2006 Beltwide Cotton Conferences, San Antonio, Texas - January 3 - 6, 2006

TIMING OF HERBICIDE APPLICATIONS MAY INFLUENCE EFFICACY OF AFLATOXIN BIOCONTROL

Nicholas Garber
The University of Arizona
Tucson, AZ
Peter J. Cotty
USDA, Agricultural Research Service
Tucson, AZ

Abstract

Biological control of aflatoxin producing fungi must function within a complex crop production system that includes management schemes directed at diverse pests. One biological control of aflatoxin producers involves applying a strain of *Aspergillus flavus* that does not produce aflatoxins (atoxigenic strain) on a sterile food source (e.g. sorghum, wheat or barley seed) to field soils. The current study used the atoxigenic strain *Aspergillus flavus* AF36 formulated on steam sterilized wheat seeds (AF36 product). AF36 is the atoxigenic strain used commercially to limit contamination of cottonseed in Arizona and Texas. Proper timing of applications allows this atoxigenic strain to grow, sporulate and disperse to other organic matter associated with the developing crop in time to competitively exclude aflatoxin producers. Competitive exclusion of aflatoxin producers from the crop results in decreased aflatoxin contamination. Atoxigenic strain applications are frequently made to fields where chemical methods are used to limit weed growth. Interactions between herbicides and AF36 have not previously been investigated. AF36 product efficacy in the field is dependant upon production of spores of AF36 on the applied colonized wheat seed. Thus fungus ability to produce spores was quantified after exposure to the common herbicides: Buctril® 4EC, Bueno® 6, Caparol® 4L, Direx 4L®, Goal® 1.6 E, Gramoxone® Extra, Prowl® 3.3 EC and Roundup® Ultra. The AF36 product was immersed in varying concentrations of herbicide, and subsequent spore production was quantified following incubation under conditions ideal for AF36 development. Spore production was greatly reduced when AF36 product was exposed to high concentrations (10X-100X recommended use rates) of all eight herbicides. Six herbicide treatments reduced sporulation greater than 30% when the product was exposed to recommended use rates: Gramoxone® Extra, Buctril® 4EC, Bueno® 6, Caparol® 4L, Prowl® 3.3 EC and Roundup® Ultra. A second set of trials was conducted with Bueno® 6 and Roundup® Ultra applied to the product in an aerosol spray that mimicked exposure that might occur from an herbicide application in the field. Bueno® 6 was used because it was the most toxic to AF36 in the first trial, and Roundup® Ultra was used because of the frequency with which it is used in Arizona. The second trial revealed that at field-use concentrations, AF36 product exposed to either Bueno® 6 or Roundup® Ultra produces fewer spores than untreated controls, but product exposed to Bueno® 6 produced the fewest spores. Reductions in AF36 sporulation after exposure to several commonly used herbicides indicates that AF36 applications should be made after all herbicide applications have completed.

Introduction

An atoxigenic strain of *Aspergillus flavus*, AF36, has demonstrated effectiveness at displacing toxigenic strains of *A. flavus* in cotton fields in Arizona and Texas (Cotty 1990). Studies continue in both states directed at understanding the optimal conditions for seeding the fields with AF36 to displace the greatest proportion of toxigenic *A. flavus* (Antilla and Cotty 2002). In order for AF36 to out-compete toxigenic strains in situ, AF36 must produce large quantities of spores (Cotty and Antilla, 2005). AF36 is labeled for 1 application of 10lbs. per acre annually. The AF36 product is wheat seed colonized by the atoxigenic strain of *Aspergillus flavus* AF36 and is labeled to produce a minimum of 2.5×10^9 spores/g product. Interactions between conventional, chemical pest control methods and biocontrol of aflatoxin contamination have not been investigated. At the request of farmers utilizing AF36 to manage contamination, the current studies were undertaken. The studies described in the current report characterized effects of herbicides that are of potential importance during use of AF36 in commercial agriculture. These studies demonstrated that many herbicides detrimentally influence sporulation and that some of the most commonly utilized herbicides can limit spore production by AF36 at recommended use rates and, as such, can be expected to reduce efficacy of atoxigenic strain applications. Recommendations for use of AF36 in relation to herbicide applications are made.

