THE ROLE OF GENETIC AND PHENOTYPIC DIVERSITY IN MAIZE AND ITS EFFECTS ON AFLATOXIN ACCUMULATION BY THE FUNGUS *ASPERGILLUS FLAVUS*

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

THE ROLE OF GENETIC AND PHENOTYPIC DIVERSITY IN MAIZE AND ITS EFFECTS ON AFALTOXIN ACCUMULATION BY THE FUNGUS *ASPERGILLUS FLAVUS*

Presented by Dana Bush

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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I would like to thank my parents, Terry and Karen Woodruff, for their support over the years. I would also like to thank the love of my life, Broderick Bush, who has stood by my side through my entire undergraduate and graduate career. Without the humor of my siblings, Derrick and Terra Woodruff, I don't think I could have kept as positive of an outlook during the last few years of graduate school. I would like to dedicate this especially to my daughter, Merrick Lynn Bush, who has been the biggest joy in my life.

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LIST OF ABBREVIATIONS

Abbreviation	Nomenclature
a1	*anthocyaninless1*
a2	*anthocyaninless2*
ABA	abscisic acid
adh1	*alcohol dehydrogenase1*
ae1	*amylose extender1*
AFB1	aflatoxin B1
aflC	*aflatoxin regulator C*
aflR	*aflatoxin regulator R*
aflJ	*aflatoxin regulator J*
Amy1	*alpha amylase 1*
ANOVA	ANalysis Of Variance
avnA	*averantin A*
avn1	*averufanin oxidoreductase1*
b1	*colored plant1*
bp	base pair
Bt	*Bacillus thuringiensis*
bt1	*brittle1*
bt2	*brittle2*
bz1	*bronze1*
bz2	*bronze2*
C	cytosine
c1	*colored aleurone1*

c2..... *chalcone synthase*2

c2-Idf..... *chalcone synthase2-inhibitor diffuse*

chi .....*chalcone isomerase*

CHIP .....chromatin immunoprecipitation

CoAS..... acetyl coA

cyp450..... *cytochrome P450*

d3 ..... *dwarf3*

D8 ..... *Dwarf*

DNA..... deoxyribonucleic acid

ELISA .....Enzyme-Linked ImmunoSorbent Assay

fas1.....*fatty acid synthase1*

fas2.....*fatty acid synthase2*

FDA ..... Food and Drug Administration

GA.....Gibberellic Acid

GC.....guanine cytosine

Gl15 .....*Glossy15*

GSH .....glutathione

GSTK..... glutathione S-transferase kappa

HCC ..... hepatocellular carcinoma

HO_2 .....hydroperoxyl ion

H_2O_2..... hydrogen peroxide ion

HPLC ..... High Performance Liquid Chromatography

IARC..... International Agency for Research on Cancer

IBM……..Intermated B73 x Mo17

InDel .....insertion deletion

kb ..... kilobase

Mb.....megabase

MIC.....minimal inhibitory concentration

myb.....*myeloblastosis-type transcription factor*

myc.....*myelocytomatosis-type transcription factor*

NCARS ..... North Carolina Agricultural Research Service

NASS ..... National Agricultural Statistical Service

nor1 .....*norsalonic acid1*

NRRL..... Northern Regional Research Laboratory

NS .....nonsignificant

$O2^{(-)}$.....radical oxygen ion

OH..... hydroxyl

omtA..... *O-methyl transferase A*

P1 ..... *pericarp color1*

Per1.....*peroxiredoxin1*

pks1 ..... *polyketide synthase1*

pksA.....*polyketide synthaseA*

pl1 .....*purple plant1*

ppb ..... parts per billion

ppm ..... parts per million

PDA……..Potato Dextrose Agar

PROC GLM ..... Procedure General Linear Model

PROC CORR ..... Procedure Correlation

r1 ..... *colored1*

Rht ..... *reduced height*

ROS ..... Reactive Oxygen Species

sh1 ..... *shrunken1*

sh2 ..... *shrunken2*

SFR ..... Super Fine Resolution

SNP ..... Single Nucleotide Polymorphism

SSR ..... Simple Sequence Repeats

su ..... *sugary*

su1 ..... *sugary1*

T ..... thymine

TA ..... thymidine adenine

US ..... United States

vbs ..... *versicolorin synthase B*

ver1 ..... *versicolorin reductase1*

whp1 ..... *white pollen1*

wx1 ..... *waxy1*

THE ROLE OF GENETIC AND PHENOTYPIC DIVERSITY IN MAIZE AND ITS EFFECTS ON AFALTOXIN ACCUMULATION BY THE FUNGUS *ASPERGILLUS FLAVUS*

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ABSTRACT

Accumulation of the potent carcinogen, aflatoxin, in maize poses a significant health risk to humans and animals. Most commercially grown maize lines are susceptible to infection by the fungus that synthesizes aflatoxin, and toxin levels can accumulate to dangerously high levels under the influence of environmental factors–such as drought, heat, and insect damage–that are typical of many corn growing regions. Aflatoxin levels can also be modulated by the type and amount of sugar in the kernels and by the presence of reactive oxygen species (ROS) and antioxidants. How these factors interact to control aflatoxin levels is poorly understood.

The objective of this research was to identify new sources of low aflatoxin maize germplasm and to discover underlying genetic mechanisms leading to low aflatoxin accumulation in maize. Aflatoxin levels were quantified in a large set of diverse maize lines in three locations–Missouri, Georgia, and Mississippi. As expected from previous studies, lines with tropical origins were found to have low aflatoxin levels, but temperate sources of low aflatoxin were also identified. Stiff stalk, popcorn, and sweet corn lines were not good sources of low aflatoxin. Correlations were sought between aflatoxin levels and a suite of previously defined, agronomically important traits for the diversity lines. Significant correlations were found for brace root traits, ear and plant height, and flowering time. A search for associations between aflatoxin levels and

DNA sequence polymorphisms for genes involved in various kernel and plant architecture traits revealed significant associations for haplotypes of *brittle2*, *dwarf3*, and *Dwarf8* (*D8*). Kernel reactive oxygen species (ROS) levels were not correlated with aflatoxin at 25 days after pollination, but significant correlations were found between aflatoxin and carotenoid levels. Significant associations between aflatoxin levels were identified in genes–*anthocyaninless1*, *colorless2*, and *white pollen1*–encoding antioxidants from the flavonoid pathway. Together, the results of these studies identify new sources of low aflatoxin germplasm and suggest avenues for further investigation of factors that regulate aflatoxin accumulation.

Chapter 1. Introduction

Overview

Aflatoxin contamination of maize kernels poses a significant risk to human health. Consequently, the United States Food and Drug Administration has strict guidelines (Compliance Policy 7126.33, section 683.100) that limit toxin levels in products for human consumption to 20 parts per billion (ppb) for grain and 0.5 ppb for milk products. Slightly higher levels are allowed for some animal feeds (200 ppb for finishing swine and 300 ppb for finishing cattle). Annual aflatoxin-related crop losses are estimated to be over $160 million (Wu, 2006). These losses accrue because all current commercially available maize germplasm is highly susceptible to aflatoxin accumulation. The goals of this study were to identify new germplasm sources and associated traits that correlate with low aflatoxin accumulation.

This chapter provides a brief overview of the health risks of aflatoxin, the fungal source of aflatoxin, the factors known to affect aflatoxin accumulation, and the maize resources used in this study.

Health Risks Associated with Aflatoxin

Aflatoxin, which is produced by *Aspergillus flavus* and *A. parasiticus*, poses a number of health risks. Dietary exposure can lead to cancer and aflatoxicosis, whereas occupational exposure to airborne fungal spores can lead to respiratory infection.

Cancer results from conversion of ingested aflatoxin to exo-8, 9-epoxide and other metabolic products by the P450 detoxifying group of liver enzymes (Smela et al.,

2001). The resulting products can damage DNA and induce mutations, specifically a GC→TA transversion in the p53 gene. In hepatocellular carcinomas (HCCs) within some populations, the p53 gene is mutated in >50% of the tumors (Bressac et al., 1991; Hsu et al., 1991). Aflatoxins have been found to act adjuvantly with the hepatitis B virus in the promotion of liver cancer (Groopman and Kensler, 1996; Montesano et al., 1997). Susceptibility to aflatoxin is determined by several factors such as liver detoxification capabilities, genetic makeup, age, and other nutritional factors.

Consuming highly aflatoxin infected grain can also cause a poisoning known as aflatoxicosis, with symptoms that include hemorrhage, liver damage, edema, and digestive difficulties. Human cases of this illness have been reported in China, India, and Africa (Bosch et al., 1999; Eaton and Groopman, 1994; Krishnamachari et al., 1975). In Kenya in July 2004, 125 of 317 reported cases of aflatoxin poisoning resulted in fatality (Centers for Disease Control and Prevention, 2004).

Occupational exposure of workers in granaries, oil mills, and other agricultural product handling facilities to contaminated grain can lead to infection by *Aspergillus* spores. Three common types of spore-caused infection are allergic bronchopulmonary aspergillosis, pulmonary aspergilloma, and invasive aspergillosis (Stevens et al., 2000).

Aspergillus flavus

A. flavus, the primary producer of aflatoxin, is an anamorphic soil-borne fungus, which can be identified by a change in colony color, from yellow to green, with age. The fungus reproduces rapidly, reaching a diameter of 6-7 cm in 10 d at 24-26^{o}C (Raper et al., 1965). The vegetative cells of *A. flavus* are haploid hyphae that extend by branching and function in the acquisition of nutrients. Although *A. flavus* is hypothesized to reproduce

sexually, it primarily reproduces through vegetatively produced spores called conidia (Figure 1). Germination occurs during hot humid weather from sclerotia or spores that have overwintered in crop debris and on the soil surface. After germination occurs, hyphae develop to a branched mycelium and then specialiazed conidiophores, which produce conida. The conidia serve as the primary inoculum for infections of plants and animals (Calvo et al., 2002). *A. flavus* has low host specificity, infecting a wide range of plants, including agricultural crops such maize, rice, peanuts, figs, pistachios, almonds, cotton, and many other crops (Raper et al., 1965; St. Leger et al., 2000).

Maize is susceptible to aflatoxin contamination after silks have emerged, during pollination, and until maize is harvested and dried down to less than 12-13% moisture (Munkvold, 2003). Spores land on the sticky silks, germinate, and grow down the silk channels to penetrate the maize kernel (Vincelli et al., 1996).

Factors That Influence Aflatoxin Accumulation

Once an agricultural product is found to be contaminated with *A. flavus* few detoxification measures are available. Thus, strategies for reducing aflatoxin contamination have focused on defining the factors that influence aflatoxin accumulation and on breeding crops that produce low aflatoxin levels. In maize, low aflatoxin accumulation in maize is a quantitative trait with large genotype by environment interaction (Campbell and White, 1995a; Williams et al., 2008). Environmental conditions, such as water stress, high temperature, and insect damage create favorable conditions for infection in the preharvest period (Calvo et al., 2002; Munkvold, 2003; Vincelli et al., 1996). Other factors known to influence aflatoxin production are carbon

source, reactive oxygen species (ROS), and antioxidants, such as flavonoids and terpenoids.

Insect damage. Fungal spores can be introduced to the maize ear through insect damage, and levels of aflatoxin accumulation in insect-damaged grain have been documented to be in the parts per million range. A study aimed at testing whether insect-resistant Bt corn accumulates less aflatoxin tested eight hybrids in one year and ten hybrids in a second year. The results indicated that Bt plants inoculated by sprayng with *A. flavus* in the presence of southwestern corn borer accumulated less toxin than non-Bt plants. However, if the plants were inoculated by needle puncture of the ears, no difference in aflatoxin content was observed between Bt and non-Bt corn (Williams et al., 2005). These data support the indirect role of Bt corn in reducing the spread of *A. flavus* by insect feeding, but show that once the fungus is present, Bt corn has no direct ability to reduce aflatoxin contamination.

Time to harvest. The time to harvest can affect the amount of aflatoxin accumulation. If grain dries slowly in the field, the moisture content remains high enough to support continued fungal growth, aflatoxin production, and sclerotiu production (Munkvold, 2003). Insects may also continue to feed during this time and spread the fungus.

Carbon source. The presence of glucose, maltose, or maltotriose is may induce aflatoxin production (Woloshuk et al., 1996). Woloshuk et al. (1996) demonstrated that readily metabolized carbon sources from the glucose and pentose phosphate pathway are the best inducers of aflatoxin production. When these carbon sources were present, degenerate mitochondria, ethanol accumulation, and increased expression of *adh1* in the

fungus were observed. Taken together, these observations suggest that the fungus may be metabolizing the sugars through alcoholic fermentation. The importance of simple sugars as a nutrient source was underscored by analysis of *A. flavus* mutants defective for *Amy1*, the single starch-degrading fungal α-amylase. When grown on a starch-containing medium, mutants lacking this enzyme showed a 50% reduction in growth and failed to produce aflatoxin (Fakhoury and Woloshuk, 1999).

This sugar-mediated effect on aflatoxin appears to be mediated by chromatin changes that alter expression of the aflatoxin genes. The 27 aflatoxin biosynthetic genes occur within a 70 kB cluster (Roze et al., 2007). *aflR* is a positive regulator of transcription of most aflatoxin structural genes (Ehrlich et al., 1998; Woloshuk et al., 1994). *aflJ* is necessary for the expression of other genes in the biosynthesis cluster (Meyers et al., 1998). Roze et al. (2007) studied regulation of the genes in the aflatoxin biosynthetic cluster using chromatin immunoprecipitation (CHIP) assays and developed a model for expression of the 27 genes. Their data suggest that reduction in the glucose/sucrose ratio initiates a signaling cascade that leads to histone H4 acetylation between the *pksA* and *nor1* genes. Histone acetylation then proceeds bi-directionally from this point throughout the aflatoxin biosynthetic gene cluster.

ROS and antioxidants. ROS also play a role in aflatoxin accumulation (Chang et al., 2007). ROS are oxygen ions (O_2^-), free radicals (HO_2), and peroxides (H_2O_2) that are highly reactive because of unpaired electrons. They are produced by metabolism in various cell compartments (Gechev et al., 2006) and, under stress conditions, can accumulate to high levels. Overproduction of free radicals can cause cell death, as is the case with plant hypersensitive response to some pathogens. Free radicals can also

oxidize amino acids and affect protein function (e.g., oxidation of cysteine to cystine and conversion of arginine and proline to glutamyl semialdehyde).

Higher levels of glutathione (GSH) S-transferase kappa (GSTK) in the fungus lowers aflatoxin levels by protecting the fungus during respiration when large amounts of ROS are being produced (Nebert and Vasiliou, 2004). Plant antioxidants such as gallic acid have been demonstrated to reduce aflatoxin levels and ROS (Mahoney and Molyneux, 2004). Mahoney and Molyneux (2004) suggest aflatoxin production is a result of increased oxidative stress and that gallic acid overrides the stress response to prevent high levels of aflatoxin accumulation. Magbanua et al. (2007) evaluated hydrogen peroxide and salicylic acid levels in maize embryos from two resistant and two susceptible maize lines. They observed a significant reduction in hydrogen peroxide in resistant compared to susceptible maize embryos along with a significant increase in salicylic acid. In addition, they observed an increase in *catalase3* activity in the resistant lines.

Research on flavonoids and *A. flavus* demonstrated that some flavonoids have antifungal properties. Flavonoids are phenolic compounds that act as potent metal chelators and free radical scavengers (Middleton et al., 2000). The role of flavonoids on *A. flavus* mycelial mass and aflatoxin levels was studied by using suspended disk cultures (Norton, 1999). Comparison of the 3-hydroxy compounds, pelargonidin to delphidin, to their 3-deoxy counterparts revealed inverse and opposite relationships on fungal growth and aflatoxin levels. The 3-hydroxy compounds showed a three-fold greater reduction of aflatoxin levels, but stimulated growth, whereas the 3-deoxy compounds had higher

aflatoxin levels, but no growth stimulation. None of the compounds tested reduced both mycelial mass and aflatoxin levels.

Gordon et al. (1980) tested the effect of isoflavonoid phytoalexins [(+)-pisatin, (-)-phaseollin, (-)-phaseollin isoflavan, (±)-demethylhomopterocarpin, (±)-maackiain, (±)-vestitol, and (±)-sativan] on various zoopathogenic fungi. Of the three compounds tested on *A. flavus* pisatin (>100 μg/ml), phaseollin (>100 μg/ml), and phaseollin (50 μg/ml) isoflavan were the minimal inhibitory concentrations (MIC) needed to prevent fungal growth. Of the seven phytoalexins tested on *A. fumigatus*, a MIC of at least 100 μg/ml was needed to inhibit growth except with pisatin which required only 50 μg/ml (Gordon et al., 1980).

The maize anthocyanin and *A. flavus* aflatoxin biosynthetic pathways share some commonalities (Figure 2). Chalcone synthase, *c2*, is a polyketide synthase that is the rate limiting step in anthocyanin biosynthesis in maize. Polyketide synthase, *pks1*, is the rate limiting step in aflatoxin synthesis in *A. flavus*. *AflR* and *P1* are both *myb* homologs. Both the aflatoxin and anthocyanin synthesis pathways begin with the addition of acetate molecules through polyketide synthase (*pksA* versus *c2*). The fungal polyketide is converted to norsaloronic acid by isomerization which is the same type of reaction catalyzed by chalcone isomerase in maize. The reduction reactions controlled by *a1* in maize and *nor1* in *A. flavus* are also similar. Later portions of the fungal pathway correspond to reactions that occur in the phlobaphene pathway (*not shown*). The similarities in both of these biosynthetic pathways led to experiments to determine if defects in anthocyanin biosynthesis in maize affect fungal aflatoxin production. Maize mutants were inoculated with *A. flavus* and aflatoxin accumulation was measured. When

c2 was defective, seven times more toxin was produced (G. Davis, *unpublished data*). A near-isogenic line containing the mutant *C2-Idf* allele had a nine-fold increase in toxin production. When *c2* was defective and the recessive loss-of-function *sugary1* allele was homozygous, a 23-fold increase in toxin production was seen. This synergism is postulated to be due to the combination of the anthocyanin mutant with a kernel containing high levels of sucrose, the preferred fungal carbon source (G. Davis, *personal communication*).

Terpenoids have antioxidant properties and are precursors to carotenoids. Terpenoids from *Trichodesma amplexicaule* were isolated and tested on four bacteria and four fungi that are common plant pathogens. The calculated activity index range was 0.34 to 0.93 for *A. flavus* growth (Singh and Singh, 2003).

Maize Resources

A large amount of molecular diversity is reflected in the natural phenotypic variation observed among maize inbreds. Molecular studies aimed at characterizing that diversity have defined a set of approximately 300 lines, called the maize diversity lines. Analysis of these lines indicates that on average two maize inbreds are as genetically distant as humans and chimpanzees (Buckler et al., 2006). The maize diversity lines capture over 80% of the simple sequence repeat (SSR) diversity in maize (Liu et al., 2003).

The finding that linkage disequilibrium in a subset of the maize diversity lines extends only about 2,000 bp on average across the genome (Remington et al., 2001) enabled development of association genetics approaches for identifying genes associated with a number of traits (reviewed in Flint-Garcia et al., 2005). An important finding from

these studies was that robust associations required population structure to be considered. Because many maize inbreds are related by through their breeding history, failure to account for alleles shared by descent can lead to false genotype to phenotype associations.

Candidates for association analysis can be readily identified utilizing the 2005 Intermated B73 x Mo17 (IBM) Neighbors genetic map which contains 33,990 markers including 1918 markers anchored to the physical map (www.maizegdb.org). A draft sequence for the 2500 Mb genome is available and can also be used to identify additional potential candidate genes underlying complex traits (http://mips.gsf.de/proj/plant/jsf/maize/index.jsp).

Goals of This Study

The overall goal of this study was to exploit the maize diversity lines to identify genes and/or agronomic traits that account for low aflatoxin accumulation in maize. Three interrelated strategies were used.

First, aflatoxin levels were measured in each of the diversity lines. Most low aflatoxin lines reported to date are of tropical origin. Our goal was to identify novel sources of low aflatoxin that might be useful for breeding programs. Results of this experiment are in Chapter 2.

Second, we took advantage of published gene sequences and agronomic trait data for the diversity lines to look for correlations with aflatoxin levels. Because aflatoxin levels are expensive and time-consumng to measure on large numbers of samples, we hoped to find surrogate phenotypes (agronomic trait or gene sequence) that could be used

in breeding programs aimed at developing low aflatoxin lines. Chapter 3 describes the results of this analysis.

Third, to explore the involvement of ROS and antioxidants in aflatoxin accumulation, we measured ROS levels in the diversity lines and used those measurements, together with published data on carotenoid levels, to look for correlations with aflatoxin levels. Results are presented in Chapter 4.

