

**MOVEMENT AND LONGEVITY OF *Aspergillus flavus* PROPAGULES  
AND FACTORS THAT CONTRIBUTE TO AND INFLUENCE THEIR  
COLONIZATION AND PRODUCTION**

A Thesis

by

BRANDON THOMAS HASSETT

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Plant Pathology

Movement and Longevity of *Aspergillus flavus* Propagules and Factors That Contribute  
to and Influence Their Colonization and Production

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## ABSTRACT

Movement and Longevity of *Aspergillus flavus* Propagules and Factors That Contribute to and Influence Their Colonization and Production. (August 2012)

Brandon Thomas Hassett, B.S., La Roche College

Chair of Advisory Committee: Dr. Thomas Isakeit

Aflatoxin contamination accounts for millions of dollars worth of losses for corn and cotton in Texas. Two atoxigenic strains of *Aspergillus flavus*, AF36 and Afla-Guard, are labeled for its management. The purpose of this study was to measure differences in the ability of these strains to sporulate and to track movement of their conidia in corn and cotton fields. Sporulation was evaluated by incubating the two strains on their commercial formulations (inoculated on cereal grains) at six constant humidity levels ranging from 0-100%, using closed chambers with saturated salt solutions. Conidial production by Afla-Guard was 3-fold greater than that of AF36 at 100% humidity. Sporulation of the two strains was also evaluated on one substrate by inoculating their conidia on sterile, hulled barley. After 3 days, there was a 234-fold increase in conidia recovered from the barley inoculated with Afla-Guard, compared with a 21-fold increase in conidia recovered from the AF36-inoculated barley. These data suggest that the Afla-Guard strain sporulates better than the AF36 strain, which may be a factor in effectiveness for biological control. An *in vitro* de-Wit competition experiment showed that sporulation by the Afla-Guard strain was not affected by co-inoculation with either AF36 or the wildtype NRRL3357 toxin producing strain.

To measure conidial movement, an Afla-Guard nitrate non-utilizing mutant colonizing autoclaved corn seed, was placed at one point in a field of cotton and corn. For detection, aliquots washed from leaf samples were plated onto a medium containing potassium chlorate. The mutant was recovered at a maximum distance of 6.4 m in corn fields along the same row and as far as 10.2 m across rows from the point source. In cotton fields, the mutant was recovered at 9.1 meters along the same row and 6.1 m

across rows from the point source. There was no recovery at 24.3 m from the point source - the maximum distance evaluated. The experiment was repeated in a second year with similar results. These data suggest that plots in field trials may not need wide separation in order to avoid cross contamination.

To assess the viability of a toxigenic and atoxigenic strain of *A. flavus* over time, polycarbonate packets containing conidia and sclerotia of both strains were buried in Ships Clay soil with the matric potential held constant at -24 kPa or -154 kPa. After 10 months, viable conidia were recovered in all treatments. After 14 months, viability of the atoxigenic strain incubated at -154 kPa  $\psi_m$  was lost, while other treatments remained viable.

Ears of corn were inoculated via silk channel at different stages of silk senescence. Sclerotia were enumerated from the same plants following harvest of the crop. Sclerotial production by *A. flavus* was greatest from ears with silks inoculated at senescence, compared with inoculation when silks were green. The isolation frequency of *Penicillium* sp. from surface-sterilized kernels at harvest was the highest from ears that were inoculated with *A. flavus* when silks were fresh, as compared with *A. flavus* inoculation of ears with senescent silks. A *Fusarium* and *Penicillium* species was isolated from harvested kernels, and their sterile Czapek-Dox broth culture filtrates were tested for their effect on development of three strains of *A. flavus* on agar. The *Penicillium* broth filtrate greatly reduced sclerotial numbers relative to the control and the *Fusarium* filtrate ( $P < 0.05$ ). When *A. flavus* was grown in the presence of autoclaved *Penicillium* culture filtrate, there was no effect on sclerotial production. The *Penicillium* filtrate increased the rate of radial hyphal growth of the *A. flavus* isolates on agar compared to the control and the *Fusarium* culture filtrate.

## DEDICATION

This work is dedicated to a man who has navigated the halls of academia with Farmall grease on his hands; to an intellectual who has tirelessly provided through the toughest of times and asked only tolerance, honesty and a few good hours of work in return; to a man who has only ever marched to the beat of his own drum, to a man I can only aspire to be – to my father.

## ACKNOWLEDGEMENTS

Above all, I wish to thank God for affording me a sporting chance to be the first to understand a miniscule of his divinely provident plan. Science and religion are not opposing ideologies.

Secondly, I would like to acknowledge my PI, Dr. Thomas Isakeit for providing a means to this research. I am very grateful for his professional advice, technical assistance, scientific insight, lab space, and time reviewing this manuscript. I am much obliged for his technical training in the isolation, identification, and diagnosing of common plant pathogens. Additionally, I would like to thank my committee members, Dr. Betsy Pierson and Dr. Charles Kenerley for their consultations, critical reviews and thoughtful suggestions of my research.

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They say, 'it takes a village to raise a child.' I believe it takes a department to produce a successful graduate student. With this in mind, I'd like to thank all the Plant Pathology graduate students and professors. Every one of these individuals had a heavy hand in contributing to my aptitude as a scientist. Specifically, I'd like to thank Dr. Dawoon Chung and Mr. David Laughlin for their help in field sampling and Mr. Eli Borrego and Mrs. Julia Cope for their assistance in PCR, primer designs, and sequence analysis. These folks are not just good colleagues, but great friends. I would like to thank Dr. Brian Shaw for his time in discussing fungal biology and complementary experiments that contributed to this research. I would also like to extend a thank you to Dr. Mike Kolomiets for exciting conversations about world-changing research and for inclusion in his projects.

Dr. Samuel Johnson, an English author during the 18<sup>th</sup> century said, "The love of life is necessary to the vigorous prosecution of any undertaking." To this end, I am grateful for a group of friends that have helped me keep the struggles of graduate school in perspective and stirred the adventurous spirit inside me when seemingly it

was all but lost. Specifically, I'd like to thank Mr. Matthew Renner and Ms. Callie Stump for showing me that happiness really is only a two-step or a rodeo away. Your friendship is invaluable. I would like to thank the Davis family –Digger, Jeannie, Jon, and Jamie—for telling their banjo tale every chance we had to visit - be it the high planes of Oklahoma or the honkytonks of Bryan; and to Dr. Brian Shaw, for breaking up the day with lunch-time banjo-mandolin jam sessions in the microscope room. I thank you all for being my friend.

Braving the tempestuous sea of graduate school alone is a tall order. I am thankful for everyone who has shared this journey with me and contributed to its successes. No man is a sclerotium.

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## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW

Politically, economically, and agriculturally, fungi and their sometimes toxic secondary metabolites have influenced history. “St. Anthony’s fire,” an epidemic occurring in Europe during the Middle Ages, associated with hallucinations, death, gangrene and loss of limbs, was the likely result of ergot alkaloid consumption. In the Early Modern Period, events leading to the Salem Witch Trials may have been a result of the consumption of secondary metabolites from ergot-contaminated rye (14). Ergot alkaloids, while recreationally (with the example of LSD, derived from ergotamine) and medicinally relevant, are just a small subset of a large group of compounds not actively involved in primary metabolic production, and thus referred to as secondary metabolites. These metabolites are produced at a particular stage of morphological differentiation in the organismal life cycle (46). For instance, ergotamine is produced by the fungus *Claviceps purpurea*, while in its sclerotial phase. Secondary metabolites have been shown to serve as important fitness factors in fungal ecology, especially in response to oxidative stress or competition by other microbes (46), which can be relevant to toxin production.

Metabolic byproducts of fungi have been used as antibiotics for treating infectious diseases. The consummate example of such a beneficial secondary metabolite is penicillin. Discovered by Alexander Fleming, penicillin was refined and came to play an important role in healthcare during the WWII era (24). Several classes of secondary metabolites are known to exist: non-ribosomal peptides, a product of non-ribosomal-peptide-synthases (NRPS) that produce chelating compounds like siderophores; terpenes, which include the plant growth hormone gibberelin and are responsible for the aromas associated with flowers; indole alkaloids such as

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This thesis follows the style of Plant Disease.

ergotamines; and polyketides, derived from polyketide synthases (PKS), structurally related to fatty-acid synthases and comprise the largest group of fungal secondary metabolites (46).

An example of a highly deleterious secondary metabolite, a product of a PKS, is aflatoxin. Loaded in warheads by the Iraqis during the Gulf War (67), and classified as a group 1 human carcinogen by the International Agency for Research on Cancer (36), aflatoxin, produced largely by fungi of the genus *Aspergillus*, is considered by many to be among the most carcinogenic substances known to man. First discovered following the death of 100,000 turkey poults in England in 1960, aflatoxin has in hindsight been indicted as the disease-causing agent of cattle, trout, dogs, swine, guinea pigs, and rats that were fed moldy grain or peanut meal (64). Although over 16 structurally related aflatoxins have been characterized (78), aflatoxins are classified into two categories, B1 and B2 and G1 and G2, referring to the blue and green fluorescence produced upon exposure to UV light, respectively. Within each group, the aflatoxin types are separated by number of hydrogen atoms; while, between groups, hydrogen and oxygen number vary: B1: C<sub>17</sub> H<sub>12</sub> O<sub>6</sub>; B2: C<sub>17</sub> H<sub>14</sub> O<sub>6</sub>; G1: C<sub>17</sub> H<sub>12</sub> O<sub>7</sub>; G2: C<sub>17</sub> H<sub>14</sub> O<sub>7</sub> (62). Of these, B1 is the most deleterious and potent hepatocarcinogen, inducing tumors in the liver, kidney, lungs and colon of mammals (4). Upon consumption, aflatoxin can intercalate into the p53 tumor suppressor gene (4), inducing mutation and rendering cells incapable of suppressing hyperplasia and regulating the cell cycle. In addition to cancer, aflatoxin has been shown in animals to impair the immune response by suppressing cell mediated immunity and reducing antibody formation (15). In 1995, following the consumption of aflatoxin- contaminated noodles in Malaysia, containing up to 3 mg of aflatoxin per serving, 20 children were hospitalized, with 4 of them dying (36). Allowable limits of aflatoxin in food are substantially lower than toxic levels, for example, the European Union allowable limit of aflatoxin in food is 4ng/g (77). Of additional concern for food safety is the ability of dairy cattle to convert aflatoxin to an isoform that accumulates in milk, aflatoxin M (64).

Aflatoxin is produced primarily by fungi belonging to *Aspergillus* section *Flavi*, including *A. nominus*, *A. pseudoamarii*, *A. bombycis*, *A. parasiticus* and *A. flavus*, *A. minisclerotigenes* and *A. arachidicola* (16, 18). Section *Flavi* is probably the most important of the sections of *Aspergillus* - not only because it contains aflatoxin-

producing species, but also for economically- important fermenting species such as *A. sojae* and *A. oryzae*. These species have been used for centuries by Asian cultures for the production of products like soy sauce. While most aflatoxin producing species belong to section *Flavi*, some species outside this section such as *A. ochraceoroseus* produce aflatoxin as well.

The name "*Aspergillus*" was derived by a Florentine priest and mycologist P.A. Micheli in 1792 (4), for resemblance of the conidiophore to the liturgical aspergillum (8) used during religious service for sprinkling holy water. *Aspergillus* is a large genus of filamentous ascomycetes, which encompasses approximately 250 species (50).

Because of its importance in medicine and agriculture, *A. flavus* has been studied extensively. *A. flavus* is a naturally-occurring saprophyte of decaying organic matter in soil (19, 18), and an opportunistic pathogen of many agriculturally- important crops, especially oil-rich seeds of crops such as corn, cotton, peanuts and walnuts (4). *A. flavus* is found in all biomes, but is more common in warmer climates, occurring frequently between 25° to 35° latitude (49). Because aflatoxins are ubiquitous in the food supply of developing countries, the strain on human health and economies is hard to quantify. However, in 1999, in South Texas, aflatoxin accounted for \$7 million in losses on cottonseed (65). In addition to producing mycotoxins, *A. flavus* can cause diseases such as otitis, cavitory pulmonary aspergillosis, aspergilloma, allergic bronchopulmonary aspergillosis, keratitis, endophthalmitis, infection of the central nervous system, and urinary tract infections (37). Disease can be caused by other secondary metabolites made by *A. flavus* such as sterigmatocystin, a compound similar to aflatoxin; cyclopiazonic acid, which inhibits calcium dependant-ATPases; and aflatrem, a potent tremorigenic mycotoxin that leads to neurological disorders (28).

The life cycle (Fig. 1) of *A. flavus* is initiated in the spring when sclerotia germinate and produce primary airborne inocula called conidia from spore bearing structures called conidiophores. Sclerotia are hard, melanized mycelial masses capable of hyphal production and/or conidiophore production when conditions are favorable for growth (71). Primary conidia are disseminated from conidiophores and will either reach a substrate conducive for growth or remain viable under appropriate environmental conditions for months. If conidia reach a conducive substrate for growth, they will germinate and form a germ-tube that later develop into hyphae. Hyphae can

continue to grow or become aerial hyphae and differentiate into conidiophores that produce secondary inoculum. Hyphae can also aggregate into mycelia and form sclerotia. Until 2009, it was believed asexuality was the extent of the life cycle. The teleomorph of *Aspergillus flavus*, *Petromyces flavus*, was found to produce cleistothecia within sclerotia (52). The cleistothecia give rise to asci, which contain ascospores. Sexual recombination occurs between vegetatively- incompatible fungi belonging to the same mating strain (39). This is important because some biocontrol strains were chosen because of vegetative incompatibility with toxigenic strains (32).

Sclerotial size has been used to functionally categorize *A. flavus* (29): large (L)-strains produce aflatoxins B1 and B2, while small (S)-strains produce aflatoxins G1 and G2, in addition to the B aflatoxins (1). Sclerotia size in *A. flavus* and *A. parasiticus* is greatly influenced by environmental factors (58).

With any plant disease management program, treatments are designed to inhibit growth and target vulnerable stages of an organism's life cycle. Specific control measures for *A. flavus* are discussed in detail later; however, the persistence of inoculum in the environment, as well as their movement to an infection court, are pivotal to the disease cycle, especially in biological control.

It is important to understand how the *A. flavus* genome is organized and how it is regulated, especially as contemporary control measures exploit differences within the species. The *A. flavus* genome contains 25 PKSs and 18 NRPSs (4). Strains of *A. flavus* are extremely diverse in their ability to produce secondary metabolites and to colonize plants (18). One measure of genetic diversity is vegetative compatibility group (VCGs), in which compatibility, and subsequent heterokaryon formation, is contingent upon identical alleles occurring at specific loci (60) within a species, specifically, the *het* locus. Vegetative incompatibility during anastomosis arises because of allelic diversity in different isolates (38). One study documented 44 distinct VCGs of *A. flavus* within one agricultural field (55). *A. flavus*, like all other *Aspergillus* species, contains 8 chromosomes; however, *A. flavus*' genome is slightly larger than that of other *Aspergillus* species, - approximately 37 MB (18).

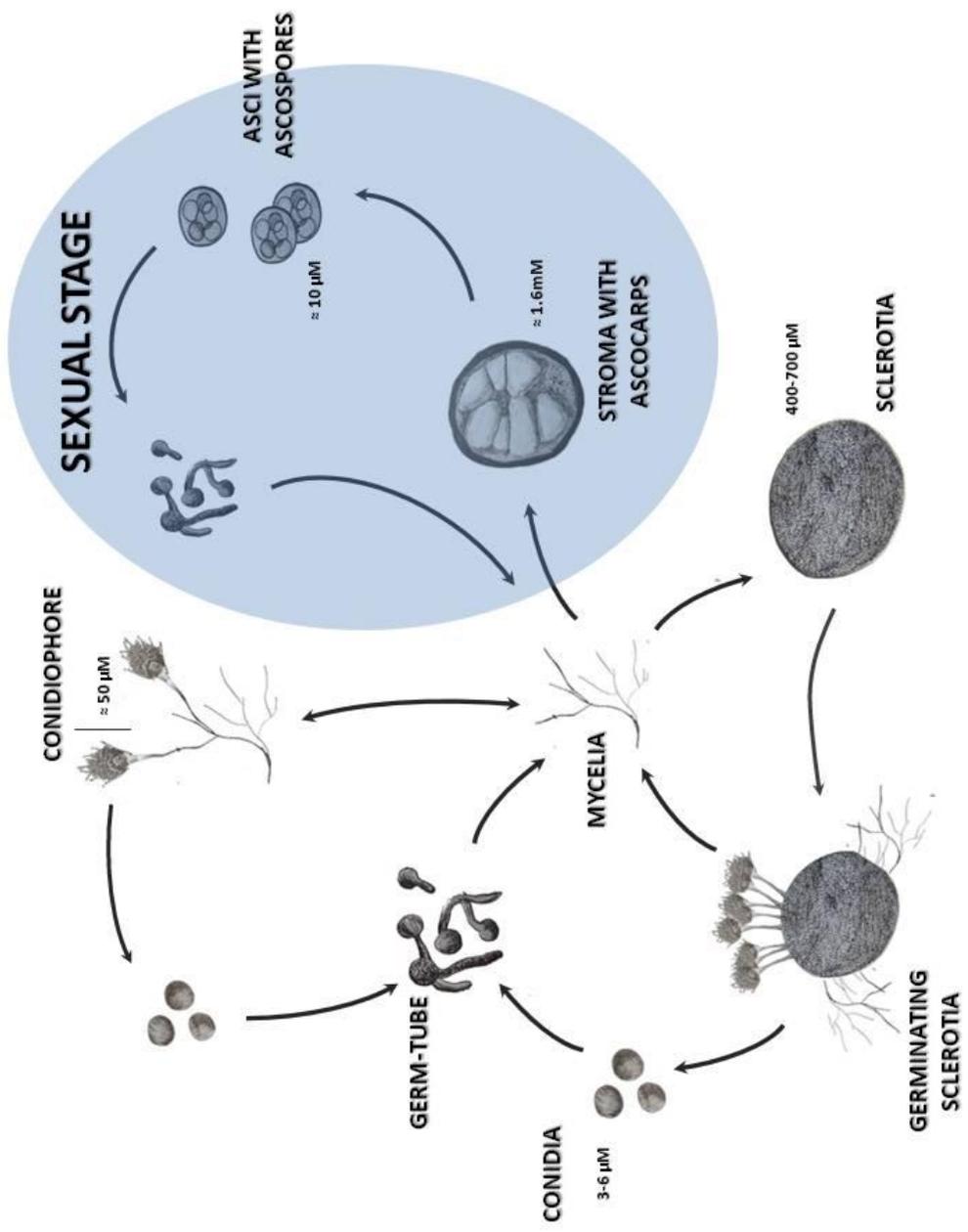


Figure 1. Life cycle of *Aspergillus flavus*. [Sketches by Ms. Erin M. Hassett]

## CHAPTER II

# CONIDIAL MOVEMENT OF *Aspergillus flavus* IN CORN AND COTTON FIELDS FROM A POINT SOURCE

### Introduction

*Aspergillus flavus* (Link:Fr.) is the anamorphic form of an ascomycete that colonizes seeds of many economically important crops in tropical and subtropical climates. Isolates of *A. flavus* produce an array of deleterious secondary metabolites. One group of them, the aflatoxins, are highly regulated due to their adverse effects on animal and human health following consumption.

Atoxigenic strains of *A. flavus* have been commercialized for the biocontrol of aflatoxin in peanut, corn and cotton. In trials, they have reduced contamination up to 95% (11, 42, 43). *Aspergillus flavus* propagates through the production of airborne, asexually- produced inocula called conidia. These spores remain viable for months in the soil and on organic substrates where they overwinter (74). In the spring, conidia colonize corn silks and grow into the ear where the fungus can colonize kernels if conditions are conducive. Likewise, atoxigenic strains reproduce and operate through the same mechanism of conidiation and colonization; however, a number of questions still remain about the use and efficacy of atoxigenic strains in the field, specifically, the earliest application timing to achieve adequate management and the efficacy of different strains in the field. To answer these questions, field experiments with comparative treatments are needed. Because field conditions such as soil types, weather patterns and farming practices are highly variable between regions - sometimes even within the same field - replications in field trials need to be in close proximity. If replicates are spaced too closely, cross- contamination becomes a problem, while a distant separation of replicates may increase variability because of the greater influence of extraneous variables, making it difficult to accurately measure treatment effects.

The objective of this research is to better understand *Aspergillus flavus* propagule movement, persistence, and production in agroecosystems (corn, cotton, and fallow fields) through the use of an *A. flavus nit* (nitrate- non-utilizing) mutant. The *nit* mutant was used because it is trackable with a semi-selective growth medium. Specifically, *nit* mutants are unable to reduce nitrate to nitrite because of a mutation in nitrate reductase (NR) (63, 23). Since chlorate is an analog of nitrate, NR is able to act on chlorate. When chlorate is reduced, the toxic ion chlorite is produced, killing or severely inhibiting the growth of organisms that reduce chlorate. In contrast, *nit* mutants are unable to reduce chlorate and thus grow faster or more profusely on chlorate-containing media. This is one molecular mechanism of action, although other mechanisms exist in different organisms like *Aspergillus nidulans* (23).

Analysis of the data from this study will lead to a better understanding of spore dispersal as a function of time and space, enable the design of better experiments, and provide a measure of the quantity of spores needed to successfully colonize the host. This information can be used to optimize the efficacy of atoxigenic strains of *A. flavus* for biological control.

## Materials and Methods

***A. flavus* Mutant and Media.** A nitrate- non-utilizing (*nit*) mutant, N3 (Isakeit, unpublished), generated from *A. flavus* NRRL 21882 on the medium of Elias and Cotty (31), was used in the experiments. Specifically, this medium contained 35 g of Czapek-Dox broth (Difco) with 25 g of potassium chlorate (Sigma-Aldrich), 10 ml of a rose bengal (Sigma) solution (5mg/ml) and 20 g of Bacto agar (Difco). This medium was also used for detection of the mutant in the field experiments.

