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Use of a Biocompetitive Agent to Control Preharvest Aflatoxin in Drought Stressed Peanuts

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ABSTRACT

A three-year study was conducted to evaluate the use of a nonaflatoxin-producing strain of *Aspergillus parasiticus* (NRRL 13539) as a biocompetitive agent for the control of preharvest aflatoxin contamination of peanuts. The agent was added to the soil of the environmental control plot facility at the National Peanut Research Laboratory and tested by subjecting peanuts to optimal conditions for the development of aflatoxin contamination. Edible peanuts from the treated soil contained aflatoxin concentrations of 11, 1, and 40 ppb for crop years 1987, 1988, and 1989, respectively, compared to untreated peanuts with 531, 96, and 241 ppb, respectively. In addition, treatment in 1989 with low and high inoculum levels of a UV-induced mutant from the NRRL 13539 strain resulted in aflatoxin concentrations of 29 and 17 ppb, respectively, in edible peanuts. Soil populations of the biocompetitive agents were not higher than populations of wild strains of *A. flavus/parasiticus* in untreated soil subjected to late-season drought stress. This is an important ecological consideration relative to the utilization of this biocontrol system.

Aflatoxins are potent hepatotoxic, carcinogenic compounds produced by *Aspergillus flavus* Link:Fr. and *Aspergillus parasiticus* Speare (*6*). When these fungi invade and grow in commodities such as peanuts, corn, and cottonseed, the resulting contamination with the aflatoxins often makes the commodity unfit for consumption. The four naturally occurring aflatoxins are designated B_1, B_2, G_1, and G_2 and will hereafter be collectively referred to as aflatoxin.

The U.S. peanut industry has identified aflatoxin contamination of peanuts as the number one problem for which a solution is needed (*11*). Because peanuts are used primarily for food, strict regulatory limits for the amount of aflatoxin allowable in finished peanut products have been established. Although the U.S. Food and Drug Administration has an action level of 20 ppb of total aflatoxins in food products, several states are considering much stricter limits. For this reason the U.S. peanut industry has a goal to ensure the delivery of aflatoxin-free peanut products by the year 2000.

Although aflatoxin contamination of peanuts can occur during postharvest curing and storage, the most significant contamination usually occurs prior to harvest during periods of late-season drought stress as peanuts are maturing (*8,9,15,17-19,21*). The only known method for controlling preharvest aflatoxin contamination in peanuts is irrigation (*8,9*), an option that is unavailable to the majority of peanut growers.

The purpose of this paper is to report results of a 3-year investigation of an alternative approach for controlling preharvest aflatoxin contamination of peanuts. The strategy is a form of biological control that is achieved through biological competition. The possibilities for biological control of aflatoxin contamination of certain crops has been reported for cottonseed and corn. It was demonstrated that the simultaneous inoculation of wounded cotton bolls with toxigenic and nontoxigenic strains of *A. flavus* resulted in lower aflatoxin concentrations in the cottonseed from those bolls than in cottonseed from bolls inoculated with a toxigenic strain alone (*12*). Similar results were obtained when developing ears of corn were wounded and coinoculated with toxigenic and nontoxigenic strains of *A. flavus* (*5*). However, the practical demonstration of biological control of aflatoxin contamination in the field is still needed.

The hypothesis tested in this investigation was that the addition of a highly competitive, nontoxigenic strain of *A. parasiticus* (biocompetitive agent) to soil would result in lower concentrations of aflatoxin in peanuts. The rationale was that the biocompetitive agent would dominate the soil microflora and prevent the buildup of native, aflatoxin-producing strains of *A. flavus/parasiticus* that normally occurs during late-season drought. In other words, the toxigenic strains found naturally in soil would be replaced by a nontoxigenic strain added to the soil. Therefore, peanuts subjected to late-season drought stress would be invaded predominantly by the biocompetitive agent, which is unable to produce aflatoxin.