The conclusions of all three experiments, together with considerations for future studies, are summarized in Chapter 5.

Finally, results from an experiment aimed at directly measuring the inhibitory effect of flavonoid compounds on growth of *A. flavus* are presented in the Appendix.

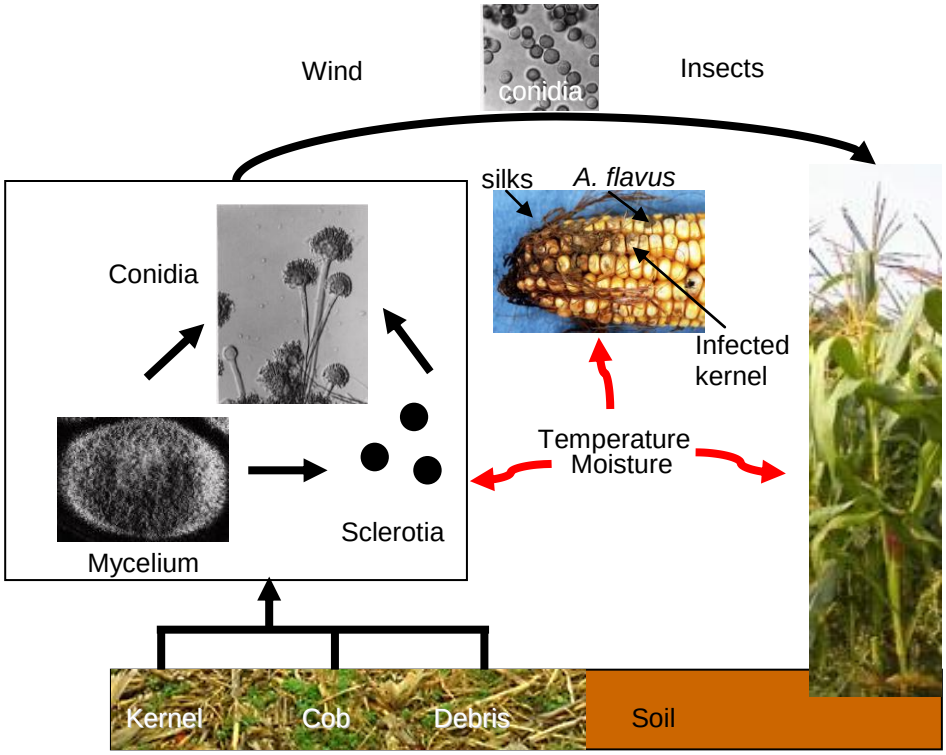


Figure 1. The life cycle of *A. flavus*.

Image adapted from http://www.aspergillusflavus.org/images/cycle.jpg.

Fungal images from: http://www.cbs.knaw.nl.

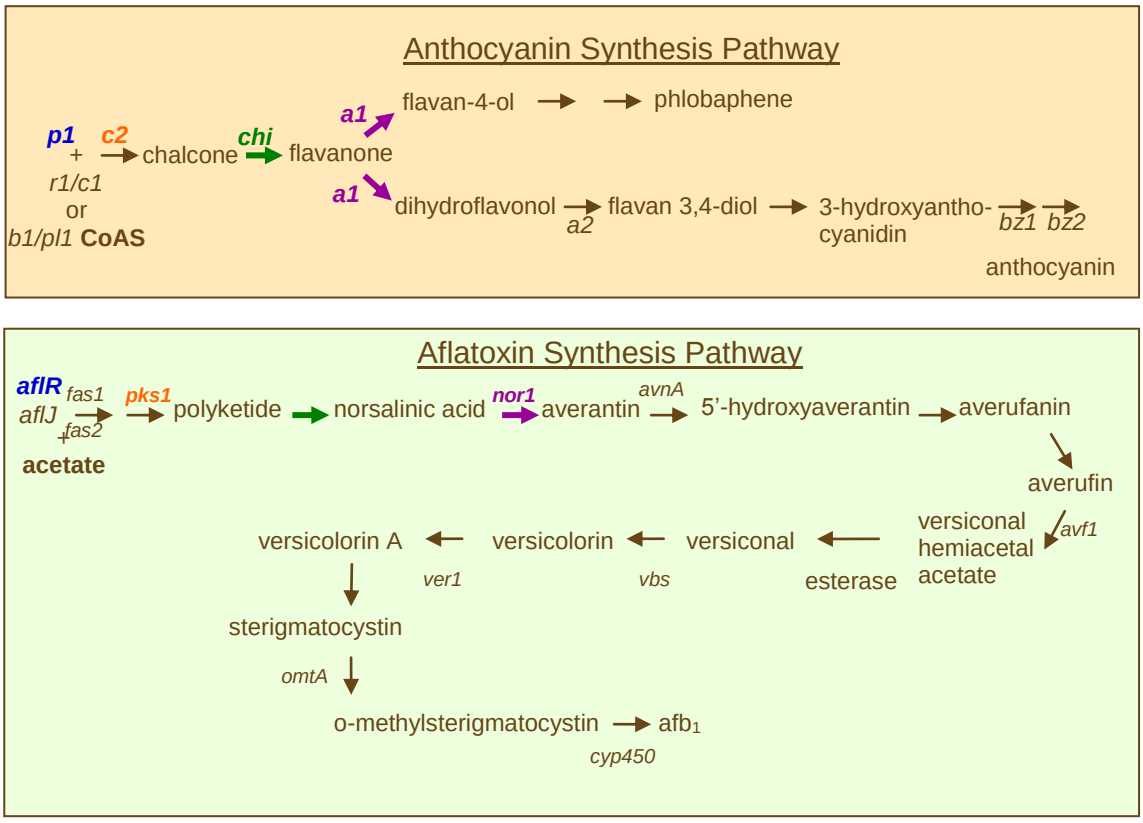


Figure 2. Upper panel: The anthocyanin biosynthetic pathway of *Zea mays* L. (Adapted from: Nueffer et al., 1997). Lower panel: The aflatoxin biosynthetic pathway from *Aspergillus flavus* (Bhatnagar et al. 2006).

Chapter 2. Temperate and Tropical Low Aflatoxin *Zea mays* L. Germplasm

Introduction

Aflatoxin is a polyketide secondary metabolite produced by some strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin consumption is associated with increased risk of liver cancer in mammals (reviewed in Hussain et al., 2007; McGlynn et al., 2003). Infants ingesting aflatoxin experience higher rates of growth impairment and stunting (Gong et al., 2004). Immune suppression is observed in both children and adults exposed to aflatoxin (Jiang et al., 2005; Turner et al., 2003). These negative health effects occur at higher rates in developing countries due to reduced dietary diversity and lack of testing for contaminants in food (Shephard, 2008). The U.S. Food and Drug Administration (FDA) has set strict limits on the maximum level of aflatoxin in maize for human (20 ng g^{-1}) and animal consumption (300 ng g^{-1} for finishing cattle; lesser amounts for poultry and swine).

Genes in both the fungus and the plant contribute to preharvest aflatoxin levels. In maize, low aflatoxin contamination is a quantitative trait with both additive and dominant gene action and significant genotype x environment interactions (Bétran et al., 2002; Campbell and White, 1995a; Williams et al., 2008). Increased temperature or limiting water have been associated with increases in aflatoxin content in maize (Rodriguez-Del-Bosque, 1996; Thompson et al., 1980; Wiatrak et al., 2005; Widstrom et al., 1978). Hawkins et al. (2008) determined that the silking interval represents the time when environmental stress most significantly impacts aflatoxin content.

A limited number of low aflatoxin maize lines have been identified. These correspond to tropical *per se* or tropically-derived germplasm and can be used in breeding programs, but they frequently introduce other undesirable agronomic characteristics (e.g. late maturity) during the breeding process. No temperate lines with low aflatoxin have been identified (Bétran et al. 2002; Campbell and White, 1995b; McMillian et al., 1993; Scott and Zummo, 1990, 1992; Williams and Windham 2001, 2006).

Maize has a high level of genetic diversity compared to other crops. Molecular evidence suggests two maize inbreds are as genetically distant on average as human and chimpanzee (Buckler et al., 2006). Yamasaki et al. (2005) observed similar levels of genetic diversity in temperate and tropical maize lines based on analysis of 1095 maize genes. These data suggest it should be possible to identify temperate maize lines with low aflatoxin content. Given the large impact of environment on aflatoxin content, the quantitative nature of the trait, and the extreme level of genetic variation that is present in maize, evaluation of a broad germplasm sample should result in a more complete understanding of the potential sources of genes for reduced aflatoxin contamination in maize.

A large amount of molecular diversity is reflected in the natural phenotypic variation observed among maize inbreds. Molecular studies aimed at characterizing that diversity have defined a set of approximately 300 lines, called the maize diversity lines. The objective of this experiment was to identify maize germplasm with low aflatoxin content within the maize diversity lines, particularly temperate sources of low aflatoxin content.

Methods and Materials

Germplasm and locations. A subset of lines from the maize diversity set were grown during the summers of 2003 and 2005 at the Genetics Research Farm, University of Missouri-Columbia, Columbia, MO. They were: 38-11, A441-5, A554, A6, A619, A632, B104, B14A, B37, B68, B73, B84, B97, CI187-2, CM105, CM174, CML10, CML52, CML69, CML91, CML103, CML228, CML247, CML358, CML261, CML277, CML287, CML322, CML333, CMV3, D940Y, EP1, F2, F7, F44, F2834T, GA209, GT112, H95, H99, HP301, I137TN, I29, IA2132, IDS28, IL101, IL14H, IL677A, K55, Kui3, Kui11, Kui21, Kui43, Kui44, Kui2007, Ky21, M162W, M37W, Mo17, Mo24W, Mp313E, Mp420, Mp714, Mp717, MS71, MS153, N192, N28Ht, NC250, NC258, NC320, NC338, NC348, NC350, NC352, NC254, NC358, ND246, Oh43, Oh7B, P39, Pa91, SA24, SC212M, SC213R, SC55, SG18, T232, T8, Tx303, Tx601, Tzi8, Tzi18, U267Y, Va26, Va35, W117Ht, W153R, W182B, and W64A. In 2005 two more resistant lines, Mp715 and Mp717, were added to the set. Plots were 6.10 m long with 0.61 m spacing and consisted of 20 plants per row. The experimental design was a randomized complete block with three replications.

In 2005 the study was also conducted at the R. R. Foil Plant Science Research Center, Mississippi State University, Starkville, MS and the Coastal Plain Experiment Station, University of Georgia, Tifton, GA. The Starkville, MS plots contained 20 plants per row with 5.1 m length and 0.96 m spacing. The experimental design was a randomized complete block with four replications. The Tifton, GA plots had 20 seeds

per row with 4.8 m length spaced 0.9 m apart in an alpha-lattice design with four replications.

Inoculation and aflatoxin measurement. *A. flavus* isolate NRRL 3357 was used as inoculum. At the Columbia, MO location inoculum was grown on sterile corn grits in 500-ml flasks, each containing 50 g grits and 100 ml H_2O, and incubated at 37^oC for 3 wk. Conidia were washed from the grits using 500 ml sterile distilled water and filtered through four layers of sterile cheesecloth. The concentrations of conidia were determined with a hemocytometer and adjusted with sterile distilled water to $1x10^6$ conidia per ml. Inoculum was kept at 4^oC until use. At the Starkville, MS location inoculum was increased on sterile corn cob grits in 500-ml flasks, each containing 50 g grits and 100 ml H_2O, and incubated at 28^oC for 3 wk. Conidia were washed from the grits using 500 ml sterile distilled water containing 20 drops of Tween 20 (Acros Organics, Fair Lawn, NJ) per liter and filtered through four layers of sterile cheesecloth. The concentrations of conidia were determined with a hemocytometer and adjusted with sterile distilled water to 9×10^7 conidia per ml. Inoculum not used immediately was refrigerated at 4^oC. At the Tifton, GA location the isolate was maintained on silica gel. To create the spore suspension silica chips were first sprinkled on potato dextrose agar (PDA) plates. After four or five days, spores were transferred to PDA tubes. A suspension was made by adding distilled water and Tween 20 and inoculating a small flask containing PDA. Sterile water and Tween 20 were subsequently added to reach the desired spore concentration of 1×10^6 spores per ml of solution.

At the Columbia, MO location, the top self-pollinated ear of each plant was inoculated with *A. flavus* conidia 19 d after pollination using the side-needle technique

(Zummo and Scott, 1989). At Starkville, MS, a needle was inserted through the husk and a 1 ml suspension containing 1 x 10^6 conidia was injected. The top ear of each plant was inoculated with *A. flavus* 7 d after midsilk using the side-needle technique (Zummo and Scott, 1989) by injecting a 3.0-ml suspension containing 3 x 10^8 conidia. At Tifton, GA, inoculations were performed 20 days after mid-silk, using a paring knife dipped into the inoculum and inserted through the husk on the side of the ear at 20 d after mid-silk.

For harvesting, lines were harvested at Columbia, MO, at maturity by hand, dried at 40.5^{o}C for 5 d and 12% humidity, shelled, and bulked within row. Plots were shelled, mixed, and 250 g was ground using a stein mill. Three 2g samples of each combination of line, replicate, and year were weighed from the 250 g sample into 15 ml tubes for aflatoxin extraction. Aflatoxin was extracted using 10 ml 70% methanol (Fisher Scientific, Fair Lawn, NJ), 30% water, and 0.5% NaCl (Fisher Scientific, Fair Lawn, NJ). Aflatoxin was quantified using a direct competitive anti-aflatoxin rabbit IgG antibody enzyme-linked immunosorbent assay (ELISA; Sigma, St. Louis, MO) that detects aflatoxin levels greater than 1 ng g^{-1} (ppb). Aflatoxin standards in ng g^{-1} (ppb) were: 0, 12.5, 25, 50, 100, 200, 400, 800, and 1600. The sensitivity of this ELISA assay is comparable to that of high performance liquid chromatography (HPLC).

At the Starkville, MS location, ears were allowed to open pollinate. All inoculated ears in each plot were harvested by hand at kernel maturity, *ca*. 63 days after midsilk, and dried at 38^{o}C for 7 d. Ears were machine shelled and grain samples from each row were poured into a sample splitter twice to mix the grain. Grain samples for aflatoxin analyses were ground using a Romer mill (Romer Laboratories Inc., Union, MO). Aflatoxin contamination in a 50 g subsample from each plot was determined using

the VICAM AflaTest® (VICAM, Watertown, MA). This procedure can detect aflatoxins (B_1, B_2, G_1, G_2) at concentrations as low as 2 ng g^{-1}.

Ears were allowed to open pollinate at the Tifton, GA location and all plots were harvested at 60 d post mid-silk. The ten inoculated ears were oven-dried with forced air for at least 3 d at 60ºC. Samples were shelled, bulked, and ground using a Romer mill. Aflatoxin concentrations were determined from a 100 g sample using the VICAM AflaTest®.

Statistical analysis. Mississippi and Missouri aflatoxin content data were transformed using the function, $y'=\ln(y+1)$, and Georgia data were transformed using the function, $y'=\sqrt{(y+1)}$, to normalize the data. Statistical analysis was conducted based on a linear mixed-model where years, replications, and subsamples were random effects and lines were a fixed effect. The model for the Missouri data was:

$$Y_{ijkl} = \mu + A_i + R_j + L_k + AR_{ij} + AL_{ik} + RL_{jk} + ARL_{ijk} + S_{l(k)} + \varepsilon_{ijkl}$$

where μ = experiment-wide mean, A_i =effect of year i, R_j =effect of replication j, L_k = effect of line k, AR_{ij} = the interaction of year i with replication j, AL_{ik} = the interaction of year i with line k, RL_{jk} = the interaction of replication j with line k, ARL_{ijk} = the interaction of year i with replication j and line k, $S_{l(k)}$ = the effect of subsample l nested within line k, and ε_{ijkl} = the residual effect.

The model for the Mississippi and Georgia data sets was:

$$Y_{ij} = \mu + L_i + R_j + \varepsilon_{ij}$$

where μ = experiment-wide mean, L_i= effect of line i, R_j = effect of replication j, and ε_{ijkl} = the residual effect. ANOVA was performed using the PROC GLM model in SAS version 9.0 (SAS Institute Inc., Cary, NC). The Student-Newman- Keul's multiple-

range-test was performed to classify each line into groups and look for significant differences among lines at the α=0.05 level. The multiple range tests were performed within the structure-based subpopulations determined by Liu et al. (2003); i.e., Stiff Stalk, Non-Stiff Stalk, Tropical-Subtropical, Sweet Corn, Popcorn, and Mixed.

Phylogenetic tree construction. All but five of the lines examined in this study were previously genotyped with ninety-four simple sequence repeat (SSR) markers by Liu et al. (2003). To incorporate the five uncharacterized linesinto the phylogenetic tree with the other lines, SSR genotyping was performed. DNA was extracted from fresh seedling tissue of the remaining five lines, SC212M, Mp313E, Mp420, Mp717, and Mp715, using DNAzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Lines B73 and Mo17 were also included in our analysis to enable direct comparison with the fragment sizes generated by Liu et al. (2003) where they were also measured. Ninety-four SSR markers evenly distributed throughout the genome were amplified across the seven maize lines and electrophoresed in 4% SFR agarose with a 100 bp molecular weight ladder until separated (Supplementary Table 1). Gel images were digitized and fragment sizes determined on an Alpha Imager (Alpha Innotech, Carlsbad, CA). Fragment sizes were adjusted if needed based on comparison of B73 and Mo17 fragments between our experiment and the Liu et al. (2003) dataset to account for small differences in DNA migration. Our data for these seven lines was integrated with data from the 102 remaining lines previously obtained by Liu et al. (2003). The DNA fragment sizes were analyzed using MSAnalyzer (Dieringer and Schlötterer, 2003) and PHYLIP (Felsenstein, 1989) and the resulting data was visualized using PhyloDraw (Choi et al., 2000).

Results and Discussion

Before this study, a large scale survey for low aflatoxin lines in maize was lacking. Previous aflatoxin germplasm surveys had examined relatively small numbers of plants with lines which were often related to one another (Betrán and Isakeit, 2004; Guo et al., 2004; Hawkins et al., 2008; Lillehoj et al., 1974, 1983, 1987). In addition, some of the prior studies used natural inoculum which makes interpretation difficult (Wiatrak et al., 2005). Allelic diversity studies have detected the same amount of diversity in temperate and tropical maize (Yamasaki et al., 2005). Earlier aflatoxin research indicated the only source of low aflatoxin maize lines are from the tropical germplasm (Bétran and Isakeit, 2004; Jones and Duncan, 1981; Jones et al., 1981); however, molecular studies suggested we should be able to identify temperate low aflatoxin lines. We analyzed preharvest aflatoxin contamination in a broad sample of lines, reflecting the majority of genetic diversity in maize, to test whether temperate low aflatoxin lines existed and to determine the genetic distances among the low aflatoxin lines.

Analysis of variance (ANOVA) indicated that genetic variation in aflatoxin contamination within the maize diversity lines was highly significant (Tables 1–3). Genetic variation represented 77% of the total variation in Mississippi, 72.3% of the total variation in Georgia, and 41% of the total variation in Missouri (Table 1–3). The lower percentage of explained variation in Missouri resulted from the removal of genotype x year variation from the line term where it is confounded in the Georgia and Mississippi analysis.

A number of low aflatoxin lines were identified among the three locations. In Georgia, none of the lines tested had aflatoxin levels less than 20 ng g^{-1} (ppb), the FDA maximum for human consumption (Table 4). Line A6 had the lowest aflatoxin content at 23.34 ng g^{-1} (ppb), but nine other lines in the Tropical group were not significantly different from line A6 in aflatoxin content. These included six lines, U267Y, Hp301, A6, Mp717, CML258, and CML 261, grown in Georgia that contained less than 300 ng g^{-1} (ppb) aflatoxin (the FDA maximum for finishing beef cattle) and three lines with greater than 300 ng g^{-1} aflatoxin. Inoculation and environmental differences may have contributed to the identification of so few maize lines with aflatoxin levels bewlo the 300 ng g^{-1} threshold in Georgia. In Mississippi, one line, Mp313E , had less than 20 ng g^{-1} of aflatoxin (Table 5) and five other lines with aflatoxin levels up to 156 ppb, Tzi18, A6, CML247, NC354, and Mp717, did not differ statistically in aflatoxin content. The Mississippie data showed 13 lines that contained less than the 300 ng g^{-1} threshold for aflatoxin content: Kui43, D940Y, I137TN, F44, NC258, Mp313E, Tzi18, A6, CML247, NC354, Mp717, Kui3 and Tx601 (Table 4). In Missouri, there were nineteen lines with less than 20 ng g^{-1} of aflatoxin (Figures 3, 4 and Table 6): U267Y, I137TN, D940Y, SC55, M162W, Ky21, CML333, CML10, Mp715, Mp313E, Mp717, Kui2007, NC304, A6, Tzi18, CML258, CML277, Kui11, and NC300. Only 31.3% of the lines tested in Missouri had greater than 300 ng g^{-1} of aflatoxin. The majority of the lines identified with low aflatoxin had not previously been tested and represent novel sources of low toxin for use in breeding.

