AFLATOXIN CONTAMINATION OF COMMERCIALLY GROWN TRANSGENIC BT COTTONSEED

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Abstract

Transgenic Bt cotton may have reduced susceptibility to aflatoxin contamination as a result of pink bollworm resistance. During 1995 and 1996, Bt cottonseed from several commercial fields in Arizona contained aflatoxin levels unacceptable for dairy use. Comparison of cottonseed with and without BGYF (bright-green-yellow fluorescence) from one highly contaminated (>6,000 ppb aflatoxin B_1) Bt seed lot indicated that most contamination probably resulted from exposure of mature cotton to high humidity. Seed exhibiting BGYF was repeatedly detected in Bt cottonseed lots but, pink bollworm exit holes were not observed in the field. A field plot test in 1996 demonstrated high resistance among Bt cultivars to both pink bollworm damage and formation of BGYF seed cotton. These observations suggest that resistance to pink bollworm will result in reduced aflatoxin contamination when pink bollworm pressure coincides with conditions conducive to *Aspergillus flavus* infection. However, Bt cultivars are not resistant to aflatoxin increases occurring after boll opening and large quantities aflatoxin can form during this period.

Introduction

Aflatoxins are toxic chemicals produced by *Aspergillus flavus*. Aflatoxin B_1 is the most common aflatoxin and one of the most potent known carcinogens. Because aflatoxins are transmitted from feed to the milk of dairy cows, the feeding of cottonseed exceeding 20 PPB aflatoxin B_1 to dairy cows is prohibited. This prevents contamination of milk with aflatoxin concentrations greater than 0.5 PPB, the maximum level permitted by law. Cottonseed containing less than 300 ppb aflatoxin B_1 can be fed to mature beef cattle. Greater aflatoxin quantities prevent cottonseed use as a feed. Failure of cottonseed to meet dairy requirements results in a decrease in cottonseed value; aflatoxin content is the most important factor determining the value of whole cottonseed.

All cotton producing regions of the U.S. may experience some aflatoxin contamination in some years. However, in most of the cotton belt occurrence of aflatoxin levels unacceptable for dairy use is infrequent (Russell, 1980). In other regions aflatoxins are a perennial concern. These regions include the production areas of Arizona, southern Texas, and the Imperial Valley of California.

Aflatoxins contaminate cottonseed when *A. flavus* infects cottonseed either during boll development or after boll maturity (Cotty, 1991). Contamination may occur when *A. flavus* infects developing bolls through wounds or cracks. In Arizona, pink bollworm exit holes are the most common factor predisposing bolls to infection during this stage. Bolls infected during this period typically produce seed with fluorescent staining (bright-green-yellow-fluorescence = BGYF) on the lint and linters. Although seed with linter BGYF typically composes less than 1% of the crop, individual BGYF seeds may contain aflatoxin in excess 500,000 PPB (Lee, *et al*., 1990). Seed infected during this first phase can contain most of the aflatoxin. The second phase of contamination occurs when mature seed is exposed to both conducive temperature [usually above 30 C (80 F)] and either high relative humidity (above 85%) or rewetting at or after boll opening. This phase is characterized by increases in aflatoxin content of seeds infected during the first phase, as well as, infection of new seed (Cotty, 1991). In areas where aflatoxin contamination is a perennial problem, seed cotton becomes associated with *A. flavus* propagules shortly after boll opening and seed infection may proceed when the seed is exposed to adequate moisture. Seed infected during the second phase often do not exhibit BGYF on either linters or lint.

The introduction of transgenic cotton cultivars that express *Bacillus thuringiensis* insecticidal proteins (Bt cotton) accompanied reports of reduced susceptibility of Bt cotton to aflatoxin contamination (Berberich, 1995). These claims stemmed from field plot comparisons of Bt and non-Bt cotton cultivars under high pink bollworm pressure. However, aflatoxin contamination is not directly correlated with pink bollworm damage and contamination may occur in the absence of damage (Ashworth, 1969; Henneberry, 1978; Russell, 1980). During the 1995 season, several growers produced Bt cottonseed with unacceptable aflatoxin content. The current report details the most severe of these events and attempts to both explain vulnerability of Bt cottons to aflatoxin contamination and the use of Bt cottons in aflatoxin management programs.

