Effects of Cultivar and Boll Age on Aflatoxin in Cottonseed After Inoculation with *Aspergillus flavus* at Simulated Exit Holes of the Pink Bollworm

P. J. COTTY, Southern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 19687, New Orleans, LA 70179

ABSTRACT

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 Disease 73:489-492.

Seven cultivars of *Gossypium hirsutum* commonly grown in Arizona, Deltapine (DP) Acala 90 (DP 90), DP 120, DP 77, DP 61, DP 41, DP 20, and McNair 235, became equally contaminated with aflatoxin B_1 when 28- to 32-day-old bolls were wounded to simulate exit holes of the pink bollworm and then inoculated with conidia of *Aspergillus flavus*. The cultivar Pima S-6 of *G. barbadense*, however, became more contaminated than the cultivars of *G. hirsutum*. In subsequent studies, Pima S-6 became more contaminated than DP 90 when bolls were inoculated at 29–36 days of age and less contaminated when bolls were inoculated at 17–21 days. Aflatoxin was predominantly found in seed from wound-inoculated locks. *A. flavus* did, however, move from inoculated locks to adjacent unwounded locks and contaminate seed produced there with low levels of toxin. Thus, undamaged locks in bolls with exit holes of the pink bollworm may be important sources of contamination in commercial fields. Boll age at inoculation influenced the contamination of both wound-inoculated and adjacent unwounded locks. Wound-inoculated locks of young bolls (13–20 days old at inoculation) became less contaminated than similar locks of bolls 25–32 days old at inoculation. The fungus, however, moved into and contaminated unwounded locks of young bolls more extensively than those of older bolls. Thus, the maturation of the intercarpellary membrane may hinder the interlock spread of *A. flavus*.

Additional keyword: mycotoxins

The contamination of cottonseed with aflatoxin is a chronic problem in Arizona that significantly reduces the market value of the crop (6,8). Aflatoxins are produced by *Aspergillus flavus* Link ex Fries during seed infection. Infection of cotton bolls by *A. flavus* has repeatedly been associated with pink bollworm damage (2,6,15), and a model system has been developed to study this host-fungus interaction utilizing simulated exit holes of the pink bollworm (8).

During boll infection, *A. flavus* produces kojic acid on the lint and aflatoxin in the seed (7,11). Host peroxidases convert kojic acid into a compound producing a bright green-yellow fluorescence (BGYF), which is easily observed under ultraviolet light and has proven to be a reliable indicator of the presence of *A. flavus* (1,11).

Little information is available on variation in susceptibility to *A. flavus* among cultivars, and no studies have been undertaken under controlled conditions. Comparisons of cultivars under such conditions should permit an evaluation of host susceptibilities without the confounding effects of fruiting habit, plant symmetry, or other factors that vary among cultivars in the field (4). Data from such studies should permit better design and interpretation of field experiments (4).

Rapid changes in cotton cultivars planted in Arizona over the last 10 yr have influenced certain disease problems (5), but it is not known how these changes may affect the aflatoxin problem. In this study, variation in susceptibility to *A. flavus* among Arizona's most important commercial cultivars was examined under carefully controlled conditions.

MATERIALS AND METHODS

A strain of *A. flavus*, AFMC 5A86, isolated from cottonseed collected in Maricopa County, Arizona, in 1986 was

Accepted for publication 27 December 1988.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1989.

used in all studies. The isolate was transferred twice serially by single spores and demonstrated to be highly virulent prior to use. Active cultures were maintained in the dark at 30 C on a medium containing 5% V-8 juice and 2% agar. For long-term storage, plugs (3 mm in diameter) of sporulating cultures were submerged in 5 ml of sterile distilled water in 15-ml vials and refrigerated at 8 C.

The cultivars Deltapine (DP) Acala 90 (DP 90), DP 120, DP 77, DP 61, DP 41, DP 20, and McNair 235 of *G. hirsutum* and Pima S-6 of *G. barbadense* were grown in a greenhouse in 3-L pots containing a 50:50 mixture of Pro-mix (Premier Brands Inc., New Rochelle, NY) and sand. After 21 days, plants were fertilized weekly with 100 ml of Miracle-Gro (Sterms Miracle-Gro Products, Inc., Port Washington, NY) at 2,000 ppm. Flowers were dated at opening.