MATERIALS AND METHODS

Biocompetitive agent

The fungus initially chosen for these studies was a strain of *A. parasiticus* (CP461; NRRL 13539) isolated from the environ-

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mental control plots at the National Peanut Research Laboratory in 1980 and subsequently shown not to produce aflatoxin (*14*). At that time, it was the only naturally occurring strain of *A. parasiticus* known that did not produce aflatoxin. However, it did produce O-methylsterigmatocystin (OMS), the immediate biosynthetic precursor to aflatoxin B_1 (*2*). Because this strain of *A. parasiticus* is the only strain of either *A. parasiticus* or *A. flavus* to have been isolated from soil used in this facility over several years that accumulates OMS, it was decided to use OMS as a chemical marker to monitor the presence of the fungus in the soil and in peanuts.

Since this strain of *A. parasiticus* (NRRL 13539) accumulates OMS, it may be unacceptable as a general-use biocompetitive agent because OMS is reported to be carcinogenic (*20*), and it possesses the dihydrobisfuran moiety reportedly responsible for aflatoxin's carcinogenicity (*6*). Therefore, for studies conducted in crop year 1989 a UV-induced mutant of the NRRL 13539 strain that does not produce OMS was also tested as a biocompetitive agent.

Cultures of the parent strain were irradiated under shortwave UV light by the method of Bennett and Goldblatt (*1*). Surviving colonies were isolated, grown on potato dextrose agar slants, and analyzed for OMS and aflatoxin by adding 3 ml chloroform to the slant tube, vortexing for 1 min, and filtering through microfiber filter paper. The filtrate was evaporated to dryness under nitrogen, redissolved in 50 µl chloroform, and mixed. A 2-µl aliquot was spotted on a silica gel 60 thin-layer chromatography plate along with standards of aflatoxin and OMS, and plates were developed in a solvent system of chloroform-acetone (93-7, vol/vol). Developed plates were viewed under longwave ultraviolet light before and after spraying with 50% ethanolic sulfuric acid. Aflatoxins were visualized as bluish or greenish fluorescent spots before spraying and as yellowish spots following spraying. OMS was a blue fluorescent spot before spraying and an intensely yellow fluorescent spot after spraying. A mutant was identified (M52) for testing in 1989, and it was subsequently found to accumulate versicolorin A, another intermediate in aflatoxin bioynthesis (*16*). Versicolorin A was visualized on thin-layer chromatography plates as an orange spot in visible light.

Soil microfloral analysis

The number of CFU of aflatoxin-producing strains of *A. flavus/parasiticus* and the biocompetitive agents in treated and nontreated soils were determined at various times. Five soil samples of approximately 250 g were taken to a depth of 5 cm at various locations in the plots. Each sample was well mixed and screened through a 20-mesh sieve. Fifteen grams of screened soil was then added to 300 ml of a sterile 0.2% agar solution in a 500-ml Erlenmeyer flask. After swirling, ten 0.5-ml portions were spread on petri plates containing the following selective medium: 20 g agar; 10 g glucose; 5 g peptone; 30 g NaCl; 1 g K_2HPO_4; 0.5 g $MgSO_4$; 6 ml of a botran solution (90 ml acetone, 30 mg botran); 5 ml of an antibiotic solution (20 ml water, 0.2 g chlortetracycline HCl, 0.2 g streptomycin sulfate); and 1 l distilled water (David M. Wilson, personal communication). For samples with colonies too numerous to count, appropriate dilutions of the soil suspension were made and the samples were replated. Plates were incubated for 4 d at 30°C.

The *A. flavus/parasiticus* colonies were then tested to determine the percentage that were aflatoxin producers, one of the biocompetitive agents, or neither. Approximately 10% of the total colonies were randomly chosen, transferred to potato dextrose agar slants, and incubated at 30°C for 7 d. Each slant was analyzed for aflatoxin and the chemical markers, OMS and versicolorin A, as described for the analysis of slants of mutated colonies. The resulting percentages were then multiplied by the total CFU per gram of soil to estimate the CFU of each type.