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Materials and Methods

Eight herbicides were used in exposure trials: Buctril® 4EC, active ingredient bromoxonil; Bueno® 6, active ingredient methanearsonate; Caparol® 4L, active ingredient prometryn; Direx 4L®, active ingredient diuron; Goal® 1.6 E, active ingredient oxyfluorfen; Gramoxone® Extra, active ingredient paraquat; Prowl® 3.3 EC, active ingredient pendimethalin; and Roundup® Ultra, active ingredient glyphosphate. The formulated herbicides used in these tests were obtained from farmer collaborators. AF36 product was both immersed in all eight herbicides, and exposed to an aerosol spray of Bueno® 6 and Roundup® Ultra. Spore yield of the product exposed to herbicides was compared to that of the product treated with buffer alone.

Immersion Trial:

Standard AF36 product (Bock and Cotty 1999) capable of producing >$2.5x10^9$ spores/g product was used. Five-fold serial dilutions were made of eight herbicides, in Butterfield's buffer. Twenty seeds of AF36 product were submerged and mixed in each solution for approximately thirty seconds. Control seeds were treated with Butterfield's buffer. The seeds were removed from the treatments with forceps and dried at ambient conditions on paper towels. Evaluation of spore yield with turbidity (below) followed.

Aerosol Trial:

AF36 product from the same manufactured batch used in the immersion trial was also used for the aerosol treatments. Two herbicides (Bueno® 6 and Roundup® Ultra) were used in this trial. Each herbicide was evaluated at three concentrations: a labeled field rate, one tenth field rate, and ten times field rate. Herbicide concentrations were made by diluting commercially formulated product in Butterfield's buffer. The control group was treated with Butterfield's buffer alone. Both herbicide dilutions and the Butterfield's buffer control were misted with a calibrated sprayer individually onto AF36 product (colonized wheat seed) spread across paper towels in a fume hood. The treated seeds were allowed to dry in the fume hood for thirty seconds. Evaluation of spore yield with turbidity followed as described below.

Evaluation of Spore Yield with Turbidity:

Spore yield of seeds treated in both immersion and aerosol trials was evaluated by placing one seed in each cell of a 24 well cell plate. In the immersion trial, replicates consisted of 12 cells (one half of each plate) and in the aerosol trial each replicate consisted of an entire 24 cell plate. Each treatment was replicated 3 times, and each trial was performed twice. Distilled water was added to fill each intercellular space of the plate to approximately half its total volume. The plates were covered, placed into sealed plastic containers to prevent evaporation, and then transferred to a 31°C water-jacketed incubator for seven days. For the immersion trial, 2 seeds were randomly selected from each replicate for turbidity measurement. Six seeds were selected from each aerosol replicate. Each seed was washed with 1 to 2 ml absolute ethanol 3 times, and the washes from two seeds were combined for each measurement and brought up to 11ml with ethanol. The combined washes were diluted with 11ml of distilled deionized water and turbidities of the final spore suspension was measured with a turbidity meter and the spore concentration was extrapolated from a standard curve (turbidity versus colony forming unit).

Visual Evaluation of Spore Yield:

All seeds incubated in the multiwell plates (above) were visually evaluated. After seven days' incubation, each seed was evaluated visually on a scale of 0 to 4: 0= no fungal growth visible; 1= visible mycelial growth with few conidia; 2= seed surface less than 1/3 covered with conidia; 3= seed surface more than 2/3 covered with conidia; 4= entire surface of the seed covered with conidia and none of the seed surface visible.

Results

Immersion Trial

The immersion trial provided initial assessment of herbicide influences on spore yield of the AF36 colonized wheat seed product. The AF36 product was immersed in a range of herbicide concentrations bracketing the recommended field application rates to quantify potential toxicity to the fungus. The immersion trial revealed that all the herbicides had the potential to reduce the spore yield of AF36 product with spore yield declining with increased herbicide concentration (Fig. 1). Results per herbicide are listed below.