It is important to note that relative rank rather than absolute toxin values should be used to compare maize lines among locations as average toxin levels differed

substantially among locations. Lines in Mississippi and Missouri were ranked very similarly as a whole (Tables 5 and 6). Maize lines grown in Georgia had a few changes in rank but some of the low toxin lines performed well in this location as well as in the two other locations (Table 4). Aflatoxin production was greatest in Mississippi followed by Georgia and then Missouri. While difference in pollination and inoculation method may have contributed to the variability in aflatoxin levels among locations, differences in weather were likely responsibly for much of the variation we observed. Mississippi and Georgia have warmer, more humid climates than Missouri. The effect of weather is further supported by the significant differences in aflatoxin accumulation observed between years in the Missouri data. Both temperature and humidity are known to directly affect fungal growth (Amer, 2005; Diener and Davis, 1967; Kulshrestha et al., 2008; Seenappa and Kempton, 1980). Changes in temperature and humidity are also correlated with changes in toxin production (Amer, 2005; da Silva et al., 2000; Diener and Davis, 1967, 1970; Jurjevic et al., 2007; Mukherjee and Lakshminarasimham, 1995; Ogundero, 1987; Seenappa and Kempton, 1980; Wilson and Jay, 1975). In addition, temperature differences have a significant impact on the level of insect pressure and therefore on the proportion of the ear with damage easily accessible to fungal invasion (Cotty and Jaime-Garcia, 2007; Kurtzman et al., 1987; Magan and Aldred, 2007; Sales et al., 2005).

To address the question of whether temperate low aflatoxin germplasm could be identified we divided the dataset into structure-based subpopulations on the basis of SSR data from Liu et al. (2003) and novel genotypes for five lines Mp313E, Mp420, Mp714, Mp717, and SC212M. Liu et al. (2003) previously used SSR data for 104 lines from the maize diversity set to evaluate the genetic relationship among lines and place them into

six structure-based sub-populations (Stiff Stalk, Non-Stiff Stalk, Tropical, Popcorn, Sweet Corn, and Mixed) each sharing common genetic histories. Two lines within the same group share alleles that are identical by descent at a higher rate than two lines from different groups. The Mixed sub-population contains lines that share less than 80% identity with any of the other five sub-populations. Based on these sub-populations we identified a number of low aflatoxin lines from temperate sub-populations, i.e. Non-Stiff-Stalk and Mixed lines. When grown in Mississippi, Kui43, D940Y, and I137TN were low aflatoxin lines from the Mixed structure-based sub-population while F44 and NC258 were from the Non-Stiff Stalk structure-based sub-population (Table 5). Line U267Y from the Mixed structure-based sub-population had less than 300 ng g^{-1} in Georgia (Table 4). When grown in Missouri lines U267Y, I137TN, D940Y, SC55, M162W, and Ky21 had less than 20 ng g^{-1} and were part of the Mixed and Non-Stiff Stalk sub-populations (Table 6). In the Mixed sub-population, lines D940Y, I137TN, and U267Y were consistently ranked with low aflatoxin content across locations. Line M162W was the only Non-Stiff Stalk line that repeatedly had less aflatoxin across locations. In the Stiff Stalk sub-population, lines B68 and N28HT had smaller amounts of aflatoxin as compared with other lines tested in the three locations. Lines Mp313E, A6, and Mp717 had some of the lowest aflatoxin levels of any lines tested and consistently ranked as less contaminated within the Tropical sub-population. These results dispute the hypothesis that tropical germplasm is the only source of low aflatoxin maize lines.

Table 7 contains a summary of the low aflatoxin lines identified at each of the three locations based on the population structure grouping determined by Liu et al. (2003). The number of lines from each structure-based sub-population was divided by

the total number of lines that were less than 300 ng g^{-1} in Georgia, 300 ng g^{-1} in Mississippi, and 20 ng g^{-1} in Missouri. The Tropical sub-population consistently had the largest percentage of low aflatoxin among the locations however they were not the only source of low aflatoxin germplasm. Sweet Corn, Popcorn, and Stiff Stalk sub-populations were poor sources of low aflatoxin maize. In general, Sweet Corn lines had poor performance with high amounts of aflatoxin in this experiment across all locations. Line IL14H accumulated less than 300 ng g^{-1} of aflatoxin in Missouri however it had substantially more aflatoxin (>7700 ng g^{-1}) in Georgia indicating it is not a suitable source of low aflatoxin alleles. In Mississippi and Missouri, lines with less than 300 ng g^{-1} of aflatoxin were also identified in both the Mixed and Non-Stiff Stalk subpopulations while in Georgia the Mixed sub-population was the only further source of such germplasm. Thus although tropical germplasm offers the greatest proportion of low aflatoxin lines, a few better adapted temperate lines were identified which could be used as new sources of genes for low aflatoxin contamination.

A phylogenetic tree was constructed to determine if low aflatoxin lines were clustered (Figure 5). The distribution of low aflatoxin lines on the phylogenetic tree indicates a single low aflatoxin progenitor line is unlikely. In addition, coupling the phylogenetic information with aflatoxin data provides an important tool for breeders seeking to find lines adapted to their respective locations. The genetic distance information can be utilized to choose pairs of less related, low aflatoxin, adapted lines for use in breeding (Supplementary Table 2).

The large amount of genetic variation surveyed in these experiments enables us for the first time to speculate about the population genetics behind the distribution of low

aflatoxin accumulating lines in temperate and tropical maize. Previous work indicates aflatoxin contamination in maize is controlled by a number of genes with favorable alleles present at low to moderate frequency. The distribution of low aflatoxin lines among the structure groups might suggest low aflatoxin alleles are present in low to moderate frequency in the ancestral gene pool and are maintained by positive selection pressure in the tropical structure group where conditions are routinely favorable for fungal infection and toxin production. The absence of low aflatoxin lines within the sweet corn and popcorn groups could result from small sample size. Alternatively selection for kernel traits in these groups could have inadvertently caused increased susceptibility to *A. flavus* or increased aflatoxin levels. The type and amount of sugar available to the fungus can alter aflatoxin levels. Glucose, sucrose, and maltose have been documented to support high levels of aflatoxin whereas peptone, lactose, and xylose do not (Buchanan and Stahl, 1984; Davis and Diener, 1968; Diener et al., 1987; Reddy et al., 1979; Wiseman and Buchanan, 1987). In the Mixed group, low toxin alleles could be maintained at low frequency. It contains lines which were bred in a multitude of environments including both tropical and subtropical environments. These hypotheses remain to be tested in part by comparison with aflatoxin content in landraces.

Conclusions

This study identified new germplasm to be tested in further field trials for low aflatoxin accumulation. The best lines are from the Mixed, Non-Stiff Stalk, and Tropical pedigrees; they are adapted to a range of environments. The adaptation of the low

aflatoxin temperate lines makes them more suitable sources for Mid-Western breeding programs. This survey information could be used for association analysis; it may also be used to identify SNPs and Indels for breeding. The aflatoxin data generated here will provide a foundation for further understanding of the genes and phenotypes contributing to low aflatoxin in maize which will reduce producer losses, keep consumer prices from rising as quickly, and help reduce or eliminate human and animal cases of aflatoxin poisoning and death.

Table 1. Analysis of variance for aflatoxin contamination in a subset of maize diversity lines grown in Starkville, Mississippi. The goodness of fit for the model was 0.81 and the coefficient of variation was 12.27%.

Source	*DF*	*SS*	*MS*	*Significance*[†]
Rep	3	25.12	8.37	**
Line	85	583.03	6.86	**
Error	196	143.81	0.73	
Total	284	753.52		

[†] ** Significant at the 0.01 probability level.

Table 2. Analysis of variance for aflatoxin content in a subset of the maize diversity lines grown in Tifton, Georgia. The goodness of fit for the model was 0.73 and the coefficient of variation was 25.40%.

Source	*DF*	*SS*	*MS*	*Significance*[†]
Line	83	38650.57	465.67	**
Rep	3	281.29	93.76	NS
Error	158	14621.38	92.54	
Total	244	53410.43		

[†] ** Significant at the 0.01 probability level. NS, not significant.

Table 3. Analysis of variance for aflatoxin content in a subset of the maize diversity lines in Columbia, Missouri. The goodness of fit for the model was 0.89 and the coefficient of variation was 24.42%.

Source	*DF*	*SS*	*MS*	*Significance*[†]
Year	1	37.68	37.68	***
Rep	2	69.66	34.83	***
Line	95	4061.22	42.75	***
Year*Rep	2	6.53	3.26	NS
Year*Line	78	976.34	12.52	***
Rep*Line	177	1652.91	9.34	***
Year*Rep*Line	120	1107.50	9.23	***
Subsample(Line)	192	129.45	0.67	NS
Error	833	1106.62	1.33	
Total	1501	9977.16		

[†] *** Significant at the 0.001 probability level. NS, not significant.

Table 4. Means analysis of aflatoxin content in a subset of the maize diversity lines grown at Tifton, GA as compared by the Student-Newman-Keul's test performed within structure-based sub-population. Means followed by the same letter are not significantly different at $\alpha = 0.05$.

Mixed			*Non-Stiff Stalk*			*Popcorn*		
Line	Mean		Line	Mean		Line	Mean	
U267Y	187.42	A	F44	352.31	A	HP301	238.89	A
D940Y	411.28	AB	GT112	620.01	AB	IDS28	649.03	A
F2834T	568.82	AB	M162W	668.22	AB	SA24	862.3	A
I137TN	805.42	AB	KY21	820.82	ABC	SG18	957.78	A
SC55	861.42	AB	SC212M	994.77	ABC	I29	1393.68	A
M37W	934.52	AB	GA209	1042.64	ABC			
A441-5	1278.06	AB	H95	1069.94	ABC			
NC320	1285.94	AB	NC258	1095.61	ABC			
W182B	1619.26	AB	B97	1131.65	ABC			
T232	2010.63	AB	PA91	1167.59	ABC			
EP1	2584.71	AB	VA35	1179.92	ABC			
MS153	3485.72	B	T8	1469.19	ABC			
OH7B	3583.22	B	WF9	1505.44	ABC			
			OH43	1568.95	ABC			
			H99	1728.06	ABCD			
			SC213R	1785.91	ABCD			
			C103	1977.58	ABCD			
			VA26	2088.49	ABCD			
			W64A	2179.02	ABCD			
			NC260	2702.96	ABCD			
			MO17	2714.41	ABCD			
			A554	2762.55	ABCD			
			W153R	3092.47	ABCD			
			A619	3416.4	ABCD			
			ND246	3846.48	BCD			
			B103	4552.2	CD			
			38-11	6055.95	D			

Table 4. *Continued.*

Stiff Stalk			*Sweet*			*Tropical*		
Line	Mean		Line	Mean		Line	Mean	
B68	456.68	A	IA2132	4438.22	A	A6	23.34	A
N28HT	677.04	AB	IL101	5452.35	A	MP717	148.84	AB
B37	1187.49	ABC	IL14H	7712.35	A	CML258	173.63	ABC
B84	1190.25	ABC				CML261	276.49	ABCD
B14A	1516.32	ABCD				MP313E	323.28	ABCD
B104	1528.03	ABCD				KI3	332.73	ABCD
NC250	1716.44	ABCD				TZI8	342.95	ABCD
A632	2459.17	ABCD				NC350	377.37	ABCD
B73	3331.6	BCD				TZI18	479.96	ABCD
CM174	4175.74	CD				KUI2007	664.81	ABCD
N192	4525.25	CD				NC338	749.34	BCD
CM105	4884.61	D				CML287	764.69	BCD
						KI11	796.14	BCD
						CML277	838.68	BCD
						NC304	874.98	BCD
						NC300	877.52	BCD
						CML91	956.36	BCDE
						KI21	963.42	BCDE
						CML333	968.83	BCDE
						NC348	1027.14	BCDE
						TX601	1037.42	BCDE
						NC298	1387.64	CDE
						CML10	1409.63	DE
						TZI10	2598.04	EF
						NC296	3129.73	F

Table 5. Means analysis of aflatoxin content in a subset of the maize diversity lines grown at Starkville, MS in 2005 as compared by the Student-Newman-Keul's test within structure-based sub-population. Means followed by the same letter are not significantly different at $\alpha = 0.05$.

Mixed			*Non-Stiff Stalk*			*Popcorn*		
Line	Mean		Line	Mean		Line	Mean	
KI43	166.96	A	F44	110.16	A	SA24	886.86	A
D940Y	181.36	AB	NC258	211.64	AB	SG18	1464.66	A
I137TN	254.75	AB	M162W	380.61	ABC	HP301	2680.01	A
T232	378.44	AB	SC213R	503.92	ABCD			
F2834T	465.85	AB	OH43	621.72	ABCDE			
U267Y	605.19	AB	B97	904.78	BCDEF			
MO24W	819.82	AB	VA35	1015.78	BCDEF			
NC320	919.76	AB	GT112	1044.45	BCDEF			
OH7B	1144.62	AB	C103	1360.03	BCDEF			
M37W	1230.64	AB	PA91	1456.97	BCDEF			
SC55	1345.68	AB	T8	1695.80	BCDEF			
F2	1640.06	AB	H95	1884.03	BCDEFG			
A441-5	3586.46	AB	K55	2101.75	BCDEFG			
MS153	4138.37	B	B103	2394.63	CDEFG			
W117HT	4400.06	B	WF9	2496.64	CDEFG			
			A619	2560.12	CDEFG			
			KY21	2698.44	CDEFG			
			W64A	2920.05	CDEFG			
			38-11	3376.53	CDEFG			
			ND246	3635.22	CDEFG			
			W153R	3961.19	CDEFG			
			H99	4361.06	CDEFG			
			MO17	5506.69	DEFG			
			CI187-2	6627.94	EFG			
			VA26	7029.41	EFG			
			NC260	8222.90	FG			
			SC212M	8791.90	FG			
			A554	21199.92	G			

Table 5. *Continued*.

Stiff Stalk			*Sweet*		*Tropical*		
Line	Mean		Line	Mean	Line	Mean	
B68	619.79	A	IA2132	9200.00	MP313E	19.51	A
N28HT	884.81	A			TZI18	23.24	AB
B84	1362.49	AB			A6	53.51	ABC
B104	2604.29	BC			CML247	82.31	ABCD
B37	3043.83	BC			NC354	135.09	ABCDE
B73	5847.26	CD			MP717	156.46	ABCDE
B14A	5982.73	CD			KI3	220.07	BCDEF
A632	7372.15	CD			TX601	226.97	BCDEF
CM105	9175.49	D			CML10	336.48	CDEF
N192	9634.41	D			KI21	384.18	CDEFG
NC250	10574.55	D			NC300	394.84	CDEFG
CM174	10623.31	D			CML277	426.09	CDEFG
					CML91	445.48	CDEFG
					MP420	519.92	CDEFG
					NC304	534.12	CDEFG
					NC352	546.68	CDEFG
					KUI2007	548.22	CDEFG
					TZI8	605.68	CDEFG
					NC298	650.84	CDEFG
					KI11	703.37	CDEFG
					NC338	709.88	CDEFG
					TZI10	1159.96	DEFG
					MP714	1339.10	EFG
					CML333	1994.00	EFG
					NC348	2679.34	FG
					NC350	2858.21	FG
					NC296	5809.95	G

Table 6. Means analysis of aflatoxin content in a subset of the maize diversity lines grown at Columbia, MO in 2005 as compared by the Student-Newman-Keul's test within structure-based sub-population.

Mixed			*Non-Stiff Stalk*			*Popcorn*		
Line	Mean		Line	Mean		Line	Mean	
U267Y	4.89	A	M162W	12.34	A	SG18	215.29	A
I137TN	9.70	AB	KY21	14.12	AB	SA24	282.16	B
D940Y	11.38	AB	GT112	24.57	ABC	HP301	290.46	B
SC55	17.02	ABC	B103	32.57	ABCD	IDS28	592.6	C
NC320	24.49	BCD	H99	39.47	ABCDE			
MO24W	28.25	BCD	SC213R	58.85	ABCDEF			
KI43	32.40	BCD	F44	64.39	ABCDEFG			
M37W	42.48	BCD	38-11	80.13	ABCDEFG			
OH7B	64.58	CDE	NC258	98.67	BCDEFGH			
CMV3	79.75	DEF	PA91	99.90	BCDEFGH			
T232	98.95	DEF	K55	106.22	BCDEFGH			
W117HT	163.40	EFG	ND246	109.24	BCDEFGH			
F2834T	245.04	FGH	CI_187-2	113.13	BCDEFGH			
A441-5	348.01	GHI	MO17	130.04	CDEFGH			
W182B	658.78	HIJ	B97	191.69	CDEFGHI			
MS153	740.37	HIJ	C103	193.81	CDEFGHI			
EP1	1053.37	IJ	H95	222.99	DEFGHI			
F2	1967.05	J	T8	230.32	DEFGHI			
F7	10492.38	K	VA35	271.11	DEFGHIJ			
			CM7	331.22	EFGHIJ			
			Wf9	337.56	EFGHIJ			
			VA26	484.27	FGHIJ			
			A619	566.76	FGHIJ			
			OH43	800.19	GHIJ			
			A554	1183.17	HIJK			
			W153R	1376.60	IJK			
			W64A	1736.84	JK			
			NC260	4386.43	K			

Table 6. *Continued.*

Stiff Stalk			*Sweet*			*Tropical*		
Line	Mean		Line	Mean		Line	Mean	
N28HT	86.68	A	IL14H	63.21	A	CML333	1.66	A
B37	89.46	A	IL101	842.28	B	CML10	2.32	AB
B68	180.20	AB	IA2132	902.16	B	MP715	2.68	ABC
NC250	381.49	BC	P39	965.81	B	MP313E	3.83	ABCD
CM105	417.22	BC	IL677A	1200.95	B	MP717	5.37	ABCDE
CM174	513.50	BCD				KUI2007	6.17	ABCDE
B14A	564.61	BCD				NC304	7.22	ABCDE
B104	582.18	BCD				A6	8.68	ABCDEF
A632	735.64	BCD				TZI18	10.52	ABCDEF
B84	849.65	BCD				CML258	11.98	ABCDEFG
N192	916.08	CD				CML277	16.14	ABCDEFGH
B73	2257.82	D				KI11	17.15	ABCDEFGH
						NC300	17.95	ABCDEFGH
						NC298	20.66	ABCDEFGH
						KI3	23.28	ABCDEFGH
						NC354	24.21	ABCDEFGH
						CML91	27.03	ABCDEFGH
						NC352	27.25	ABCDEFGH
						CML247	37.67	BCDEFGH
						TX601	42.25	CDEFGH
						NC350	49.46	DEFGH
						NC338	51.46	DEFGH
						NC348	74.47	EFGH
						KI21	75.30	EFGH
						TZI8	111.12	FGH
						TZI10	147.34	GH
						NC296	174.35	H

Table 7. Percentage of low aflatoxin lines within a structure-based sub-population as a percentage of all low aflatoxin lines within each location (Mississippi, Georgia, and Missouri). Lines with less than 300 ng g^{-1} at Mississippi and Georgia and less than 20 ng g^{-1} at Missouri were considered low aflatoxin.

Structure Group	Mississippi	Georgia	Missouri
Non-stiff stalk	15.4	0.0	10.5
Stiff stalk	0.0	0.0	0.0
Tropical	61.5	66.7	68.4
Popcorn	0.0	16.7	0.0
Sweet corn	0.0	0.0	0.0
Mixed	23.0	16.7	21.1

Figure 3. Mean aflatoxin content across years in maize diversity lines grown at Columbia, MO within the Stiff Stalk, Sweet Corn, and Tropical pedigree structure-based sub-populations. The allowable toxin limit of 20 ppb is indicated with light gray shading and the aflatoxin maximum of 300 ppb for finishing beef cattle indicated is indicated with dark gray shading.