Susceptibility of cultivars. Bolls 28–32 days old were wounded with a cork borer (3 mm in diameter) to simulate exit holes of the pink bollworm, as previously described (8). Wounds were inoculated with 10 μl of 0.001% Triton X-100 containing approximately 5,000 conidia of *A. flavus*. The plants were maintained at all times in complete randomized blocks. The treatments each consisted of four to six plants (four to 10 bolls) and were replicated six times. In each experiment, all varieties were inoculated on the same day in a random manner. Each variety was tested in at least two experiments, and DP 90 was used as a standard in all experiments. Bolls were harvested 3 wk after inoculation and dried at 60 C for 72 hr, and wound-inoculated locks were separated from adjacent unwounded locks. Lint from each unwounded lock was observed under ultraviolet light for BGYF (1) and rated on a scale of 0 (no BGYF) to 3 (intense BGYF). Uninoculated locks were then ginned with a small roller gin. The inoculated locks failed to fluff out fully at maturation; therefore, seed from these locks was delinted manually.

Boll age at inoculation and development of aflatoxin. Experiments were also performed to determine whether differences between the susceptibilities of DP 90 and Pima S-6 are dependent on boll age at inoculation, because the susceptibility of bolls to aflatoxin contamination was previously found to be age-dependent (8,9,17). Fifty plants of each cultivar were grown in 10 alternate rows in the greenhouse as described above. Bolls 9–36 days old from each cultivar were inoculated in a random manner on the same date; 28 days later the bolls were harvested and grouped by cultivar (main plot) and age (subplot). Bolls in the same subplot were divided into three replicates prior to analysis.

Toxin quantification. Aflatoxin was extracted and quantified with thin-layer chromatography (TLC) by a modification of the method of the Association of Official Analytical Chemists (15). Dried, pulverized seed from each replicate was placed in a 250-ml jar containing 200 ml of 85% acetone, and the jar was shaken for 15 sec. The next morning the mixture was filtered through number 4 Whatman paper. A 100-ml portion of the filtrate was mixed with 100 ml of an aqueous solution of 0.22 M $Zn(CH_3COO)_2$ and 0.008 M $AlCl_3$. Diatomaceous earth (5 g) was added to the mixture, which was shaken, left to settle for 1–2 hr, and filtered through number 4 Whatman paper. A 100-ml sample of the filtrate was extracted twice with 25 ml of methylene chloride. Fractions were pooled and concentrated to dryness. Residues were dissolved in methylene chloride. Samples of the solutions (1–16 μl) were applied to TLC plates coated with 250-μm-thick Silica Gel 60 (Merck). The plates were developed in a solution of diethyl ether, methanol, and water (96:3:1, v/v). The fluorescence of the aflatoxin was quantified with a Shimadzu TLC densitometer (model CS-930, Shimadzu Scientific Instruments, Inc., Tokyo) and compared with standards. Aflatoxins B_1 and B_2 are produced by *A. flavus*. Only B_1 was quantified in the current study, because the fungus produces minor quantities of B_2 in cottonseed (8).

Statistical analysis. Analyses were performed either manually or with the Statistical Analysis System (SAS Institute, Inc., Cary, NC). All multiple comparisons were first subjected to analysis of variance. Toxin values were log-transformed (log $X + 1$) when necessary to homogenize variances among treatments.

Aflatoxin concentrations in bolls of DP 90 and Pima S-6 inoculated at various ages were log-transformed and subjected to split-split-plot analysis, in which the experiments were the main plots, the cultivars were the subplots, and boll ages at inoculation were the sub-subplots. Significant differences between treatment means were determined with the LSD test for split-plot analyses (12).

RESULTS

In all tests, the predominant aflatoxin detected in seed from inoculated bolls was aflatoxin B_1. Only trace levels of aflatoxin B_2 were found.

When 28- to 32-day-old bolls were inoculated with *A. flavus*, more than 50 μg of aflatoxin B_1 was detected per gram of seed from inoculated locks of all cultivars (Table 1). The quantities of B_1 detected in the cultivars of *G. hirsutum* did not differ at $P = 0.05$, according to Tukey's Studentized range test (Table 1). However, significantly higher levels of B_1 were detected in seed from inoculated locks of Pima S-6 (*G. barbadense*) than in DP 61 and DP 90 (*G. hirsutum*) in the first test (Table 1). In subsequent direct comparisons of Pima S-6 and DP 90, significantly higher concentrations of B_1 were consistently detected in Pima S-6 when bolls were inoculated at 29–32 days of age (Table 2). However, less B_1

Table 1. Production of aflatoxin B_1 and bright green-yellow fluorescence (BGYF) by *Aspergillus flavus* in bolls of various cotton cultivars inoculated 28–32 days after flowering