Buctril® 4EC influenced spore yield of AF36 even at low concentrations. At a concentration of bromoxonil within the labeled rate ($4.8x10^{-3}$g/ml), the spore yield was less than half the minimum spore yield ($2.5x10^{9}$spores/g) warranted on the label for AF36 product. Exposure to $4.8x10^{-2}$g/ml bromoxonil, (10x label use rate) resulted in production of only $3.4x10^{7}$spores/g, about one hundredth the spore yield ($2.9x10^{9}$spores/g) of controls not exposed to herbicide. At concentrations of $4.8x10^{-5}$g/ml and $4.8x10^{-4}$g/ml bromoxonil, smaller influences on spore yield ($2.5x10^{9}$spores/g and $1.9x10^{9}$spores/g, respectively) were observed. At the lowest concentration tested, $4.8x10^{-6}$g/ml bromoxonil, AF36 had a spore yield within 10% of the untreated controls.

Of the tested herbicides, Bueno® 6 had the greatest influence on AF36 spore yield. Immersion in $7.2x10^{-3}$g methanearsonate/ml, within the concentration range labeled for field use, resulted in over a 1,000 fold reduction in spore yield of the AF36 product. At the lowest concentration tested, $7.2x10^{-6}$g methanearsonate/ml, spore production was 79% that of the control. At $7.2x10^{-2}$g methanearsonate/ml, ten times a recommended field use concentration, only $1.9x10^{5}$spores/g AF36 product was produced.

Caparol® 4L had only minor influences on spore production by AF36 product when applications were made within recommended field use concentrations of prometryn. Indeed, immersion in a Caparol® 4L concentration of $4.8x10^{-4}$g prometryn/ml, slightly higher than the 1.5pt/20gal tank mix rate, resulted in only a 25% reduction in spore yield and immersion in a 10 fold greater concentration resulted in only a 35% reduction.

Among the herbicides tested, Direx 4L® was the least detrimental to spore production by AF36. Although spore yield was inversely related to diuron concentration, at low herbicide concentrations ($4.8x10^{-5}$ to $4.8x10^{-6}$g) treated product yielded up to 11% more spores than untreated controls. At $4.8x10^{-3}$g diuron/ml, within the label recommendation for sandy soils, spore yield was reduced 9% compared with untreated controls. The highest concentration tested, $4.8x10^{-2}$g diuron/ml, reduced spore yield 55%.

Goal® 1.6E reduced spore yield 72% at the highest concentrated tested ($1.9x10^{-2}$g oxyfluorfen/ml), a concentration within the labeled application recommendations. However, it stimulated spore production at $1.9x10^{-6}$g and $1.9x10^{-5}$g oxyfluorfen/ml.

Concentrations of Gramoxone® Extra at or above field application rates reduced spore yield of AF36 product. A paraquat dichloride concentration of $3.0x10^{-2}$g, within the labeled application recommendations, reduced spore yield 98%. Active ingredient concentrations equal to or less than $3.0x10^{-5}$g paraquat dichloride/ml had no detrimental effect on sporulation.

Both Prowl® 3.3 EC and Roundup® Ultra reduced spore yield of AF36 at all concentrations tested. A concentration of $4.0x10^{-2}$g pendimethalin (Prowl®)/ml resulted in over a 70% reduction in AF36 spore yield; the herbicide label recommends application of between $3.0x10^{-3}$ and $1.2x10^{-2}$g pendimethalin/ml. Similarly, at a concentration within its labeled application rate ($4.8x10^{-3}$g glyphosphate/ml) Roundup® Ultra reduced AF36 spore yield by 75%.