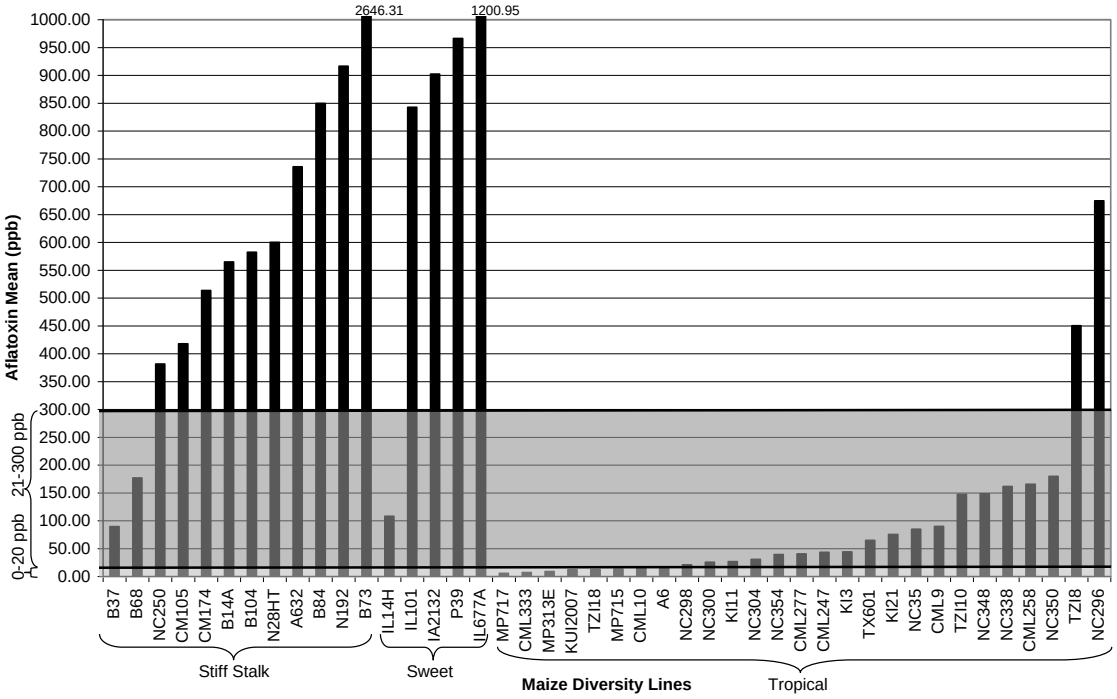


Figure 4. Mean aflatoxin content across years in maize diversity lines grown at Columbia, MO within the Mixed, Non-Stiff Stalk, and Popcorn structure-based sub-populations. The allowable toxin limit of 20 ppb is indicated by light gray shading and the aflatoxin maximum of 300 ppb for finishing beef cattle indicated is indicated by dark gray shading.

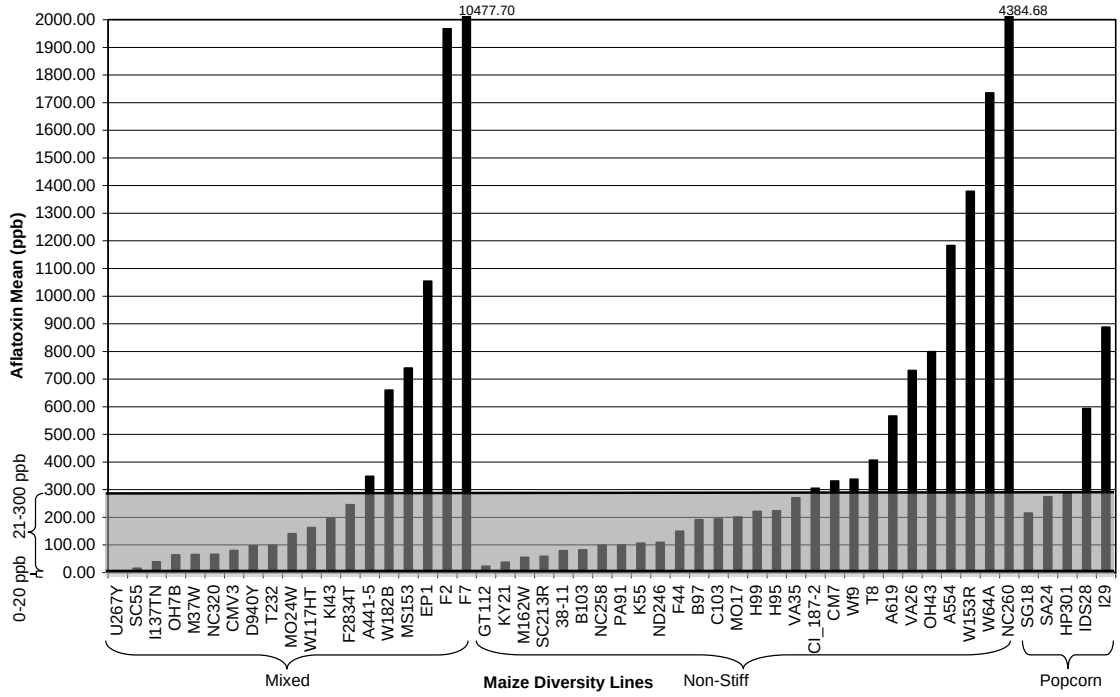
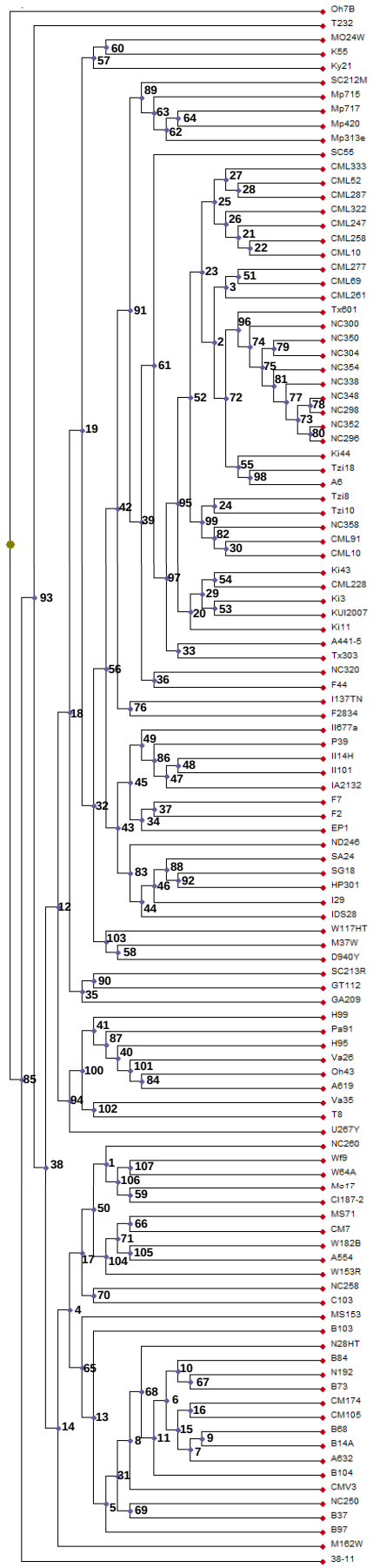


Figure 5. The phylogenetic tree based on 94 SSR markers (Supplementary Table 1) depicting genetic relationships among the maize diversity lines test for aflatoxin contamination. Numbers at the nodes represent reference numbers used to find branch length or genetic distance between lines on the dendogram based on Nei's distances given in Supplementary Table 2.



Supplementary Table 1. SSR markers used to determine fragment size and construct the phylogenetic tree with their associated bin location and forward and reverse primer sequences.

SSR Locus	*Bin*	*Forward Primer*	*Reverse Primer*
bnlg1014	1.01	CACGCTGTTTCAGACAGGAA	CGCCTGTGATTGCACTACAC
bnlg1017	2.02	ATTGGAAGGATCTGCGTGAC	CAGCTGGTGGACTGCATCTA
bnlg1018	2.04	CGAGGTTAGCACCGACAAAT	CGAGTAAATGCTCTGTGCCA
bnlg1022	3.05	GTGTTGTCGATCCACTCCCT	GCAAAGATCTGTGAGGGGAC
bnlg1028	10.0	AGGAAACGAACACAGCAGCT	TGCATAGACAAAACCGACGT
bnlg1043	6.00	TTTGCTCTAAGGTCCCCATG	CATACCCACATCCCGGATAA
bnlg1046	5.03	TGAGCCGAAGCTAACCTCTC	GATGCAAAGGAGGTTCAGGA
bnlg105	5.02	GACCGCCCGGGACTGTAAGT	AGGAAAGAAGGTGACGCGCTTTTC
bnlg1065	8.06	TTGCCTCTCGTCTTCCAACT	TGATGCTCGTTGCTTACCTG
bnlg1070	7.03	TTCCAGTAAGGGAGGTGCTG	TAAGCAACATATAGCCGGGC
bnlg1074	10.0	CATGCTAATAGCCTACCGGG	TTTCCCCCTGATTCGTTATG
bnlg1079	10.0	CGTACGTCGTTGCTGTCTGT	CAGTACGTGCAGTCCCTCCT
bnlg1094	7.02	GTGAAGAACGATGACGCAGA	CAGCAACGCTCTCACATTGT
bnlg1108	3.08	GGATTCCTTTATGACGGGGT	AGTAACAACCAAGGCATCGG
bnlg1129	9.05	GAGAGTATGCTACTCGCCGC	GACGAGTTTGGAGTGCCATT
bnlg1131	8.09	TTAGTTGGGTAAACGTGCAC	GCATCAGGGGGTAGTTGAGA
bnlg1138	2.06	TGCTCTAGCCGACCTCAATT	ATGCCTGAACCGTGATTAGG
bnlg1176	8.05	ACTCCTCAAAACCTAGGTGACA	CACCGATGATGGTGAGTACG
bnlg118	5.07	CTTCCAGCCGCAACCCTC	CCAACAACGCGGACGTGA
bnlg1182	3.09	AGCCGAGTCAGTTCGAGGTA	CAGGGGCTTGAGGTGAGTTA
bnlg1189	4.07	CGTTACCCATTCCTGCTACG	CTTGCTCGTTTCCATTCCAT
bnlg1191	9.07	AATCATGCGTAGGCGTAGCT	GCCAGAGGAAAAAGAAGGCT
bnlg1194	8.01	GCGTTATTAAGGCAAGCTGC	ACGTGAAGCAGAGGATCCAT
bnlg1208	5.03	GCTGTGATGGTGAGACGAGA	GCAGGCACTACTAAAACCGC
bnlg1209	9.05	GTCCCGGGCAGAATAATACC	TTCCTCCTTGAAGTGCTCGT
bnlg1237	5.05	TGGCGCGATTTTCTTCATAT	AAAGAGCAACCTTCAACGGA
bnlg1257	3.09	CGGACGATCTTATGCAAACA	ACGGTCTGCGACAGGATATT
bnlg1265	4.05	GGTTGTCCGTAAAGGCAAGA	TGTGAAGGCCAGACAGTCAG
bnlg1287	5.04	GCCCTACCTGTTCTGTCTCG	TGTCCCATACCTCAACGTGA
bnlg1305	7.03	GCACGGGCATCAGAGAGAG	CATGGGTAAGTTGCTGAAAGTTT
bnlg1325	3.03	CTAAATGCGCAGCAGTAGCA	TGCTCTGCAACAACTTGAGG
bnlg1329	2.08	ATAGAATGGGATGTGGGCAA	TCCGATCATATCGGGAGATC
bnlg1360	10.0	TCTGCTCATCCACAACTTGC	AGAACGTGAAGCTGAGCGTT
bnlg1371	6.02	TTGCCGATAAGAACCAAACA	ACGACCGGTGTGGTTACATT
bnlg1429	1.02	CTCCTCGCAAGGATCTTCAC	AGCACCGTTTCTCGTGAGAT
bnlg1449	3.06	AGTCAACGTAGCTGGCGAGT	TTCACGACGGGTCTCTCTCT
bnlg1456	3.05	CTCTAGGTGGTTAAGATTAACTCATT	TTCATGAGGACCGTGTTGAA
bnlg1484	1.03	GTAAAAGACGACGACATTCCG	GACGTGCACTCCGTTTAACA
bnlg149	1.00	CATCCTCCAAAAGCACTACGT	CAGCTGTCCGACACTTATTCTGTA
bnlg1520	2.09	TCCTCTTGCTCTCCATGTCC	ACAGCTGCGTAGCTTCTTCC
bnlg1523	3.03	GAGCACAGCTAGGCAAAAGG	CTCGCACGCTCTCTCTTCTT
bnlg1526	10.0	ACGAGCGAGTGGAGAATAGG	AGCCCAGTACGTGGGGTC
bnlg1538	6.01	CAGCCGAAGACGAAGCC	GTGGTGAACGAACGAGCAA
bnlg1605	3.07	TCCTGCCCCCTTTGTTTTC	CACCTCTGAACCCCTGTGTT
bnlg1662	2.08	GCACCCACATGAAGTATCCC	TTGTTTTTGCAGTGCCTCAG
bnlg1720	1.09	CAACCCGGATGTCTCAAGTT	TTCGATGCGTATGTACTCAGC
bnlg1732	6.05	AACTTTTGGCATTGCACTGG	CGTAAGTGCACACGGCATTA
bnlg1740	6.07	TTTTCTCCTTGAGTTCGTTCG	ACAGGCAGAGCTCTCACACA
bnlg1782	8.05	CGATGCTCCGCTAGGAATAG	TGTGTTGGAAATTGACCCAA
bnlg1784	4.07	GCAACGATCTGTCAGACGAA	TTGGCATTGGTAATGGGTCT

Supplementary Table 1. *Continued.*

bnlg1808	7.02	CTTTTCTCTTCTAGTAATGAACAGTC	GCATGATCGAACGAAGGC
bnlg1831	2.05	TCGCTCATTTGCATACACCT	TAGGAACATGCCAGCAGTTG
bnlg1834	8.03	AAGGTTGGGTGTTGCTATGC	TAGCTCTGCCACTGGACATG
bnlg1866	1.03	CCCAGCGCATGTCAACTCT	CCCCGGTAATTCAGTGGATA
bnlg1890	4.11	ACCGGAACAGACGAGCTCTA	GTCCTGCAAAGCAACCTAGC
bnlg1904	3.04	AGGAGCATGCACTTGGTTCT	ACTCAACTGATGGCCGATCT
bnlg1917	4.10	ACCGGAACAGACGAGCTCTA	TTTGCTTCCAACTCACATGC
bnlg1940	2.08	CCTTTTGTTTCAGGCCGTTA	CAGCAGCCTGATGATGAACA
bnlg2047	3.04	CATGCATCCATCCTTTTCCT	ATCCATCGGCAACTACAAGC
bnlg2086	1.05	CGGAACCTGCTGCAGTTAAT	GAGATGCAGGAATGGGAAAA
bnlg2122	9.01	TCATCTGGCAAAACCTAGCC	CTTGCCAACTTGAGGACATG
bnlg2132	7.00	GGCGAGAGAGGCAAAGTTAA	GTCGCACAAGGGGATCAC
bnlg2238	1.04	TGCCACTCAAGCCTTCTTTT	TTCTGATTGCAGTGCAGACC
bnlg2259	7.04	ACCATTGATTTCATGGTATTGG	GCGGATAATGACATTGGGTC
bnlg2305	5.07	CACCTTGAAAGCATCCTCGT	GTATCACACCCTCTGCTGCA
bnlg244	9.02	GATGCTACTACTGGTCTAGTCCAGA	CTCCTCCACTCATCAGCCTTGA
bnlg252	4.06	CGTTCTCCGTACAGCACAGACCAACG	CTCAGATGAACTCCTCAGCAGCTG
bnlg426	6.01	TGCATTAATTAGAAGGCTATCAAA	GGTTTGGTGACTGGACTGACTT
bnlg589	4.11	GGGTCGTTTAGGGAGGCACCTTTGGT	GCGACAGACAGACAGACAAGCGC
bnlg615	1.07	CTTCCCTCTCCCCATCTCCTTTCCAA	GCAACCTGTCCATTCTCACCAGAG
bnlg619	9.07	ACCCATCCCACTTTCCACCTCCTCCT	GCTTTCAGCGAATACTGAATAACG
dupssr14	8.09	AGCAGGTACCACAATGGAG	GTGTACATCAAGGTCCAGATTT
dupssr28	4.08	GAAGGAAGCCTTTGTTACAAGT	CTGGAGTGCTGGTCTTGTTAT
nc004	4.03	TGCGAAGAAGCAGTAGCAAA	TGGAGGTAGAAGACGCACG
nc009	6.04	CGAAAGTCGATCGAGAGACC	CCTCTCTTCACCCCTTCCTT
phi017	9.02	CGTTGGCGACCAGGGTGCGTTGGAT	TGCAACAGCCATTCGATCATCAAA
phi021	4.03	TTCCATTCTCGTGTTCTTGGAGTGGTC	CTTGATCACCTTTCCTGCTGTCGCC
phi024	5.01	ACTGTTCCACCAAACCAAGCCGAGA	AGTAGGGGTTGGGGATCTCCTCC
phi033	9.01	ATCGAAATGCAGGCGATGGTTCTC	ATCGAGATGTTCTACGCCCTGAAG
phi037	1.08	CCCAGCTCCTGTTGTCGGCTCAGAC	TCCAGATCCGCCGCACCTCACGTC
phi050	10.0	TAACATGCCAGACACATACGGACAG	ATGGCTCTAGCGAAGCGTAGAG
phi051	7.05	GGCGAAAGCGAACGACAACAATCTT	CGACATCGTCAGATTATATTGCAG
phi061	9.02	GACGTAAGCCTAGCTCTGCCAT	AAACAAGAACGGCGGTGCTGATTC
phi064	1.11	CCGAATTGAAATAGCTGCGAGAACCT	ACAATGAACGGTGGTTATCAACAC
phi065	9.03	AGGGACAAATACGTGGAGACACAG	CGATCTGCACAAAGTGGAGTAGTC
phi072	4.00	ACCGTGCATGATTAATTTCTCCAGCC	GACAGCGCGCAAATGGATTGAACT
phi078	6.05	CAGCACCAGACTACATGACGTGTAA	GGGCCGCGAGTGATGTGAGT
phi089	6.08	GAATTGGGAACCAGACCACCCAA	ATTTCCATGGACCATGCCTCGTG
phi093	4.08	AGTGCGTCAGCTTCATCGCCTACAAG	AGGCCATGCATGCTTGCAACAATG
phi099	3.02	TACAAAAATCAGGACTGCGAAAAAC	GTCGGTGTGTGATCCTTCCAC
phi101	5.06	TGTTCGCCGTCTAGCCTGGATT	TCATCAGCAACGACGACTACTCC
phi116	7.06	GCATACGGCCATGGATGGGA	TCCCTGCCGGGACTCCTG
phi119	8.02	GGGCTCCAGTTTTCAGTCATTGG	ATCTTTCGTGCGGAGGAATGGTCA
phi120	1.11	GACTCTCACGGCGAGGTATGA	TGATGTCCCAGCTCTGAACTGAC

Supplementary Table 2. Nei's distances (D) between branch points corresponding to the phylogenetic tree presented in Figure 5.