**Inoculum for the Field.** Corn kernels (3000 cc) were autoclaved with 3 l water in a 45 x 38 x 13 cm. polypropylene tray for 20 minutes at 121°C and then inoculated with an aqueous suspension of the *A. flavus* mutant. The suspension was prepared as follows: the mutant was grown on 10 plates of Czapek-Dox agar for 5 days and then plates were blended in 500 ml distilled water in a Waring blender for 30 seconds. The inoculated

corn was then incubated in a clear plastic bag for 4 days in a greenhouse (26°C to 43°C).

**Field Locations.** The experiments were conducted within corn and cotton fields on the Texas AgriLIFE Experiment Station in Burleson County, Texas. Specifically, the corn fields were located at 30° 30' 30" N, 96° 25' 15" W and 30° 31' 58" N, 96° 25' 38", while the cotton field was located at 30° 31' 56", 96° 25' 21". Both crops were planted on approximately 0.75 m centers.

**Field Application.** Colonized corn inoculum was placed on the ground in an area of approximately 0.6 x 0.4 m within a field. Inoculum was placed approximately 200 m apart at different places in the corn field on three dates: 05/05/11 (125 Julian Day (JD)), 05/31/11 (151 JD), and 06/09/11(160 JD) and in the cotton field on 07/02/11 (183 JD), 07/19/11 (170 JD), and 08/18/11 (230 JD) (Fig. 2). Before inoculum placement, 5L of water was poured on the ground to promote sporulation. To avoid contamination, the outside of bags containing inoculum was sprayed with alcohol before walking in and out, and contaminated bags were rebagged before leaving the field. When leaving the field, a different route was taken to reduce any possible contamination. In 2012, inocula were placed in fields on 05/14/12 (135 JD), 05/15/12 (136 JD), and 05/16/12 (137 JD). Corn and cotton heights were assessed at the time of inoculum placement by measuring from the ground to the flag leaf in corn and from the ground to the top of the plant in cotton.

**Weather Data.** Data was retrieved from USDA/ARS Field 14 Weather Station Located: Latitude 30° 31' 28.8192"N, Longitude 96° 24' 7.5888"W accessible online (5).



**Figure 2. Field application of point source containing the mutant *A. flavus* inoculum.**

**Leaf and Air Sampling.** Corn and cotton leaves were sampled at the following distances from the point source: 0.30m, 0.60m, 0.91m, 1.21m, 1.52m, 1.8m., 2.7m., 3.7m., 6.4m., and 9.1m down rows; and 1, 2, 3, 4, 5, 6, 7, and 10 rows across, corresponding to 0.76m, 1.52m, 2.28m, 3.04m, 3.81m, 4.57m, 5.33m, and 7.62m respectively. Plants were spaced approximately 30 cm apart and row spacing was approximately 0.75m. Sampling was performed every three days for 12 days after inoculum placement. Sampling was done at the point source location both before (as a control) and after placement of the inoculum. In 2011, samples consisted of approximately three, 23 cm -long portions of the 8<sup>th</sup> leaf, as measured from the tip. In 2012, samples consisted of two, 23 cm-long portions of the 8<sup>th</sup> leaf and one from the 2<sup>nd</sup> leaf. The 8<sup>th</sup> leaf was chosen because it was closest to the infection court (ear). Three leaves were sampled on each date and location and placed in plastic bags. In the

laboratory, leaves were washed within the bag with 20 ml of distilled water. A 0.2 ml aliquot of the rinse solution was spread onto modified Rose Bengal agar medium. Plates were then incubated for 5 days at 27°C prior to enumeration of colonies. If colonies on a plate were too numerous to count, a conservative estimate of 300 colonies was assigned to the plate.

With cotton, six leaves were sampled from each plant: two from the top, two from the middle, and two from the bottom. Cotton plants were approximately 1m tall. For the three experiments, plant maturities were different. Plants were in the first flower stage during the first experiment and at open boll in the final experiment. Leaves were processed in the laboratory as previously described. Samples were taken at 3, 6, 9, 12, and 15 days after inoculum placement. The experiment was repeated 3 times in both cotton and corn fields.

In two cotton experiments, two Burkard cyclone spore traps (Hertfordshire, England), sampling air at a rate of 16.5 l/min, were placed 18.3 m from the point source along rows and at 7.6m across (Fig. 3). In the first experiment, the spore traps were placed at the west and north end of the point source. In the second experiment, the traps were placed on the south and east sides of the point source. Air samples were collected in 1.5 ml Eppendorf tubes and were changed every 3 days in both experiments. Conidia were enumerated by rinsing Eppendorf tubes with 0.6 ml of distilled water and plating 0.3ml onto two plates of Rose Bengal agar and incubating as previously described. In 2012, air samplers were used in corn fields and placed 7.6m to the west end of the field and 18.3m away from the point source to the north end of the field.



**Figure 3. Burkard spore trap setup and utilization in 2012 in a corn field.**

**Corn Ear Sampling.** The same fields used for leaf and air sampling were used for ear sampling. Sampling was done by pulling back husks and swabbing entire ears with autoclaved cotton swabs at several distances from the point source: .30m, 0.60m, 0.91m, 1.21m, 1.52m, 1.8m., 2.7m., 3.7m., 6.4m., 9.1m along rows; and 0.76m, 1.52m, 2.28m, 3.04m, 3.81m, 4.57m, 5.33m, and 7.62m across. Plants were spaced approximately 30 cm apart within rows and approximately 0.75 m across rows. In the first experiment, sampling was 56 days after placement of a point source placed on

05/05/11(125 JD) and sampled on 07/01/11 (182 JD), in the second experiment, sampling was 43 days after placement of a point source: placed on 5/31/11(151 JD) and sampled on 7/13/11 (194 JD), and in the third experiment, sampling was 26 days after placement of a point source: placed on 6/9/11 (160 JD) and sampled on 7/6/11 (187 JD).

Swabs were stored in individual plastic bags until they were processed in the lab. Cotton swabs were rubbed onto modified Rose Bengal agar and incubated for 5 days at 27° C.

**Statistical Analysis.** Data were analyzed with Microsoft Office Excel 2007. Regression analysis was run exclusively on raw data. For movement down rows in corn, data were log transformed using the following regression equation to linearize the data:

$$\log y = a + b \log x.$$

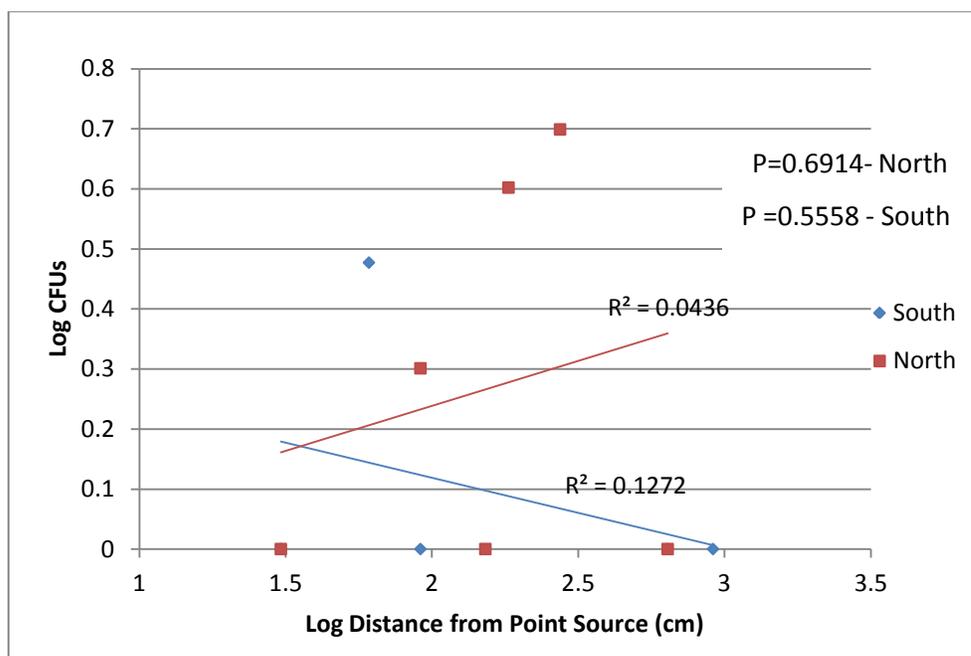
Transformed data was fit with a linear line while field data was fit with a power curve to generate R<sup>2</sup> values. The significance of difference between two population proportions was done with VassarStats: <<http://vassarstats.net/>>.

## Results

Conidia of the mutant were recovered with regular frequency from leaves in both corn and cotton fields, although not consistently over time at the same sample location. In 2011, over the course of three experiments, spores were recovered from 57 of 367 sampling locations in corn and 21 of 396 in cotton which differed significantly (P<0.001). Relative colony counts indicated a general gradient diminishing from the point source that extended to terminal sampling points down the rows (10.6m). In 2011, no clear gradient of movement was observed across rows in either corn or cotton fields, as mutant recovery was low for all sampling dates: 1 to 3 CFU in cotton and 1 to 4 CFU in corn. There was no significant (P=0.05) difference in the frequency of recovery in perpendicular vs. parallel direction in corn based on differences of proportions.

In 2012, the mutant was detected in 33% (n=432) of the sampled sites. As in 2011, there was no significant ( $P=0.05$ ) difference in the frequency of recovery between directions perpendicular and parallel to rows. Total recovery in 2012, was significantly higher than the total recovery in 2011 ( $P<0.0002$ ). In contrast to 2011, a significant gradient was established in both perpendicular and parallel directions in corn fields in 2012.

The overall recovery of spores in cotton fields was low compared to that of corn fields. In cotton fields, mutant recovery was greater down rows (Fig. 4&5) rather than across rows (Fig. 6). No significant ( $P=0.05$ ) gradient was observed in cotton fields, either across or down rows, even when all points from each experiment were combined. When each directional movement was examined separately, P-values generally increased, with the exception of movement to the east. In cotton fields, a maximum of 5 spores were recovered from leaf samples in any one location.



**Figure 4. Recovered *nit* mutant CFUs from washed leaves in the north and south direction from a cotton field in 2011. Data points represent the combined total of three – two week field experiments. P-values represent the probability of the slope.**

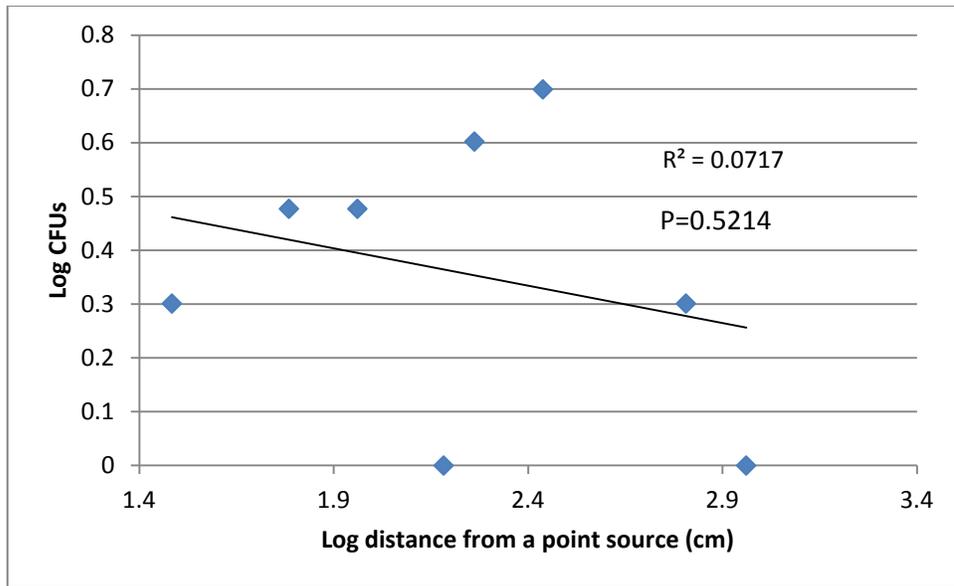


Figure 5. Log transformed data of recovered *nit* mutant CFUs from parallel row movement in a cotton field in 2011. Data points represent the combined total of three – two week field experiments.

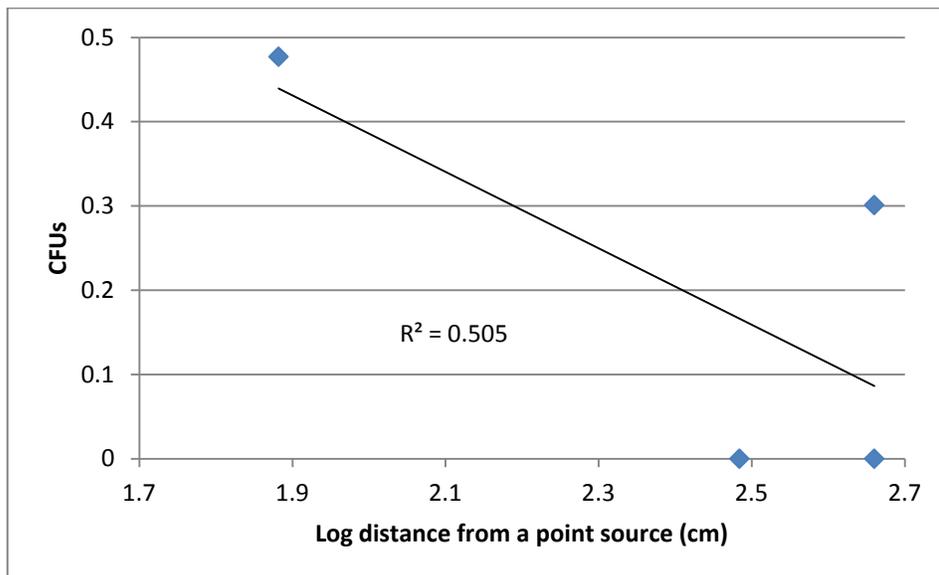


Figure 6. Log transformed data of recovered *nit* mutant CFUs from movement across rows in a cotton field in 2011. Data points represent the total of three – two week field experiments.

Perpendicular movement of conidia across rows in cotton fields was even more marginal than movement along the row. There was insufficient numbers for statistical testing. The mutant was detected at only 4 locations over the course of three experiments. Of these 4 locations, the maximum recovery from one location was 3 CFU. In 2011, the mutant *A. flavus* placed in corn fields was recovered not only more frequently than in cotton fields, but also more abundantly (Figs., 7, 8). In cotton fields there was a total of 30 CFU recovered compared to 440 CFU in corn fields. In 2011, the maximum recovery from one corn field sample was 101 CFU compared to only 4 CFU from a cotton field. In 2012, plates were too numerous to count for 5 sampling intervals.

In 2011, in corn fields, the mutant was detected at a maximum distance of 6.4m down rows (Fig. 7&8). When data from all three corn experiments were combined, there was a significant ( $P=0.0768$ ) gradient of diminishing movement within corn rows from the point source when data was linearized (Fig. 8). As with the cotton experiments, movement to perpendicular rows in corn fields was minimal at all distances and no gradient was observed (Figs. 9). With the exception of one sampling point in perpendicular movement, mutant recovery was consistently below 5 CFU in all replicates in corn (Fig.10).

In 2011, in corn fields, the mutant was detected at a maximum distance of 7.6m across (Fig. 10). With leaf sampling, the mutant was only detected at the maximum distance once at 7.6m across rows in corn (Fig. 10) and only twice at 9.1 m in cotton. Once the mutant was detected at the maximum distance of either 9.1 m or 7.6m, leaves were additionally sampled perpendicularly at every row out to 15.2m and within rows at 0.91 m intervals out to 24.4 m in corn. In a parallel row, one CFU was detected by leaf sampling at 21.6m in the second corn experiment. In 2011, a total of 353 CFU were recovered along rows as compared with 80 CFU across rows. In 2012, 3226 CFU were recovered along rows in contrast to only 621 CFU across rows.

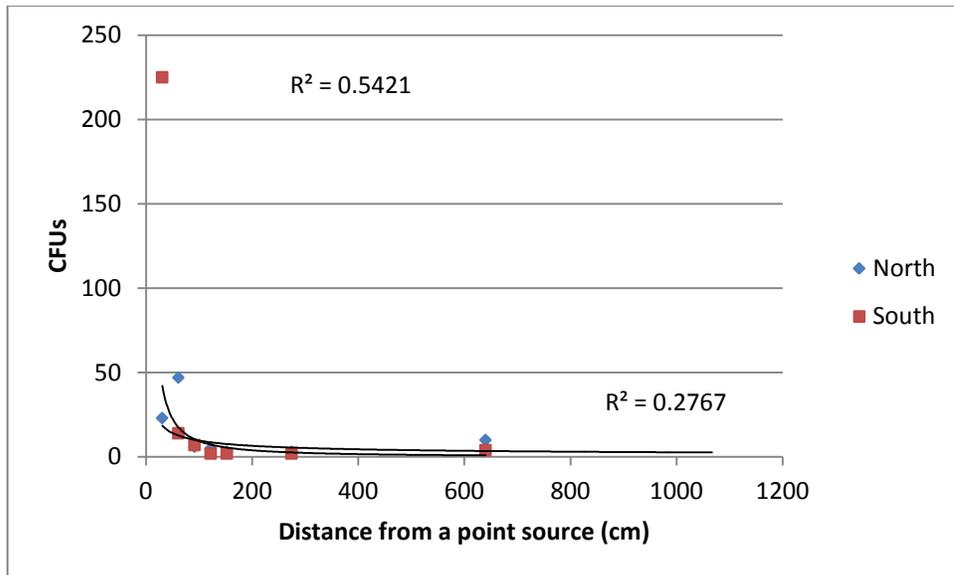


Figure 7. Recovered *nit* mutant CFUs from washed leaves in the north and south direction from a corn field in 2011. Data points represent the total of three – two week field experiments.

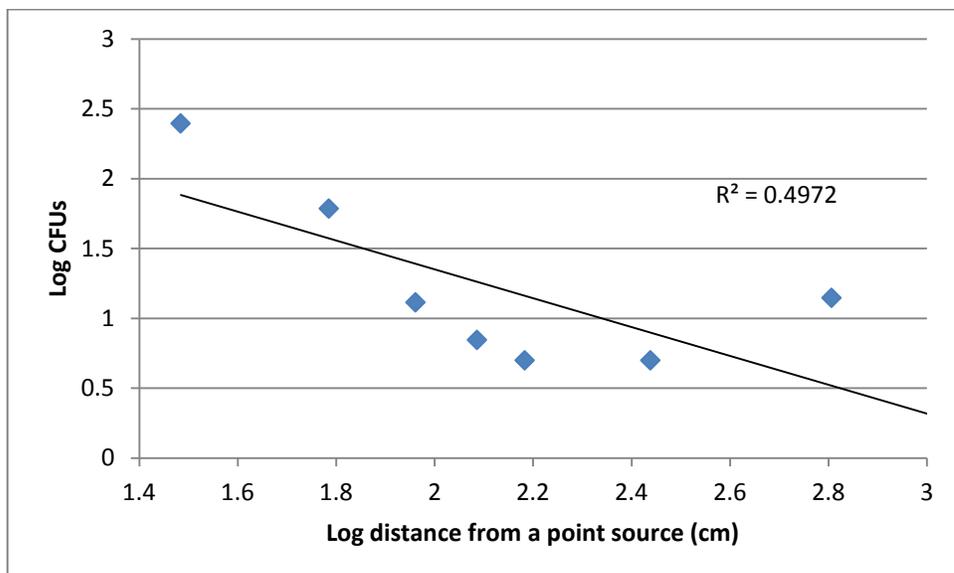


Figure 8. Log transformed data of CFUs recovered from parallel row movement in a corn field in 2011. Data points represent the total of three – two week field experiments.

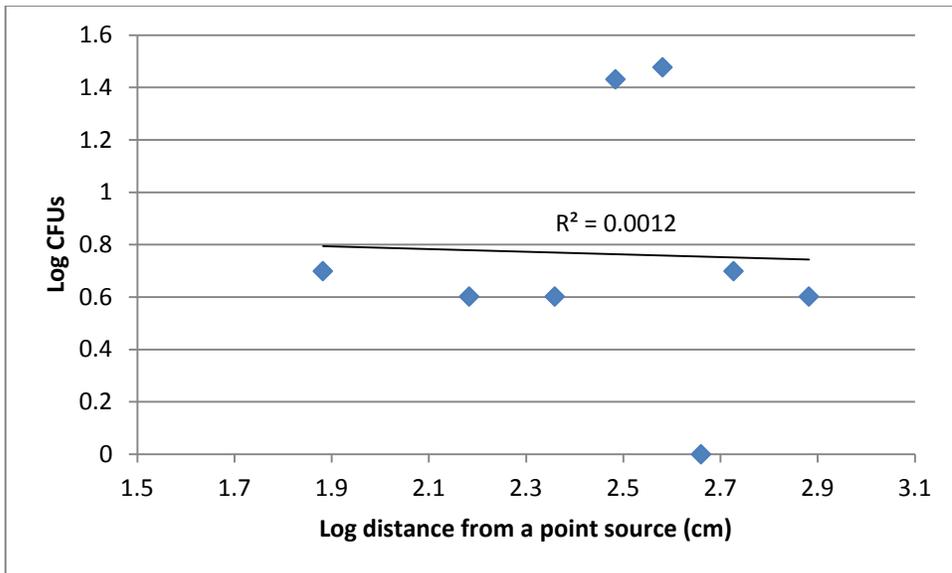


Figure 9. Log transformed data of across row movement from a corn field in 2011. Data points represent the total of three – two week field experiments.

