenic *Aspergillus flavus* for field release. Biocontr. Sci. Technol. 5:175-184.

Davidson, J.I., R.F. Hudgins, and C.T. Bennett. 1981. Some design and performance characteristics of a small peanut sheller. Oléagineux 36:433-438.

Diener, U.L., R.J. Cole, T.H. Sanders, G.A. Payne, L.S. Lee, and M.A. Klich. 1987. Epidemiology of aflatoxin formation by *Aspergillus flavus*. Ann. Rev. Phytopathol. 25:249-270.

Dorner, J.W. 2002. Simultaneous quantitation of *Aspergillus flavus/A. parasiticus* and aflatoxin in peanuts. J. Assoc. Off. Anal. Chem. 85:911-916.

Dorner, J.W., and R.J. Cole. 1988. Rapid determination of aflatoxins in raw peanuts by liquid chromatography with postcolumn iodination and modified minicolumn cleanup. J. Assoc. Off. Anal. Chem. 71:43-47.

Dorner, J.W., and R.J. Cole. 1993. Variability among peanut subsamples prepared for aflatoxin analysis with four mills. J. Assoc. Off. Anal. Chem. 76:983-987.

Dorner, J.W., and R.J. Cole. 2002. Effect of application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* on subsequent aflatoxin contamination of peanuts in storage. J. Stored Prod. Res. 38:329-339.

Dorner, J.W., R.J. Cole, and P.D. Blankenship. 1992. Use of a biocompetitive agent to control preharvest aflatoxin in drought stressed peanuts. J. Food Prot. 55:888-892.

Dorner, J.W., R.J. Cole, and P.D. Blankenship. 1998. Effect of inoculum rate of biological control agents on preharvest aflatoxin contamination of peanuts. Biol. Control 12:171-176.

Dorner, J.W., R.J. Cole, W.J. Connick, D.J. Daigle, M.R. McGuire, and B.S. Shasha. 2003. Evaluation of biological control formulations to reduce aflatoxin contamination in peanuts. Biol. Control 26:318-324.

Dorner, J.W., R.J. Cole, T.H. Sanders, and P.D. Blankenship. 1989. Interrelationship of kernel water activity, soil temperature, maturity, and phytoalexin production in preharvest aflatoxin contamination of drought-stressed peanuts. Mycopathologia 105:117-128.

Dorner, J.W., R.J. Cole, and D.T. Wicklow. 1999. Aflatoxin reduction in corn through field application of competitive fungi. J. Food Prot. 62:650-656.

Hill, R.A., D.M. Wilson, W.W. McMillian, N.W. Widstrom, R.J. Cole, T.H. Sanders, and P.D. Blankenship. 1985. Ecology of the *Aspergillus flavus* group and aflatoxin formation in maize and groundnut, pp. 79-95. *In* J. Lacy (ed.) Trichothecenes and Other Mycotoxins. John Wiley & Sons Ltd., Chichester, UK.

Horn, B.W., and J.W. Dorner. 1998. Soil populations of *Aspergillus* species from section *Flavi* along a transect through peanut-growing regions of the United States. Mycologia 90:767-776.

Horn, B.W., R.L. Greene, V.S. Sobolev, J.W. Dorner, J.H. Powell, and R.C. Layton. 1996. Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus*, *A. parasiticus*, and *A. tamarii*. Mycologia 88:574-587.

Horn, B.W., R.L. Greene, R.B. Sorensen, P.D. Blankenship, and J.W. Dorner. 2001. Conidial movement of nontoxigenic *Aspergillus flavus* and *A. parasiticus* in peanut fields following application to soil. Mycopathologia 151:81-92.

Joshua, H. 1993. Determination of aflatoxins by reversed-phase high-performance liquid chromatography with post-column in-line photochemical derivatization and fluorescence detection. J. Chromatogr. A 654:247-254.

Kurtzman, C.P., B.W. Horn, and C.W. Hesseltine. 1987. *Aspergillus nomius*, a new aflatoxin-producing species related to *Aspergillus flavus* and *Aspergillus tamarii*. Antonie van Leeuwenhoek 53:147-158.

Lamb, M.C., and D.A. Sternitzke. 2001. Cost of aflatoxin to the farmer, buying point, and sheller segments of the southeast United States peanut industry. Peanut Sci. 28:59-63.

McGuire, M.R., and B.S. Shasha. 1992. Adherent starch granules for encapsulation of insect control agents. J. Econ. Entomol. 85:1425-1433.

Official Methods of Analysis. 1995. 16th Ed., Method 970.44, sec. 49.2.02, Assoc. Off. Anal. Chem., Gaithersburg, MD.

Official Trade Rules. 2000. Amer. Peanut Shellers Assoc., Albany, GA.

Payne, G.A. 1992. Aflatoxin in maize. Crit. Rev. Plant Sci. 10:423-440.

Peanut Production Field Guide. 1997. Univ. of Georgia Coop. Ext. Serv., Bull. no. 1146.

Sanders, T.H., R.J. Cole, P.D. Blankenship, and R.A. Hill. 1985. Relation of environmental stress duration to *Aspergillus flavus* invasion and aflatoxin production in preharvest peanuts. Peanut Sci. 12:90-93.

Shane, S.M. 1994. Economic issues associated with aflatoxins, pp. 513-527. *In* D.L. Eaton and J.D. Groopman (eds.) The Toxicology of Aflatoxins. Academic Press, Inc., San Diego, CA.

Tran-Dinh, N., J.I. Pitt, and D.A. Carter. 1999. Molecular genotype analysis of natural toxigenic and nontoxigenic isolates of *Aspergillus flavus* and *A. parasiticus*. Mycol. Res. 103:1485-1490.

van Egmond, H.P. 1995. Mycotoxins: Regulations, quality assurance and reference materials. Food Addit. Contam. 12:321-330.