Between	And	Nei's D.	Between	And	Nei's D.	Between	And	Nei's D.	Between	And	Nei's D.
85	OH7B	0.31534	96	74	0.00462	86	P39	0.30984	1	NC260	0.3377
85	93	0.0575	74	NC300	0.35117	86	47	0.00139	1	16	0.04192
93	T232	0.36068	74	75	0.06193	47	48	0.07981	106	17	0.1471
93	38	0.01959	75	79	0.09277	48	IL14H	0.21502	107	Wf9	0.12261
38	12	0.00266	79	NC350	0.23161	48	Il101	0.22482	107	W64A	0.1449
12	18	0.00362	79	NC304	0.24359	47	IA213	0.30144	106	59	0.12047
18	19	0.00072	75	81	0.02419	45	34	0.03541	59	MO17	0.18694
19	57	0.02061	81	NC354	0.28928	34	37	0.02194	59	CI187	0.18899
57	60	0.02691	81	77	0.03193	37	F7	0.33505	50	14	0.0065
60	MO24W	0.3531	77	NC338	0.25196	37	F2	0.34311	104	71	0.00437
60	K55	0.3208	77	73	0.0291	34	EP1	0.35313	71	66	0.04179
57	KY21	0.37164	73	78	0.16491	43	83	0.01131	66	MS71	0.32206
19	32	0.00177	78	NC348	0.04502	83	ND246	0.3868	66	CM7	0.29206
32	56	0.00276	78	NC298	0.0537	83	44	0.05926	71	15	0.04041
56	42	0.00523	73	80	0.06576	44	46	0.01956	105	W182B	0.29659
42	91	0.00415	80	NC352	0.1468	46	88	0.08323	105	A554	0.29226
91	89	0.14257	80	NC296	0.14941	88	SA24	0.21518	104	W153R	0.35497
89	Sc212	0.3987	72	55	0.00666	88	92	0.07683	17	70	0.16613
89	63	0.02206	55	KUI44	0.35862	92	SG18	0.13827	70	NC258	0.20926
63	Mp715	0.37136	55	98	0.0182	92	HP301	0.13967	70	C103	0.18456
63	62	0.02323	98	TZI18	0.37193	46	I29	0.32324	4	65	0.00964
62	64	0.03902	98	A6	0.33742	44	IDS28	0.31496	65	MS153	0.38275
64	Mp717	0.33111	52	99	0.00775	32	13	0.0165	65	13	0.0055
64	Mp420	0.31889	99	24	0.0062	103	W117H	0.36107	13	B103	0.36363
62	Mp313	0.37783	24	Tzi8	0.39831	103	58	0.20491	13	5	0.00265
91	39	0.0015	24	Tzi10	0.38575	58	M37W	0.21384	5	31	0.01989
39	61	0.00085	99	82	0.00406	58	D940Y	0.1958	31	8	0.01619
61	SC55	0.41124	82	NC358	0.38552	18	35	0.02325	8	68	0.028
61	97	0.00447	82	30	0.02646	35	90	0.16215	68	N28HT	0.31051
97	95	0.0041	30	CML91	0.37362	90	SC213	0.20605	68	11	0.05101
95	52	0.00514	30	CML10	0.36652	90	GT112	0.20832	11	6	0.02858
52	23	0.00805	95	20	0.02923	35	GA209	0.38042	6	10	0.03704
23	25	0.01035	20	29	0.00593	12	94	0.00839	10	B84	0.2204
25	27	0.01005	29	54	0.01603	94	10	0.00182	10	67	0.05589
27	CML33	0.37695	54	KUI43	0.34722	100	41	0.03751	67	N192	0.12885
27	28	0.02587	54	CML22	0.35727	41	H99	0.33995	67	B73	0.14132
28	CML52	0.34464	29	53	0.32421	41	87	0.00886	6	15	0.07151
28	CML28	0.34295	53	KUI3	0.06522	87	PA91	0.31167	15	16	0.14532
25	26	0.01002	53	KUI20	0.06128	87	40	0.07209	16	CM174	0.01421
26	CML32	0.3932	20	KUI11	0.35793	40	H95	0.25576	16	CM105	0.00992
26	21	0.01934	97	33	0.00746	40	11	0.07867	15	7	0.04453
21	CML24	0.37819	33	A4415	0.40119	101	VA26	0.20678	7	9	0.04787
21	22	0.01354	33	Tx303	0.37788	101	84	0.08614	9	B68	0.09047
22	CML25	0.36409	39	36	0.02798	84	OH43	0.10133	9	B14A	0.0641
22	CML10	0.35207	36	NC320	0.36682	84	A619	0.11211	7	A632	0.11693
23	2	0.00417	36	F44	0.36634	100	12	0.21541	11	B104	0.25555
2	3	0.00591	42	76	0.02418	102	VA35	0.12406	8	CMV3	0.34121
3	51	0.02132	76	I137T	0.37564	102	T8	0.15532	31	69	0.23001
51	CML27	0.37873	76	F2834	0.37195	94	U267Y	0.39012	69	NC250	0.16015
51	CML69	0.36446	56	43	0.00757	38	14	0.001	69	B37	0.16469
3	CML26	0.3874	43	45	0.02964	14	4	0.00756	5	B97	0.36986
2	72	0.00771	45	49	0.06046	4	17	0.01021	14	M162W	0.36825
72	96	0.0135	49	IL677	0.31082	17	50	0.01051	85	38-11	0.30732
96	Tx601	0.38498	49	86	0.02573	50	1	0.02517			

Chapter 3. The Correlation of Agronomic Traits with Aflatoxin Accumulation

Introduction

A number of agronomic and morphological traits have been reported to affect aflatoxin accumulation in maize. These traits include ear husk coverage (Betrán and Isakeit, 2004), husk tightness (Clements and Williams, 2005), silking date (Bétran and Isakeit, 2004), planting date (Rodriquez-del-Bosque, 1996), grain moisture (Lillehoj et al., 1987), seed type or seed constituents (Lillehoj et al., 1983; McMillian et al., 1982), heat and/or drought tolerance (Hawkins et al., 2008; Odvody et al., 1997), insect damage (Windham et al., 1999), and time of harvest (Wiatrak et al., 2005).

Bétran and Isakeit (2004) observed a negative correlation between silking date and aflatoxin content in 25 maize hybrids. They suggest that avoidance may play a role in reducing aflatoxin levels in late maturing lines. Bétran and Isakeit (2004) also observed a positive correlation between loose ear husks and high aflatoxin. Rodriguez-del-Bosque (1996) observed an increase in aflatoxin associated with late planting. Lillehoj et al. (1987) surveyed one maize line (Pioneer 3369A) at five moisture concentrations and saw a significant effect of kernel moisture on aflatoxin. Odvody et al. (1997) observed significantly higher rates of kernels infection with *A. flavus* and *Fusarium moniliforme* under drought conditions as compared to non-stressed conditions. Hawkins et al. (2008) evaluated the effect of climatic conditions on aflatoxin accumulation in three maize hybrids during six time intervals. Their data indicated that temperature stress from 65 to 85 days after planting had the highest correlation with increased aflatoxin levels as compared with other time points.

Numerous studies have investigated the role of seed type and/or seed constituents on aflatoxin production. Darrah et al. (1987) evaluated 29 diverse populations across the US and Mexico and their data suggested that populations with dent-type endosperm accumulated more aflatoxin. Russin et al. (1997) observed an association between kernel wax levels and wax type with aflatoxin contamination. Mellon et al. (2005) monitored kernel starch, sugar, triglyceride, and zein levels following infection in maize. They observed a significant immediate reduction in sucrose and raffinose followed by a reduction in triglycerides indicating that the fungus utilizes the simplest carbon source first. This result suggests that starch composition may affect aflatoxin levels.

Because the majority of these studies relied on small sample sizes, and the samples within a study often had a narrow genetic base, we decided to examine the relationship of a number of agronomic traits with aflatoxin levels across the diverse germplasm studied in Chapter 2. In addition to agronomic trait measurements collected in Missouri, additional publicly available trait measurements for these lines (http://www.panzea.org) were used to identify traits that are correlated with aflatoxin content. The objective of this experiment was to assess the relationship of aflatoxin content with agronomic traits in the maize diversity lines in order to identify biological or biochemical processes and their underlying genes which might be further tested for their relevance in reducing aflatoxin contamination.

Methods and Materials

The mean aflatoxin content across years from the Columbia, MO location (Chapter 2) was used to identify traits correlated with aflatoxin content. The methods for plant growth, inoculation, and aflatoxin analysis on a subset of lines from the maize diversity set were described in Chapter 2.

Agronomic traits. Two replicates of 101 lines from the maize diversity set were grown during the summers of 2004 and 2005 in Columbia, MO. Individual plots consisted of ten plants per line per replicate. Traits examined were ear husk coverage, husk number, husk tightness, area of the fifth leaf, brace root angle, total number of brace roots, number of brace roots at nodes one, two, three, and four; number of nodes from root to ear, number of nodes from ear to tassel, and plant height. Husk coverage was rated on four plants per plot as 1 or 0; a score of 1 indicated husk covered ears and a score of 0 indicated exposed ear tips. Husk tightness was rated on four plants per plot using a binary score, where 1 indicated tightly rolled husks, and 0 loosely rolled husks. The area of the fifth leaf was determined on four plants per plot as the product of length and width multiplied by 0.75 (Stewart and Dwyer, 1999). Brace root angle was determined from digital images using Image J (http://rsb.info.nih.gov/ij/) by measuring the angle at which an adventitious root came off of the stalk of the mature maize plant. Brace roots were counted at each node above the soil after removal of the lower leaves. Plant height (mm) was measured from the soil to the tip of the tassel.

Additional trait data for this set of lines was retrieved from the Panzea database (www.panzea.org) (Zhao et al., 2008). The Panzea data was collected from several

locations depending on year and trait. The locations used in this analysis were: North Carolina Agricultural Research Station, Clayton, NC; 27 Farms, Homestead, FL; Purdue Agronomy Farm, West Lafayette, IN; and the Agronomy Farm, University of Illinois, Urbana, IL. The climate in each of these locations is very different; the growing season length ranked from longest to shortest is 27 Farms, North Carolina Agricultural Research Station, West Lafayette, and University of Illinois. The traits examined were 10 kernel length, 10 kernel thickness, 10 kernel width, 10 kernel weight, kernel halo, kernel protein, kernel starch, kernel color, kernel moisture, cob color, cob weight, cob diameter, ear height, ear weight, ear diameter, ear length, ear row number, seed set length, days to silk, days to tassel, plant height, nodes from ear to tassel, and nodes from root to ear.

Statistical analysis. Spearman's correlations were determined between the aflatoxin means over years and agronomic trait means for individual years, and over years, using (PROC CORR) SAS version 9.0 (SAS Institute Inc., Cary, NC). Significant correlations were further analyzed to evaluate the effect of population structure by re-testing subpopulations based on the structure groupings of Liu et al. (2003). Association analysis was performed using the publicly available *brittle2 (bt2), dwarf3 (d3),* and *Dwarf8 (D8)* gene sequence obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) or Panzea (http://www.panzea.org/) according to the methods outlined in Bradbury et al. (2007).

Results and Discussion

Seed trait correlations. Previous literature suggests that kernel composition may affect aflatoxin accumulation (Lillehoj et al., 1983; McMillian et al., 1982). However, kernel starch and protein levels were not significantly correlated with aflatoxin (Table 8). Within the endosperm, starch is the largest component and is positively correlated with seed size. When analyzed across the entire diversity set, kernel thickness, width, and weight were negatively correlated with aflatoxin level at one or more locations (Table 8). However when examined among the subpopulations, only kernel width within the mixed group had a significant -31.3% ($P<0.05$) correlation with aflatoxin levels (Table 9) suggesting that population structure has a significant effect on the relationship of these traits. These results indicate that neither the physical properties of the kernel, nor the kernel composition were important factors in determining aflatoxin levels in these experiments.

Whole ear trait correlations. Ear husk coverage, husk number, and husk tightness all affect the ability of insects and *A. flavus* to access maize kernels (Bétran and Isakeit, 2004; Williams et al., 2002, 2005; Windham et al., 1999). We observed a negative correlation between husk tightness and aflatoxin levels at $P < 0.001$ before accounting for population structure (Table 10). Similarly husk coverage and husk number were negatively correlated with aflatoxin levels with values of -0.278 ($P<0.01$) and -0.226 ($P < 0.05$), respectively. However, only husk tightness within the Stiff Stalk group was significant after population structure was taken into account (Table 11). Significant negative correlations between cob weight and diameter relative to aflatoxin

content were observed across all lines (Table 12) but this relationship was not significant after accounting for population structure (Table 13). Ear height was significantly correlated with aflatoxin levels in the Mixed (-55.3%, P<0.001) and Non-Stiff Stalk groups (-33.5%, P<0.05) when each structure based sub-population was examined (Table 13).

Maturity-related correlations. Across all lines, days to silk (-46.2%, P < 0.001) and days to tassel (-46.4%, P < 0.001) were highly correlated with aflatoxin levels (Table 12). However, when this correlation is examined by structure based subpopulation, only the Mixed and Non-Stiff Stalk groups have significant correlations between days to silk and aflatoxin and days to tassel and aflatoxin (Table 13). This result suggests that maturity is not a consistent factor for determining aflatoxin levels and that for some populations such as the Tropical and Sweet Corn lines other factors play a greater role. These results validate the use of broader germplasm set and help explain why traits reported as affecting aflatoxin in a narrow germplasm pool sometimes do not associate in a different germplasm sample.

Number of brace roots, number of nodes from root to ear, from ear to tassel, and plant height were negatively correlated with aflatoxin content when analyzed without regard for population structure (Table 10). After accounting for population structure, significant negative correlations were observed between plant height and aflatoxin content within the Mixed and Popcorn groups, -47.6% (P<0.05) and -90.0% (P<0.05), respectively (Table 14). The number of nodes from tassel to ear and from the soil line to the top ear node was negatively correlated with aflatoxin levels in the Mixed group (Table 14). The total number of brace roots and the number of nodes from the soil line to

the upper ear node were correlated with reduced aflatoxin in the Non-Stiff Stalk group in 2004.

Taken together these data suggest a number of the phenotypic trait correlations with aflatoxin are highly affected by population structure. To further examine this phenomenon, Panzea and Missouri plant trait correlations generated from this aflatoxin research were compared with R^2 values indicative of population structure generated by Flint-Garcia et al. (2005). R^2 values were expressed as a percentage of phenotypic variation explained by population structure. Traits with low R^2 values are less influenced by population structure than those with high R^2. Ear height was moderately affected by population structure (R^2=16.4) while days to silk (R^2=32.9) and days to tassel (R^2=35) were highly influenced by population structure (Flint-Garcia et al., 2005). A one-way ANOVA was conducted to determine the proportion of aflatoxin variation control by structure based subpopulation; the R^2 value was 21.3. This analysis indicates that aflatoxin levels are moderately influenced by population structure. The association of aflatoxin accumulation with population structure greatly hinders the ability to determine the biological significance of correlated traits if those traits are also determined by population structure.

A putative role of gibberellic acid. Is there a common biological theme to the traits that remain correlated with aflatoxin, after accounting for population structure? Virtually all of the agronomic traits that were significantly correlated with aflatoxin content in our analysis are traits reported to be affected by gibberellic acid (GA). GA controls plant germination, elongation, and flowering (Schwechheimer, 2008). In maize GA deficient dwarfs display altered plant height, internode length, flowering time, tassel

branching, kernel row number, and sex determination (Bensen et al., 1995). In maize, brace root number and position are altered in both GA sensitive and insensitive dwarfs (M. Gerau, *personal communication*). *Rht* (*reduced height*) wheat lines contain mutations in the ortholog of the maize *D8* gene (Gale and Youssefian, 1985). These mutants display reductions in leaf and internode length (Hoogendoorn et al., 1990; Keyes et al., 1989; Pinthus et al., 1989). In wheat, reduction of GA by ectopic expression of GA-20-oxidase gave rise to plants with increased tillering, and reduced height, ear rachis length, seed set, and seed size (Appleford et al., 2007). A significant reduction in α-amylase activity was also observed in these wheat lines. GA induction of α-amylase has been observed in both plants and fungi (Jacobsen et al., 1970).

As an initial test of the hypothesis that GA may play a role in aflatoxin content, association analysis was performed on aflatoxin phenotype data and sequence data from a variety of gibberellic acid pathway genes, *sh1*, *sh2*, *bt1*, *bt2*, and *ae1*, genes involved in starch biosynthesis. Only significant associations are displayed (Table 15). The *bt2* gene had a minor effect on aflatoxin levels. This was the only starch gene polymorphism that was significant in agreement with our correlation data that kernel starch levels are not correlated with aflatoxin (Table 8). The *d3* gene that had 15 significant polymorphisms (Table 15); this gene encodes a cytochrome P450 that acts at an early step in gibberellin biosynthesis. In addition, we observed one significant association of aflatoxin with the *D8* gene. *D8* is a negative regulator of GA response and may regulate *d3* expression.

Our data suggest that the majority of agronomic trait correlations we observed with aflatoxin are affected by GA and by population structure. This is consistent with a hypothesis that GA is involved either indirectly through changes in plant architecture or

flowering time or by regulation of α-amylase activity in altering aflatoxin contamination in maize. We hypothesize that selection for a modern maize architecture altered GA regulated traits. These alterations may have created maize plants that are both more easily colonized by *A. flavus* and more susceptible to aflatoxin production, for example through decreased husk coverage with the increased opportunity for insect damage and fungal entry.

Aflatoxin is an expensive and time consuming trait to measure. Our data indicate that several easily measured traits such as days to silk, days to tassel, plant height, ear height, number of nodes, ten kernel weight, and ten kernel width are correlated with aflatoxin. A low aflatoxin line might have the following phenotypes: tall ear height, large seed size, an ear that is completely covered with thick leaves to the tip of the ear, and many plant nodes. The problem is that most of the traits correlated with lower aflatoxin levels, increased ear height, increased husk cover, increased nodes, and later maturity are considered negative traits to temperate maize breeders. Therefore the utility of using these correlated traits for improving aflatoxin levels is probably limited.

Table 8. Spearman's correlation coefficients across all lines for kernel traits with mean aflatoxin in Missouri across years.

Trait	*Location*	*Correlation coefficient*	*Significance Level*
10 kernel length	27 farms	-0.046	NS
10 kernel length	NCARS	-0.071	NS
10 kernel length	West Lafayette	0.136	NS
10 kernel length	All locations	-0.029	NS
10 kernel thickness	27 farms	-0.129	NS
10 kernel thickness	NCARS	-0.399	***
10 kernel thickness	West Lafayette	-0.197	NS
10 kernel thickness	All locations	-0.247	***
10 kernel width	27 farms	-0.363	***
10 kernel width	NCARS	-0.392	***
10 kernel width	West Lafayette	-0.249	*
10 kernel width	All locations	-0.303	***
10 kernel weight	NCARS	-0.444	***
10 kernel weight	West Lafayette	-0.189	NS
10 kernel weight	All locations	-0.281	***
Kernel halo	NCARS	-0.127	NS
Kernel protein	27 farms	0.063	NS
Kernel protein	NCARS	0.073	NS
Kernel protein	University of Illinois	-0.222	*
Kernel protein	West Lafayette	0.000	NS
Kernel protein	All locations	-0.015	NS
Kernel starch	27 farms	0.073	NS
Kernel starch	NCARS	-0.058	NS
Kernel starch	University of Illinois	0.072	NS
Kernel starch	West Lafayette	0.087	NS
Kernel starch	All locations	-0.037	NS
Kernel color	NCARS	0.095	NS

*, *** Significant at the 0.05 and 0.001 probability levels. NS, not significant.

Table 9. Spearman's correlation coefficients for kernel traits with mean aflatoxin in Missouri across years analyzed within structure-based subpopulation.

Trait	*Subpopulation*	*Location*	*Correlation Coefficient*	*Significance Level*
10 Kernel Thickness	Mixed	All locations	-0.034	NS
10 Kernel Thickness	Non-Stiff Stalk	All locations	-0.164	NS
10 Kernel Thickness	Popcorn	All locations	-0.345	NS
10 Kernel Thickness	Stiff Stalk	All locations	-0.216	NS
10 Kernel Thickness	Sweet	All locations	-0.040	NS
10 Kernel Thickness	Subtropical-Tropical	All locations	-0.103	NS
10 Kernel Width	Mixed	All locations	-0.313	*
10 Kernel Width	Non-Stiff Stalk	All locations	-0.072	NS
10 Kernel Width	Popcorn	All locations	-0.114	NS
10 Kernel Width	Stiff Stalk	All locations	-0.003	NS
10 Kernel Width	Sweet	All locations	-0.264	NS
10 Kernel Width	Subtropical-Tropical	All locations	-0.116	NS
10 Kernel Weight	Mixed	All locations	0.149	NS
10 Kernel Weight	Non-Stiff Stalk	All locations	-0.120	NS
10 Kernel Weight	Popcorn	All locations	-0.700	NS
10 Kernel Weight	Stiff Stalk	All locations	0.214	NS
10 Kernel Weight	Sweet	All locations	0.100	NS
10 Kernel Weight	Subtropical-Tropical	All locations	-0.216	NS

* Significant at the 0.05 probability level. NS, not significant.

Table 10. Spearman's correlation coefficients and significance levels for agronomic trait data collected in the Missouri location with mean aflatoxin across years within a subset of 94 maize diversity lines by structure-based subpopulation. Year indicates the season in which the agronomic trait data were collected.

Trait	*Year*	*Correlation coefficient*	*Significance*
Ear husk coverage	2004	-0.278	**
Husk number	2004	-0.226	*
Husk tightness	2004	-0.426	***
Leaf area	2004	0.219	*
Brace root angle	2004+2005	-0.006	NS
Total brace roots	2004	-0.156	**
Total brace roots	2005	-0.197	***
Total brace roots node 1	2004+2005	-0.180	**
Total brace roots node 2	2004+2005	-0.329	***
Total brace roots node 3	2004+2005	-0.276	**
Total brace roots node 4	2004+2005	-0.064	NS
Total brace roots node 5	2004+2005	0.043	NS
Nodes from root to ear	2004	-0.553	***
Nodes from ear to tassel	2004	-0.419	***
Plant height	2004	-0.380	***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels. NS, not significant.

Table 11. Spearman's correlation coefficients and significance level for ear associated trait data collected at the Missouri location vs. mean aflatoxin in Missouri analyzed within subpopulations based on structure analysis. Year indicates the season in which the agronomic trait data was collected.