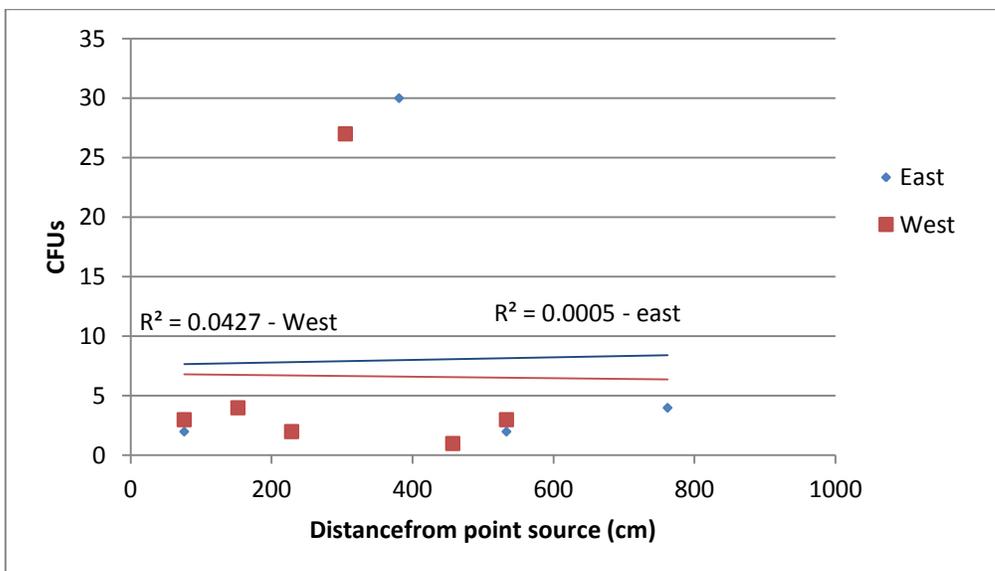


Figure 10. Recovered *nit* mutant CFUs from washed leaves in the north and south direction from a corn field in 2011. Data points represent the total of three – two week field experiments.

In 2012, as in 2011, the mutant was detected rarely at maximum distances from the point source by leaf sampling. In 2012, the mutant was detected twice at the maximum distance of 10.6 m down rows (Figs. 11 & 12) that when log transformed produced significant slopes ( $P < 0.05$ ) (Fig. 12). The mutant was detected 4 times at terminal points in perpendicular rows (Figs. 13 & 14). When perpendicular movement data was log transformed, unlike 2011, movement data fit a negative power curve that was significant when log transformed (Fig 13 & 14). However, with the exception of experiment 1, conidia were never recovered twice at the same terminal point. As in 2011, parallel row movement produced a negative power curve where recovery decreased rapidly with increasing distance from a point source.

In the first corn experiment, the *A. flavus* mutant was found in 45% ( $n=10$ ) of ears sampled along the row, in comparison with an incidence of 20% ( $n=4$ ) ears sampled across rows (Table 1). In the second corn experiment, the mutant was recovered from 18% ( $n=4$ ) of ears along the rows and 20% ( $n=4$ ) of ears across rows (Table 2). In the third experiment, the mutant was recovered from 9% of ears ( $n=2$ ) along the row and from 20% ( $n=4$ ) of ears across rows (Table 3).

Burkard spore traps showed a higher frequency and recovery yield than leaf sampling (Tables 4, 5, 6). In 2011, 17 colonies were recovered from the spore traps from a three day sampling period. With the exception of one sampling location, conidia were recovered during each sampling period at every location. In cotton fields, conidia were recovered by leaf sampling at a maximum distance down rows at 9.1m and at a maximum of 4.5m from the point source. In 2012, 66 CFU were recovered with one sampling in a corn field (Table 6).

In 2011, the height of corn was different in the three experiments: 86 cm, 134 cm, and 114 cm, corresponding to the 1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup> experiments, respectively. These heights were all significantly ( $P < 0.01$ ) different from each other. In 2012, the average corn height was 221cm, which was significantly different from the average corn height in 2011 ( $P < 0.01$ ).

Graphs represent that sum total of all movement over 2 weeks. Movement as a function of time as well as maximal distance spores were recovered over time can be assessed in the following movement maps (Figs. 15, 16, 17, 18, 19, 20, 21, 22, & 23).

Weather data from 2011 and 2012 is presented in the appendix.

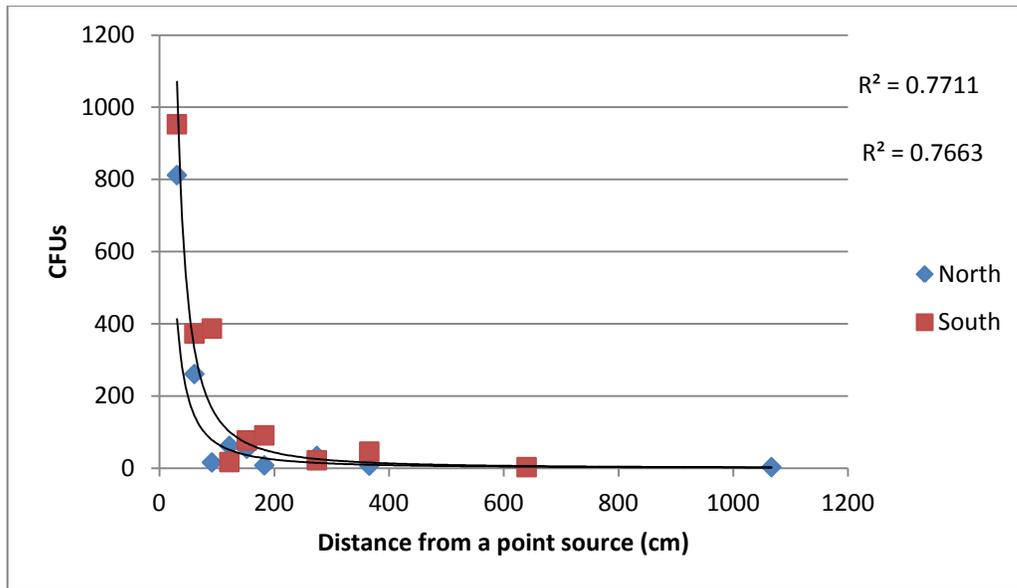


Figure 11. 2012. Recovered *nit* mutant CFUs from washed leaves in the north and south direction from a cotton field in 2012. Data points represent the total of three – two week field experiments.

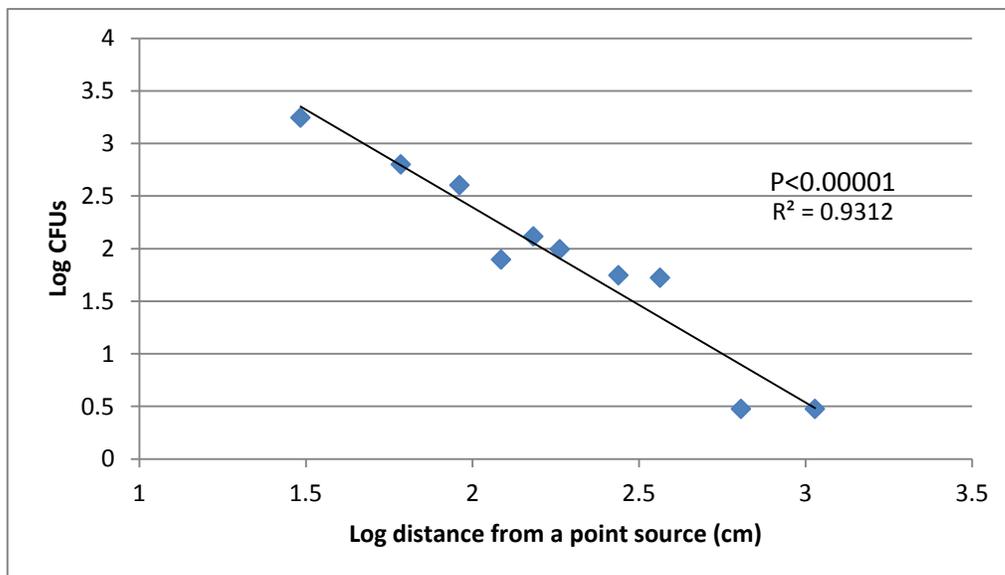


Figure 12. 2012. Log transformed data of parallel row movement of a *nit* mutant recovered by leaf sampling from a corn field in 2012. Data points represent the total of three – two week field experiments.

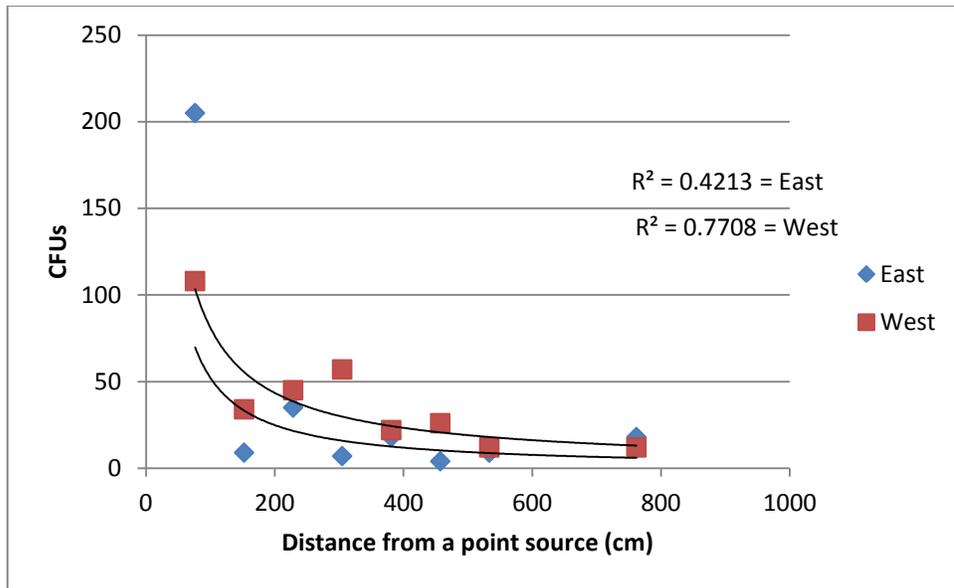


Figure 13. 2012 Recovered *nit* mutant CFUs from washed leaves in the north and south direction from a corn field in 2012. Data points represent the total of three – two week field experiments.

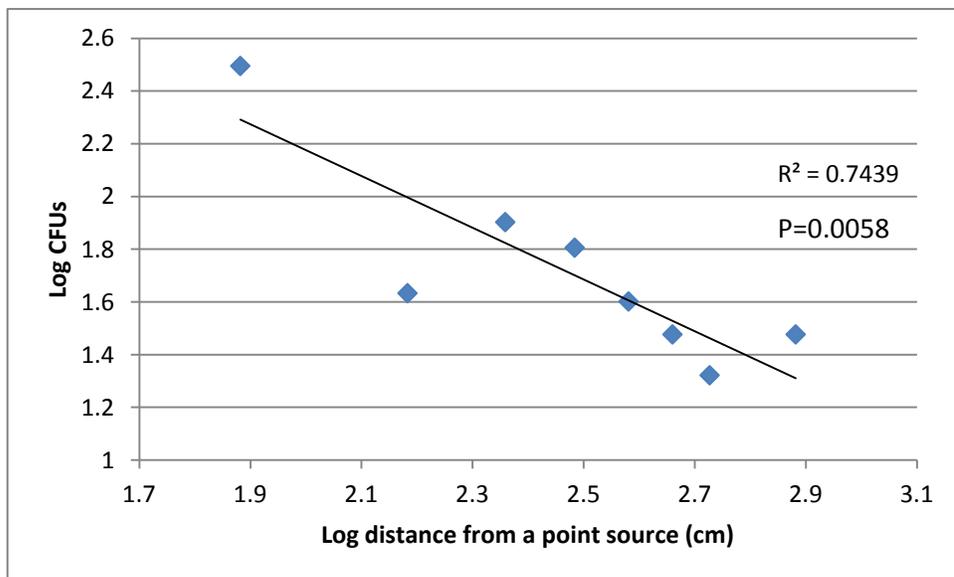


Figure 14. 2012. Log transformed data of perpendicular row movement of a *nit* mutant recovered by leaf sampling from a corn field in 2012. Log transformed data. Data points represent the total of three – two week field experiments.

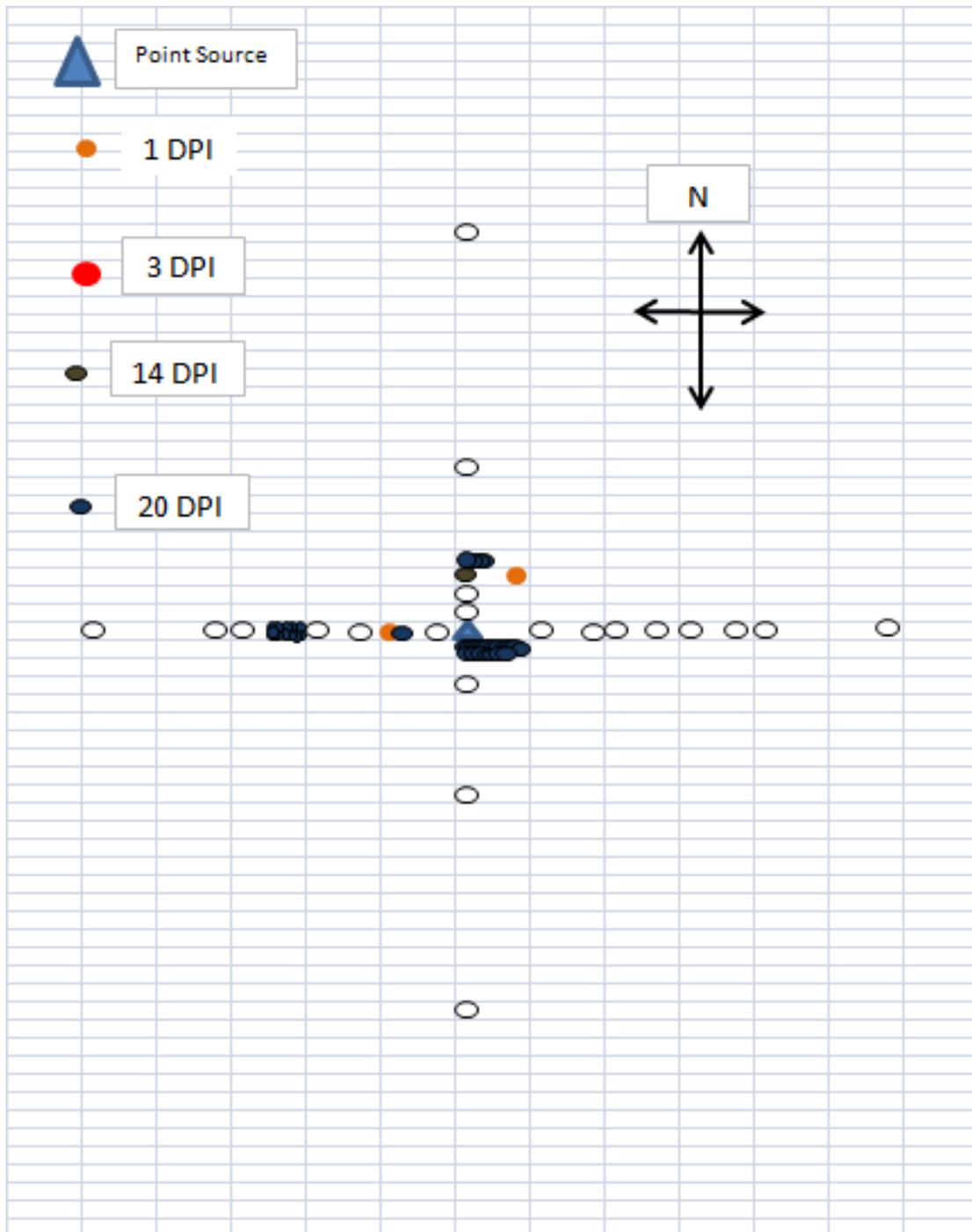


Figure 15. Movement of *A. flavus* mutant from a point source in corn in experiment 1. Each rectangle represents 30.5cm vertical and two corn rows (2 m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.

**Table 1. Qualitative recovery of *nit* mutant from inside corn ears at several distances from a point source 56 days after placement of a point source during the period of 05/05/11 (125 JD) through 07/01/11 (182 JD)– Corn experiment 1.**

North		South		East		West	
Distance	Result	Distance	Result	Distance	Result	Distance	Result
0.30m	+	0.30m	+	0.76m	-	0.76m	-
0.60m	-	0.60m	-	1.52m	-	1.52m	-
0.91m	-	0.91m	+	2.28m	-	2.28m	-
1.21m	+	1.21m	-	3.04m	-	3.04m	-
1.52m	-	1.52m	-	3.81m	+	3.81m	-
1.82m	-	1.82m	-	4.57m	+	4.57m	+
2.74m	+	2.74m	-	5.33m	-	5.33m	+
3.65m	+	3.65m	+	7.62m	-	7.62m	-
6.40m	-	6.40m	+				
10.66m	+	10.66m	-				
18.20m	+	18.20m	-				

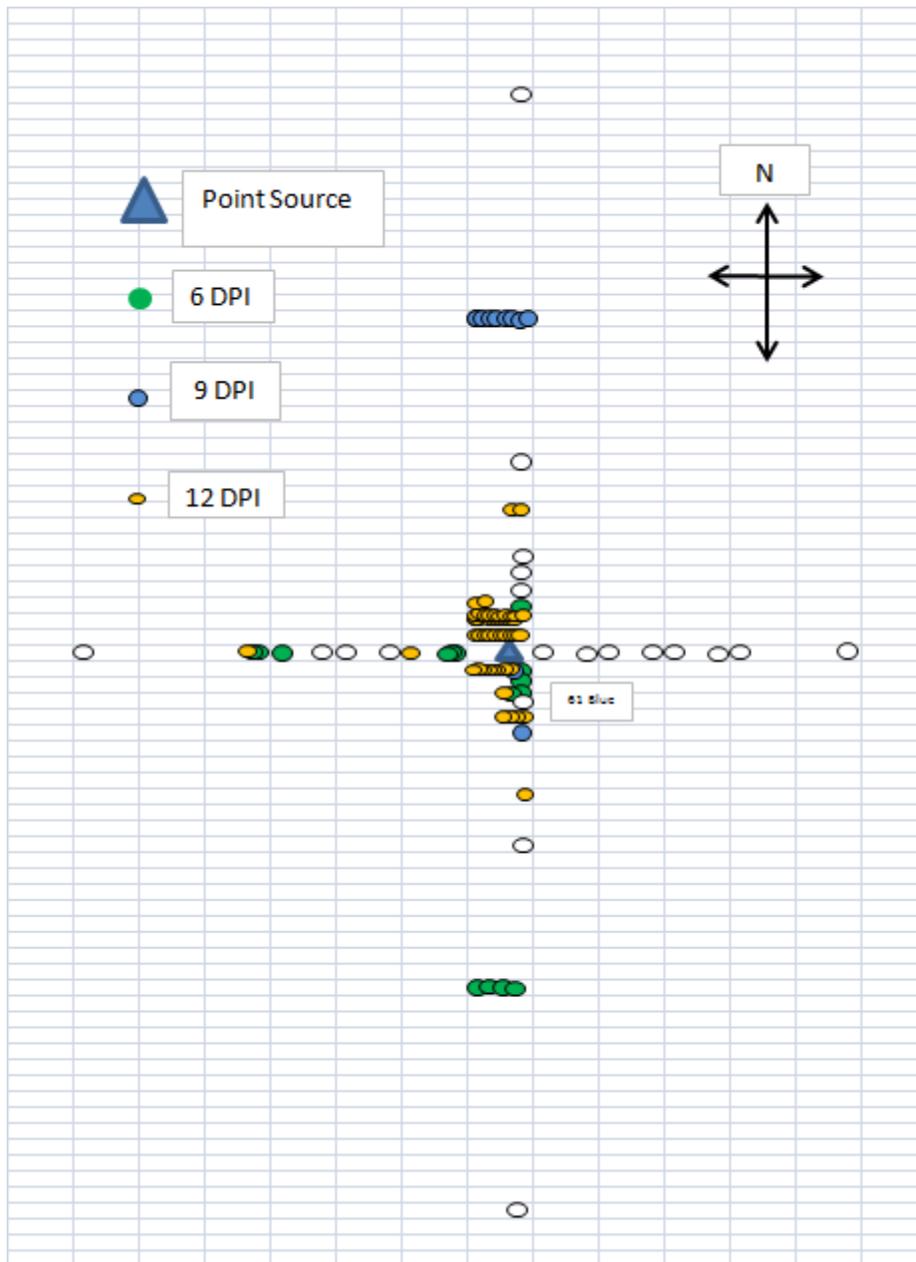


Figure 16. Movement of *A. flavus* mutant from a point source in corn in experiment 2. Each rectangle represents 30.5cm vertical and two corn rows (2 m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.