Materials and Methods

Commercial seed. Information about the incidence of contamination in Bt cottonseed produced in Arizona during 1995 and 1996 was provided by gins and other cotton industry sources. A 100 pound sample of one Bt cottonseed lot (NuCOTN 33^B) was supplied by a cooperating cotton gin. The seed, initially part of a seed increase program, had been rejected for planting seed due to excess free fatty acid content (= 1.69%). A single commercial aflatoxin analysis

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revealed an aflatoxin content of 7,000 ppb. Six 2 kg subsamples of the cottonseed were taken. The seed was sorted by hand into three categories: 1) seed exhibiting bright-green-yellow-fluorescence under UV light; 2) seed with portions of the seedcoat free of linters (bald seed); 3) seed neither bald nor fluorescent. The percent (by weight) of the crop composed of the various seed categories and the aflatoxin concentration within seed in different categories were determined as previously described (Cotty, 1991).

Field plots. A variety trial planted March 23, 1996 at the Yuma Valley Ag Center had Deltapine NuCOTN 33^{B} and DP 5415, a similar variety without the Bt gene. A vigourous pink bollworm control program was not followed and, as a result, pink bollworm infestation was severe. Prior to harvest, 10 plants from each replicate for each cultivar were evaluated for pink bollworm damage. Open bolls were examined for evidence of pink bollworm infestation on the lint and on the carpel walls. A total of 100 green bolls were taken from each variety on October 4, 1996 and examined for pink bollworm larvae. Most bolls were infested at this time and the percent of bolls with live and dead pink bollworm larvae was determined. The field plot was mechanically harvested with a spindle picker on October 14, 1996 and seed cotton yield was determined.

Results

Unacceptable aflatoxin levels were reported in Bt cottonseed commercially produced in Arizona in both 1995 and 1996. Several Bt cottonseed lots contained seed which exhibited BGYF on linters under uv light. Only 0.5 % of the Bt cottonseed lot with the highest aflatoxin content (6,000 ppb<) exhibited BGYF (Table 1).

Analysis of the distribution of aflatoxin within the highly contaminated lot (Table 1) revealed that although BGYF cottonseed contained a very high aflatoxin concentration (130,000 ppb), most of the aflatoxin (89%) was contained in non-fluorescent seed. Bald seed (seed with portions of the seedcoat free of linters) contained quantities of aflatoxin similar to other non-fluorescent seed.

In field plot tests under severe pink bollworm pressure, BGYF was less common in seed cotton from Bt cotton cultivars than that from cultivars without Bt genes (Table 2). BGYF seed cotton from Bt cultivars contributed less than 3 ppb aflatoxin B_1 to the average aflatoxin content of the overall seed cotton. For cultivars without the Bt genes, BGYF seed cotton contributed 61 to 152 ppb to the overall seed cotton aflatoxin content. Pink bollworm larvae penetrated either into the carpel walls or into the locules of all cultivars. However, larvae died at immature stages in the transgenic pink bollworm resistant cultivars.

Discussion

Pink bollworm exit holes are important avenues through which aflatoxin producing fungi infect developing cottonbolls (Ashworth, *et. al.*, 1971; Russell, *et. al.*, 1976; Cotty and Lee, 1989). Therefore, prevention of pink bollworm damage to the developing crop is an important component of programs directed at managing aflatoxin in areas where the environment predisposes the crop to contamination. Cultivars resistant to pink bollworm, such as certain transgenic Bt cottons, may facilitate pink bollworm management. However, there is not a direct correlation between pink bollworm damage and aflatoxin contamination (Henneberry, *et. al.*, 1978; Russell, *et al.*, 1976). Unacceptable concentrations of aflatoxins may contaminate seed from fields with no pink bollworm exit holes (Russell, 1980), so, other factors must also play decisive roles in determining the quantity of aflatoxin in the crop.