1987 Crop year

Six rows, spaced 0.9 m apart, of cv. Florunner peanuts were grown in an environmental control plot (5.5 m x 12.2 m) at the National Peanut Research Laboratory (*3*), which provided the late-season drought and soil temperature conditions previously determined to be optimum for preharvest aflatoxin contamination of peanuts (*4,10*). The plot contained soil from crop year 1986 studies that supported extensive aflatoxin contamination. Cultural practices recommended by the Georgia Cooperative Extension Service were used until 100 d after planting (DAP), at which time the peanuts received a final irrigation. The stress period was started at 107 DAP.

The biocompetitive agent (*A. parasiticus*, NRRL 13539) was grown in six 2.8-L Fernbach flasks on liquid YES medium (15% sucrose; 5% mycological broth, pH 4.8; 2% yeast extract) for 2 weeks at 27°C. The cultures were combined and homogenized in 15 L of water plus 0.05% Tween 20 with an Ultra-Turrax homogenizer. The homogenate was strained through cheesecloth and applied over three of the six rows of peanuts at 32 DAP using a garden sprinkler. A similar application was made 100 DAP, the day of the final irrigation. Each of the treated and nontreated rows was divided into sections so that four random samples of each were harvested after 23, 30, 37, and 44 d of stress. Each sampling used approximately one-fourth of the peanut plants from both the treated and nontreated sections and yielded an average of 2.5 kg of peanuts. Samples were shelled with a Federal State Inspection Service sample sheller and sized into commercial-size categories (jumbo, medium, No. 1, other-edible, and oil stock). Damaged and visibly molded kernels were removed from each category and combined as a single category.

Jumbo, medium, No. 1, and other-edible size peanuts were ground to pass a 20-mesh sieve. Aflatoxin analyses of each category were carried out on 75% of the ground samples using the high-performance liquid chromatography (HPLC) method of Dorner and Cole (*13*). Oil stock and damaged peanut samples were relatively small and thus were analyzed for aflatoxin only. The remaining 25% of the ground samples was analyzed for OMS with an HPLC method developed for this study. The ground peanut samples were extracted with chloroform, filtered through microfiber filter paper, evaporated to an oil on a rotary evaporator, and applied to a Florisil Sep PAK (Waters Chromatography Division, Millipore Corp., Milford, MA) in cyclohexane. The Sep PAK was washed with 10 ml volumes of cyclohexane and cyclohexane-ethyl acetate (60-40, vol/vol), and OMS was eluted with 10 ml of methanol. The methanol fraction was evaporated to dryness and redissolved in 1 ml of ethyl acetate-hexane (85-15, vol/vol). Fifty microliters was injected into an HPLC system consisting of a Waters silica cartridge (5 mm x 10 cm) in a radial compression module, eluted with a mobile phase of ethyl acetate-hexane (85-15, vol/vol), and detected at 310 nm with a Waters Model 490E programmable UV detector. Quantitation was achieved with a Waters Model 730 data module that compared peak areas of samples to areas of OMS standard solutions. Recoveries of OMS from spiked samples using this method averaged 82.3% with a limit of detection of 2 ppb.

1988 Crop year

Because of the positive results of the 1987 study, it was continued in 1988 in the same environmental control plot with several modifications. Preplant soil microfloral analysis indicated that a large population of the biocompetitive agent remained in the soil from the previous year's study, including the area that was not treated with the biocompetitive agent in 1987. Therefore, soil was removed from one-half of the plot to a depth of 1 m and replaced with new soil. A barrier was placed between the two halves of the plot, and the half containing the new soil served as an untreated control, while the half with the old soil, containing *A. parasiticus*,

was used to determine the effectiveness of the biocompetitive agent for the second year. No additional biocompetitive agent was added to the soil.

Florunner peanuts were grown and subjected to late-season drought stress as in the previous year. The final irrigation was applied 98 DAP, the stress period started 105 DAP, and all peanuts were harvested 154 DAP after 49 d of stress. Peanuts from treated soil were analyzed for aflatoxin and OMS as in 1987, but only edible peanuts grown in the new soil were analyzed for aflatoxin.