**Table 2. Qualitative recovery of mutant from inside corn ears at several distances from a point source 43 days after placement of point source – from 5/31/11 (151 JD) through 7/13/11 (194 JD) - Corn experiment 2.**

<b>North</b>		<b>South</b>		<b>East</b>		<b>West</b>	
<b>Distance</b>	<b>Result</b>	<b>Distance</b>	<b>Result</b>	<b>Distance</b>	<b>Result</b>	<b>Distance</b>	<b>Result</b>
0.30m	+	0.30m	-	0.76m	-	0.76m	-
0.60m	-	0.60m	+	1.52m	+	1.52m	-
0.91m	-	0.91m	-	2.28m	+	2.28m	-
1.21m	-	1.21m	-	3.04m	+	3.04m	+
1.52m	-	1.52m	-	3.81m	-	3.81m	-
1.82m	-	1.82m	-	4.57m	-	4.57m	-
2.74m	-	2.74m	+	5.33m	-	5.33m	-
3.65m	+	3.65m	-	7.62m	-	7.62m	-
6.40m	-	6.40m	-				
10.66m	-	10.66m	-				
18.20m	-	18.20m	-				

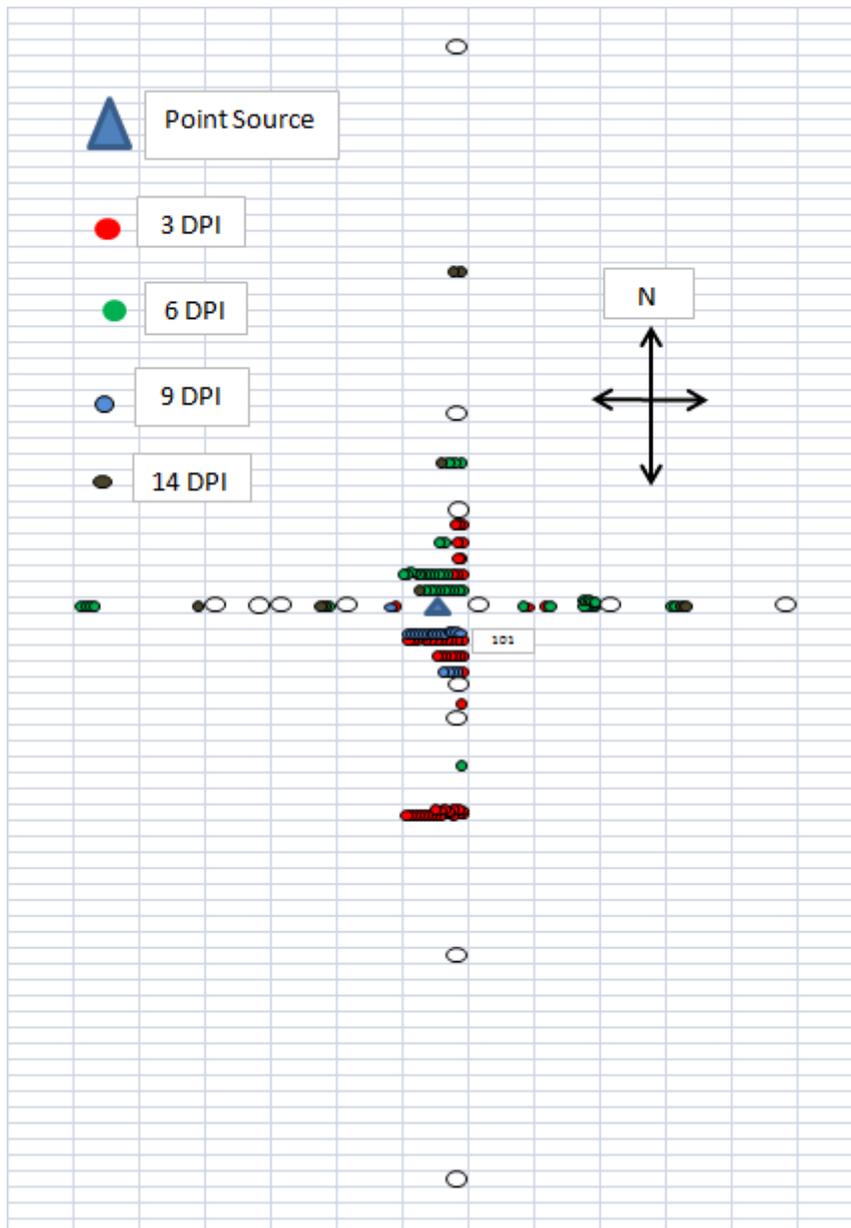


Figure 17. Movement of *A. flavus* mutant from a point source in corn in experiment 3. Each rectangle represents 30.5cm vertical and two corn rows (2 m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.

**Table 3. Qualitative recovery of mutant from inside corn ears at several distances from a point source 26 days after placement of point source – from 06/09/11 (160 JD) through 07/06/11 (187 JD) – Corn experiment 3.**

<b>North</b>		<b>South</b>		<b>East</b>		<b>West</b>	
<b>Distance</b>	<b>Result</b>	<b>Distance</b>	<b>Result</b>	<b>Distance</b>	<b>Result</b>	<b>Distance</b>	<b>Result</b>
0.30m	-	0.30m	-	0.76m	-	0.76m	-
0.60m	-	0.60m	-	1.52m	-	1.52m	-
0.91m	-	0.91m	-	2.28m	+	2.28m	-
1.21m	-	1.21m	-	3.04m	+	3.04m	-
1.52m	-	1.52m	+	3.81m	-	3.81m	-
1.82m	-	1.82m	-	4.57m	-	4.57m	-
2.74m	-	2.74m	-	5.33m	+	5.33m	+
3.65m	-	3.65m	-	7.62m	-	7.62m	-
6.40m	+	6.40m	-				
10.66m	-	10.66m	-				
18.20m	-	18.20m	-				

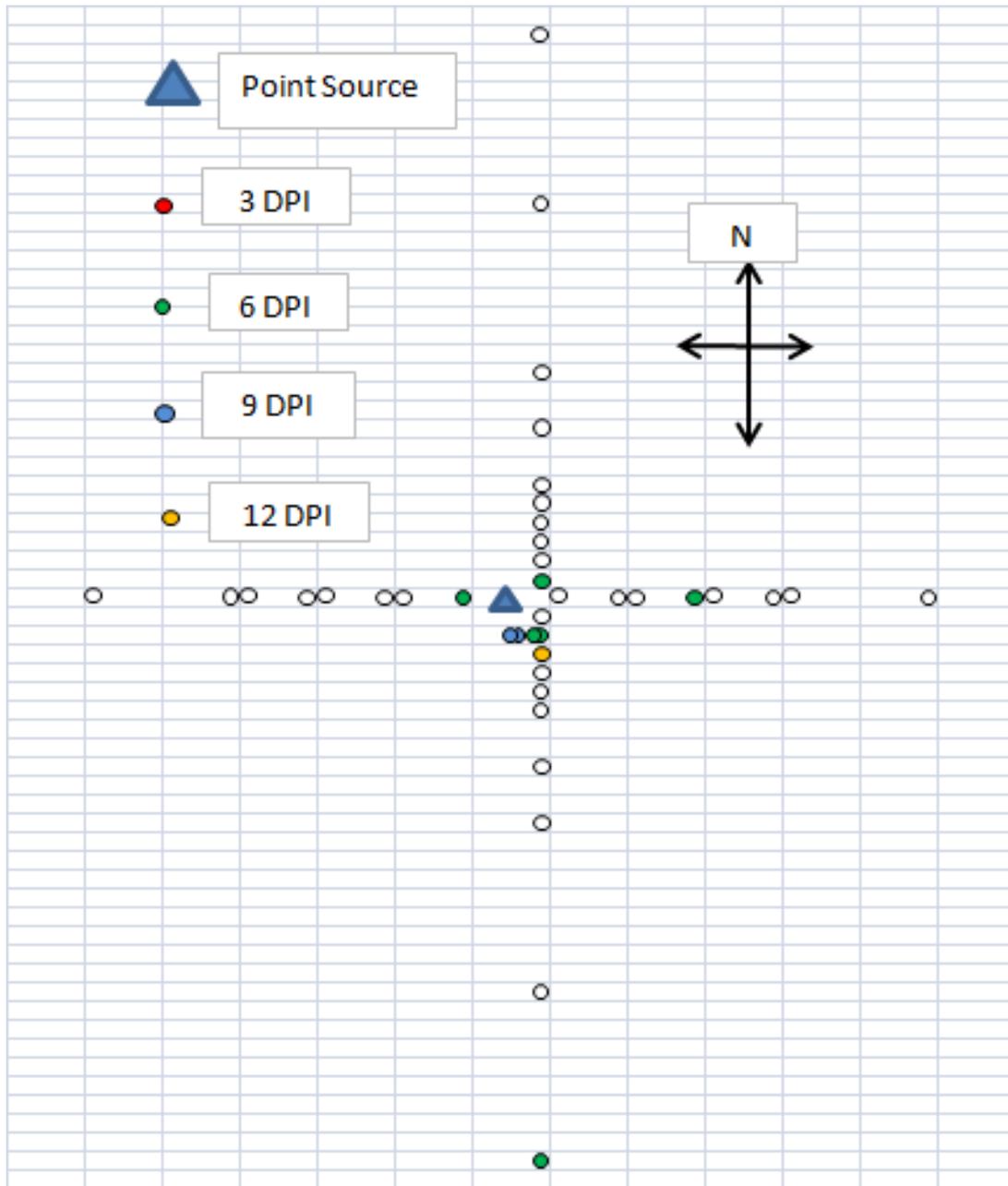


Figure 18. Movement of *A. flavus* mutant from a point source in cotton in experiment 1. Each rectangle represents 30.5cm vertical and two corn rows (2 m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.

**Table 4. *A. flavus nit* mutant with a Burkard spore traps placed at 18.2m to the north and 7.6m to the west of a point source in cotton experiment 1 at sequential days post inoculation.**

<b>Total <i>A. flavus</i> mutant</b>	
<b>Cotton 1</b>	<b>CFU</b>
3DPI West	10
3DPI North	11
6DPI West	3
6DPI North	5
9DPI West	1
9DPI North	5
12 DPI West	8
12 DPI North	4

**Table 5. *A. flavus nit* mutant recovery with a Burkard spore traps placed at 18.2m to the north and 7.6m to the west from a point source in cotton experiment 2 at sequential days post inoculation.**

<b>Total <i>A. flavus</i> mutant</b>	
<b>Cotton 2</b>	<b>CFU</b>
3 DPI East	11
3DPI South	0
6 DPI East	17
6 DPI South	3
12 DPI East	1
12 DPI South	2

**Table 6. *A. flavus* mutant recovery with a Burkard spore trap placed at 18.2m to the north and 7.6 to the west from a point source in corn experiment 3 at sequential days post inoculation.**

<b>Corn 3</b>	<b>Total <i>A. flavus</i> mutant CFUs</b>
3DPI West	10
3DPI North	7
6DPI West	66
6DPI North	10
9DPI West	30
9DPI North	21
12 DPI West	0
12 DPI North	0

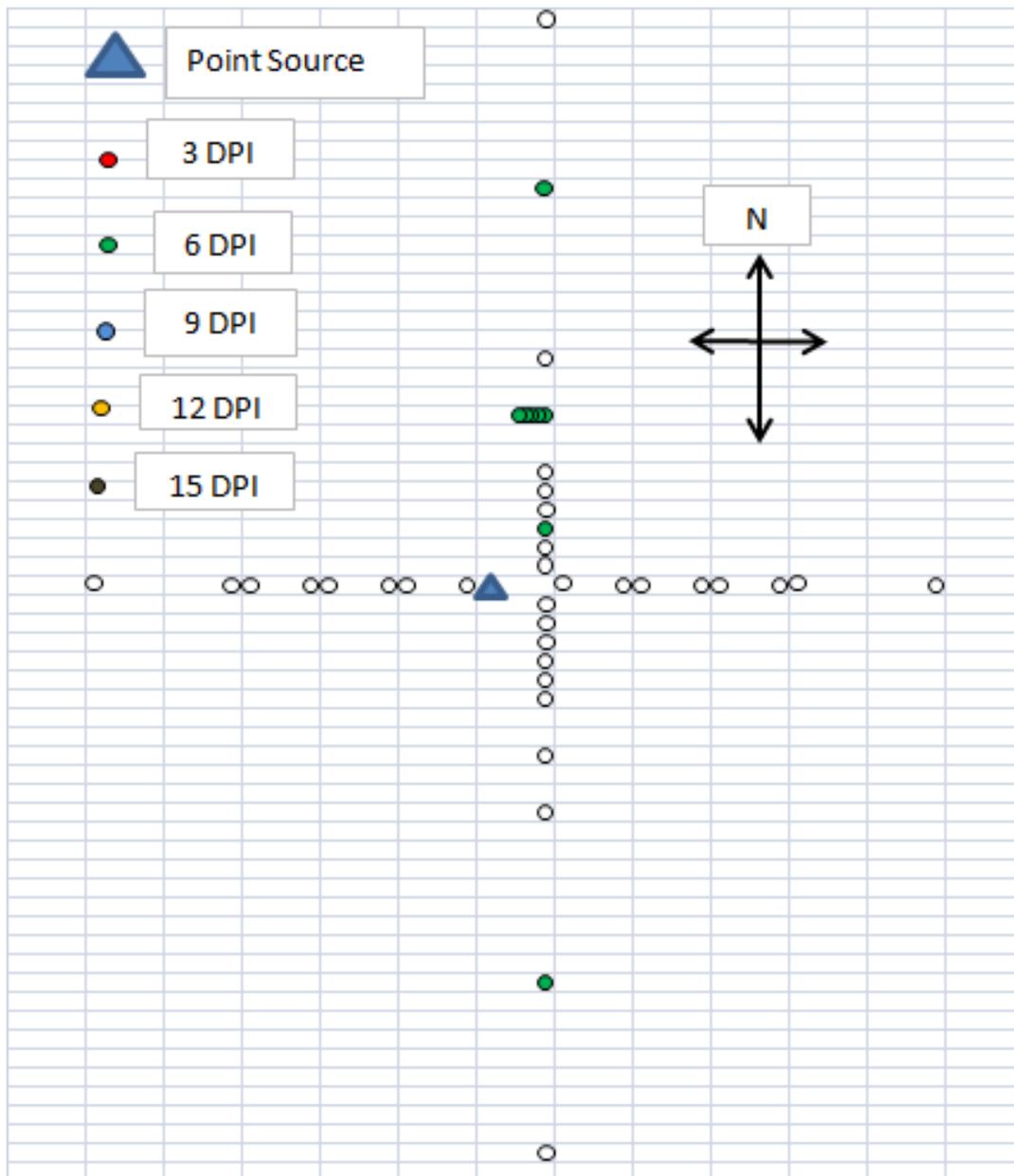


Figure 19. Movement of *A. flavus* mutant from a point source in cotton in experiment 2. Each rectangle represents 30.5cm vertical and two corn rows (2 m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.

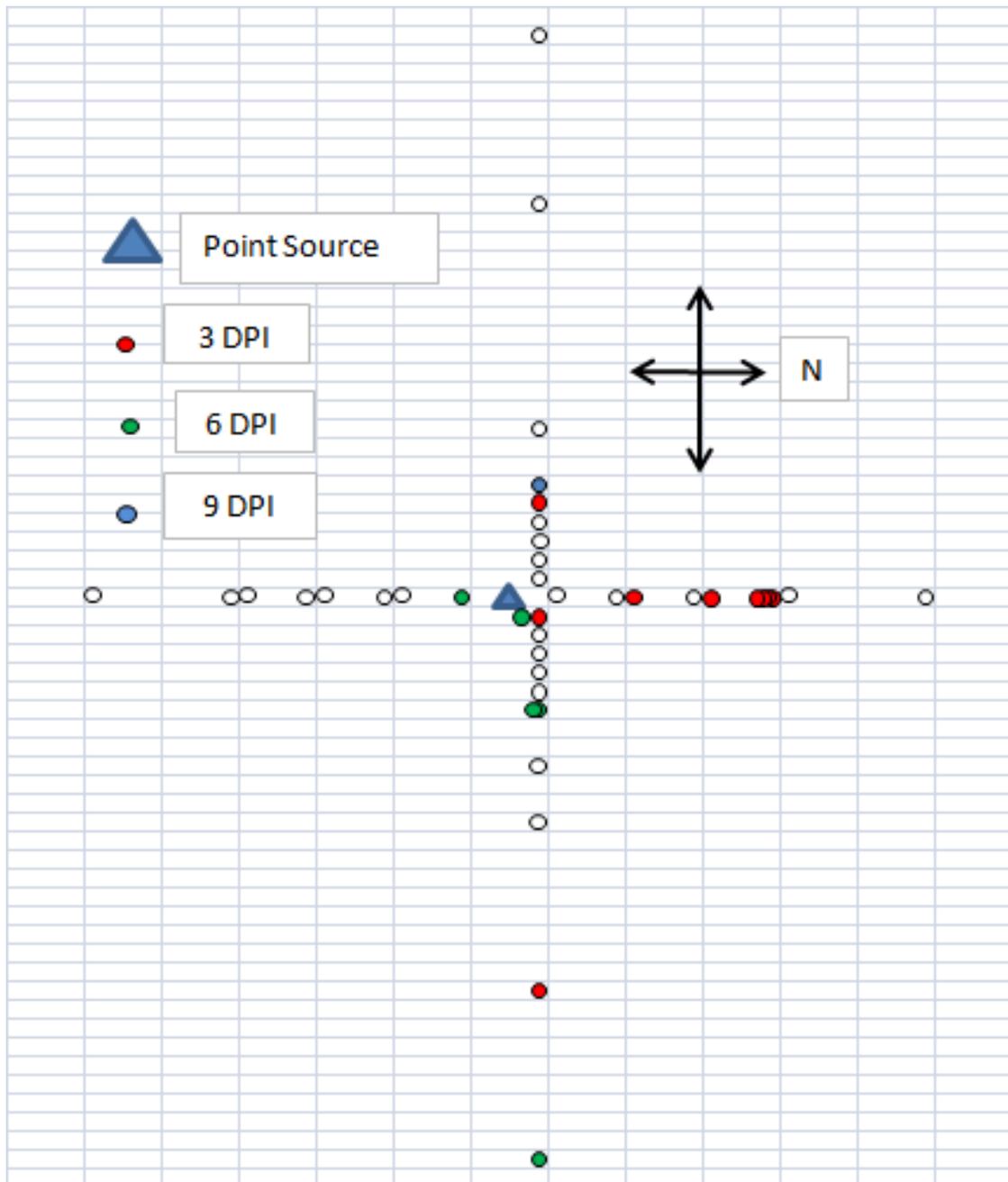


Figure 20. Movement of *A. flavus* mutant from a point source in cotton in experiment 3. Each rectangle represents 30.5cm vertical and two corn rows (2m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.

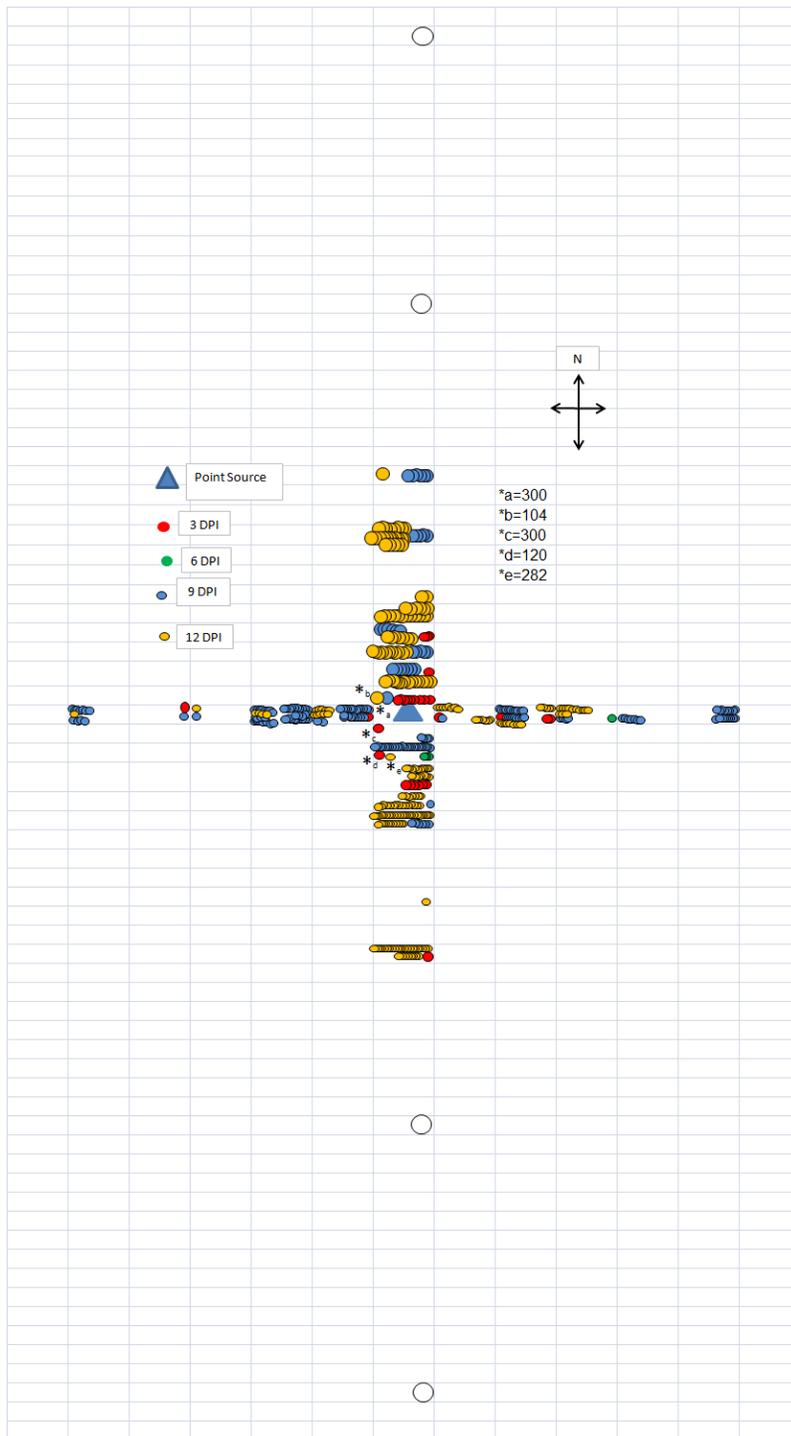
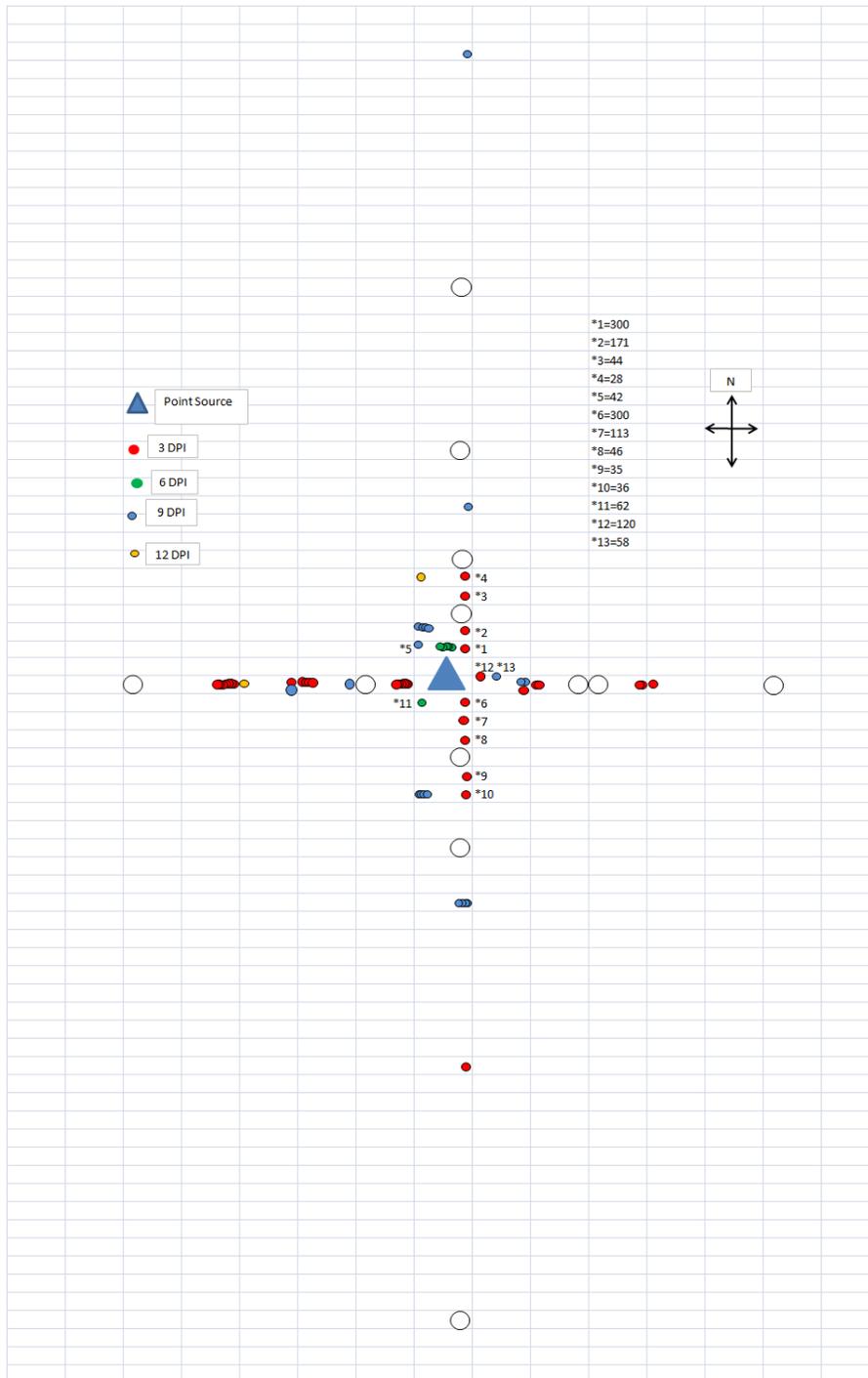