Aerosol Trial

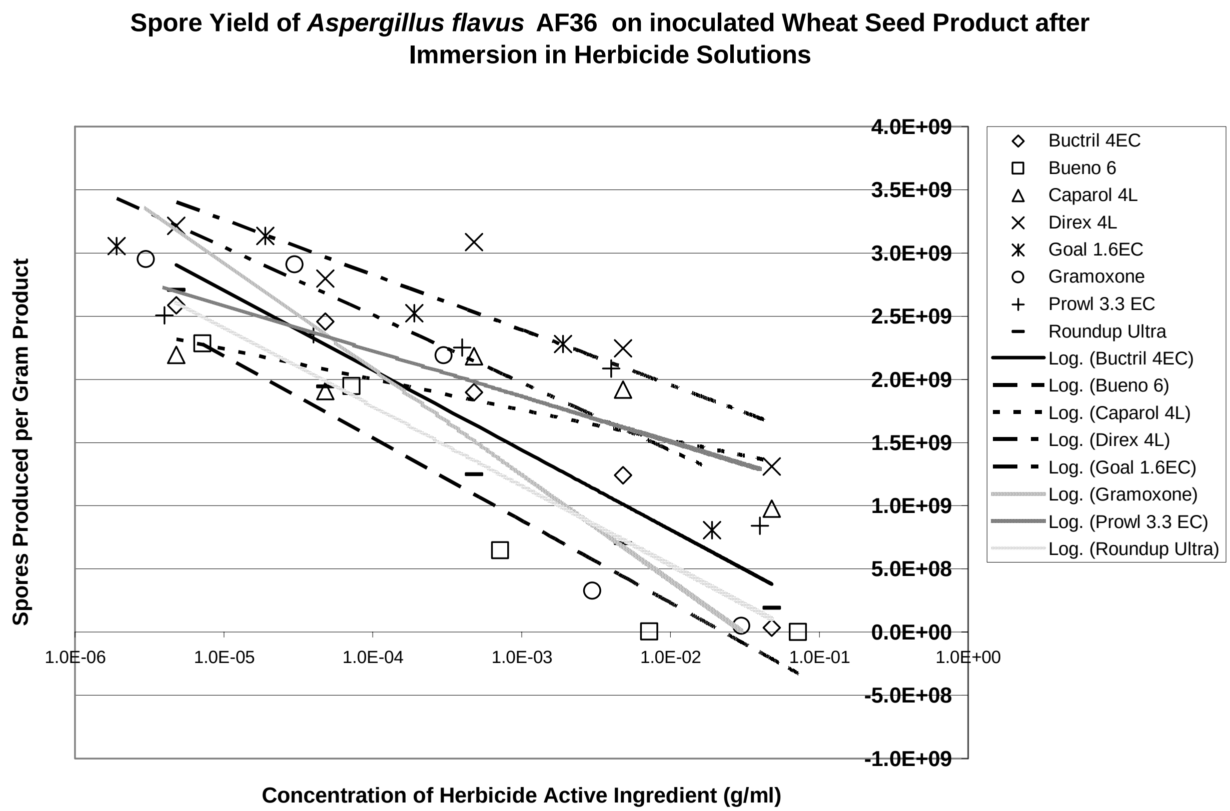
A second set of tests was performed utilizing an aerosol mist to better imitate the type of herbicide exposure the AF36 produce would receive under field conditions. Using a calibrated sprayer, and distributing AF36 product evenly over a uniform area, aerosol sprays of Bueno® 6 and Roundup® Ultra were applied to the product. Bueno® 6 was selected for the aerosol trial because this herbicide reduced spore yield more than any other herbicide in the immersion trial. Roundup® Ultra was selected because of the frequency with which this herbicide is applied within regions where AF36 is also used. Both herbicides reduced spore yield of the AF36 product to which they were applied, with Bueno® 6 causing the greatest reduction (Fig. 2). Results per herbicide are listed below.