In addition to the preplant soil microfloral analysis, soil samples were also taken at harvest to compare propagule levels of the biocompetitive agent and wild strains of *A. flavus/parasiticus* with those determined prior to planting.

1989 Crop year

The 1988 study was essentially repeated. Soil was again replaced in the untreated half of the plot to serve as the control plot for this year. No additional biocompetitive agent was added to the treated half. This soil was last inoculated with the biocompetitive agent on August 5, 1987.

Additional studies were conducted to determine the effectiveness of the M52 mutant from *A. parasiticus* NRRL 13539 as a biocompetitive agent. Four environmental control plots (5.5 m x 6-m), each containing new soil on which peanuts had not been grown for several years (identical to soil in control plot), were inoculated with two different inoculum levels of the mutant to determine the effect of inoculum level on fungal soil populations and aflatoxin contamination. To determine the number of CFU in a Fernbach flask after homogenization with water and 0.05% Tween 20, serial dilutions of three flasks were plated and the average determined to be approximately 120 x 10^9 CFU per flask. Therefore, it was decided to broadcast the homogenized and cheesecloth-strained contents of five Fernbach flasks (600 x 10^9 CFU) over each of two plots to serve as the high inoculum treatment. Half the contents of one flask (60 x 10^9 CFU) were broadcast over each of the other two plots to serve as low inoculum treatment. These plots were inoculated on March 1, 1989, 57 d before peanuts were planted. The day following inoculation plots were irrigated with 4 cm of water.

Peanuts for this and the continuing study were grown as in previous years with final irrigation occurring 92 DAP, stress period beginning 99 DAP, and harvesting 147 DAP (48 d of stress).

Peanuts were harvested and handled as previously described, except that peanuts were analyzed for aflatoxin only. Soil samples for microfloral analysis were collected from all plots on the day after planting, 99 DAP (beginning of stress period), and on the day of harvest.

The design of these studies did not allow for replicated treatments and statistical analysis of data. Prior experience with this environmental control plot facility has shown that preharvest aflatoxin contamination is extremely variable. Subdividing the 5.5 m x 12.2-m environmental plots in order to obtain replications has proven unacceptable because of the extreme variability encountered. Because conditions in the environmental plots can be controlled so exactly, it has proven more desirable to treat all the peanuts in a plot as a population and analyze all the peanuts from that population to avoid sampling errors. In achieving control of aflatoxin contamination in edible peanuts, the goal must be to come as close as possible to zero aflatoxin in the population that has been exposed to optimal conditions for aflatoxin contamination. That has been the objective in this investigation.

RESULTS AND DISCUSSION

1987 Crop year

Results of aflatoxin and OMS analyses are presented in Table 1. By 23 stress d, aflatoxin concentrations were already high in the inedible peanuts (other-edible, oil stock, and damaged peanuts). Aflatoxin has been shown consistently to appear first and achieve higher concentrations in these high risk categories (9). Therefore, in milling and processing operations, most of these peanuts are removed from peanuts destined for edible use, regardless of aflatoxin concentration. Aflatoxin concentrations were unacceptably high in both treated and untreated edible peanuts (jumbo, medium, and No. 1) from the 30 stress-day sampling. However, in subsequent samplings (37 and 44 stress days) aflatoxin concentrations were lower in edible peanuts from soil that was treated with the biocompetitive agent than in peanuts from nontreated soil. Results of OMS analyses indicated that the biocompetitive agent was actively contaminating peanuts, but it had not excluded all wild, aflatoxigenic strains of *A. flavus/parasiticus*. These results warranted continuation of the study for another year.