	BGYF[x,y]			Aflatoxin B_1 (μg/g)[y,z]		
Cultivar	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Deltapine 20	ND	0.21 a	0.50 a	ND	114 a	336 a
Deltapine 41	ND	0.24 a	0.37 a	ND	194 a	170 a
Deltapine 61	0.04 b	ND	0.52 a	97 b	ND	290 a
Deltapine 77	ND	0.32 a	0.82 a	ND	138 a	255 a
Deltapine Acala 90	0 b	0.07 a	0.56 a	58 b	150 a	244 a
Deltapine 120	ND	0.24 a	0.22 a	ND	164 a	249 a
McNair 235	ND	0.41 a	0.93 a	ND	133 a	304 a
Pima S-6	1.04 a	ND	ND	148 a	ND	ND

[x] BGYF on lint of uninoculated locks indicates the spread of *A. flavus* within bolls. BGYF per lock was rated on a scale of 0 (no BGYF) to 3 (intense BGYF).

[y] Mean values in the same experiment followed by the same letter are not significantly different ($P = 0.05$) by Tukey's Studentized range test. ND = not determined.

[z] Aflatoxin content of seed produced in inoculated locks. Values were log-transformed to homogenize variances in tests 2 and 3.

Table 2. Aflatoxin B_1 concentrations in seed of *Gossypium barbadense* 'Pima S-6' and *G. hirsutum* 'Deltapine Acala 90' from locks inoculated with *Aspergillus flavus* via simulated exit holes of the pink bollworm

Boll age at inoculation (days)[y]	Aflatoxin B_1 (μg/g)[z]	
	Pima S-6	Deltapine Acala 90
33–36*	288 a	93 b
29–32*	669 a	165 a
25–28	548 a	225 a
21–24	202 b	174 a
17–20*	11 b	89 b
13–16	8 c	16 b

[y] Asterisk indicates boll ages at which values for Pima S-6 and Deltapine Acala 90 differ significantly by the LSD test (12).

[z] Values are averages of six observations made during two experiments. Values within a column followed by the same letter are not significantly different by the LSD test for split-plot analyses (12). Values were log-transformed prior to analysis.

occurred in Pima S-6 than DP 90 when bolls were inoculated at 17–24 days of age.

Seed from unwounded locks adjacent to wound-inoculated locks contained relatively small quantities (0–0.9 μg/g) of aflatoxin B_1 when bolls 28–32 days old were inoculated. However, lint from such locks of all cultivars exhibited BGYF under ultraviolet light. No differences among cultivars were observed with respect to B_1 content; however, more BGYF was observed on lint from Pima S-6 than on lint from DP 90 and DP 61 in the first test (Table 1).

Aflatoxin concentrations in seed from bolls of DP 90 and Pima S-6 inoculated at various ages were determined in two experiments. The experiment variable was not significant and did not interact with cultivar or boll age. Therefore, data from the two experiments were pooled. For inoculated locks, the variables boll age and cultivar were both significant ($P < 0.001$ and $P < 0.04$, respectively), and cultivar and boll age interacted ($P = 0.05$). For uninoculated locks only boll age was significant ($P < 0.001$). Inoculation of bolls less than 20 days old led to less contamination of wound-inoculated locks and more contamination of adjacent unwounded locks than inoculation of bolls 25–32 days old (Table 3).

The weight of seed from wound-inoculated locks of both DP 90 and Pima S-6 increased with boll age at inoculation (Fig. 1). BGYF on lint from unwounded locks adjacent to wound-inoculated locks was also influenced by boll age at inoculation and increased as boll age at inoculation decreased in both cultivars (Fig. 2).

DISCUSSION

Cotton cultivars compared earlier appeared equally likely to become contaminated with aflatoxins under field conditions (4). Cultivars currently grown in Arizona, however, have not been compared. The cultivars evaluated in the

Table 3. Aflatoxin B_1 concentrations in seed from uninoculated locks of cotton bolls inoculated with *Aspergillus flavus* via simulated exit holes of the pink bollworm

Boll age at inoculation (days)	Aflatoxin B_1 (ng/g)[z]
33–36	14 c
29–32	199 c
25–28	3,451 b
21–24	3,191 b
17–20	16,755 a
13–16	26,171 a

[z]Values are averages of 12 observations of two cultivars, Deltapine Acala 90 and Pima S-6, made during two experiments. Values followed by the same letter are not significantly different by the LSD test for split-plot analyses (12). Values were log-transformed prior to analysis.