Trait	*Subpopulation*	*Year*	*Correlation Coefficient*	*Significance*
Husk tightness	Mixed	2004	-0.472	NS
Husk tightness	Non-Stiff Stalk	2004	-0.345	NS
Husk tightness	Popcorn	2004	-0.400	NS
Husk tightness	Stiff Stalk	2004	-0.730	**
Husk tightness	Sweet	2004	0.051	NS
Husk tightness	Subtropical-Tropical	2004	0.000	NS
Ear Coverage	Mixed	2004	-0.442	NS
Ear Coverage	Non-Stiff Stalk	2004	-0.198	NS
Ear Coverage	Popcorn	2004	-0.707	NS
Ear Coverage	Stiff Stalk	2004	-0.131	NS
Ear Coverage	Sweet	2004	-0.354	NS
Ear Coverage	Subtropical-Tropical	2004	Non-estimable	Non-estimable
Husk Number	Mixed	2004	-0.056	NS
Husk Number	Non-Stiff Stalk	2004	-0.061	NS
Husk Number	Popcorn	2004	-0.300	NS
Husk Number	Stiff Stalk	2004	0.021	NS
Husk Number	Sweet	2004	0.100	NS
Husk Number	Subtropical-Tropical	2004	-0.301	NS

** Significant at the 0.01 probability level. NS, not significant.

Table 12. Spearman's correlation coefficients for Panzea plant traits with Missouri aflatoxin data combined across years.

Trait	*Location*	*Correlation coefficient*	*Significance*
Cob color	NCARS	-0.395	**
Cob weight	27 farms	-0.468	**
Cob weight	NCARS	-0.227	*
Cob weight	West Lafayette	-0.173	NS
Cob weight	All locations	-0.228	***
Cob diameter	27 farms	-0.279	**
Cob diameter	NCARS	-0.157	NS
Cob diameter	West Lafayette	0.014	NS
Cob diameter	All locations	-0.137	*
Ear height	27 farms	-0.461	***
Ear height	NCARS	-0.516	***
Ear height	All locations	-0.409	***
Ear weight	27 farms	-0.231	*
Ear weight	NCARS	-0.048	NS
Ear weight	West Lafayette	-0.074	NS
Ear weight	All locations	-0.122	NS
Ear diameter	27 farms	-0.197	NS
Ear diameter	NCARS	-0.118	NS
Ear diameter	West Lafayette	0.061	NS
Ear diameter	All locations	-0.082	NS
Ear length	27 farms	-0.139	NS
Ear length	NCARS	-0.166	NS
Ear length	West Lafayette	-0.161	NS
Ear length	All locations	-0.115	NS
Ear row number	27 farms	0.077	NS
Ear row number	NCARS	0.287	***
Ear row number	West Lafayette	0.253	*
Ear row number	All locations	0.262	***
Seed set length	NCARS	0.049	NS
Days to silk	27 farms	-0.629	***
Days to silk	NCARS	-0.727	***
Days to silk	All locations	-0.462	***
Days to tassel	27 farms	-0.663	***
Days to tassel	NCARS	-0.709	***
Days to tassel	All locations	-0.464	***
Plant height	27 farms	-0.051	NS
Plant height	NCARS	-0.563	NS
Plant height	All locations	-0.041	NS
Number of Nodes from ear to tassel	NCARS	-0.255	*
Number of Nodes from root to ear	NCARS	-0.529	***

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels. NS, not significant.

Table 13. Spearman's correlation coefficients for Panzea plant traits with Missouri aflatoxin data analyzed within structure-based subpopulation.

Trait	*Subpopulation*	*Location*	*Correlation Coefficient*	*Significance*
Cob Color	Mixed	All locations	-0.169	NS
Cob Color	Non-Stiff Stalk	All locations	-0.302	NS
Cob Color	Popcorn	All locations	Non-estimable	Non-estimable
Cob Color	Stiff Stalk	All locations	-0.151	NS
Cob Color	Sweet	All locations	Non-estimable	Non-estimable
Cob Color	Subtropical-Tropical	All locations	Non-estimable	Non-estimable
Cob Weight	Mixed	All locations	-0.169	NS
Cob Weight	Non-Stiff Stalk	All locations	-0.302	NS
Cob Weight	Popcorn	All locations	Non-estimable	Non-estimable
Cob Weight	Stiff Stalk	All locations	-0.151	NS
Cob Weight	Sweet	All locations	Non-estimable	Non-estimable
Cob Weight	Subtropical-Tropical	All locations	Non-estimable	Non-estimable
Ear Height	Mixed	All locations	-0.553	***
Ear Height	Non-Stiff Stalk	All locations	-0.335	*
Ear Height	Popcorn	All locations	-0.566	NS
Ear Height	Stiff Stalk	All locations	0.050	NS
Ear Height	Sweet	All locations	0.049	NS
Ear Height	Subtropical-Tropical	All locations	-0.148	NS
Days to Tassel	Mixed	All locations	-0.601	***
Days to Tassel	Non-Stiff Stalk	All locations	-0.347	**
Days to Tassel	Popcorn	All locations	-0.333	NS
Days to Tassel	Stiff Stalk	All locations	-0.065	NS
Days to Tassel	Sweet	All locations	0.074	NS
Days to Tassel	Subtropical-Tropical	All locations	0.071	NS
Days to Silk	Mixed	All locations	-0.442	***
Days to Silk	Non-Stiff Stalk	All locations	-0.230	***
Days to Silk	Popcorn	All locations	-0.100	NS
Days to Silk	Stiff Stalk	All locations	-0.850	NS
Days to Silk	Sweet	All locations	-0.007	NS
Days to Silk	Subtropical-Tropical	All locations	-0.028	NS

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels. NS, not significant.

Table 14. Spearman's correlation coefficients and significance level for plant architecture trait data collected in Missouri with Missouri aflatoxin data analyzed within structure-based subpopulations. Year indicates the season in which the agronomic trait data was collected.

Trait	*Subpopulation*	*Year*	*Correlation Coefficient*	*Significance*
Leaf Area	Mixed	2004	0.275	NS
Leaf Area	Non-Stiff Stalk	2004	-0.072	NS
Leaf Area	Popcorn	2004	0.100	NS
Leaf Area	Stiff Stalk	2004	0.252	NS
Leaf Area	Sweet	2004	0.100	NS
Leaf Area	Subtropical-Tropical	2004	0.137	NS
Plant Height	Mixed	2004	-0.476	*
Plant Height	Non-Stiff Stalk	2004	-0.260	NS
Plant Height	Popcorn	2004	-0.900	*
Plant Height	Stiff Stalk	2004	0.014	NS
Plant Height	Sweet	2004	-0.100	NS
Plant Height	Subtropical-Tropical	2004	-0.061	NS
Nodes from tassel to ear	Mixed	2004	-0.588	**
Nodes from tassel to ear	Non-Stiff Stalk	2004	-0.206	NS
Nodes from tassel to ear	Popcorn	2004	-0.300	NS
Nodes from tassel to ear	Stiff Stalk	2004	-0.280	NS
Nodes from tassel to ear	Sweet	2004	-0.224	NS
Nodes from tassel to ear	Subtropical-Tropical	2004	-0.213	NS
Nodes from root to ear	Mixed	2004	-0.607	**
Nodes from root to ear	Non-Stiff Stalk	2004	-0.507	**
Nodes from root to ear	Popcorn	2004	-0.400	NS
Nodes from root to ear	Stiff Stalk	2004	-0.035	NS
Nodes from root to ear	Sweet	2004	-0.300	NS
Nodes from root to ear	Subtropical-Tropical	2004	0.188	NS
Total Brace Roots	Mixed	2004	-0.141	NS
Total Brace Roots	Non-Stiff Stalk	2004	-0.288	***
Total Brace Roots	Popcorn	2004	-0.284	NS
Total Brace Roots	Stiff Stalk	2004	-0.087	NS
Total Brace Roots	Sweet	2004	-0.062	NS
Total Brace Roots	Subtropical-Tropical	2004	0.049	NS
Total Brace Roots	Mixed	2005	-0.108	NS
Total Brace Roots	Non-Stiff Stalk	2005	-0.185	**
Total Brace Roots	Popcorn	2005	-0.009	NS
Total Brace Roots	Stiff Stalk	2005	-0.158	NS
Total Brace Roots	Sweet	2005	-0.205	NS
Total Brace Roots	Subtropical-Tropical	2005	-0.031	NS

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability levels. NS, not significant.

Table 15. Summary of the significant polymorphisms in the *bt2*, *d3*, and *D8* genes affecting aflatoxin content in maize based on association analysis.

Locus	*Site*	*df*	*F*	*p*	*Model df*	*Error df*	*Error MS*
bt2 subunit	145	1	14.1201	3.40 E-04	3	74	6.04E-04
bt2 subunit	312	1	7.7576	0.0068	3	73	0.0087
bt2 subunit	314	1	7.7576	0.0068	3	73	0.0087
bt2 subunit	316	1	7.7576	0.0068	3	73	0.0087
bt2 subunit	330	1	7.7576	0.0068	3	73	0.0087
d3 genomic	10	1	7.6824	0.0089	3	35	0.2015
d3 genomic	177	1	7.4645	0.0098	3	35	0.6139
d3 genomic	448	1	7.4645	0.0098	3	35	0.6139
d3 genomic	1795	1	7.4645	0.0098	3	35	0.6139
d3 genomic	1983	1	7.4645	0.0098	3	35	0.6139
d3 genomic	1899	1	7.3614	0.0103	3	35	1.2005
d3 genomic	554	1	7.3283	0.0107	3	33	0.1795
d3 genomic	2926	1	6.5386	0.0150	3	35	0.0074
d3 genomic	2932	1	6.5386	0.0150	3	35	0.0074
d3 genomic	2972	1	6.2367	0.0174	3	35	0.0102
d3 genomic	2280	1	5.2322	0.0283	3	35	0.7385
d3 genomic	532	2	3.7178	0.0347	4	34	0.5128
d3 genomic	163	1	4.8359	0.0355	3	31	2.1408
d3 genomic	2450	1	4.6311	0.0384	3	35	2.5565
d3 genomic	239	1	3.8925	0.0564	3	35	0.0304
D8 genomic	3386	1	4.5532	0.0366	3	65	0.4182

Chapter 4. Antioxidants But Not ROS Levels Affect Aflatoxin Production

Introduction

Genes in both the fungal and maize genomes control the amount of aflatoxin produced by the fungus during infection of maize grain. Aflatoxin is a polyketide secondary metabolite produced by *A. flavus*. The genes responsible for aflatoxin biosynthesis in the fungus have been cloned (reviewed in Bhatnagar et al., 2006). The 27 aflatoxin biosynthesis genes are clustered in a 70 kb region (Roze et al., 2007). Regulatory genes within the cluster include *aflR, myb* (Ehrlich et al. 1998; Woloshuk et al., 1994) and *aflJ* (Meyers et al., 1998). Numerous inhibitors of aflatoxin biosynthesis have been identified, primarily through *in vitro* studies (reviewed in Holmes et al., 2008). Among these are a preponderance of compounds capable of scavenging reactive oxygen species (ROS) including both phenylpropanoids, e.g. flavonoids (Ververidis et al., 2007) and terpenoids, e.g. carotenoids (Sies and Stahl, 1995). Other biological markers of oxidative stress such as lipid peroxidation and antioxidant activity like superoxide dismutase have also been associated with changes in aflatoxin biosynthesis (Kim et al., 2008; Narasaiah et al., 2006). Conversely, many of the compounds that strongly stimulate aflatoxin production are oxidizing agents (De Luca et al., 1995). Plants that are unable to efficiently combat ROS often experience cell death (Zhang and Xing, 2008). While cell death is a necessity for normal plant development (reviewed in Hong-bo et al., 2008) it can also be detrimental as in the case of pathogen-induced necrosis (reviewed in Apel and Hirt, 2004). Stressed plants accumulate higher ROS levels compared with healthy plants (reviewed in Apel and Hirt, 2004).

A small number of studies have linked changes in aflatoxin content to oxidative stress or antioxidant levels *in planta*. However they suffer from some of the pitfalls mentioned in the previous chapter, namely narrow germplasm samples or small sample sizes. Our investigation of the relationship of aflatoxin to various agronomic traits indicates that many of these relationships do not hold across all maize lines after population structure is accounted for. Instead, individual subpopulations may have significant correlations that are not observed in the general germplasm (see Chapter 3). Taken together, these results indicate that a broad germplasm sample is necessary to assess the generality of the relationships of individual genes or traits with aflatoxin content. Based on the large body of literature suggesting a link between aflatoxin content and ROS levels, we carried out a survey of kernel ROS levels in the maize diversity lines and tested for a relationship between kernel ROS and aflatoxin content as well as the relationship of kernel ROS levels to other agronomic traits with significant correlations to aflatoxin content (see Chapter 3).

Methods and Materials

ROS quantification. A set of 109 maize lines from the maize diversity set described in Chapter 2 was grown during the summers of 2006 and 2007 at the Genetics Research Farm, University of Missouri-Columbia, Columbia, MO. In 2007, 15 additional lines were added to the set. Plots consisted of 20 plants per row that were 6.10 m long with 0.61 m spacing. The experimental design was a randomized complete block with two replications.

Two randomly chosen self-pollinated ears were sampled 25 days after pollination. Whole in-husk ears were harvested and the ear shank was placed in ice until sampling. Ten *in tact* kernels were removed from the middle third of the maize ear. The ten kernels from each ear were bulked to create one sample per ear. The kernels were frozen in liquid nitrogen, ground, and stored at -20^{o}C. A tissue sample of 30 mg was weighed into liquid nitrogen cooled 1.5 ml eppendorf tubes (Shin and Schachtman, 2004). ROS was extracted from the tissue by adding 100 µl 20 mM K_2HPO_4 buffer pH 6.5 to each sample and spinning the sample in a centrifuge at 10,000 rpm for 10 min at 4^{o}C. The supernatant was removed and 50 µl was used to measure ROS levels. ROS was measured using an Amplex® red hydrogen peroxide/peroxidase assay kit (Molecular Probes, Carlsbad, CA). The one-step assay uses 10-acetyl-3,7-dihydroxyphenoxazine with horseradish peroxidase to measure H_2O_2 released from samples. More red color from resorufin production equates to more H_2O_2 release. Standards were 10 µM, 5 µM, 2 µM, 1 µM, 0.5 µM, 0.1 µM, 0.05 µM, and 0 µM H_2O_2. Absorbance readings were taken after 30 min at 560 nm using a µQuant (Bio-tek Instruments Inc., Mississauga, ON). The log of the standards was plotted against the log of the absorbance reading to construct a standard curve. Using this information a linear equation can be calculated to determine ROS levels of samples.

ROS statistical analysis. Statistical analysis was performed using SAS version 9.0 (PROC GLM) (SAS Institute Inc., Cary, NC) on the raw ROS values. ANOVA was conducted based on a linear mixed-model where years, replications, and climate groupings were random effects while lines were a fixed effect. The model for this experiment was:

$$Y_{ijkl} = \mu + A_i + R_j + L_k + C_l + AR_{ij} + AL_{ik} + RL_{jk} + ARL_{ijk} + \varepsilon_{ijkl}$$

where μ = overall mean, A_i =effect of year i, R_j =effect of replication j, L_k = effect of line k, C_l = the effect of climate l, AR_{ij} = the interaction of year i with replication j, AL_{ik} = the interaction of year i with line k, RL_{jk} = the interaction of replication j with line k, ARL_{ijk} = the interaction of year i with replication j and line k, and ε_{ijkl} = the residual variation. The Student-Newman- Keul's multiple-range-test was performed to look for significant differences among lines at the 0.05 significance level.

Agronomic trait correlations with aflatoxin and ROS. Spearman's correlations were also performed using (PROC CORR) SAS version 9.0 (SAS Institute Inc., Cary, NC) between aflatoxin content across years in Columbia, Missouri (described in Chapter 2) and mean ROS levels, as well as, between mean ROS levels and the agronomic trait data described in the previous chapter. Traits mentioned in Chapter 3 were used to assess relationships with kernel ROS levels.

Results and Discussion

Germplasm survey of ROS levels. ROS levels have been postulated to affect aflatoxin accumulation *in vitro* (Kim et al., 2006; Narasaiah et al., 2006) and *in planta* (Magbanua et al., 2007). Kernel tissue was collected and sampled for ROS- levels in the maize diversity lines in 2006 and 2007 in Columbia, MO. There was significant genetic variation for kernel ROS levels among the maize diversity lines representing 30.3% of the total variation in kernel ROS levels (Table 16). Kernel ROS levels were also significantly different among years. Differences among years are expected due to annual

variations in climate. Variation among structure-based subpopulations was not statistically significant. A Duncan's multiple range test was performed to assess the differenceds among lines using mean ROS levels (Table 17). Tzi8, an African line, had the highest kernel ROS at 0.218 µM/mg while H99, a temperate line, had the lowest at 0.016 µM/mg. No significant correlation was observed between mean aflatoxin content and ROS levels (Figure 6). Line A6, which has among the highest ROS content was among the lowest in aflatoxin content (Table 6). In contrast, lineTzi8 had both high kernel ROS and aflatoxin content. These data were collected 25 d after pollination and results might have been different if ROS data were examined over the course of kernel development at different times.

Agronomic trait correlations with ROS data. As plants grow and reproduce some ROS is beneficial to aid programmed cell death, response to environmental stimuli, and growth (Bailey-Serres and Mittler, 2006). Correlations were conducted between agronomic trait data described in the previous chapter and kernel ROS data. Brace root angle was significantly correlated with lower ROS levels (-0.245, $P < 0.01$) (Table 18). ROS has been associated with root growth and root growth under water stress (Liszkay et al., 2004; Schopfer and Liszkay, 2006; Zhu et al., 2007). Although no specific studies of the relationship between brace root angle and ROS have been published, measuring brace root angle may aid in choosing plants that grow better root systems and are better adapted to handle water stress. Kernel ROS was mainly correlated with maturity-related traits such as days to silk, days to tassel, and plant height (Table 19). ROS also had a 12.7% correlation with kernel protein levels. Correlations were also performed with the starch haplotype data from Wilson, et al. (2004) described in the previous chapter to determine

if the presence of a particular starch polymorphism affected ROS levels. Shifts in carbohydrate metabolism have been suggested to occur when plants are under stress and ROS levels are often altered as well. GA and ABA act antagonistically regulating gluconeogenesis in the aleurone layer. GA and ABA also control the biosynthesis and synthesis of starchy endosperm hydrolases (Eastmond and Jones, 2005). The presence of the *Sh1-775(C)* haplotype was significantly correlated with kernel ROS levels (Table 20).

Significant differences in ROS levels among lines within the maize diversity set were observed (Table 17). However, ROS levels and aflatoxin content were not significantly correlated indicating that ROS levels *per se* are not responsible for differences in aflatoxin accumulation among lines. ROS levels were significantly correlated with kernel protein, kernel starch, ear height, days to silk, days to tassel, and plant height (Table 19). Forward regression analysis of starch, carotenoids, and ROS indicated that amylose activity and starch viscosity explained significant portions of the variation in aflatoxin content (Table 21). Comparison of aflatoxin content with agronomic traits in the previous chapter yielded a model in which selection occurred for agronomic traits controlled by GA. We observed a significant association between afltoxin contamination and two genes involved in GA biosynthesis suggesting alterations in GA levels may play a role in aflatoxin contamination. This hypothesis remains to be tested through the manipulation of endogenous or exogenous GA levels. Alterations in allele frequency in genes likely affecting GA levels may affect aflatoxin production through the regulation of α-amylase activity. Changes in GA content may also be responsible for the results we observed *in planta* for the flavonoids and carotenoids. GA has been shown to stimulate chalcone synthase gene expression and to regulate floral

pigmentation in petunias (Weiss et al., 1992). ABA, a product of the carotenoid biosynthetic pathway, prevents activation of chalcone synthase by GA in petunias (Weiss et al., 1995). Phytoene desaturase catalyzes the oxidation reaction in carotenoid synthesis. Mutation in the phytoene desaturase gene has recently been shown to negatively affect both carotenoid and gibberellin biosyntheses, suggesting a complex feedback mechanism may occur between the two (Qin et al., 2007). Our results indicate that ROS levels are not correlated with aflatoxin content *in planta*. However some GA regulated antioxidants have a significant relationship with aflatoxin content (Chapter 3). Future studies investigating aflatoxin content in single and double mutants involved in GA, ABA, carotenoid, and flavonoid synthesis would help to further elucidate the role of GA in both aflatoxin contamination and in the relationship between antioxidants and aflatoxin.

Table 16. The analysis of variance for ROS levels among maize diversity lines grown in Missouri in 2006 and 2007. The goodness of fit was 0.90 and the coefficient of variation was 40.33%.

Source	*DF*	*SS*	*MS*	*Significance*
Line	96	0.563	0.006	***
Rep	1	0.008	0.008	**
Year	1	0.007	0.007	**
Subpopulation	1	0.000	0.000	NS
Line*Rep	39	0.260	0.004	***
Line*Year	39	0.286	0.007	***
Rep*Year	1	0.018	0.018	***
Error	141	0.176	0.001	
Total	356	1.855		

, * Significant at the 0.01 and 0.001 probability levels. NS, not significant.