TABLE 1. *Aflatoxin and O-methylsterigmatocystin (OMS) concentrations (ppb) in peanuts from soil treated and not treated with the biocompetitive agent in crop year 1987.*

Stress period		Aflatoxin		OMS
(days)	Treatment	Edible[1]	Inedible[2]	Edible
23	Treated	4	577	15
	Untreated	1	739	7
30	Treated	222	2,534	31
	Untreated	97	4,775	41
37	Treated	19	11,783	120
	Untreated	106	12,688	5
44	Treated	11	7,035	94
	Untreated	531	21,692	81

[1] Values are the weighted average for jumbo, medium, and No. 1 sizes.

[2] Values are the weighted average for the other-edible, oil stock, and damaged categories.

1988 Crop year

Results of the preplant and harvest soil microfloral analyses of soil treated with the biocompetitive agent in 1987 are presented in Table 2. Populations of both aflatoxin-producing strains of *A. flavus/parasiticus* and the biocompetitive agent approximately doubled during the season. However, the population of the biocompetitive agent far outweighed that of wild-type aflatoxin producers. The final population of the biocompetitive agent was comparable to levels commonly seen for *A. flavus/parasiticus*

TABLE 2. *Soil populations (CFU per gram) of aflatoxigenic strains of* A. flavus/parasiticus *and biocompetitive agent prior to planting and at harvest for the 1988 crop year in soil treated with biocompetitive agent in 1987.*

Sampling	*A. flavus/parasiticus*	Biocompetitive agent
Preplant	207	5,233
Harvest	442	10,925

in peanut soils exposed to late-season drought stress (unpublished data). This demonstrated that the high levels of the biocompetitive agent in the soil prevented the wild, native strains of *A. flavus/parasiticus* from increasing to such similar high levels.

Aflatoxin concentrations in edible, treated peanuts in 1988 were the lowest ever observed during 9 years of research using the environmental control plots (Table 3). Together, edible peanuts contained only 1 ppb of aflatoxin compared to 96 ppb in edible peanuts from untreated soil. The OMS concentration in edible peanuts from treated soil was 172 ppb, providing strong evidence that the biocompetitive agent had invaded peanuts and proliferated to a far greater extent than aflatoxigenic strains. The inedible peanuts still had high amounts of aflatoxin, but these too were much lower than had been observed in previous years and were much lower than the amounts of OMS present.

TABLE 3. *Crop year 1988 aflatoxin and OMS concentrations (ppb) in peanuts from soil treated in 1987 with the biocompetitive agent and new, untreated soil. All peanuts were subjected to 49 d of drought stress.*

Category	Aflatoxin	OMS
Treated soil		
Jumbo	0	45
Medium	0	238
No. 1	4	98
Edible weighted average	1	172
Other-edible	0	1,288
Oil stock	68	1,776
Damage	3,908	13,311
Inedible weighted average	515	2,945
Untreated soil		
Edible[1]	96	NA[2]

[1] All edible category peanuts (jumbo, medium, No. 1) were analyzed together.
[2] Peanuts from untreated soil were not analyzed for OMS.

1989 Crop year

Results of soil microfloral analyses conducted during 1989 appear in Table 4. As previously observed in new soil, wild strains of *A. flavus/parasiticus* were relatively low prior to planting (40 propagules per g) and increased dramatically by harvest time (4,386 propagules per g). In the continuing study, populations of aflatoxin producers and the biocompetitive agent remained fairly constant throughout the growing season, indicating that this soil could not support a significant buildup of either type. In comparing the populations at harvest of the untreated soil with those of the soil from the continuing study, it was interesting that the wild *A. flavus* propagules and biocompetitive agent propagules were essentially reversed in the two treatments. This shows that introduction of a relatively large fungal population did not result in a final population that was higher than that of wild strains of *A. flavus/parasiticus* under these conditions. In the low and high inoculum treatments with the M52 mutant, much better control of wild *A. flavus/parasiticus* populations was achieved at harvest with the high inoculum treatment. This provides an indication that the amount of inoculum is an important consideration in achieving control through biological competition.