Figure 21. 2012. Movement of *A. flavus* mutant from a point source in corn in experiment 1. Each rectangle represents 30.5cm vertical and two corn rows (2m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.



**Figure 22. 2012. Movement of *A. flavus* mutant from a point source in corn in experiment 2. Each rectangle represents 30.5cm vertical and two corn rows (2 m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.**

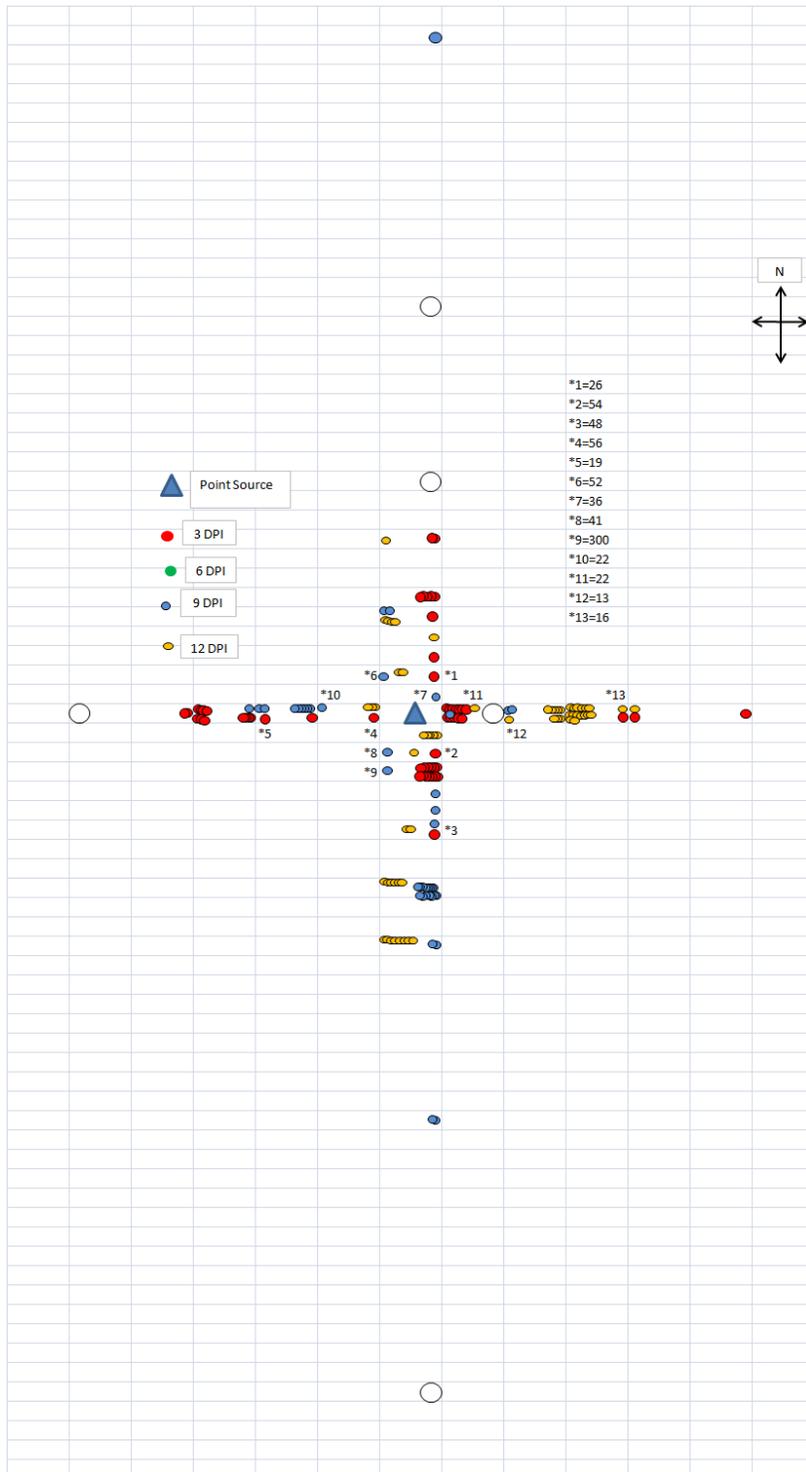


Figure 23. 2012. Movement of *A. flavus* mutant from a point source in corn in experiment 3. Each rectangle represents 30.5cm vertical and two corn rows (2 m) horizontally. Circles represent sample locations, with an open circle denoting no detection and a colored circle representing at least one CFU.

## Discussion

The objective of this study was to determine if a concentration gradient existed for movement of conidia from a point source in a corn and cotton field. Establishing a concentration gradient of spore movement will permit a sounder plot design that minimizes cross contamination and allows repeatability in future field experiments. Based on previous work, it was hypothesized that spore movement would remain under 10m in any given direction (61), and that the dispersal gradient would be steep from the point source (40). Based on two seasons of field sampling in corn, a general trend was established: the concentration gradient from a point source was very steep and followed a negative power curve when moving along or across rows in corn fields. This trend was not as apparent in 2011 as it was in 2012. As was hypothesized, movement was marginal or was undetectable by leaf sampling beyond terminal sampling points (10 rows, 10.6m). In experiments using a Burkard spore trap, in addition to leaf sampling, conidia were detected beyond the maximum leaf sampling distances with the trap, but recovery was low. In all corn experiments, the frequency of ear colonization by the mutant was lower the later inoculum was placed in fields in relation to crop maturity, decreasing from 45% to 9%. In cotton fields, no significant movement gradient was established.

Inoculum was placed in the fields at different times. Thus, the frequency at which the mutant could be recovered from within ears as a function of distance from the point source as well as application dates could be examined. Timing of application is important as kernels are most prone to *A. flavus* infection between two weeks after flowering and the end of kernel maturation (3). High evening temperatures during this maturation period are an additional predisposition factor for *A. flavus* colonization (72).

Dispersal gradients in corn are comparable to the point source movement of *A. flavus* reported by Olanya et al. (61), who found that movement generally did not exceed 8.5 m. Likewise, our data also show that direction from the point source had a significant effect on number of conidia recovered. Our results differ from Olanya et al, in that our curves were negative power curves rather than linear. These results are closer to the data presented by Horn et al, (40) in peanut fields where the dispersal gradient of *A. flavus* was very steep. However, our results are in contrast to movement

of atoxigenic *A. flavus* inoculum reported by Bock et al. (10). Bock et al. suggested that based on differences in the proportions of S and L strains at the local site vs. that captured by the air sampler, that either long distance movement occurred or there was preferential dispersal. However, sclerotial size can be influenced by other factors like water activity (59).

The shortcomings of epidemiological studies of *A. flavus* to date have been the lack of a trackable isolate. *A. flavus* is a ubiquitous fungus and so any positive result could potentially be the detection of a natural-occurring isolate. The use of a *nit* mutant circumvents this problem. However, there are still questions about the efficiency of recovery of isolates. Leaf and ear sampling had a low recovery, in comparison with the quantity of spores recovered from air sampling. To address this problem, more samples should be taken in future experiments.

Movement of *Aspergillus* in the field is only relevant to disease or disease control if the fungus is able to colonize kernels following movement. *A. flavus* has been shown to reach the base of ears in as little as 4 days from silks (25). In one study, the greatest number of air-borne conidia occurs simultaneously with corn silking (61), which is the optimum time for plant infection by *A. flavus* (54). Corn silks can harbor viable conidia (33) that can later colonize the ear (54). Thus, the window for atoxigenic conidia of *A. flavus* on the ground to reach the silks would be 7 to 10 days.

## CHAPTER III

# EFFECT OF HUMIDITY ON SPORULATION OF TWO ATOXIGENIC STRAINS OF *Aspergillus flavus* AND COMPARISON OF COMPETITIVENESS OF THESE STRAINS IN VITRO

### Introduction

*Aspergillus flavus* belongs to a ubiquitous genus of ascomycetes. It is a saprophyte in soils of every biome, including the arctic (57), and is a weak plant pathogen with a propensity for colonizing many oil-rich seed crops such as corn and cotton (4). Capable of producing an array of deleterious secondary metabolites, including aflatoxin, a potent carcinogen and highly regulated metabolite, *A. flavus* is responsible for significant annual economic losses of crops worldwide. In 1998 alone, aflatoxin accounted for approximately \$85 to \$100 million in crop losses to corn producers in Texas, Louisiana and Mississippi (9).

A number of methods have been employed for managing both pre- and post-harvest contamination of aflatoxin: elimination of overwintering organic substrate from fields, planting regionally adapted hybrids, reducing plant stress by adjusting planting dates, using a balanced fertility program, controlling insects in the field, applying antifungal agents to harvested corn, ammoniation of harvested corn, treating feed with clay binders, and breeding for drought tolerance and other traits that reduce colonization by the pathogen (17).

The utilization of non-toxin producing natural variants of *A. flavus* ("atoxicogenic") for biological control is one of the more recent innovations in aflatoxin management. These biocontrol strains occupy the same ecological niche and competitively exclude native, toxigenic *A. flavus* strains (20, 22). Atoxicogenicity is a result of an alteration of the toxin producing gene cluster. Deletions within the aflatoxin gene cluster are common in the southern United States and frequently result in atoxicogenic strains (18). By occupying the same ecological niche and competing for the same resources, the

atoxicogenic strains can displace toxigenic strains, reducing aflatoxin in the field and even having a carry-over activity during storage (26). A number of atoxicogenic strains have been isolated and developed for the control of aflatoxin. In Texas, two atoxicogenic strains are labeled for aflatoxin management on corn, AF36 (NRRL 18543) and Afla-Guard (NRRL21882).

AF36 is a formulation of autoclaved wheat colonized by the fungus, then dehydrated. Atoxicogenicity of this strain is the result of an opal mutation in a polyketide synthase gene (*pksA*) that renders AF36 unable to produce aflatoxin (30). However, AF36 is able to produce the mycotoxin cyclopiazonic acid (CPA) (2), shown to have multiple effects on organisms, including inhibition of calcium dependant ATPases in muscle tissue (35). In comparison, Afla-Guard consists of hulled barley coated with conidia. In contrast to AF36, Afla-Guard is unable to produce CPA, or any other intermediate of the aflatoxin pathway, as it lacks the aflatoxin biosynthesis cluster from the *hexA* locus to the telomeric region on the chromosome (4).

The overall research objective is to understand variables that contribute to the ability of atoxicogenic strains to competitively displace toxigenic *A. flavus*, as well as examine the relative competitiveness of the two strains labeled as biopesticides in Texas. The specific objectives of this study are to compare the production of conidia by the two atoxicogenic strains at several relative humidities, as well as to test their ability to compete relative to each other and a toxin- producing strain.

## Materials and Methods

**Fungal Mutant.** A nitrate- non-utilizing (*nit*) mutant, N3 (Isakeit, unpublished), generated from *A. flavus* NRRL 21882 on the medium of Elias and Cotty (31), was used in the experiments.

**Formulation Substrate Sporulation Tests.** A constant relative humidity was maintained in a sealed polypropylene container (14.5cm X 14.5 cm X 4.5 cm) using saturated salt solutions. The solutions were prepared as previously described (75) and consisted of tap water, (KH<sub>2</sub>PO<sub>4</sub>), (KCl), (NaCl), glucose, and silica gel, providing

humidity of 100%, 93%, 84%, 75%, 55%, and 0%, respectively. Relative humidity was measured with a Watchdog Model 150 data logger (Spectrum Technologies, Plainfield, IL).

AF36 and Afla-Guard formulations were incubated separately in moist chambers at the different humidity regimes. Colonized seeds were incubated on Parafilm above the salt solutions for 3 and 9 days at 27°C. Four replicates were used per container and each replicate consisted of four seeds. Seeds were weighed prior to incubation. Following incubation, each replicate was vortexed in 9 ml of 0.1% Tween 20 in distilled water for 30 seconds and conidia were enumerated with a hemocytometer.

**Standardized Substrate Sporulation Tests:** strains of AF36 (NRRL18543) and Afla-Guard (NRRL21882) were grown on Czapek-Dox agar in petri dishes for 5 days. Autoclaved hulled barley was then placed in the petri dishes and shaken vigorously for a minute to coat them with conidia. To determine the number of conidia coating the seed, four barley seeds were removed and vortexed in 9mL of 0.1% Tween solution for 30 seconds. Conidia were enumerated with a hemocytometer. This was repeated 3 times.

AF36 and Afla-Guard strains on the autoclaved hulled barley standardized substrate were incubated in a moist chamber—as described above—with a relative humidity of 100% for 3 days. Spores were enumerated as described previously. Differences were determined by subtracting the initial spore counts from final spore counts.

**Formulation Moisture.** The moisture content of formulations was determined following oven drying four groups of 4 kernels for 24 hours at 100°C.

**de Wit Competition Experiment.** de Wit competition experiment comparing paired growth of organisms was based on the approach of Weaver and Kenerley (76). The conidia of the Afla-Guard *nit* mutant (N3), AF36 and wildtype NRRL3357 strains, or a mixture of these strains, were added to 10mL of molten Czapek-Dox agar in 100 mm polystyrene petri dishes. Conidia were harvested from 5- day-old cultures grown on Czapek-Dox agar and adjusted to a concentration of  $1 \times 10^6$  spores/ml. Conidia of N3 and one strain of either AF36 or NRRL 3357 were added to make a total density of

$1 \times 10^6$  spores per plate, using ratios of 1:9 to 9:1. Plates were incubated for 3 days at 27° C. Following incubation, the contents of plates were blended in 200 ml of deionized water using a Waring blender, diluted  $10^{-3}$ , and 250  $\mu$ l was spread-plated onto chlorate-containing medium to enumerate the N3 mutant. There were four replicates per treatment and three experiments were conducted. The relative production of conidia was calculated by dividing yields of each mixed culture plate by the yield of the N3 mutant plated by itself.

**Statistical Analysis.** Microsoft Office Excel 2007 data analysis was used to calculate averages, standard deviations T-tests, and to graphically represent data.

## Results

Following 3 days of incubation, Afla-Guard produced significantly ( $P < 0.05$ ) more conidia than AF36 over all relative humidities tested, except 0% (Fig. 24). The greatest difference was four-fold at 100% relative humidity (RH). Sporulation was observed to begin at 93% RH and increased exponentially to 100% at both 3 days post incubation (DPI) and at 9 DPI. More spores were produced following 9 days than after 3 (Figs. 24&25).

The rate of conidial production by AF36 between 3 and 9 DPI was greater than that of Afla-Guard. At 100% RH, the increase by AF36 was seven-fold, in comparison with Afla-Guard, which was 1.4-fold. After 9 days, there was no significant difference ( $P < 0.05$ ) in the amount of sporulation of both formulations at all relative humidities tested (Fig. 25).

During the incubation period, AF36 produced noticeably more mycelia than the Afla-Guard.

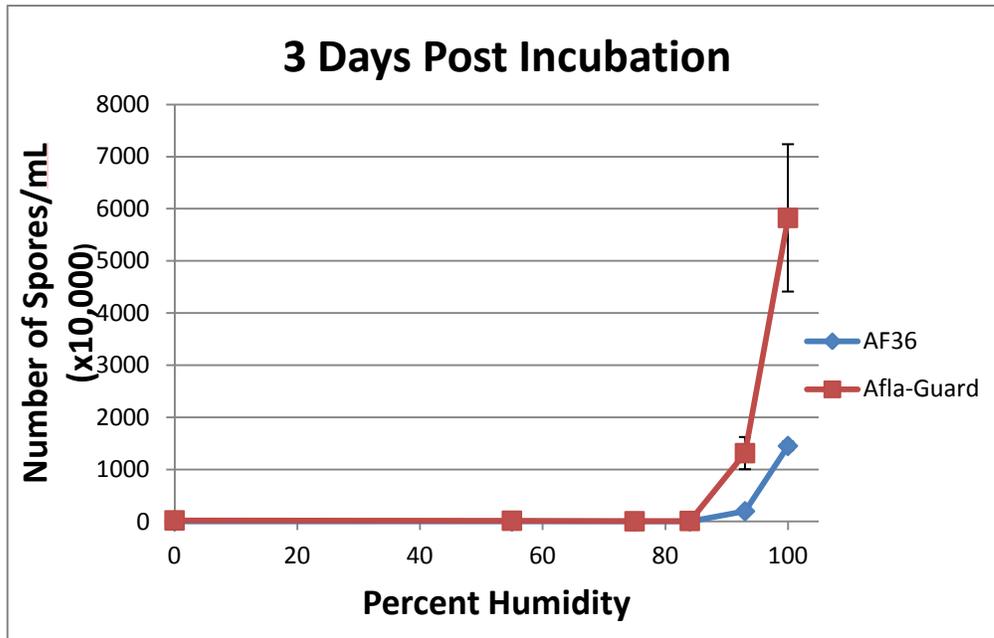
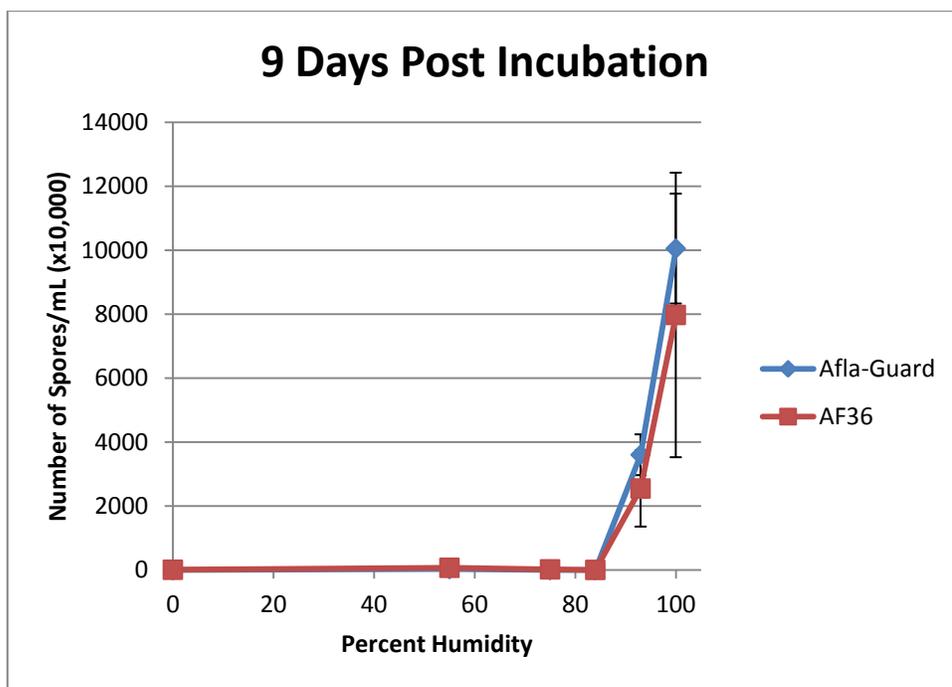


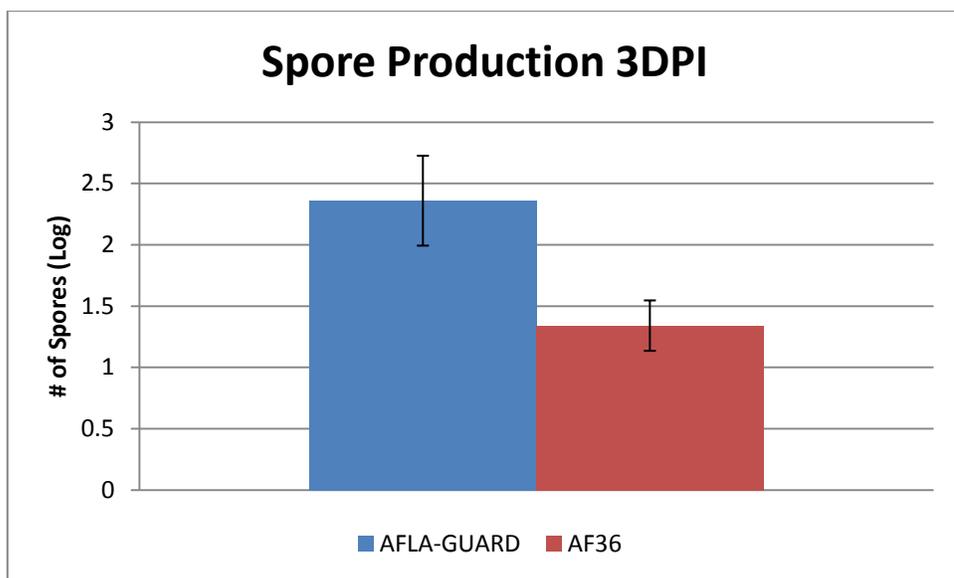
Figure 24. Conidia production of two atoxigenic biocontrol formulations following 3 days incubation under variable relative humidity.