Although pink bollworm resistant cultivars can be expected to have less aflatoxin contamination than susceptible cultivars when pink bollworm pressure is high, these cultivars are not resistant to aflatoxin contamination per se. Physical damage may predispose developing bolls to aflatoxin contamination. Insects other than pink bollworm and certain types of physiological stress (*i.e.*, heat stress-induced suture cracking) may also predispose bolls. Furthermore, a second phase of infection occurs when mature seed (open bolls through ginned seed) is exposed to adequate humidity and temperature to permit aflatoxin producing fungi to grow and contaminate the seed. This second phase apparently led to unacceptable aflatoxin contamination of several commercial lots of Bt cottonseed in 1995 and 1996. This is supported by observations on one highly contaminated commercial seed lot (Table 1) where most contamination occurred in seed without BGYF. Typically, some or all seed produced in locks infected during the first phase of contamination will exhibit some linter BGYF. As bolls fluff-out and dry, the capacity of lint and linters to support BGYF formation is lost. Thus, aflatoxin contaminated seed lacking BGYF may reflect infection subsequent to boll splitting. The commercial seed lot examined here also had a free fatty acid content (1.69%) that was elevated compared to the norm for the region. Free fatty acid increases are also associated with exposure of seed to high humidity (Conkerton, et al., 1989).

BGYF formation in Arizona cotton is frequently associated with pink bollworm exit holes (Cotty and Lee, 1989; Cotty, 1991). However, in the current study, BGYF was repeatedly found on linters of Bt cottonseed, but pink bollworm exit holes were not observed in the developing bolls. Because pink bollworms do form entry holes in Bt cotton bolls, it was speculated that *A. flavus* might colonize these minute wounds in order to gain access to the developing seed. In mid-October we examined twenty immature Bt cotton bolls with multiple entry holes, but none were fungal infected. Furthermore, ten live worms from immature cotton bolls (not Bt cotton) from a high aflatoxin

area were found not to be contaminated with *A. flavus*. This supports the previous contention that entry holes are not significant entry points for *A. flavus* (Russell, 1980). BGYF forms on lint when developing cotton bolls are wound inoculated (Cotty, 1989) and may also form on a smaller percentage of suture inoculated bolls (Ashworth and McMeans, 1966; Lee, 1988). The BGYF observed on linters of Bt cottonseed in the current study may have resulted from suture infections or from infection through wounds caused by insects other than the pink bollworm. These avenues of infection of developing bolls may have increased importance with the advent of Bt cotton.

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References

Ashworth, L. J., Jr., and McMeans, J. L. 1966. Association of *Aspergillus flavus* and aflatoxins with a greenish yellow fluorescence of cotton seed. Phytopathology 56:1104-1105.

Ashworth, L. J., Jr., McMeans, J. L., and Brown, C. M. 1971. The relationship of insects to infection of cotton bolls. Phytopathology 61:488-493.

Ashworth, L. J., McMeans, J. L., and Brown, C. M. 1969. Infection of cotton by *Aspergillus flavus*: Time of infection and the influence of fiber moisture. Phytopathology 59:383-385.

Berberich, S. 1995. Is cotton containing the bollguard gene safe? Oil Mill Gazetteer 101:18-19.

Conkerton, E. J., Schneider, G. R., and Bland, J. M. 1989. Prevention of deterioration of cottonseed in the field and during storage. Oil Mill Gazetteer 95:34-38.

Cotty, P. J. 1989. Effects of cultivar and boll age on aflatoxin in cottonseed after inoculation with *Aspergillus flavus* at simulated exit holes of the pink bollworm. Plant Dis. 73:489-492.