TABLE 4. *Fungal soil populations (CFU per gram) from crop year 1989 sampled prior to planting, 97 d after planting (DAP), and at harvest.*

Treatment	Prelant		97 DAP		Harvest	
	AF[1]	BA[2]	AF	BA	AF	BA
Untreated	40	ND[3]	100	ND	4,386	255
Continuing[4]	403	3,597	150	3,000	385	4,785
High inoculum (M52)	ND	21,200	ND	34,400	500	25,300
Low inoculum (M52)	ND	3,800	ND	3,550	3,470	5,400

[1] Wild strains of aflatoxin-producing *A. flavus/parasiticus*.
[2] Biocompetitive agent. For the untreated soil and continuing study, this is the parent NRRL 13539 strain. For the high and low inoculum treatments, this is the M52 mutant strain.
[3] No CFU detected.
[4] Refers to the study initiated in 1987 and continued in 1988 and 1989.

Results of aflatoxin analyses for the 1989 studies are presented in Table 5. A reduction in aflatoxin concentrations was again seen in peanuts grown in soils treated with a biocompetitive agent. The greatest effect was again seen in edible peanuts. The 40 ppb found in all edible peanuts from soil last treated in 1987, an increase from the 1 ppb found in 1988, indicated that the biocompetitive agent may have been losing its effectiveness after being in the soil for 3 years. Over a 10-fold reduction in aflatoxin was found in edible peanuts grown in soil treated with the high inoculum level of the M52 mutant compared to untreated soil (17 versus 241 ppb). Soil treated with the low inoculum level of the M52 mutant also resulted in a lower aflatoxin concentration in edible peanuts (29 ppb). The results from these treatments with the M52 mutant are more nearly like those seen from the 37 and 44 stress-day samplings taken in 1987, and coupled with the results from 1988 may indicate that maximum control is achieved in the year following inoculation.

The strain of *A. parasiticus* (NRRL 13539) initially chosen for these studies was selected because it met several criteria. First, it occupies the same ecological niche as *A. flavus/parasiticus*. For an organism to be successful as a biocompetitive agent for aflatoxin control, it must be able to compete under the environmental conditions that lead to aflatoxin contamination; i.e., hot, dry peanut soil during periods of late-season drought stress. Second, it has been a stable, nonproducer of aflatoxin over a period of several years. Third, *A. parasiticus* was shown to be more persistent in soil than *A. flavus* (7). Fourth, this particular strain is a prolific producer of sclerotia, which may enhance its survivability and competitiveness in the soil. It is possible that nontoxigenic strains of *A. flavus* as well as other

TABLE 5. *Crop year 1989 aflatoxin concentrations (ppb) in peanuts from untreated soil, soil treated with a biocompetitive agent in 1987 (continuing), and soil treated with high and low inoculum levels of the M52 mutant.*

Treatment	Jumbo	Medium	No. 1	Edible Weighted avg	Other Edible	Oil Stock	Damage	Inedible Weighted avg
Untreated	232	132	393	241	474	3,774	107,158	26,876
Continuing	92	10	74	40	255	1,170	28,527	7,588
High inoculum	6	22	15	17	179	90	6,786	2,022
Low inoculum	8	25	38	29	302	40	21,875	7,071

species (e.g., *Aspergillus niger*) could be useful as biocompetitive agents. Studies are currently under way to evaluate other possibilities.

CONCLUSIONS

Sound management of aflatoxin contamination of peanuts should begin in the field prior to harvest, where the toxigenic fungi first become associated with the crop and the contamination process begins. Currently, consumer concerns related to chemical residues in the food supply are increasing; therefore, it is imperative to identify and develop alternative methods of pest control. This investigation demonstrated that biological control of preharvest aflatoxin contamination of peanuts through the use of a biocompetitive agent has great potential. These results justify further studies to determine the maximum control achievable. Refinement of this approach should include evaluation of other potential biocompetitive strains, determination of optimum inoculation rates and timing, and determination of the best formulation and method for applying inoculum. Although aflatoxin contamination probably will not be completely eliminated with this approach, coupling such an approach with other available technologies could go a long way toward achieving the goal of ensuring an aflatoxin-free supply of edible peanuts.

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