Table 17. Duncan's multiple range test for the 2006 and 2007 combined ROS data in the maize diversity lines. Means followed by the same letter(s) are not significantly different at α=0.05.

Line	*ROS Mean (μM/mg)*	*Duncan's Multiple Range Test Rank*
H99	0.016	A
B84	0.0205	AB
Il14H	0.024	ABC
CML258	0.02525	ABC
W182B	0.02567	ABCD
A632	0.02625	ABCD
NC304	0.029	ABCDE
NC352	0.02967	ABCDE
B37	0.03367	ABCDEF
B68	0.03375	ABCDEF
F7	0.0345	ABCDEFG
Tx601	0.0345	ABCDEFG
Kui21	0.03567	ABCDEFGH
CML287	0.03633	ABCDEFGH
CML228	0.0385	ABCDEFGHI
MS71	0.04125	ABCDEFGHIJ
EP1	0.043	ABCDEFGHIJK
Mp717	0.04425	ABCDEFGHIJK
SC55	0.04475	ABCDEFGHIJK
F2	0.04567	ABCDEFGHIJK
TX303	0.0475	ABCDEFGHIJK
Ia2132	0.0485	ABCDEFGHIJK
NC354	0.049	ABCDEFGHIJK
W64A	0.04967	ABCDEFGHIJK
Kui43	0.051	ABCDEFGHIJK
Il101	0.059	ABCDEFGHIJKL
Wf9	0.059	ABCDEFGHIJKL
N192	0.0594	ABCDEFGHIJKL
NC296	0.062	ABCDEFGHIJKL
B14A	0.062	ABCDEFGHIJKL
F2834T	0.062	ABCDEFGHIJKL
Kui2007	0.062	ABCDEFGHIJKL
NC250	0.06233	ABCDEFGHIJKL
NC260	0.06267	ABCDEFGHIJKL
HP301	0.06333	ABCDEFGHIJKL
NC258	0.06475	ABCDEFGHIJKLM
CML69	0.0655	ABCDEFGHIJKLM
N28Ht	0.0655	ABCDEFGHIJKLM
Mp714	0.0655	ABCDEFGHIJKLM
SG18	0.0668	ABCDEFGHIJKLM
Oh43	0.067	ABCDEFGHIJKLM

NC300	0.0675	ABCDEFGHIJKLM
M162W	0.0675	ABCDEFGHIJKLM
CMV3	0.0685	ABCDEFGHIJKLM
U267Y	0.071	ABCDEFGHIJKLMN
NC298	0.07125	ABCDEFGHIJKLMN
CM174	0.07157	ABCDEFGHIJKLMN
ND246	0.07167	ABCDEFGHIJKLMN
B73	0.072	ABCDEFGHIJKLMN
D940Y	0.072	ABCDEFGHIJKLMN
CML322	0.076	ABCDEFGHIJKLMN
T232	0.07617	ABCDEFGHIJKLMN
CML247	0.0776	ABCDEFGHIJKLMN
SA24	0.078	ABCDEFGHIJKLMNO
I29	0.0784	ABCDEFGHIJKLMNO
A554	0.0785	ABCDEFGHIJKLMNO
Mp313e	0.07875	ABCDEFGHIJKLMNOP
K55	0.07929	ABCDEFGHIJKLMNOP
Va026	0.08	ABCDEFGHIJKLMNOPQ
CML333	0.0806	ABCDEFGHIJKLMNOPQ
Kui3	0.08171	ABCDEFGHIJKLMNOPQ
NC338	0.083	ABCDEFGHIJKLMNOPQ
B97	0.08367	ABCDEFGHIJKLMNOPQ
Tzi18	0.08414	ABCDEFGHIJKLMNOPQ
Va35	0.0865	ABCDEFGHIJKLMNOPQ
NC348	0.09	ABCDEFGHIJKLMNOPQR
NC358	0.09267	BCDEFGHIJKLMNOPQR
GA209	0.0935	BCDEFGHIJKLMNOPQRS
T8	0.09575	BCDEFGHIJKLMNOPQRS
W117HT	0.098	CDEFGHIJKLMNOPQRS
SC212M	0.09825	CDEFGHIJKLMNOPQRS
M37W	0.10075	DEFGHIJKLMNOPQRS
NC350	0.10375	EFGHIJKLMNOPQRST
H95	0.1052	FGHIJKLMNOPQRST
W153R	0.107	FGHIJKLMNOPQRSTU
CI_187-2	0.10767	FGHIJKLMNOPQRSTU
CM105	0.10975	GHIJKLMNOPQRSTUV
A619	0.11	HIJKLMNOPQRSTUV
CML52	0.1125	IJKLMNOPQRSTUV
Pa91	0.1135	IJKLMNOPQRSTUV
A441-5	0.116	JKLMNOPQRSTUV
B104	0.11657	JKLMNOPQRSTUV
IDS28	0.1174	KLMNOPQRSTUV
MS153	0.11825	KLMNOPQRSTUV
38-11	0.134	LMNOPQRTUVW
F44	0.139	LMNOPQRTUVW
I137TN	0.144	NOPQRSTUVWX

CML91	0.152	OPQRSTUVWXY
Kui11	0.15275	PQRSTUVWXY
Ky21	0.1535	QRSTUVWXY
Mo24W	0.161	RSTUVWXY
Il677a	0.166	STUVWXY
P39	0.16625	STUVWXY
CML277	0.1735	TUVWXY
Hi27	0.178	UVWXY
SC213R	0.18067	VWXY
C103	0.19525	WXY
A6	0.19933	WXY
NC320	0.20733	XY
Mo17	0.20975	XY
Tzi8	0.21767	Y

Table 18. Spearman's correlations for average ROS level across years and trait data collected in Missouri on the maize diversity lines.

Trait	*Location*	*Correlation*	*Significance*
Husk Tightness	Missouri	0.030	NS
Number of Husks	Missouri	0.006	NS
Exposed Ear	Missouri	0.021	NS
Aflatoxin	Missouri	-0.122	NS
Leaf Area	Missouri	0.049	NS
Brace Root Angle	Missouri 2004+2005	-0.245	**
Brace Root Angle	Missouri 2004	-0.165	NS
Brace Root Angle	Missouri 2005	-0.322	**

** Significant at α=0.01. NS, not significant.

Table 19. Spearman's correlations for average ROS content across years with trait data from the Panzea database.

Trait	*Location*	*Correlation*	*Significance*
10 kernel weight	All locations	-0.085	NS
10 kernel thickness	All locations	-0.013	NS
10 kernel width	All locations	0.104	NS
Kernel Color	All locations	-0.102	NS
Kernel Halo	All locations	0.146	NS
Kernel Protein	All locations	0.127	*
Kernel Protein	27 Farms	0.045	NS
Kernel Protein	NCARS	0.088	NS
Kernel Protein	UOI	0.135	NS
Kernel Protein	West Lafayette	0.315	**
Kernel Starch	All locations	-0.041	NS
Cob Color	27 Farms	0.042	NS
Cob Diameter	All locations	0.028	NS
Cob Weight	All locations	0.054	NS
Ear Diameter	All locations	0.065	NS
Ear Height	All locations	0.256	***
Ear Height	27 Farms	0.265	*
Ear Height	NCARS	0.319	**
Ear Length	All locations	0.036	NS
Days to Silk	All locations	0.184	***
Days to Silk	27 Farms	0.290	***
Days to Silk	NCARS	0.226	**
Days to Tassel	All locations	0.155	**
Days to Tassel	27 Farms	0.247	**
Days to Tassel	NCARS	0.183	**
Plant Height	All locations	0.264	***
Plant Height	27 Farms	0.277	**
Plant Height	NCARS	0.322	**
Number of nodes from root to ear	NCARS	0.200	NS
Number of nodes from ear to tassel	NCARS	0.094	NS

*,**,*** Significant at $P < 0.05, 0.01, 0.001$, levels. NS, not significant.

Table 20. Spearman's correlations for average ROS content across years and the presenceof several starch alleles from Wilson et al. (2004) in the maize diversity lines.

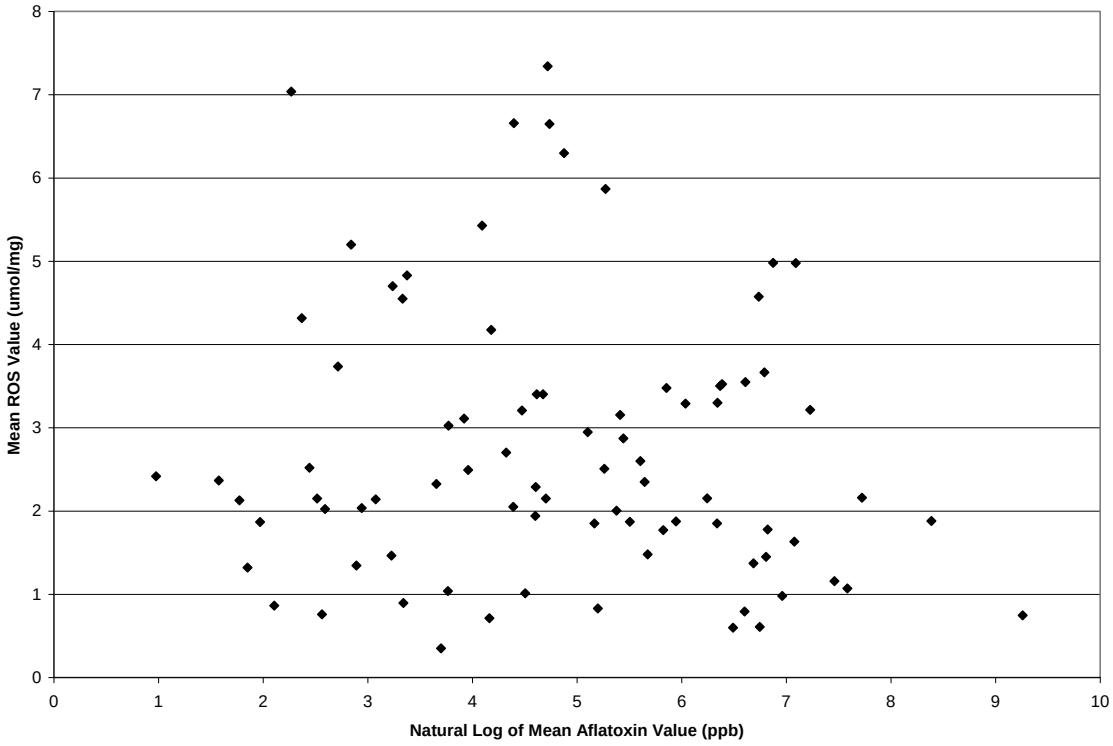
Allele	*Correlation*	*Significance*
sh1-1210G	0.014	NS
sh1-775C	-0.248	*
sh2-3674-1	0.177	NS
bt2-925T	-0.105	NS
ae1-1509G	0.017	NS
sh2-3842G	-0.045	NS
ae1-1689C	0.056	NS

*Significant at $P < 0.05$. NS, not significant.

Table 21. Summary of the forward regression analysis of starch, carotenoids, and ROS data on aflatoxin content in the maize diversity lines.

Variable	*Partial R^2*	*Model R^2*	*P-Value*
Starch Trough Viscosity	0.182	0.182	0.005
Amylose	0.086	0.268	0.038

Figure 6. The relationship between mean aflatoxin content and mean kernel ROS at 25 days after pollination.



Chapter 5. Conclusion

We utilized the maize diversity lines to assess the distribution of low aflatoxin lines in structure-based subpopulations, identified agronomic traits correlated with low aflatoxin, and developed a hypothesis regarding the underlying genetic mechanism controlling the low aflatoxin phenotype. We identified temperate sources of low aflatoxin maize refuting previous research suggesting that tropical maize was the only source of low aflatoxin. Analysis of the relationship of aflatoxin contamination with a large number of traits correlations suggests that GA might influence aflatoxin contamination. This relationship was further supported by significant association between aflatoxin contamination and haplotypes in the *d3* and *D8* genes involved in GA biosynthesis with the larger effect on aflatoxin content observed for the *d3* gene. *d3* encodes the cytochrome P450 CYP88A1 (Winkler and Helentjaris, 1995). It is tightly linked to *wx1* and *gl15* on chromosome 9 and is expressed in the roots, vegetative meristems and developing leaves. This expression pattern may help explain why we identified significant correlations between aflatoxin and traits involving root, leaf, and vegetative meristem tissue. Furthermore, allelic variation at the *d3* locus has been proposed as the basis of a quantitative trait locus for a naturally occurring height variant in maize.

Kernel composition, in particular genes in the starch pathway, have undergone extensive selection during the domestication and subsequent improvement of maize. GA induces α-amylase production, leading to starch breakdown. Changes in the ratio of glucose to sucrose can induce transcription of aflatoxin biosynthesis genes in addition to providing better carbon sources for fungal growth. We observed a significant association

between aflatoxin content and haplotypes in the *bt2* gene. In addition, the sweet corn germplasm pool contained only high aflatoxin lines. The *shrunken2* gene in sweet corn has been demonstrated to increase the production of α-amylases. These data suggest that changes in the sugar profile either through direct selection on kernel composition or indirectly through GA-mediated changes in expression could lead to unwanted increases in aflatoxin content.

Kernel ROS levels were not significantly correlated with aflatoxin levels as predicted by the literature. This could be due to use of limited germplasm pools in previous studies or to an inappropriate sampling time in this study. Further research at multiple developmental timepoints and under stressed conditions will be needed to differentiate these possibilities. We observed significant associations between aflatoxin content and three flavonoid genes, suggesting that antioxidants could play a role in aflatoxin contamination. Significant correlations with several carotenoids, another class of antioxidant, were also found. GA is known to affect the expression of both flavonoid and carotenoid genes. Further experiments are needed to determine whether these relationships are the results of direct effects of antioxidants on aflatoxin levels or indirect effects related to GA.

This research suggests a previously untested area of aflatoxin research, namely the relationship of GA and GA-related traits to aflatoxin content. Among the possible areas to be addressed are investigation of aflatoxin contamination in the landraces in relationship to GA and GA related traits. In addition, evaluation of the haplotypes with respect to GA and starch genes in teosinte x maize introgression lines for aflatoxin content could also be helpful. In addition, assays of aflatoxin contamination in GA,

carotenoid, flavonoid, and α-amylase mutants individually and in combination are warranted. Our results suggest future attempts to understand aflatoxin contamination in maize on a genetic level should focus on defining the changes in haplotypes within GA genes and their consequences for: 1) GA content in the plant; 2) α-amylase activity and starch degradation products; 3) changes in plant architecture at traits that relate to altered aflatoxin content; and 4) the interaction of GA and ABA relative to ROS content.

Appendix I

How Flavonoids Affect the Growth and Aflatoxin Production of *A. flavus*

Introduction

The anti-aflatoxigenic activity of eugenol reduces microsomal lipid peroxidation (Jayashree and Subramanyam, 1999). Reverberi et al. (2008) documented an increase in *A. parasiticus* superoxide dismutase, catalase, and glutathione peroxidase and a decrease in aflatoxin production on exposure to *Lentinula edodes* (shitake mushroom) extracts. In maize a seven-fold increase in aflatoxin content was observed in a maize line carrying a mutation in chalcone synthase, the rate limiting step in the flavonoid biosynthesis (G. Davis, *personal communication*). Based on our prior results with the chalcone synthase mutant, we report testing of ten flavonoid compounds *in vitro* to observe their effects on *A. flavus* growth and aflatoxin inhibition in culture.

Materials and Methods

Preparation of flavonoids. Daidzein, daidzin, genistein, genistin, diosmin, hesperetin, hesperidin, naringenin, quercetin, rutin, and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Absolute ethyl alcohol was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KY, USA). 10.2 mg daidzein, 16.6 mg daidzin, 10.8 mg genistein, 17.3 mg genistin, 24.3 mg diosmin, 24.4

mg hesperidin, and 24.42 mg rutin were dissolved in 1500 ml DMSO to create 100x stock solutions of each chemical. 12.1 mg hesperitin, 10.89 mg naringenin, and 13.53 mg quercetin were dissolved in ethyl alcohol to create a 1500 mL 100x stock solution of each chemical.

Culture. *A. flavus* NRRL3357 was obtained from the Agricultural Research Service Culture Collection in Peoria, IL. This strain was selected for its consistency in growth and aflatoxin production. Cultures were grown on Czapek-Dox agar supplemented with 10 g/L sodium chloride.

Treatment application and culture conditions. Media were prepared from 49 g/L Czapek-Dox agar supplemented with 10 g/L NaCl. 25 mL increments of media were poured into 100 x 25 mm petri dishes. Daidzein, daidzin, genistein, genistin, diosmin, hesperetin, hesperidin, naringenin, quercetin, and rutin at concentrations of 50 μM, 100 μM, 150 μM, 200 μM, and 250 μM were supplemented after autoclaving when medium temperature was less than 60^{o}C. Controls were Czapek-Dox agar supplemented with 10 g/L NaCl, Czapek-Dox agar supplemented with 10 g/L NaCl and 80 μl DMSO or 80 μl ethanol depending on the compound solvent (equivalent to amount of solvent used in 50 μM treatments). The center of each plate was marked and inoculated with a 1 cm square of *A. flavus* NRRL3357 mycelia under sterile conditions. Cultures were grown in a 37^{o}C incubator.

Growth measurements and analysis. Fungal growth was measured as the diameter of the mycelium intersecting the marked plate center. Growth was measured at 2, 4, 6, 8, 10, 12, and 14 days after inoculation. At 14 days, the fungus and Czapek-Dox

agar were harvested from the petri dishes, placed in 50 ml tubes, and frozen at –20^{o}C for future analysis.

The experimental design consisted of four replications of five plates each in a randomized complete block design. Each compound was analyzed separately due to heterogeneity in error variances. A preliminary analysis of variance (ANOVA) was performed using the PROC GLM module of SAS version 8.2 (The SAS Institute, Raleigh, NC) on each compound, with replications, compound concentrations, days, and concentration x day as classification variables to determine if data from different time points could be analyzed together. The final analysis of variance was performed by day within compound because of unequal error variances among days. Least squares means were used to compare mean compound differences within each concentration level.

Aflatoxin quantification and analysis. Two plates were randomly combined within each concentration x replication for a given compound for aflatoxin analysis resulting in two samples per concentration in each replicate for a total of eight samples per concentration across the four replicates. Aflatoxin was extracted from the medium using 100 ml 80% methanol (Fisher Scientific, Fair Lawn, NJ), 20% water, 5 g NaCl and liquefied in a blender for 1 min. Aflatoxin was quantified using a direct competitive anti-aflatoxin rabbit IgG antibody enzyme-linked immunosorbent assay (ELISA) (Sigma, St. Louis, MO) that detects aflatoxin levels greater than 1 ppb according to the manufacturers protocol. ELISA plates were read on a spectrophotometer at 490 nm wavelength. Aflatoxin standards (ppb) of 0, 12.5, 25, 50, 100, 200, 400, 800, and 1600 were used to construct a standard curve. Only standard curves with an R^2 value of at least 0.95 were considered acceptable for determining aflatoxin content of the unknown

samples. Samples with aflatoxin content near either end of the standard curve were re-diluted and reassayed by ELISA to increase the accuracy of aflatoxin determination.

The aflatoxin data obtained from interpolation of the standard curve was transformed using the function, $y' = \sqrt{y}$, to normalize the data. Statistical analysis was conducted based on a general linear model where compounds, replications, and levels were fixed effects. The model for the aflatoxin analysis was:

$$Y_{ijkl} = \mu + C_i + R_j + L_k + CR_{ij} + CL_{ik} + RL_{jk} + CRL_{ijk} + \varepsilon_{ijkl}$$

where μ = overall mean, C_i =effect of compound *i*, R_j =effect of replication *j*, L_k = effect of line *k*, AR_{ij} = the interaction of compound *i* with replication *j*, AL_{ik} = the interaction of compound *i* with level *k*, RL_{jk} = the interaction of replication *j* with level *k*, ARL_{ijk} = the interaction of compound *i* with replication *j* and level *k*, and ε_{ijkl} = the residual variation effect. Interaction terms that were found to be non-significant were removed from the model effectively increasing the error degrees of freedom and the data were re-analyzed with the new model.

Association analysis. Association analysis was performed on the available *a1*, *c2*, and *whp1* gene sequence with the aflatoxin data described in Chapter 2 according to the methods outlined in Bradbury et al. (2007). These genes were chosen for association analysis because of the fact that they are part of biochemical pathways that were identified as potentially affecting aflatoxin levels based previous on experiments.