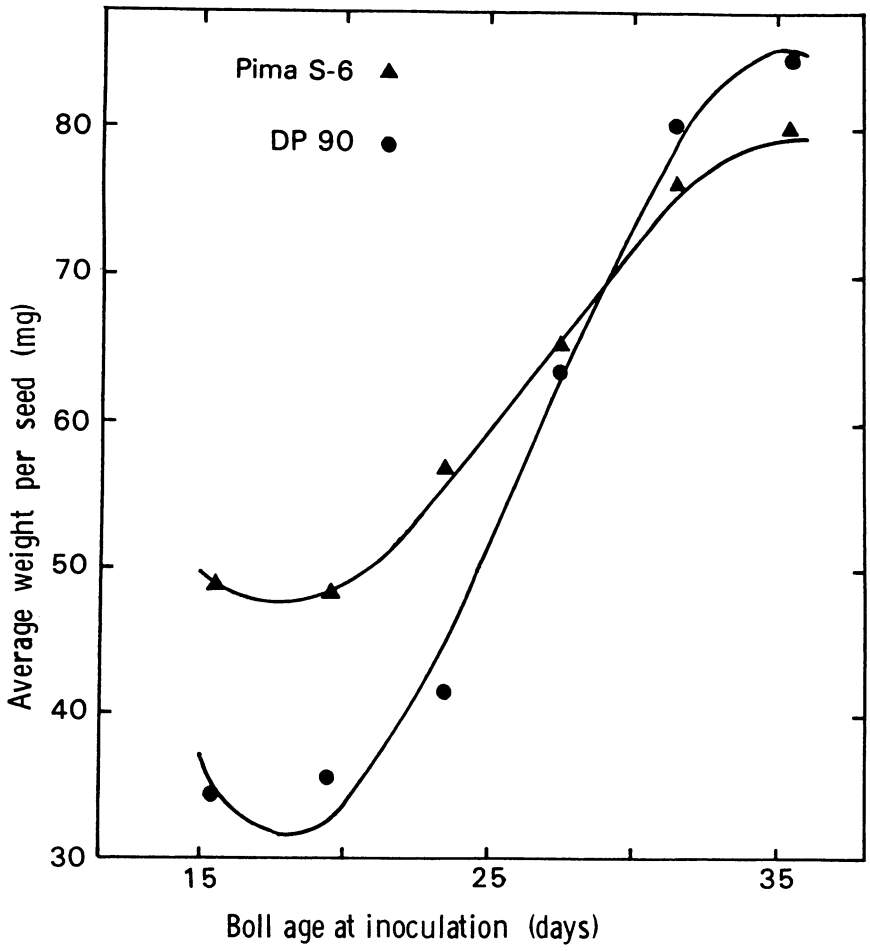

Fig. 1. Average dry weight of seed from locks wound-inoculated with *Aspergillus flavus* at various boll ages. Cubic regressions were significant for both Deltapine Acala 90 (DP 90) (with $P < 0.01$, $r^2 = 0.997$) and Pima S-6 (with $P < 0.01$, $r^2 = 0.991$).