**Figure 25. Conidia production of two atoxigenic biocontrol formulations following 9 days incubation under variable relative humidity.**

To determine whether the differences in sporulation could be attributed to differences in the substrate (i.e. wheat compared to barley), rather than strain differences, conidia from both strains were inoculated onto sterile, hulled barley. This method of inoculation resulted in an initial mean quantity of  $3.6 \times 10^6$  ( $\pm 6.5 \times 10^5$ ) and  $2.0 \times 10^7$  ( $\pm 2.9 \times 10^6$ ) conidia per 4 seeds for Afla-Guard and AF36, respectively. Following three days of incubation at 100% relative humidity, Afla-Guard produced approximately 55-fold more conidia than AF36 ( $P=0.0137$ ) (Fig. 26). Both experiments showed similar results.

The moisture content of Afla-Guard was 13.2%, which was significantly ( $P=0.0137$ ) greater than that of AF 36, which was 9.4%.



**Figure 26. Sporulation of two strains of *A. flavus* on hulled barley following 3 days of incubation at 100% relative humidity.**

In two experiments comparing competition between the Afla-Guard *nit* mutant and the AF36 strain, the relative yield of the *nit* mutant was linearly and negatively correlated with proportion of the competition strain ( $R^2=0.7219$ ,  $P=0.0683$  and  $R^2=0.9212$ ,  $P=0.0096$ ) (Figs. 27 and 28). In one experiment, one point deviated from the expected location (Fig. 27). This deviation is explained by increased variability in the data (Figs. 29 & 30). When the *nit* mutant was paired with NRRL3357, a toxin-producing strain, the relative yield was linear in one experiment ( $R^2=0.9445$ ,  $P=0.0056$ ) (Fig. 31), but not in the second experiment ( $R^2=0.443$ ,  $P=0.2273$ ) (Fig. 32). This inconsistency in data is explained by the variability in data from the second experiment, as compared with the first experiment (Figs. 33&34).

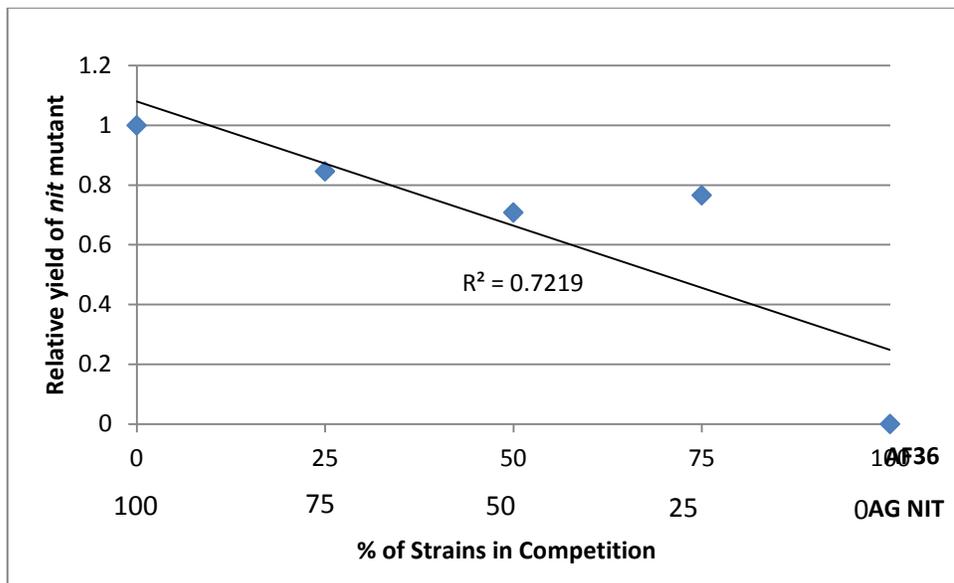


Figure 27. Experiment 1—de Wit competition between an Afla-Guard strain *nit* mutant and the *Aspergillus flavus* AF36 atoxigenic strain. Data points represent the mean relative yields of 4 replicates.

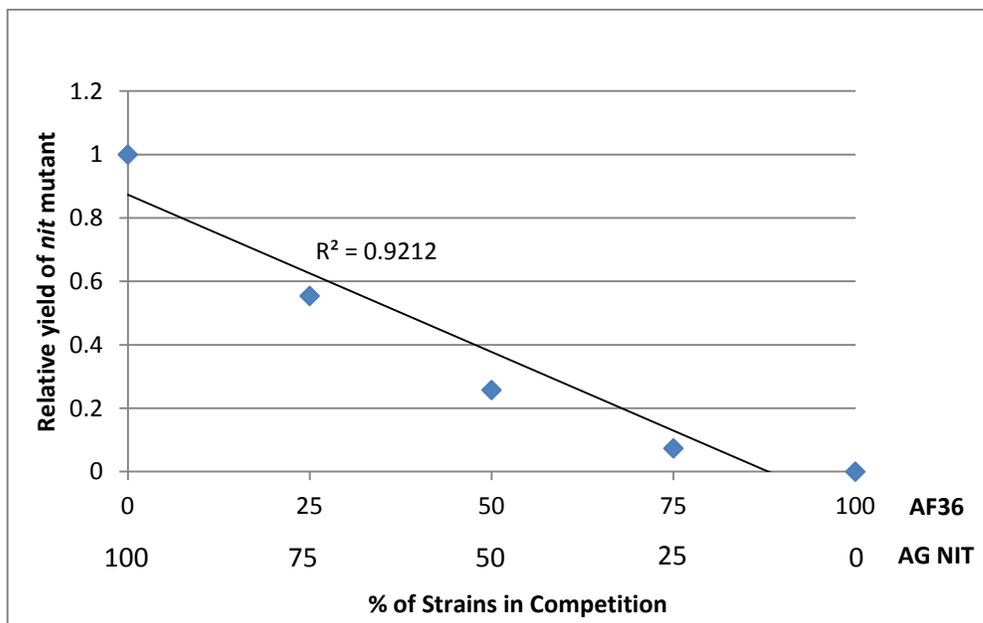


Figure 28. Experiment 2—de Wit competition between an Afla-Guard strain *nit* mutant and the *Aspergillus flavus* AF36 atoxigenic strain. Data points represent the mean relative yields of 4 replicates.

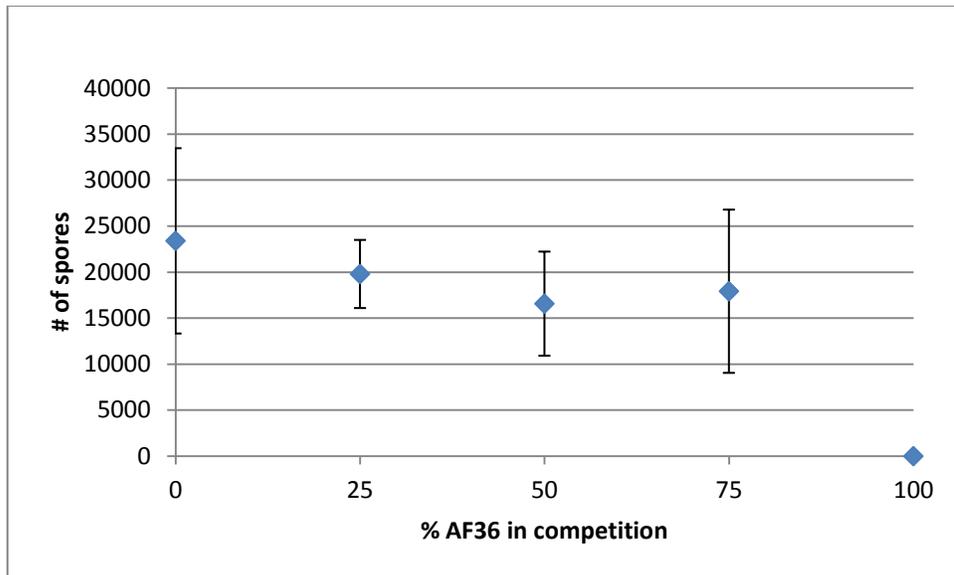


Figure 29. Experiment 1 with error bars—de Wit competition between the Afla-Guard strain *nit* mutant and AF36. Data points represent the mean yields of 4 replicates with error bars depicting the positive and negative standard deviation.

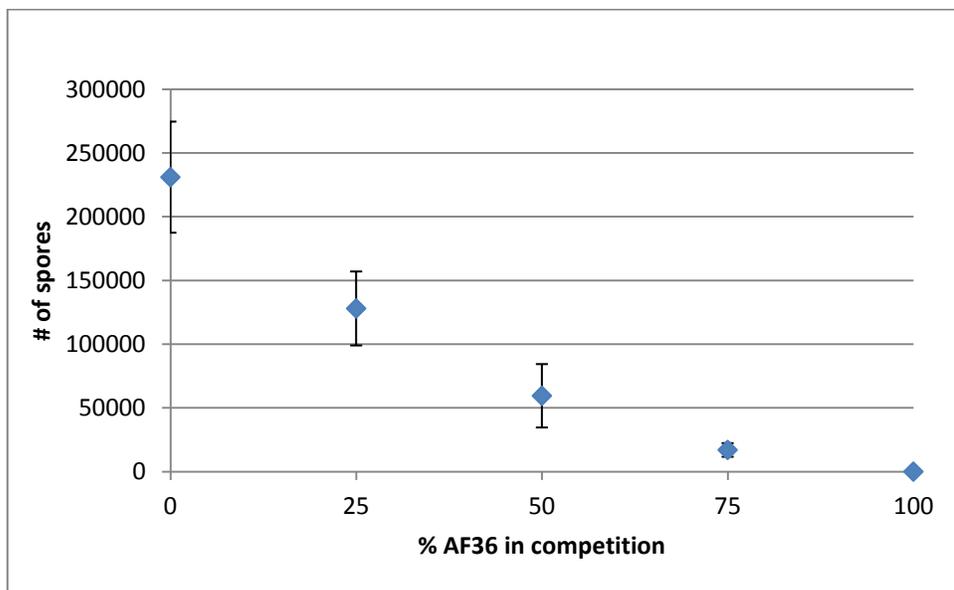


Figure 30. Experiment 2 with error bars—de Wit competition between Afla-Guard strain *nit* mutant and AF36. Data points represent the mean yields of 4 replicates with error bars depicting the positive and negative standard deviation.

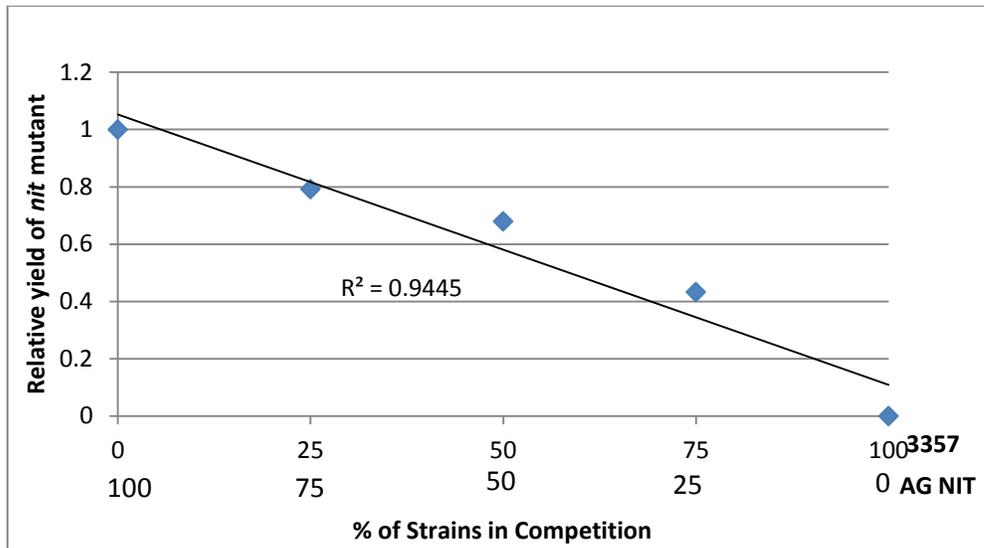


Figure 31. Experiment 1—de Wit competition between an Afla-Guard strain *nit* mutant and the *Aspergillus flavus* NRRL3357 toxin producing strain. Data points represent the mean of the relative yields of 4 replicates.

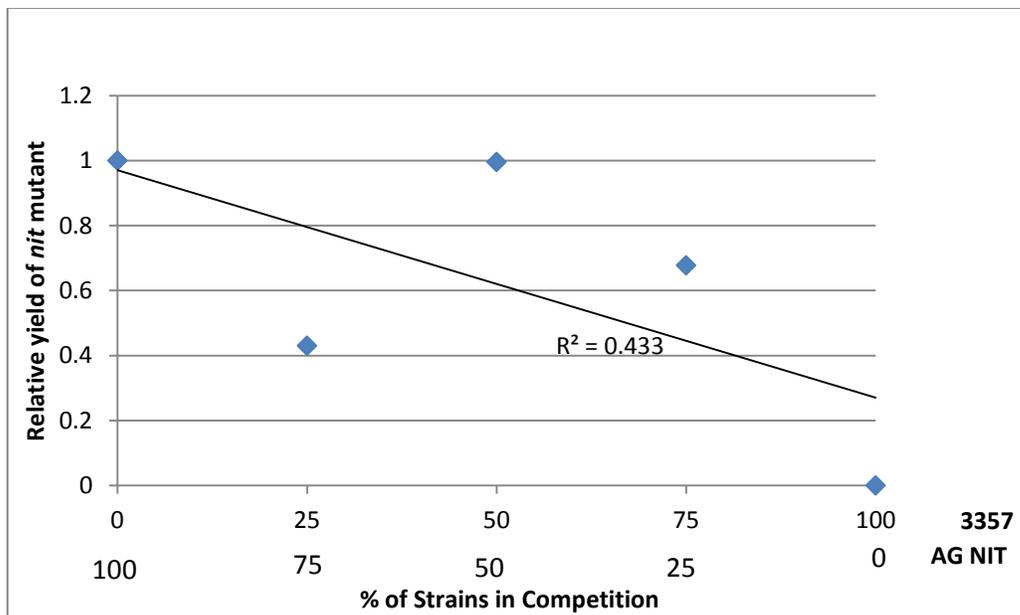


Figure 32. Experiment 2—de Wit competition between an Afla-Guard strain *nit* mutant and the *Aspergillus flavus* NRRL3357 toxin producing strain. Data points represent the mean of the relative yields of 4 replicates.

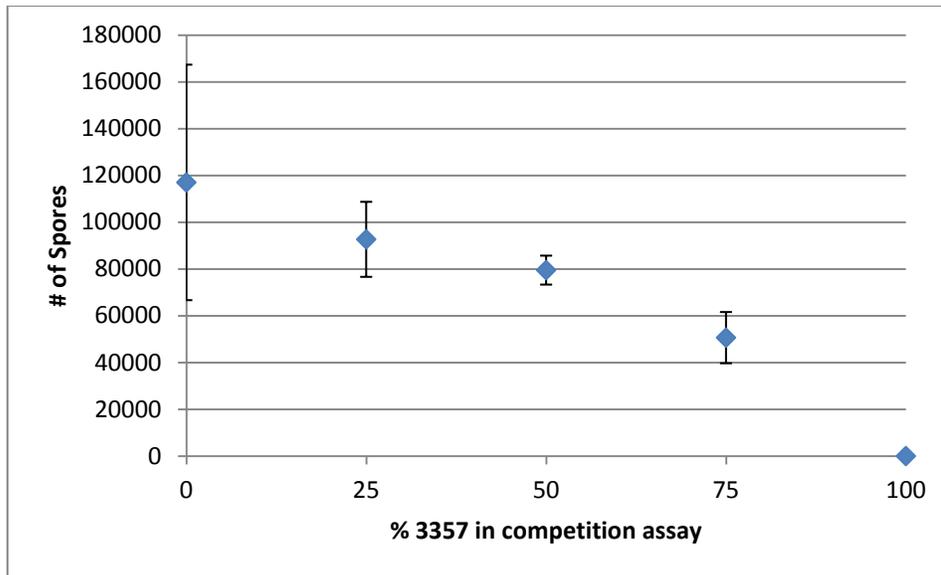


Figure 33. Experiment 1 with error bars—de Wit competition between an Afla-Guard strain *nit* mutant and the *Aspergillus flavus* NRRL3357 toxin producing strain. Data points represent the mean yields of 4 replicates with error bars depicting the positive and negative standard deviation.

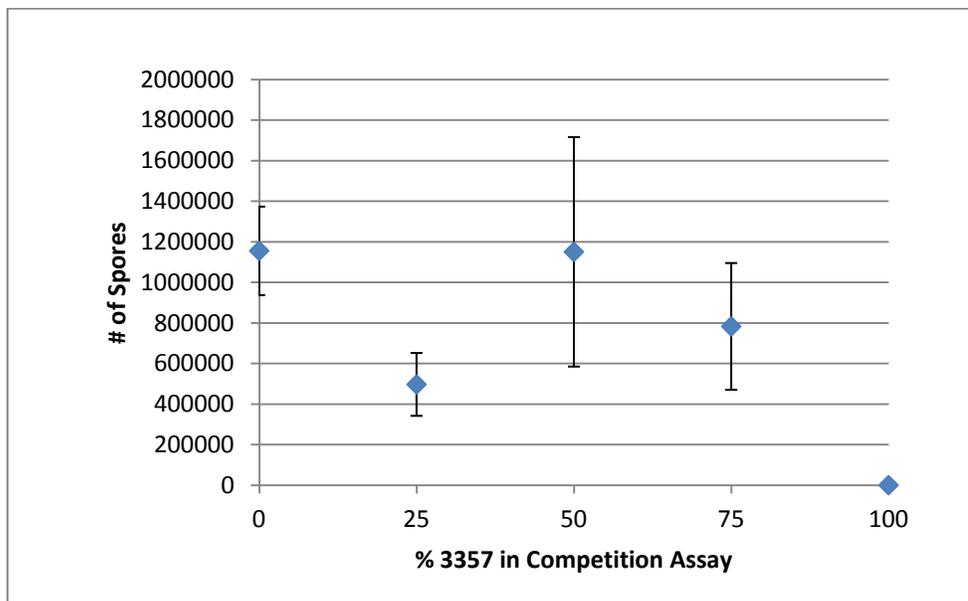


Figure 34. Experiment 2 with error bars—de Wit competition between an Afla-Guard strain *nit* mutant and the *Aspergillus flavus* NRRL3357 toxin producing strain. Data points represent the mean yields of 4 replicates with error bars depicting the positive and negative standard deviation.

## Discussion

The objectives of this study were to identify atoxigenic strain or formulation qualities that could contribute to the efficacy of biological control. The in vitro experiments suggest that, the Afla-Guard strain is just as good of a competitor as AF36. However, on its formulation of hulled barley, the Afla-Guard strain produces a greater number of spores more quickly over a greater range of relative humidities than AF36 on its formulation of sterilized wheat. Some of this difference may be explained by differences in moisture contents of the two formulations: the hulled barley formulation has 13.2%, while the sterilized wheat has 9.4%. This additional moisture in the Afla-Guard formulation may give that strain a head start in an environment where moisture is limited. However, when compared on the same substrate, hulled barley, the sporulation of the Afla-Guard strain was still greater than that of the AF36 strain, suggesting that there are strain differences.