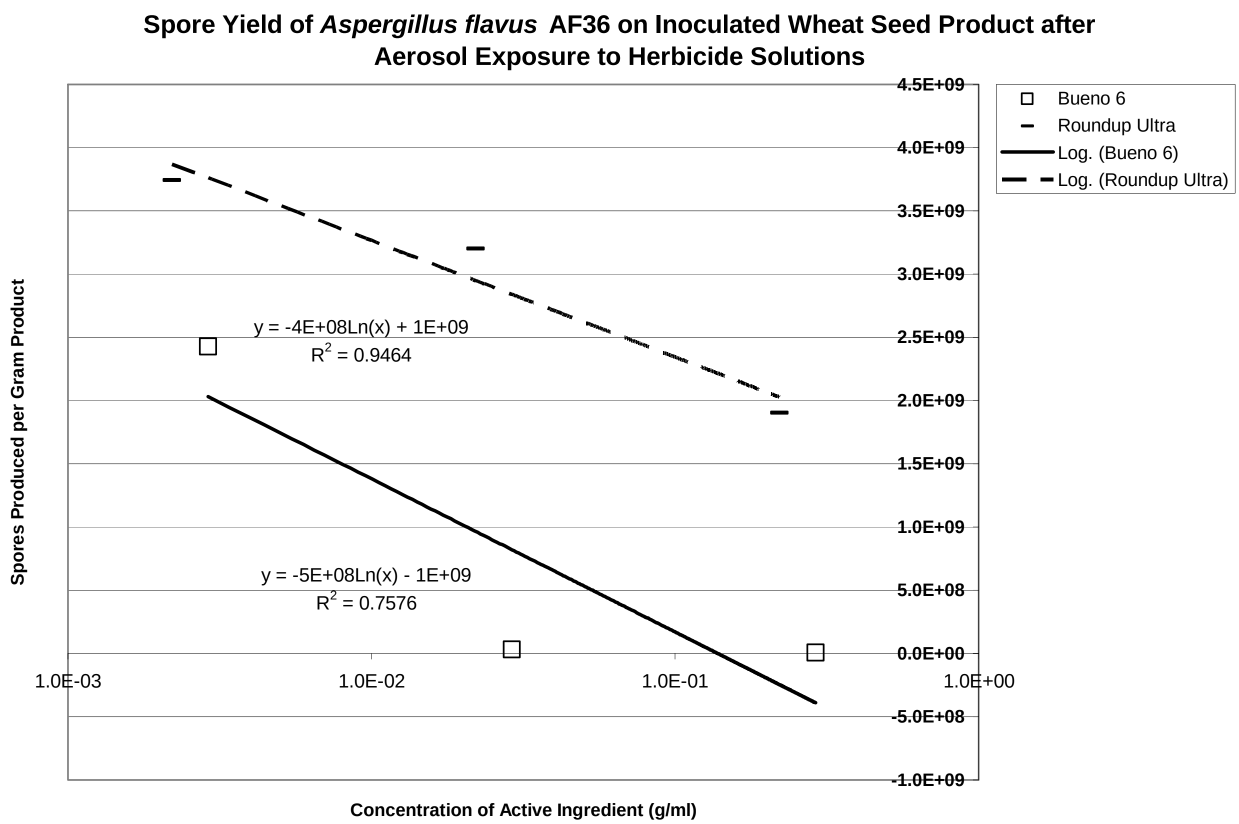
Aerosol treatments of Bueno® 6 at the label rate for a 40-gallon per acre treatment (delivering $2.9x10^{-2}$g methanearsonate/ft^2) reduced sporulation of AF36 99% compared with the Butterfield's buffer control, which yielded $3.8x10^9$spores/g of the AF36 wheat seed product. At a concentration 10% the label rate ($2.9x10^{-3}$g methanearsonate/ft^2) spore yield was $2.4x10^9$spores/g, which is slightly under the AF36 label minimum. With an application rate of $2.9x10^{-1}$g methanearsonate/ft^2 (10 times the labeled rate), $5.8x10^6$spores/g AF36 were produced.

Roundup® Ultra aerosol treatments inhibited sporulation to a lesser extent than Bueno® 6. At the labeled field rate of $2.2x10^{-2}$g glyphosphate/ft^2, spore production was reduced 16% compared with the buffer control to $3.2x10^9$spores/g. Exposure to an application rate of $2.2x10^{-1}$g glyphosphate/ft^2 (10x the recommended use rate), however dropped AF36 spore yield to $1.9x10^9$spores/g, below the minimum warranted on the AF36 label. With an application rate of $2.2x10^{-3}$g glyphosphate/ft^2, (one tenth the recommended use rate) the spore yield was $3.7x10^9$spores/g, only slightly below that of the buffer control.