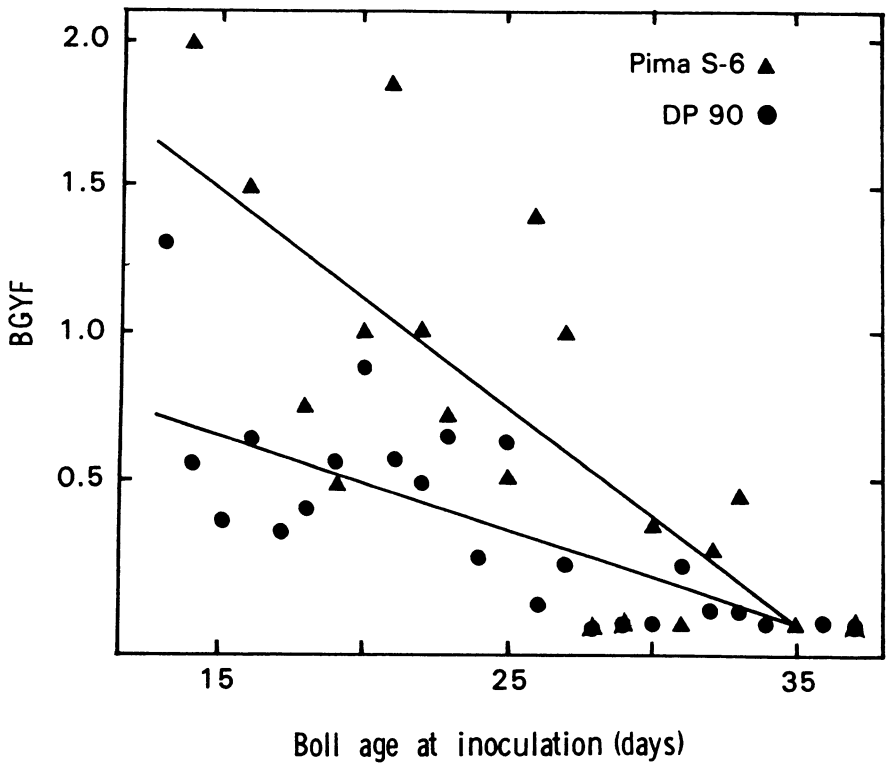

Fig. 2. Average rating of bright green-yellow fluorescence (BGYF) per lock, on lint of unwounded locks of bolls wound-inoculated with *Aspergillus flavus* at various boll ages. BGYF was rated on a scale of 0 (no BGYF) to 3 (intense BGYF). Linear regressions were significant for both Deltapine Acala 90 (DP 90) (with $P < 0.001$, $r^2 = 0.640$) and Pima S-6 (with $P < 0.001$, $r^2 = 0.583$).

current study have occupied over 85% of Arizona's cotton acreage since 1983. The cultivars of *G. hirsutum* tested here became equally contaminated with aflatoxins when 28- to 32-day-old bolls were inoculated with *A. flavus* via simulated exit holes of the pink bollworm in the greenhouse. The cultivar Pima S-6 of *G. barbadense* became more contaminated than the cultivars of *G. hirsutum*. However, these comparisons must be interpreted cautiously, because cotton boll susceptibility is age-dependent (8,9,16).

Cotton seeds change during maturation in their tendency to become contaminated with aflatoxins during boll infection by *A. flavus*; the rate at which this change occurs is cultivar-dependent. Thus, higher concentrations of aflatoxin B_1 occur in seed from inoculated locks of DP 90 than similar seed of Pima S-6 when the bolls are relatively young (17–20 days old) at inoculation; however, when older bolls (29–36 days old) are inoculated, Pima S-6 seed becomes more contaminated than that of DP 90. Boll maturity at inoculation must be considered when cultivars are compared with respect to aflatoxin contamination. In commercial fields, the pink bollworm forms exit holes mostly in bolls older than 23 days and not in bolls 21 days old or younger (10). Therefore, the higher susceptibility of Pima S-6 may be a factor in the field.

The weight of seed from inoculated locks increases with boll age at inoculation. This effect may be attributed to both greater decay of immature seed and inhibition of seed development by the fungus. The lower susceptibility of young bolls to aflatoxin contamination of inoculated locks, therefore, is apparently not attributable to resistance to seed infection and deterioration.

Cottonseed brokers have informed us that Pima cottonseed has historically been low in aflatoxins; however, in 1987 some shipments of Pima cottonseed from low elevations in Arizona contained high aflatoxin levels (greater than 0.5 μg/g). In recent years, Pima acreage in Arizona has spread rapidly from high to low elevations (3). Historically, cottonseed from low elevations has contained much greater average levels of aflatoxins than cottonseed from high elevations (13). Results of tests presented here suggest that increases in Pima acreage in low-elevation desert valleys may result in aflatoxin contamination of Pima cottonseed at levels equal to or greater than those of *G. hirsutum* cultivars. However, factors not tested here, such as fruiting habit and earliness, may reduce the risk of aflatoxin contamination of Pima cottonseed.

The movement of *A. flavus* from wound-inoculated locks to adjacent unwounded locks resulted in BGYF on the lint of unwounded locks and low aflatoxin levels in seed from those locks. Under field conditions where bolls are left on plants and exposed to the environment for months after maturation, greater contamination of undamaged locks may occur in bolls damaged by the pink bollworm. Lee et al (8) failed to find aflatoxin in unwounded locks of bolls inoculated by the same technique. However, they detected lower aflatoxin levels in wounded locks than were found here. Thus, they may have used a much less aggressive strain of *A. flavus*.

Locks that are damaged by the pink bollworm and infected by fungi often do not fully fluff out and thus are not efficiently picked by conventional spindle-type pickers used in Arizona (14). Adjacent undamaged locks typically either fully or partially fluff out (17) and are therefore more likely to be picked. Moreover, seed contained in damaged locks may disintegrate (17). Thus, the yield of seed from damaged locks is reduced, and contamination of seed from undamaged locks becomes more significant.