Because inoculation is the first step in the disease cycle, the ability of an organism to produce infective propagules is a good indication of disease potential under ideal conditions. Biocontrols that work through competitive exclusion will occupy and compete for resources in the same ecological niche as the organism that they are displacing (20). So, in the case of atoxigenic strain of *A. flavus* used as a biocontrol, acting in the same manner as its toxigenic counterpart, the ability to sporulate could be an initial measure of efficacy for the displacement of toxigenic producing strains and thus, the control of aflatoxin. However, other characteristics of a strain, such as the ability to colonize, may ultimately be more important than sporulation.

At lower humidities - 0% and 55% RH – conidia were recovered from formulations, indicating that recovery was either a function of initial spore coating, or sporulation that was initiated from formulation kernel moisture, or both. Conidia were readily recovered from formulations upon vortexing, although at a lower amount than that recovered after incubation in humidity chambers. This indicated that kernel moisture could be a variable in sporulation even at sub-optimal moisture conditions.

In in vitro pairing with both NRRL 3357 and AF36 isolates, the Afla-Guard mutant was recovered at an expected rate relative to predetermined inoculation ratios. This suggests that neither NRRL3357 nor AF36 is more competitive than Afla-Guard

under these conditions. Because NRRL3357 or AF36 were not enumerated, it is not known whether the Afla-Guard strain alters the expected rate of recovery of other strains. Based on this data and its limitations, it appears that Afla-Guard is minimally just as good of a competitor as NRRL3357 or AF36. Data counts are neither above nor below what was expected, indicating that there is neither mutualism nor inhibition in the presence of the competition strain. As so, data suggests it is a foot-race to colonize the active site as there is no interaction between the paired strains at any ratio.

Under the conditions of these experiments, the Afla-Guard formulation appears to be superior in supporting sporulation than the AF36 formulation. Innate strain qualities that lead to rapid, profuse sporulation may be a useful quality in a formulation for the reduction of aflatoxin. Because profuse conidiation of the fungus is contingent upon prolonged periods of high humidity or moisture, strains capable of rapid sporulation are advantageous for use in dry-land cropping systems or in climates with limited periods of moisture, such as Texas. Other factors, like the differences in the production of mycelia or the longevity of viability of inoculum propagules between strains were not examined in this study but could potentially influence the overall ability of the fungus to compete.

Because *A. flavus* is so diverse genetically, there may be natural variants that have more advantageous qualities than those displayed by the Afla-Guard strain. With more desirable traits, these strains would theoretically out-compete the atoxigenic strain once in the infection court. To this end, the perfect strain might never be found. Therefore, it could be to the advantage of farmers to blend strains to capitalize on innate fungal qualities. Given the recent discovery of the sexual stage in *A. flavus*, it might even be possible to make crosses to produce a more desirable strain. However, a highly competitive atoxigenic strain in the field could potentially recombine and acquire the aflatoxin gene cluster potentially leading to a highly competitive toxigenic strain. Additional research into strains that either are unable to recombine or even the utilization of other organisms should be examined further.

## CHAPTER IV

# LONGEVITY OF *Aspergillus flavus* PROPAGULES AT DIFFERENT MATRIC POTENTIALS IN SOIL

### Introduction

*Aspergillus flavus* is a filamentous ascomycete that reproduces asexually through the production of airborne conidia and overwintering structures called sclerotia. Sclerotia are able to germinate and produce hyphae and conidiophores (71). Most recently with the discovery of the teleomorph of *A. flavus*, *Petromyces flavus* (39), sclerotia have been shown to produce ascospores following the recombination of two distinct mating types

Given the overwintering capacity of sclerotia, they are believed to be a greater contributor to the source of primary inoculum the following growing season than conidia (25). Additionally, *A. flavus* overwinters on crop debris as conidia and potentially as dormant hyphae (25). Corn cobs have more than 190-fold *A. flavus* inoculum than surrounding soil (44), as *A. flavus* is a principal colonizer of corn cobs (81). Corn cobs comprise a large portion of cornfield biomass—approximately 1,500 kg/ha (44). Based upon the average number of propagules from each corn cob, it is possible that on average  $4.8 \times 10^{11}$  propagules could exist per hectare in a cornfield (44). To date, ascospores have not been shown to be involved in the infection process.

Conidia can remain viable for up to 36 months in soil (74) and up to 22 years on a bran base stored in the laboratory (56). As is the case with *Fusarium* spp., viability of *A. flavus* propagules could be affected by different crop rotations, soil types, and geographic locations (53). The effect of Texas soil types and environmental conditions on survival of *A. flavus* has not been addressed to date. Knowledge of propagule longevity is clearly important if crop rotation is part of a strategy for managing aflatoxin.

The purpose of this study is to examine the effect of two soil matric potentials on the viability of *A. flavus* conidia and sclerotia, using a Texas soil. Two strains, one toxigenic, the other atoxigenic, were evaluated.

## Materials and Methods

**Preparation of Propagules.** Conidia and sclerotia of two strains were used, NRRL 3357 and the Afla-Guard strain. Conidia were harvested from 3-day-old cultures grown on 1/10 X potato-dextrose agar (PDA) by washing with sterile, distilled water. Conidia were washed three times by centrifugation for 10 minutes at  $1438 \times g$  (3500 RPM) and resuspended in distilled water to obtain a concentration of  $1 \times 10^6$ /ml. A 500  $\mu$ l aliquot was placed in a 3x3cm polycarbonate membrane filter (0.4  $\mu$ m pore-size) (Nucleopore Track-Etch Membrane, Corning). The polycarbonate membrane was then folded and sealed with 732 multi-purpose sealant (Corning) along the margins to form sealed packets.

Sclerotia were harvested from 3 day old PDA plates and rinsed for 15 minutes in 53  $\mu$ m sieve with tap water. The sclerotia were then transferred to a 9 ml 0.1% Tween 20 solution and vortexed 30 seconds. The sclerotia were then rinsed again in a 53  $\mu$ m sieve with tap water and blotted dry with paper towels. Ten sclerotia were sealed in polycarbonate membranes as described above. To assure that conidia were removed from sclerotia, five cleaned sclerotia were selected randomly and placed in 5 ml of sterile water and vortexed. A 0.2ml aliquot of the solution was plated onto ½ strength PDA and incubated for 5 days at 28° C.

**Preparation of Soil.** The soil used was a non-sterile Ships Clay from the Texas AgriLIFE Agricultural Experiment Station near College Station, TX. Two constant matric potentials (-24 kPa & -154 kPa for wet and dry, respectively) were maintained using 97 mm diameter Buchner funnels with fritted glass plates of fine porosity as tension plates (27). The plates were covered with 1 cm soil. The membranes were placed on the plates, and then covered with an additional 1 cm soil and left at room temperature

**Determination of Viability.** At weekly intervals, membranes were removed from soil, rinsed with tap water and incubated overnight on Czapek-Dox agar. Membranes were then removed, stained with a solution of 1% rose bengal in 5% phenol, blotted dry and opened and examined for growth with a light microscope.

## Results

Aliquots plated from extensively- cleaned sclerotia rinsed with sterile water resulted in complete coverage of plates, indicating that sclerotia were not free of conidia. Because sclerotia have previously been reported to retain viability longer than conidia (74), their presence was not considered to interfere with the sclerotia longevity assay.

After 16 weeks, conidia and sclerotia of both isolates incubated at both matric potentials were still viable. After 10 months, all packets were found to contain viable conidia. After 14 months, 6 out of 6 packets containing sclerotia of the atoxigenic strain incubated at the low matric potential had no structural integrity. When visible parts of the sclerotia were plated, they did not germinate. However, there was still growth on the plate, possibly originating from conidia. Of the 10 atoxigenic sclerotia incubated at the high matric potential, 7 of 10 sclerotia were structurally intact and five of them were viable. With the toxigenic strain, 7 of 10 sclerotia at the low matric potential and 10 of 10 sclerotia at the high matric potential were viable.

All conidial treatments remained viable following 10 months of incubation.



**Figure 35. Germinating *Aspergillus flavus* following staining with Rose Bengal in phenol on polycarbonate membrane.**

## Discussion

The objective of this study was to compare longevity of conidia and sclerotia of atoxigenic and toxigenic isolates of *A. flavus* under two matrix conditions using a Texas soil. Aflatoxins have repeatedly been associated with sclerotia (7): i.e. sclerotia contain very high levels of aflatoxin (21) and exist in higher quantities compared to conidia (73). A number of studies have investigated an evolutionary purpose for aflatoxin (34), including a response to oxidative stress (45). Previously, there have been no studies comparing the longevity of atoxigenic propagules to that of toxigenic strains. If toxigenicity influenced longevity, this could have implications for the management

approach of altering the population structure of *A. flavus* in the field through the introduction of atoxigenic strains (44). Achieving adequate displacement of toxigenic strains may necessitate repeated applications of atoxigenic strains. Based on previous work, it was hypothesized that in a Texas soil, conidia and sclerotia would remain viable for at least a few weeks and sclerotia for years - this study shows that is not the case with sclerotia.

A study in Georgia and Illinois (74) showed that conidia remained viable through 18 months of incubation in the soil. However, the same study showed that after 36 months, approximately 90% of sclerotia remained viable. These observations were in contrast to this research that shows all Afla-Guard sclerotia lost viability following 14 months of incubation. However, both temperature and matric potentials differed between the studies.

In the current experiment, propagules were incubated at a constant temperature and matric potential. It would be informative to repeat this experiment with other *A. flavus* strains, both toxigenic and atoxigenic to see if there is variation both within groups of toxigenic and atoxigenic strains but also to explore the purpose of aflatoxin in viability of propagules. Additionally, repeating the experiment under a range of different matric potentials at different temperatures could more closely mimic field conditions in Texas.

Considering the current data, in dry climates like Texas, some atoxigenic strains might not be suitable for long-term management of aflatoxin, if sclerotia are intended as the primary inoculum producers for the following season (25).

Because conidia remained viable through 16 weeks in soil, the role of soil-borne conidia should be examined for their role in aflatoxin contamination. The longevity experiments with conidia need to be expanded to account for at least a 11-12 month period between release of conidia into the environment at harvest and the flowering of the next crop. If conidia survive during this interval, survival of conidia over a longer interval reflecting crop rotation should be examined. With this in mind, crop rotation or changing the rate of atoxigenic strain application following a particularly bad year of aflatoxin contamination should be considered and examined for potential efficacy.

## CHAPTER V

### SCLEROTIAL PRODUCTION OF *Aspergillus flavus* IN RELATION TO OTHER EAR-COLONIZING FUNGI

#### Introduction

*Aspergillus flavus* is a seed-colonizing fungus that is capable of producing highly deleterious and toxigenic secondary metabolites. The reproductive propagules of *A. flavus* include asexually-produced conidia and sclerotia, and rarely, ascospores. Of these propagules, overwintering structures called sclerotia have been shown most recently to play a larger role in the life cycle of the fungus than previously thought with the discovery of the sexual stage (39). Sclerotia are hard mycelia masses, usually formed when growth conditions are unfavorable for the fungus.

Sclerotial formation in fungi is influenced by factors such as light, water activity, nutrients, pH, and plant phenolics (69, 59, 66, 21, 58). Given the competitive nature of microorganisms, as well the array of microbes that colonize corn (79, 72), the presence of some microbes may be unfavorable for growth of *A. flavus*. The hypothesis of this study was that the presence of some microbes, specifically fungi, could influence sclerotial formation in *Aspergillus flavus*. Extracts of fungi of *Penicillium*, *Fusarium* and *Aspergillus* inhibit *A. flavus*, in disk inhibition assays (48), but the effect of these or other fungi on sclerotial formation of *A. flavus* is not known.

Factors that influence sclerotial formation could influence the long-term survival of *A. flavus* in the soil or in crop debris. A reduction in survival could potentially reduce aflatoxin pressure on the crop. The objective of this study is to examine the relationship of corn silk senescence to the frequency of fungal taxa colonizing kernels and their possible influence on the production of sclerotia of *A. flavus*.

## Materials and Methods

**Inoculation and Harvesting.** The experiment was conducted at the Texas AgriLIFE Experiment Station near College Station, TX. On June 9, 2011, corn plants at different stages of flowering were identified and flagged. Corn was labeled based on the appearance of the silk: 100% green, approximately 50% green, and 100% brown. Corn was silk-channel inoculated (80) with 5 ml of a  $1 \times 10^6$  spore/ml suspension of either NRRL3357 or NRRL21882, toxigenic and atoxigenic strains, respectively. On August 20, corn ears were harvested and stored at 4° C.

**Enumeration of Sclerotia.** Corn ears were individually husked and shelled over a nested 1  $\mu$ m- 0.250  $\mu$ m sieve combination. Kernels and husks on the sieves were then rinsed under running tap water for approximately 5 minutes. Sclerotia on the 0.250  $\mu$ m sieve were rinsed onto #2 Whatman filter paper and then transferred to a 10 ml test tube of 0.5% agar. The suspension was vortexed for 30 seconds and a 1 ml aliquot was transferred onto Whatman, filter paper for enumeration of sclerotia with a dissecting microscope.

Kernels from each ear sample were collected following sclerotial enumeration and retained for assay of fungal colonization.

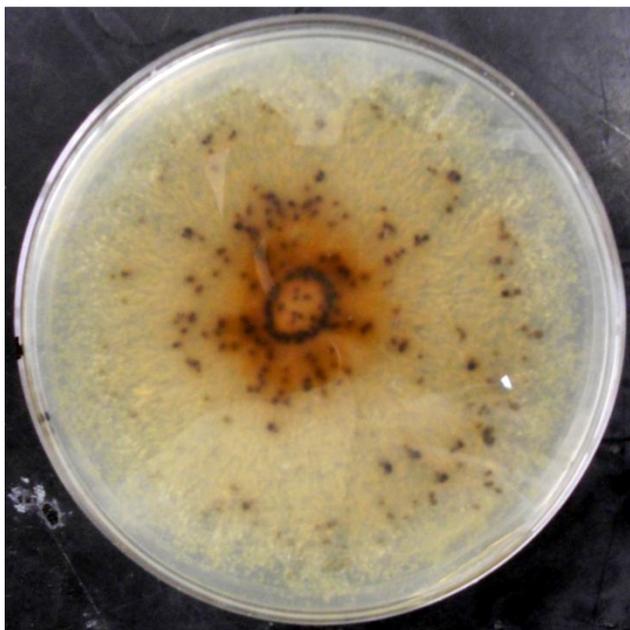
**Assay and Isolation of Fungi.** Ten kernels from each of eight ear samples from each maturation group were surface sterilized for 2 minutes with a 20% bleach solution, rinsed twice with sterile distilled water for 2 minutes, and were blotted dry with autoclaved paper towels. Kernels were then incubated on sterile wet paper towels placed in a 22 x 22 x 5 cm pan in a moist chamber for 5 days at 32°C. Following 5 days, fungal growth on kernels was assessed. The experiment was repeated three times.

A *Penicillium* species and a *Fusarium* species were isolated and stored at 4°C on Czapek-Dox agar.

**In vitro Sclerotia Assay.** The *Penicillium* and *Fusarium* isolates, as well as Afla-Guard and NRRL3357. *A. flavus* isolates were grown in 100 ml of Czapek-Dox broth in 250 ml Erlenmeyer flasks at ambient room temperature (approximately 23°C)- and light for 14

days. The broth culture was lightly swirled daily. Following 14 days, the broth culture was filtered with miracloth and the filtrate was sterilized with a 0.20  $\mu\text{m}$  sterile syringe filter (Corning). Culture filtrates were stored aseptically in the dark until used.

Ten ml of either *Fusarium* or *Penicillium* culture filtrate was added to 200 ml cooled but molten Czapek-Dox agar. Although the fungal culture filtrates were pH 5.0 to 5.5, the addition of these filtrates to the agar did not change the pH, which was 7.0 (data not shown). Three days later, 20  $\mu\text{l}$  of a  $1 \times 10^6$  conidial suspension from 3-day-old cultures of three strains of *A. flavus*, AF36 strain, the Afla-Guard strain, and the NRRL3357 strain, was placed at the center of the amended Czapek-Dox agar plates. Plates were incubated for two weeks in the dark at 32°C and then sclerotia were enumerated. Replicates varied from 7 – 10 replicates/treatment. In the autoclaved *Penicillium* assay, replicates varied from 3-7.



**Figure 36. Negative plate used for enumeration of sclerotia.**

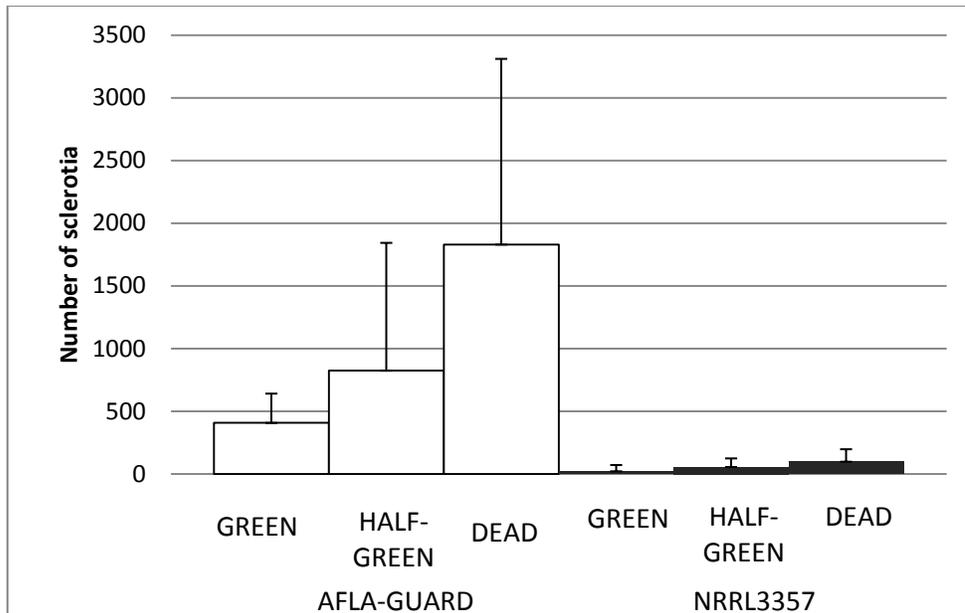
**Radial Growth Assay.** A 10 $\mu$ l aliquot of a 1x10<sup>6</sup> spore suspension from 3- day- old cultures of three strains of *A. flavus* was placed at the center of the amended Czapek-Dox agar plates. Plates were incubated at 32°C. Radial growth was assessed daily.

**Statistical Analysis.** Microsoft Office Excel 2007 was used for basic statistical analysis. Tukey's test was done with JMP 9 software.

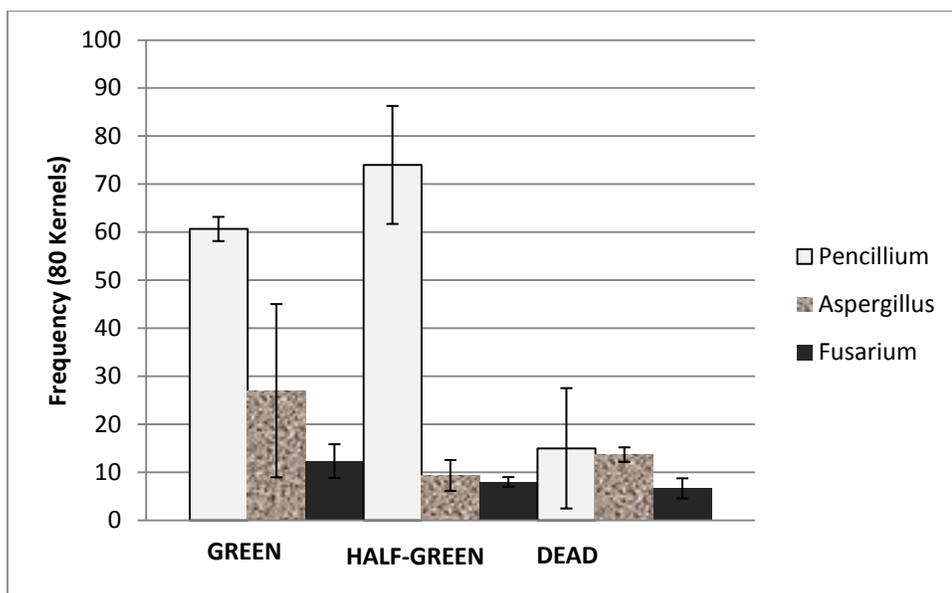
## Results

The Afla-Guard isolate produced significantly ( $P=0.0057$ ) more sclerotia in ears than the toxigenic NRRL 3357 isolate (Fig. 37). With both isolates, sclerotia production was greater in ears with inoculated as silks were senescent (dried) as compared to inoculation when silks were fresh. The difference between fresh and senesced was significantly ( $P =0.05$ ) pronounced with the Afla-Guard isolate, but not significantly different ( $P=0.05$ ) with NRRL 3357 (Fig. 37).

The predominant fungal taxa growing from surface-disinfested kernels were *Aspergillus*, *Penicillium* and *Fusarium* (Fig. 38). The frequency of *Penicillium* spp. was significantly greater than *Aspergillus* ( $P=0.0327$ ) and *Fusarium* ( $P<0.0001$ ) and occurred more frequently than any other isolates in the case of ears inoculated with *A. flavus* at fresh and half-fresh silk.