Discussion

The eight herbicides in the current study can be grouped into three categories according to effects on spore yield of AF36 on wheat seed (AF36 product). Group I herbicides (Direx 4L®, Goal® 1.6E and Gramoxone® Extra) had little effect. These three herbicides increased AF36 spore production from that of the control at low concentrations, and reduced the quantity of AF36 spores produced by less than 90% when the AF36 product was immersed in concentrations within labeled use range. Group II herbicides (Caparol® 4L and Prowl® 3.3EC) moderately reduced spore yield by AF36 product. These two herbicides reduced AF36 spore yield less than 30% at concentrations within the recommended use range, but at one tenth recommended use concentrations spore yield reductions were also observed. Group III herbicides (Buctril® 4EC, Bueno® 6 and Roundup® Ultra) caused reductions in AF36 spore yield greater than 30% at one tenth recommended rates of use. At one tenth the active ingredient concentrations recommended for use in the field, these herbicides reduced AF36 sporulation by 35% (Buctril® 4EC), 57% (Roundup® Ultra) to 78% (Bueno® 6). Bueno® 6 was the most toxic to AF36. However, Buctril® 4EC and Roundup® Ultra probably are the most likely to interfere with AF36 sporulation in commercial fields, and thus efficacy of AF36, because they are labeled for use with transgenic crops that allow for over-the-top applications. Concentrations as low as 200 µl/ml glyphosphate-ammonia (glyphosphate is the active ingredient in Roundup® Ultra) have been shown to reduce mycelial dry weight and aflatoxin B_1 production of AF13, a high aflatoxin producing strain of *Aspergillus flavus* (Tubajika and Damann 2002). Results from the immersion trial in the current study, show similar effects of glyphosphate on growth of AF36, although toxin levels could not be measured because AF36 does not make aflatoxins (is atoxigenic). Previous research also suggests that metabolism of *Aspergillus flavus* might be effected by the surfactants used to formulate herbicides for field use (Rodriguez and Mahoney 1994). AF36 product treated with aerosol applications of Bueno® 6 and Roundup® Ultra had smaller reductions in spore yield than when the AF36 product was immersed in the same herbicide concentrations, but AF36 spore yield was significantly reduced by aerosol treatments. The toxicity of herbicides to formulations of atoxigenic strains is an important consideration when developing recommendations for pest management systems seeking to utilize atoxigenic strains as biological control agents for the reduction of mycotoxins in crops. Further studies might consider effects of both herbicide combinations and herbicide residues in a variety of soil textures.


Spore Yield of *Aspergillus flavus* AF36 on inoculated Wheat Seed Product after Immersion in Herbicide Solutions
Spores Produced per Gram Product
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
-5.0E+08
-1.0E+09
1.0E-06
1.0E-05
1.0E-04
1.0E-03
1.0E-02
1.0E-01
1.0E+00
Concentration of Herbicide Active Ingredient (g/ml)
Buctril 4EC
Bueno 6
Caparol 4L
Direx 4L
Goal 1.6EC
Gramoxone
Prowl 3.3 EC
Roundup Ultra
Log. (Buctril 4EC)
Log. (Bueno 6)
Log. (Caparol 4L)
Log. (Direx 4L)
Log. (Goal 1.6EC)
Log. (Gramoxone)
Log. (Prowl 3.3 EC)
Log. (Roundup Ultra)


Spore Yield of *Aspergillus flavus* AF36 on Inoculated Wheat Seed Product after Aerosol Exposure to Herbicide Solutions
Spores Produced per Gram Product
4.5E+09
4.0E+09
3.5E+09
3.0E+09
2.5E+09
2.0E+09
1.5E+09
1.0E+09
5.0E+08
0.0E+00
-5.0E+08
-1.0E+09
1.0E-03
1.0E-02
1.0E-01
1.0E+00
y = -4E+08Ln(x) + 1E+09
R^2 = 0.9464
y = -5E+08Ln(x) - 1E+09
R^2 = 0.7576
Concentration of Active Ingredient (g/ml)
Bueno 6
Roundup Ultra
Log. (Bueno 6)
Log. (Roundup Ultra)

Conclusion

The current study demonstrates that commonly used herbicides can reduce sporulation of the AF36 colonized wheat seed product typically used as a biological control to manage aflatoxin contamination in commercial fields. The three herbicides that did not reduce sporulation at the lowest tested concentrations: Direx 4L®, Goal® 1.6 E and Gramoxone® Extra, did reduce spore yield of AF36 at concentrations close to those used in field applications. The remaining herbicides, Buctril® 4EC, Bueno® 6, Caparol® 4L, Prowl® 3.3 EC and Roundup® Ultra reduced spore yield of AF36 to such levels that efficacy of this biocontrol agent in the field would be reduced. The results of these studies on herbicide influences on atoxigenic strains of *A. flavus* reveal that for the greatest spore yield per gram of product in the field, and thus the greatest atoxigenic strain efficacy, exposure to herbicides must be reduced to the minimum practical. Exposure to herbicides can be limited by timing the application of the AF36 product so that fields are treated after the final herbicide applications for that crop have been made.

Acknowledgements

This work was supported in part by grants from the Cotton Foundation, the Texas State Support Program of Cotton Incorporated, the Texas Cottonseed Crushers Association and the USDA Multi-Crop Aflatoxin Working Group.

Disclaimer

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