With maturation, bolls of both DP 90 and Pima S-6 become less susceptible to the movement of *A. flavus* from inoculated locks to adjacent unwounded locks. This results in a decrease in BGYF on lint from uninoculated locks with increased boll age at inoculation. Apparently the intercarpellary membrane, which delimits the locks, becomes more efficient at limiting the spread of *A. flavus* with age. Cotton cultivars in which the intercarpellary membrane matures more rapidly may have a lower risk of aflatoxin contamination.

ACKNOWLEDGMENTS

I thank Lisa A. Williams and Brad L. Colvin for technical assistance and Bryan T. Vinyard for statistical assistance.

LITERATURE CITED

1. 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.
2. Ashworth, L. J., Jr., Rice, R. E., McMeans, J. L., and Brown, C. M. 1971. The relationship of insects to infection of cotton bolls by *Aspergillus flavus*. Phytopathology 61:488-493.
3. Brantner, R., Rutz, J., Manjeimers, S., Crisp, N., Macaulay, P., Hoel, S., Hoffman, L., Dye, E., and Bryant, A. 1987. 1986 Arizona agricultural statistics. Bull. S-22 Ariz. Agric. Stat. Serv. 108 pp.
4. Brown, C. M., Ashworth, L. J., Jr., and McMeans, J. L. 1975. Differential response of cotton varieties to infection by *Aspergillus flavus*. Crop Sci. 15:276-277.
5. Cotty, P. J. 1987. Evaluation of cotton cultivar susceptibility to Alternaria leaf spot. Plant Dis. 71:1082-1084.
6. Henneberry, T. J., Bariola, L. A., and Russell, T. 1978. Pink bollworm: Chemical control in Arizona and relationship to infestations, lint yield, seed damage, and aflatoxin in cottonseed. J. Econ. Entomol. 71:440-442.
7. Lee, L. S., Conkerton, E. J., Ehrlich, K. C., and Ciegler, A. 1983. Reducing sugars and minerals from lint of unopened cotton bolls as a substrate for aflatoxin and kojic acid synthesis by *Aspergillus flavus*. Phytopathology 73:734-736.
8. Lee, L. S., Lacey, P. E., and Goynes, W. R. 1987. Aflatoxin in Arizona cottonseed: A model study of insect-vectored entry of cotton bolls by *Aspergillus flavus*. Plant Dis. 71:997-1001.
9. Lillehoj, E. B., Wall, J. H., and Bowers, E. J. 1987. Preharvest aflatoxin contamination: Effect of moisture and substrate variation in developing cottonseed and corn kernels. Appl. Environ. Microbiol. 53:584-586.
10. Lukefahr, M. J. 1962. Pink bollworm development in relation to age of squares and bolls with notes on biology. J. Econ. Entomol. 57:876-877.
11. Marsh, P. B., Simpson, M. E., Ferretti, R. J., Merola, G. V., Donoso, J., Craig, G. O., Trucksess, M. W., and Work, P. S. 1969. Mechanism of formation of a fluorescence in cotton fiber associated with aflatoxins in the seeds at harvest. J. Agric. Food Chem. 17:468-472.
12. Milliken, G. A., and Johnson, D. E. 1984. Analysis of Messy Data. Van Nostrand Reinhold, New York. 468 pp.
13. Russell, T. E. 1979. Aflatoxin in Arizona cottonseed 1977–78. Pages 27-28 in: Proc. Beltwide Cotton Prod. Res. Conf. 314 pp.
14. Russell, T. E., von Bretzel, P., and Easley, J. 1981. Harvesting method effects on aflatoxin levels in Arizona cottonseed. Phytopathology 71:359-362.
15. Russell, T. E., Watson, T. F., and Ryan, G. H. 1976. Field accumulation of aflatoxin in cottonseed as influenced by irrigation termination dates and pink bollworm infestation. Appl. Environ. Microbiol. 31:711-713.
16. Stoloff, L., and Scott, P. M. 1984. Natural poisons. Pages 477-500 in: Official Methods of Analysis of the Association of Official Analytical Chemists, 14th ed. S. Williams, ed. Association of Official Analytical Chemists, Arlington, VA. 1,141 pp.
17. Sun, S., Jividen, G. M., Wessling, W. H., and Ervin, M. L. 1978. Cotton cultivar and boll maturity effects on aflatoxin production. Crop Sci. 18:724-726.