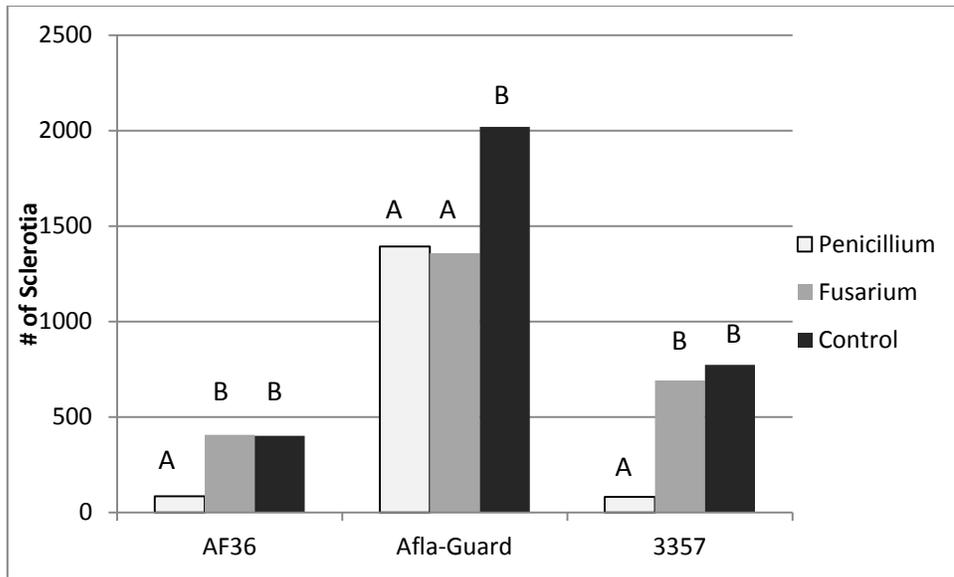
**Figure 37. Mean sclerotial counts following silk-channel inoculation of two isolates of *A. flavus* into ears of corn at three stages of silk senescence (green, half-green, dead). Error bars represent standard deviation.**



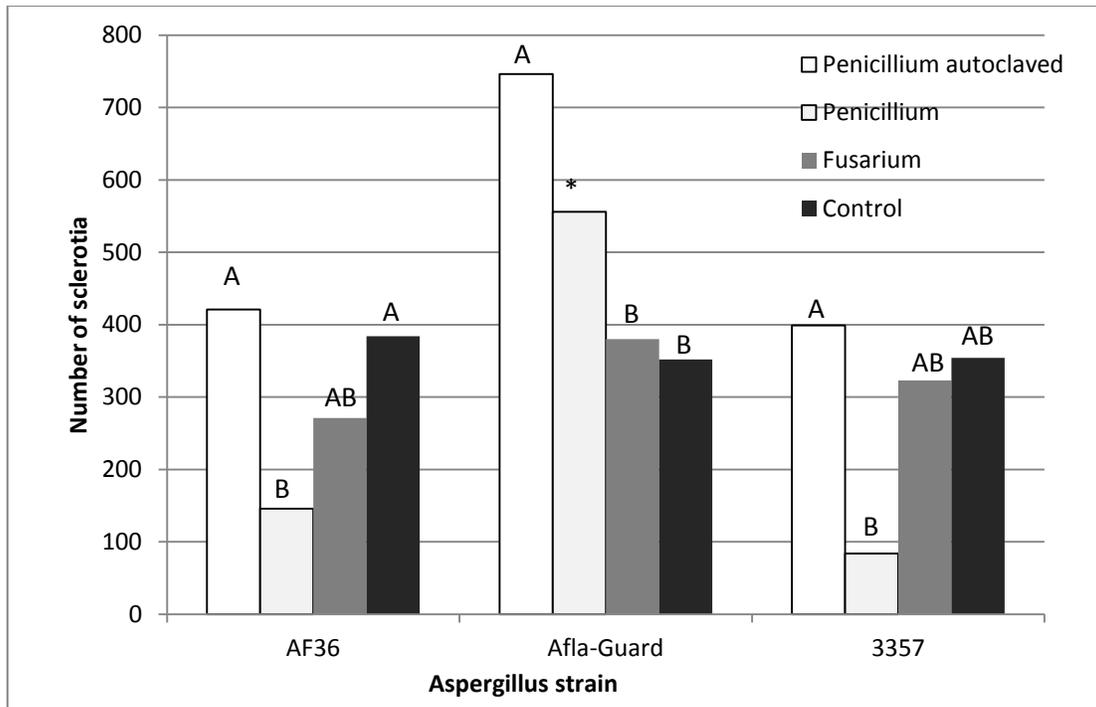
**Figure 38.** Incidence of select fungal taxa colonizing harvested corn kernels as a function of silk senescence at the time of inoculation ears with *A. flavus* mean of 80 kernels over 3 replicates. Bars indicate standard error.

The presence of a filtrate from a broth culture of *Penicillium* sp. significantly ( $P=0.05$ ) reduced sclerotial production by all three isolates of *A. flavus* on Czapek-Dox agar (Fig. 39). The *Fusarium* sp. culture filtrate reduced sclerotial production of the Afla-Guard isolate, but not the other two isolates (Fig. 39)

The addition of autoclaved *Penicillium* sp. culture filtrate did not reduce sclerotial production of any of the isolates (Fig. 40). In the case of the Afla-Guard isolate, the assessment of sclerotial production was based on one replicate.



**Figure 39. Sclerotial counts of three *A. flavus* isolates when grown in the presence of *Penicillium* or *Fusarium* culture filtrate that were isolated from corn. Letters on bars indicate significant ( $P=0.05$ ) differences among treatments of each isolate using Tukey's test.**



**Figure 40. Three strains of *Aspergillus flavus* grown in the presence of select culture filtrate, including autoclaved *Penicillium* filtrate.**

<sup>a</sup> The measurement of one replicate.

Because sclerotia are hard hyphal aggregates, the hypothesis was that if *Penicillium* sp. culture filtrate inhibits sclerotial formation, then it would also inhibit hyphal growth. To test this hypothesis, radial hyphal growth was measured over the course of 4 days. In the presence of the *Penicillium* culture filtrate, radial hyphal growth rates exceeded those of the control and those grown in the presence of *Fusarium* sp. (Figs. 41, 42, 43).

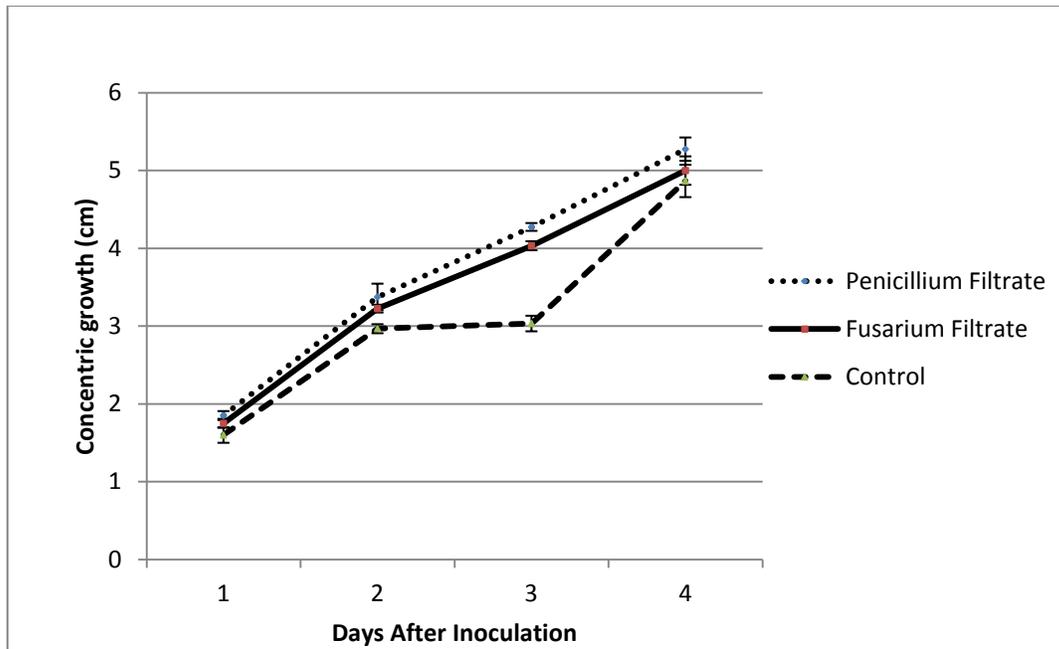


Figure 41. Hyphal growth of AF36 measured at the longest diameter over the course of 4 days. Error bars represent positive and negative standard deviation.

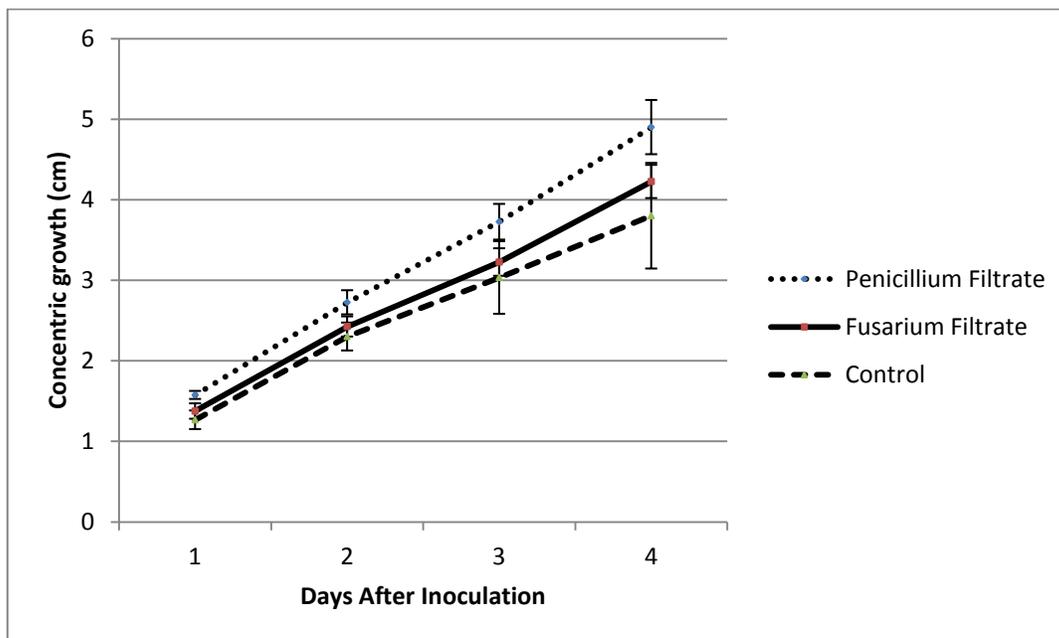
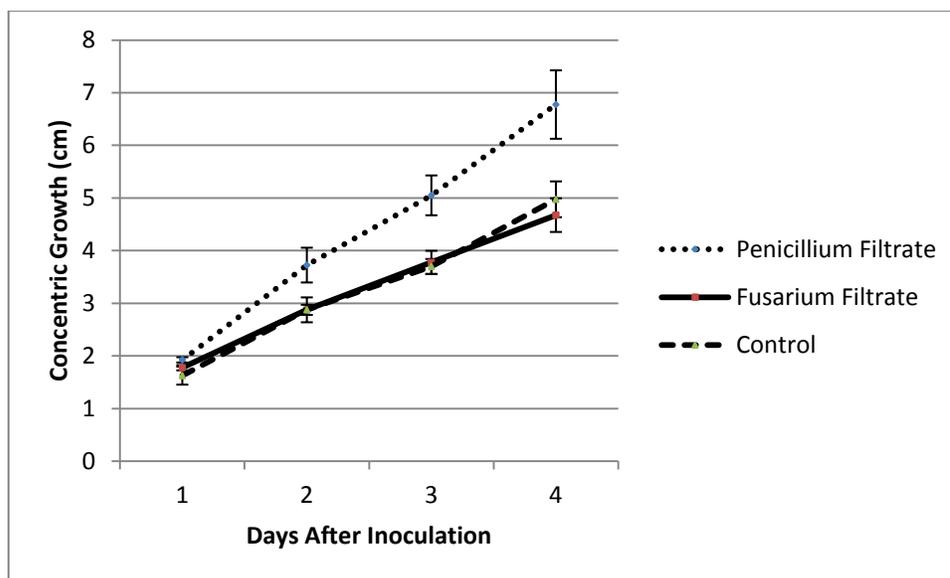


Figure 42. Hyphal growth of Afla-Guard measured at the longest diameter over the course of 4 days. Error bars represent positive and negative standard deviation.



**Figure 43.** Hyphal growth of NRRL3357 measured at the longest diameter over the course of 4 days. Error bars represent positive and negative standard deviation.

## Discussion

There was a greater amount of sclerotial production of the Afla-guard isolate when it was inoculated into ears with more senescent silks, as compared with fresher silks. There was a smaller, non-significant trend with the NRRL 3357 isolate. This was correlated with a higher incidence of *Penicillium* spp. colonization of ears inoculated with *A. flavus* when silks were less senescent. A culture filtrate of a *Penicillium* sp. isolate, but not the *Fusarium* sp. isolate, reduced sclerotial production, but not hyphal growth, of all three *A. flavus* isolates *in vitro*. The autoclaved culture filtrate did not reduce *in vitro* production of sclerotia.

The difference in the growth stage of corn when silks are fresh, as compared with when they are senescent, ranges from 7 to 10 days. Yet, this time period may reflect significant physiological changes in the developing kernels. For example, the lipid composition of corn kernels changes throughout development. Triglycerides accumulate rapidly through 52 days after silking while their main constituent, linoleates,

-increase uniformly throughout development from 52% to 62% (68). In the context of sclerotial production by *A. flavus*, it has been suggested that linoleates may affect sclerotial production of *A. flavus* in corn, as *A. flavus* cultures grown in the dark, treated with linoleic acid, produce significantly more sclerotia than controls (13). The change in lipid composition could serve as one explanation for the increased production of sclerotia.

The presence of other microbes in the developing ear may also affect sclerotial production by *A. flavus*. This is suggested by in vitro inhibition with *Penicillium* sp. culture filtrates. The active component(s) of the filtrate is heat labile. It can't be said the inhibitory agent was proteinaceous, as secondary metabolites can be photoreactive (51) and have different degrees of thermostability (41).

Within *Aspergillus* and *Penicillium*, the conserved global regulator, veA, controls aflatoxin and sclerotial production in *Aspergillus parasiticus* as well as sclerotial production, aflatoxin, aflatrem, and cyclopiazonic acid in *Aspergillus flavus* (28). In *A. nidulans* veA mutants, conidiation is increased and promoted (12). Likewise, when VeA is deleted in *A. flavus*, sclerotial production is inhibited completely (28). The VeA gene is needed for the negative regulation of the VeA protein in *A. nidulans* (47). Because *Penicillium* species contain a VeA homolog (6), it is possible that the *Penicillium* culture filtrate could negatively regulate VeA expression in *Aspergillus* and thus regulate sclerotial production. If this is the case, the denaturation of the VeA protein by autoclaving would result in no decrease in sclerotial production. However, it is also possible that other chemicals, such as heat-labile antibiotics could be involved. The implications of this microbial interaction for aflatoxin contamination or use of atoxigenic strains for aflatoxin management are not known.

## CHAPTER VI

### CONCLUSIONS

The management of aflatoxin contamination is a function of control of the fungus. One management strategy is to alter the ear-colonizing populations of *A. flavus* through the introduction of atoxigenic biocontrol strains grown on seed substrate. Because these are biological agents, a number of factors influence both the short and long-term efficacy and viability of these strains. These factors can be examined from the context of the plant disease cycle. In this paradigm, short-term efficacy begins with the movement of conidia from the formulated seed substrate to the active site, the corn ear. This research showed that the greater the distance an ear is from a point source of the atoxigenic strain, the fewer conidia will likely reach it. This trend was seen during two growing seasons. Another factor that would influence inoculation is the quantity of inoculum produced per unit substrate under variable environmental conditions. This research showed that one labeled atoxigenic strain, Afla-Guard, produced a great deal more conidia at variable humidities than another strain, AF36. While conidial production is just the first step for biological control, producing vast quantities of inoculum is not necessarily sufficient if the propagules are unable to compete with other biological entities once inside the ear. For biological control to work at the active site, the ability of an atoxigenic strain to outcompete toxigenic strains is important. This research showed that when Afla-Guard was paired with a toxin-producing strain of *A. flavus* or the AF36 strain, the growth of the Afla-Guard strain was not impaired, indicating that it is able to compete for nutrients just as well as the two strains tested.

When conidia reach the active site, in addition to encountering other strains of *Aspergillus*, they can encounter other microbes. Some of these microbes are antagonistic to *A. flavus* growth and can influence growth and survival. This has implications for the long-term and short-term management of aflatoxin and local soil populations of *A. flavus*. This research showed that in the presence of *Penicillium in vitro*, three strains of *A. flavus* grew faster and produced significantly less sclerotia

compared to controls. A diminished production of sclerotia could affect long-term survival of the fungus.

The effect of two matric potentials on long-term viability of sclerotia and conidia in soil was assessed. This research showed that after 10 months, viable conidia were found with all treatments. After 14 months, all sclerotia of the atoxigenic isolate incubated at the low matric potential lost viability, while sclerotia with other treatments remained viable. This suggests that matric potential needs to be considered as a factor affecting the long-term survival of some atoxigenic strains.

Ultimately, the use of atoxigenic strains can be beneficial in the management of aflatoxin. A number of questions still remain as to the best use of atoxigenic formulations. This research has provided new insight into the appropriate spacing for field plot designs, differences in the characteristics of the two available atoxigenic strain for grower use, and provides new information on other factors that affect *A. flavus* biology.

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## APPENDIX

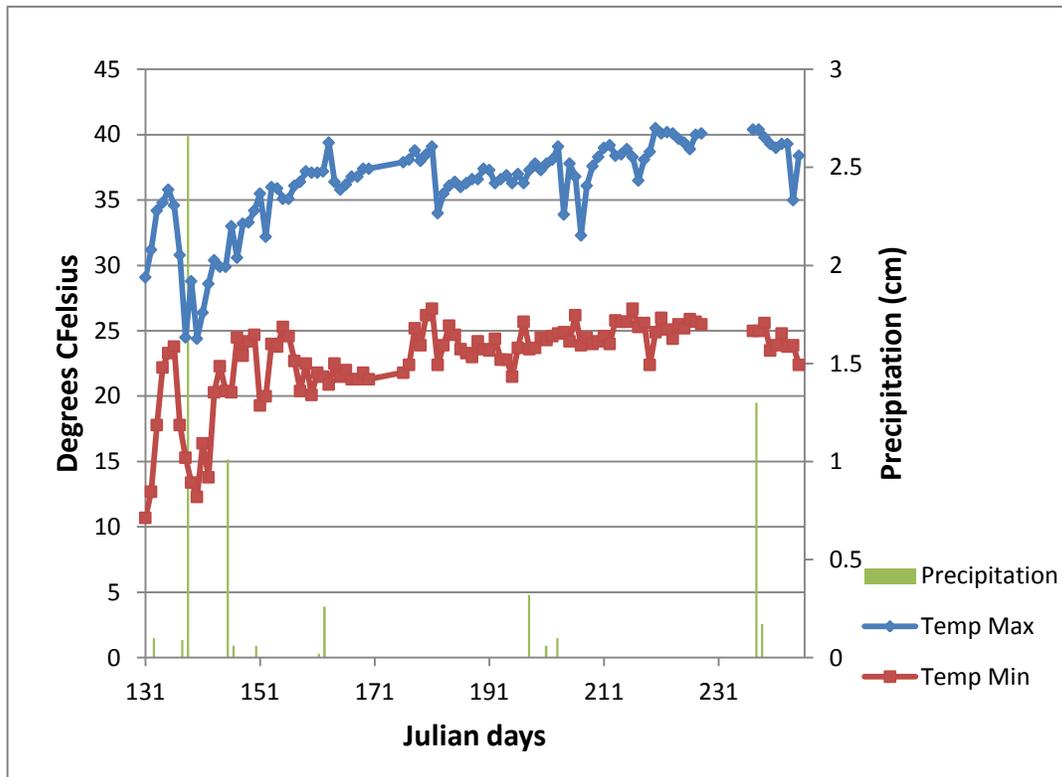


Figure 44: 2011 weather data.

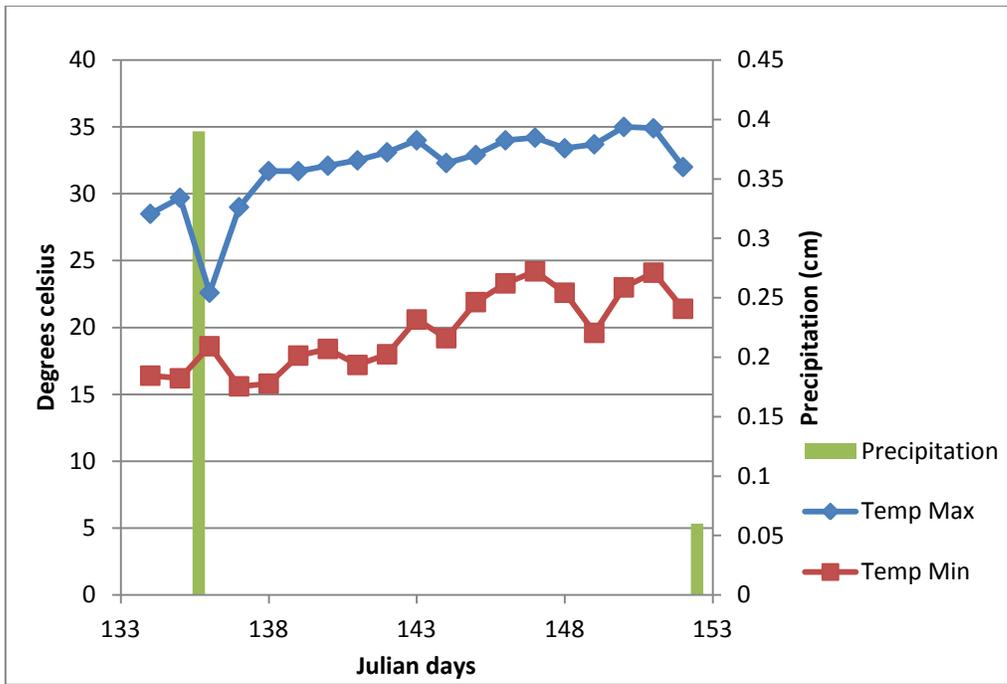


Figure 45. 2012 weather data.

## VITA

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