Cotty, P. J. 1990. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. Plant Disease 74:233-235.

Cotty, P. J. 1991. Effect of harvest date on aflatoxin contamination of cottonseed. Plant Disease 75:312-314.

Cotty, P. J., and Lee, L. S. 1989. Aflatoxin contamination of cottonseed: comparison of pink bollworm damaged and undamaged bolls. Trop. Sci. 29:000-000

Cotty, P. J., Bayman, P., Egel, D. S., and Elias, K. S. 1994. Agriculture, aflatoxins, and *Aspergillus*. Pages 1-27 in: The Genus *Aspergillus*: From Taxonomy and Genetics to Industrial Applications. K. A. Powell, A. Renwick, and J. F. Peberdy, eds. Plenum Press, New York.

Henneberry, T. J., Bariola, I. A., and Russell, T. E. 1978. Pink bollworm: chemical control in Arizona and relationship to infestations, seed damage, and aflatoxin contamination. J. Econ. Entomol. 71:440-448.

Lee, L. S. 1988. Aflatoxin in Arizona cottonseed: Lack of toxin formation following *Aspergillus flavus* inoculation at sutures. J. Am. Oil Chemists Soc. 65:127-128.

Lee, L. S., J. H. Wall, P. J. Cotty, and P. Bayman. 1990. Integration of ELISA with conventional chromatographic procedures for quantitation of aflatoxin in individual cotton bolls, seeds, and seed sections. J. Assoc. Official Analytical Chemists. 73:581-584.

Russell, T. E. 1980. Aflatoxins in cottonseed. Publication Q422. University of Arizona, Cooperative Extension Service, Tucson.

Russell, T. E., Watson, T. F., and Ryan, G. F. 1976. Field accumulation of aflatoxin in cottonseed is influenced by irrigation termination dates and pink bollworm infestation. Appl. Environ. Microbiol. 31:711-713.

Table 1. Distribution of aflatoxin within a commercially grown lot of transgenic BT cottonseed cultivar Deltapine Nucotn 33

Seed Category (Q)	Incidence (% Crop)	Aflatoxin (ppb)	Contribution (% Total Aflatoxin)
Fluorescent	0.53%c (R)	139,410a	10.87%
Bald	11.62%b	14,437b	24.67%
Remainder	87.85%a	4,990b	64.47%
All Seed	100.00%	6,800	100.00%

(Q) Fluorescent = seed exhibiting bright-green-yellow fluorescence (BGYF) under UV light; bold = seed with portions of the seedcoat free of linters; remainder = seed neither fluorescent nor bold.

(R) Values are averages of three to six replicate. Values within a column not followed by a common letter differ significantly (p=0.05 by Tukey's hsd test).

Table 2. Comparison of certain Bt cottons (NUCOTN33B and Hartz 1220 BG) with similar cultivars without Bt genes

Cultivar	Seed Cotton with BGYF (U)	Larvae dead (V)	Aflatoxin from BGYF (W)	Overall Aflatoxin (X)	Yield (Y)
NUCOTN33B	0.22% b (Z)	100%	0 b	2 b	5,485 a
DPL5415	5.80% a	4%	61 a	79 a	3,422 c
Hartz1220 BG	0.81% b	88%	2 b	331 a	4,672 b
Hartz1560	8.45% a	0%	152 a	156 a	3,784 c

(U) Percent of seed cotton locks by weight exhibiting bright-green-yellow fluorescence under uv light.

(V) Percent larvae from 100 green bolls that were dead upon examination. Data not replicated for statistical comparison.

(W) Quantity of aflatoxin B_1 in total seed contributed by seed from locks exhibiting bright-green-yellow fluorescence in ppb. Limit of detection = 1 ppb.

(X) Quantity of aflatoxin in total seed in ppb.

(Y) Seed cotton yield in pounds per acre.

(Z) Values within a column followed by the same letter do not differ significantly (P=0.05) by Tukey's HSD test.