Results

Growth results for diosmin, daidzin, daidzein, genistin, and genistein. The flavanoids diosmin, daidzin, daidzein, genistin, and genistein, as well as controls had highly significant differences in fungal growth across days (Table 22). Concentrations also significantly affected fungal growth for all compounds (Table 22). A highly significant interaction of concentration x day was identified in the daidzin, daidzein, and genistein experiments but not for diosmin or genistin. Replications were significantly different at the 1% level in the DMSO control, daidzin, daidzein, diosmin, and genistin, the 5% level in genistein, and not significantly different in the control.

Five concentrations were examined for each flavonoid in this experiment. Diosmin showed the largest reduction in growth at all levels tested (Figure 7). Smaller growth differences were observed for genistin at 50, 100, and 150 μM, for genistein at 200 and 250 μM and for daidzin at 250μM.

Another important variable examined in this experiment was the effect that individual flavonoids had on growth over time. Different patterns of growth over time were observed with each flavonoid (Figure 8). Diosmin exhibited the most striking reduction in fungal growth over all time points. The reduction in growth ranged from 13 % on day 2 to 30 % on day 14 compared to the DMSO control. Genistin and genistein also reduced growth throughout the time course but not as dramatically. Daidzin reduced growth compared to the control after day 6 (Figure 8).

Concentration x day interactions were observed for several flavonoids (Figures 9 and 10). Genistein significantly reduced growth beginning on day 6 at the 200 μM and

250 μM concentrations (Figure 9). Daidzin significantly reduced growth on day 2 at the 250 μM concentration (Figure 10).

Growth results for hesperetin, hesperidin, naringenin, rutin, and quercetin. Replications were significantly different at the 1% level in the DMSO control, hesperetin, and naringenin treatments (Table 23), and at the 5% level for the rutin treatment (Table 24). All flavonoids and controls demonstrated highly significant differences in *A. flavus* growth across days after inoculation (Tables 23 and 24). Concentrations had a highly significant effect on *A. flavus* growth for hesperetin, naringenin, and rutin (Tables 23 and 24). The most noticeable differences in fungal growth among concentrations occurred for naringenin and rutin (Figure 11). The interaction of concentration by day had a highly significant effect on *A. flavus* growth for hesperetin, naringenin, and rutin supplemented media. Hesperetin had a significantly lower mean fungal growth at all concentrations compared to the control and this effect appeared to be concentration independent (Figure 11). Naringenin had a significant reduction in mean fungal growth at the highest concentration tested. Quercetin and hesperidin showed no significant difference in mean *A. flavus* growth across concentrations.

The effect that the individual flavonoids had on fungal growth over time was another important component examined in this experiment (Figure 12). Rutin and quercetin increase early fungal growth as compared to the control whereas naringenin and hesperetin slow growth of the fungus during day two through six of the experiment (Figure 12).

Naringenin significantly reduced growth on day 2 through 6 at all levels except 250 μM (Figure 13). Rutin significantly reduced growth on day 4 through 6 at 200 μM

(Figure 14). A highly significant concentration by day interaction for fungal growth on media supplemented with hesperetin was also detected.

Aflatoxin analysis. The ANOVA for aflatoxin analysis had an overall R^2 value of 0.86 and the CV was 33.33 (Table 25). Flavonoid, concentration, and flavonoid x concentration were significant at α=0.001. Replication was not statistically significant nor were any of the interactions with replication. Since significant differences were observed among flavonoids for aflatoxin content Duncan's multiple range test at $P < 0.05$ was used to further study the effect of different compounds (Table 26). Diosmin was demonstrated to significantly reduce *A. flavus* growth at all time points in previous experiments but had the highest aflatoxin level on average among the compounds tested. Only 50 μM and 100 μM diosmin had significantly less aflatoxin as compared to the experimental controls (*data not shown*). Higher levels of diosmin caused the production of more aflatoxin. Aflatoxin levels of cultures grown on media supplemented with at least 150 μM diosmin were comparable to the control and DMSO control. There was no relationship between fungal growth and aflatoxin levels across the compounds we tested. Naringenin and hesperetin inhibited early growth of the fungus but aflatoxin levels were not significantly different from compounds such as quercetin and rutin that failed to inhibit fungal growth. A one-way ANOVA was completed for each compound where level was the independent variable and aflatoxin was the dependent variable (*data not shown*). Daidzin, diosmin, genistin, and genistein each had a one-way ANOVA model that was significant at $P < 0.01$. The one-way ANOVA model for quercetin was significant at $P < 0.05$. The Duncan's multiple range test for each compound did not

validate that increasing or decreasing the amount of flavonoid added would affect aflatoxin levels.

Anti-oxidant trait correlations. Holmes et al. (2008) review the inhibitory effects of carotenoids and flavonoids on aflatoxin production in *A. flavus*. In an effort to extend these results to whole plants we evaluated correlations between cob and kernel pigmentation as well as carotenoid content with aflatoxin levels in the maize diversity set. Previous experiments using single gene mutants in various enzymes in the anthocyanin pathway assessed changes in aflatoxin content. Only mutations in the *c2, chalcone synthase* gene were found to significantly affect aflatoxin levels (G. Davis, *unpublished data*). These mutants display wide variation in pigmentation, however, no relationship with aflatoxin content was observed suggesting that anthocyanin composition rather than overall pigmentation affects aflatoxin content. In this study kernel color was not significantly correlated with aflatoxin content. These findings are in agreement with the results from the anthocyanin mutant study. However, cob color was measured at the North Carolina Agricultural Research Station and there was a significantly negative correlation (-0.395, $P < 0.01$) with aflatoxin levels (Table 12).

In vitro studies by Norton et al. (1997) suggested that some carotenoids which are present in maize can inhibit the production of aflatoxin B_1. Harjes et al. (2008) evaluated carotenoid levels in the maize diversity lines to address the problem of vitamin A deficiencies in developing countries. Carotenoids that were measured by HPLC analysis were: lutein, zeaxanthin, cryptoxanthin, α-carotene, β-carotene, and total carotenoids levels. The correlations between the measured carotenoids and aflatoxin levels were assessed (Table 27). Zeaxanthin, cryptoxanthin, and β-carotene were correlated with

reducing aflatoxin levels by 40.9-50.5% (P<0.001); these compounds are all part of the carotenoid and abscisic acid (ABA) pathways. Carotenoid and aflatoxin correlations were subsequently analyzed within a structure-based subpopulation to see if the population structure would affect these correlations. The data indicate that population structure does affect the correlations we observed.

Association analysis with flavonoid genes. Association analysis identified three polymorphisms in the *a1* gene, one in the *c2* gene, and five in the *whp1* gene associated with changes in aflatoxin content (Table 28). All of the individual polymorphisms explained very small portions of the variance in aflatoxin content.

Discussion

In maize, flavonoids and carotenoids represent two major classes of antioxidant molecules. In addition, flavonoids have been recognized to have a variety of health promoting activities. Hesperetin, quercetin, and rutin are currently not documented in commercial maize varieties but are present in a number of other vegetable sources. Of the flavonoids we tested *in vitro*, only naringenin is present in maize. Hesperetin and naringenin are present in citrus. Black tea, apple peels, and onions are all sources of quercetin (Yang et al., 2001). Rutin has found to be present in buckwheat. Hesperetin and naringenin lower plasma cholesterol *in vivo* by decreasing apoB (apolipoprotein B) accumulation that is associated with LDL (low density lipoprotein) cholesterol (Wilcox et al., 2001). Naringenin has been identified as a potential PI3K (phosphoinositide-3-kinase) inhibitor and MEK (mitogen-activated protein kinase extracellular signaling-

regulated kinase) inhibitor that blocks basal and insulin-stimulated glucose uptake in breast cancer cells (Harmon and Patel, 2004). Quercetin applied topically has been demonstrated to markedly reduce skin carcinoma (Verma et al., 1988). Rutin research supports hypotheses that it possesses effective pharmacological actions on cardiovascular systems (Satoh and Nishida, 2004).

Norton (1999) evaluated the ability of several flavonoids to reduce fungal growth using a suspended disk culture. Several of the compounds tested reduced aflatoxin levels but not fungal growth. Flavonoids can possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic properties. They are metal chelators and free radical scavengers (Middleton, et al., 2000). Previous studies conducted by Norton (1999) using suspended disk cultures examined the role of flavonoids in decreasing *A. flavus* mycelial mass and aflatoxin levels. Pelargonidin and delphinidin significantly reduced aflatoxin levels, but propelled fungal growth. 3-OH compounds such as pelargonidin and delphinidin had three times more activity than related 3-deoxy compounds, however, the 3-deoxy compounds did not provoke fungal enlargement as compared to 3-OH compounds. Malvidin, cyanidin, kaempferol, luteolin to a lesser degree also inhibited aflatoxin B_1 production but stimulated growth. This experiment did not find any compounds that considerably suppressed mycelial mass and aflatoxin levels. Concentrations of compounds tested ranged from 0 to 9.78 mM. The majority of the upper levels tested are not feasible to attain endogenously in maize.

Daidzin, daidzein, genistein, and genistin (all isoflavones) are present in soybean and diosmin (a flavone) is present in citrus (Caristi et al., 2003; Marin and Del Rio, 2001), but none have been documented in corn varieties. These compounds have been

associated with a number of health benefits. Genistein has been reported to cause G2-M cell cycle arrest and inhibition of *cdc2* kinase activity (Su et al., 2000). Genistein was also reported to inhibit lung tumor nodule formation in mice (Menon et al., 1998; Su et al., 2000). Zhang et al. (2003) demonstrated that daidzein, genistein, and diosmin were active in stably transfected Hepa-1 cells exhibiting weak aryl hydrocarbon antagonist activities. Genistin and daidzin were demonstrated to be effective in suppressing rat prostate cancer tumors (Kato et al., 2000). Adlercreutz et al. (1993) found seven to 110 times higher levels of daidzein, genistein, and other isoflavonoids in the plasma of Japanese men than those of Finnish men, providing a possible explanation for the low mortality rate from prostate cancer in Japan. Diosmin is used to help treat chronic venous insufficiency (Simka and Majewski, 2003).

Of the compounds we tested, diosmin was the best at reducing the growth of *A. flavus in vitro* at all concentrations and time points. Diosmin has the same carbon attachment site as luteolin and kaempferol which were found to reduce aflatoxin levels (Norton, 1999). In contrast, aflatoxin levels were the highest for diosmin of all the compounds tested in this experiment. Levels of diosmin above 150 µM were toxic to the fungus causing greater aflatoxin production as compared to the 50µM and 100 µM levels. The treatment levels tested here are within the range that have been observed *in planta*. The effective concentrations are slightly higher than those documented to inhibit fungal growth by Gordon et al. (1980). Although we observed a 30% reduction in *A. flavus* growth with diosmin, only 50 µM and 100 µM had significantly less aflatoxin as compared to controls. Higher levels of diosmin caused the production of more aflatoxin. Aflatoxin levels are often uncorrelated with fungal growth except in the case of complete

inhibition of fungal growth. Complete inhibition growth and aflatoxin production might be achieved with diosmin in combination with another flavonoid.

Some of the compounds tested such as naringenin and hesperitin reduced only the early growth of *A. flavus* over time. Other important places for the inhibition compound to be present are silks, kernels, pediduncle, and cob tissue. Fungal growth may be inhibited or the infection process may be slowed if an inhibitory compound were present in the silks. An inhibitory compound present in the pedicel or "tip cap" would be important in preventing the infection of other kernels sharing the same vascular tissue. Another imperative place for fungal inhibition to take place to avoid the spread of *A. flavus* is the cob tissue.

Table 22. Analysis of variance for *A. flavus* growth in control, DMSO control, daidzein, daidzin, diosmin, genistein, and genistin treated cultures.

Flavanoid	Variable[b]	DF	Mean Square	Significance[a]
Control	Day	6	534.3484	**
Control	Rep	3	0.0481	NS
DMSO Control	Day	6	579.5143	**
DMSO Control	Rep	3	0.3305	**
Daidzein	Day	6	486.6810	**
Daidzein	Conc	4	0.0973	**
Daidzein	Conc*Day	24	0.0517	**
Daidzein	Rep	3	0.09478	**
Daidzin	Day	6	419.4606	**
Daidzin	Conc	4	12.4444	**
Daidzin	Conc*Day	24	0.7528	**
Daidzin	Rep	3	1.3081	**
Diosmin	Day	6	172.7930	**
Diosmin	Conc	4	80.9036	**
Diosmin	Conc*Day	24	1.7033	NS
Diosmin	Rep	3	243.8218	**
Genistein	Day	6	488.7828	**
Genistein	Conc	4	13.7151	**
Genistein	Conc*Day	24	2.7978	**
Genistein	Rep	3	1.2672	*
Genistin	Day	6	382.8771	**
Genistin	Conc	4	13.2765	**
Genistin	Conc*Day	24	0.9716	NS
Genistin	Rep	3	6.4909	**

[a] *, ** Levels of significance $P < 0.05$ and $P < 0.01$. NS, not significant.

[b] Conc. , cconcentration.

Table 23. Analysis of variance for *A. flavus* growth in control, DMSO control, hesperetin, naringenin, and quercetin treated cultures.

Flavonoid	Variable[b]	DF	Mean Square	Significance[a]
Control	Day	6	542.6055	**
Control	Rep	3	0.1699	NS
Ethanol Control	Day	6	434.6694	**
Ethanol Control	Rep	3	0.0344	NS
Hesperetin	Day	6	698.0053	**
Hesperetin	Conc	4	0.0156	NS
Hesperetin	Conc*Day	24	0.0086	NS
Hesperetin	Rep	3	0.0992	**
Naringenin	Day	6	560.2714	**
Naringenin	Conc	4	0.8343	**
Naringenin	Conc*Day	24	0.7666	**
Naringenin	Rep	3	0.1456	**
Quercetin	Day	6	505.0955	**
Quercetin	Conc	4	0.188064	NS
Quercetin	Conc*Day	24	0.0665	NS
Quercetin	Rep	3	0.1090	NS

[a] **, Level of significance $P < 0.01$. NS, not significant.

[b] Conc, concentration.

Table 24. Analysis of variance for *A. flavus* growth in control, DMSO control, and rutin supplemented cultures.

Compound	Variable[b]	DF	Mean Square	Significance[a]
Control	Day	6	542.6055	**
Control	Rep	3	0.1699	NS
DMSO Control	Day	6	103.6551	**
DMSO Control	Rep	3	0.0481	**
Rutin	Day	6	441.3309	**
Rutin	Conc	4	0.2000	**
Rutin	Conc*Day	24	0.0613	**
Rutin	Rep	3	0.0066	NS

[a] **, Level of significance is $P < 0.01$. NS, not significant.

[b]Conc = concentration.

Table 25. Analysis of variance for aflatoxin content following 14 days of growth on media supplemented with daidzein, daidzin, diosmin, genistein, genistin, hesperetin, hesperidin, naringenin, quercetin, or rutin at 0, 50, 100, 150, 200, and 250 µM.

Source	DF	SS	MS	Significance
Flavonoid	9	37.380	4.153	***
Concentration	6	5.298	0.883	***
Replication	3	0.909	0.303	NS
Flavonoid*Concentration	48	34.204	0.713	***
Flavonoid*Replication	27	6.818	0.253	NS
Replication*Concentration	18	4.389	0.244	NS
Flavonoid*Replication*Conc	104	20.911	0.201	NS
Error	118	19.781	0.167	
Total	333	143.656		

***, Level of significance is $P < 0.001$. NS, not significant.

Table 26. Duncan's multiple range grouping for mean aflatoxin content of cultures supplemented with several flavonoid compounds. The aflatoxin mean is the average of all concentrations tested. Means followed by the same letter are not statistically different.

Flavonoid	Aflatoxin Mean (ppb)	Duncan's Multiple Range Grouping
Hesperedin	0.497	A
Rutin	0.750	AB
Naringenin	0.863	B
Quercetin	0.995	B
Hesperetin	1.523	C
Daidzin	1.606	C
Daidzein	1.753	C
Genistein	2.596	D
Genistin	3.602	E
Diosmin	3.700	E

Table 27. Spearman's correlation of carotenoid content from Harjes et al. (2008) with average Missouri aflatoxin content.

Trait	Correlation	Significance
Lutein	0.165	NS
Zeaxanthin	-0.462	***
Cryptoxanthin	-0.505	***
α-carotene	-0.061	NS
β-carotene	-0.409	***
Total Carotenoids	-0.350	**

****, ***,** Significant at $P < 0.001$ and $P < 0.0001$, respectively. NS, not significant.

Table 28. Summary of the results from association analysis of the maize *a1*, *c2*, and *whp1* genes with mean Missouri aflatoxin level across years.

Locus	Site	df	F	p	Model df	Error df	Error MS	R^2 model	R^2 marker
a1	453	1	4.9186	0.0307	3	55	0.0485	0.9831	0.0015
a1	76	1	4.3974	0.0405	3	56	0.0030	0.9989	8.33E-05
a1	96	1	4.1238	0.0470	3	56	0.0074	0.9974	1.89E-04
c2	756	1	4.1544	0.0476	3	44	0.3574	0.8761	0.0117
whp1	44	1	7.9195	0.0080	3	35	0.1952	0.9450	0.0125
whp1	626	1	5.0729	0.0288	3	49	0.1259	0.9607	0.0041
whp1	523	1	4.9053	0.0316	3	48	1.8209	0.4203	0.0592
whp1	526	1	4.7422	0.0343	3	49	1.8515	0.4227	0.0559
whp1	739	1	3.9555	0.0527	3	46	0.2204	0.9231	0.0066

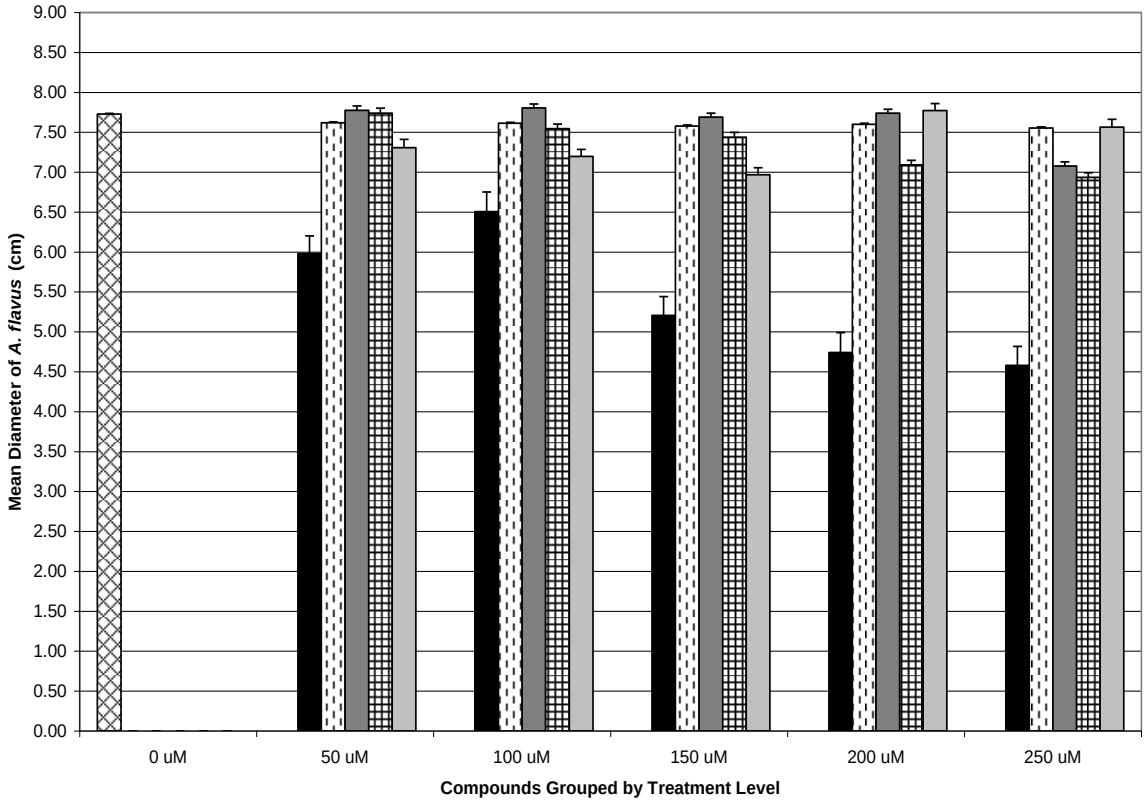


Figure 7. Mean colony diameter of *A. flavus* at different concentrations for diosmin, daidzein, daidzin, genistein, and genistin. Error bars represent the standard error of each LS Mean. Treatments: (■) diosmin; (◳) daidzein; (▩) daidzin; (▤) genistein; genistin (▢); control (▨).

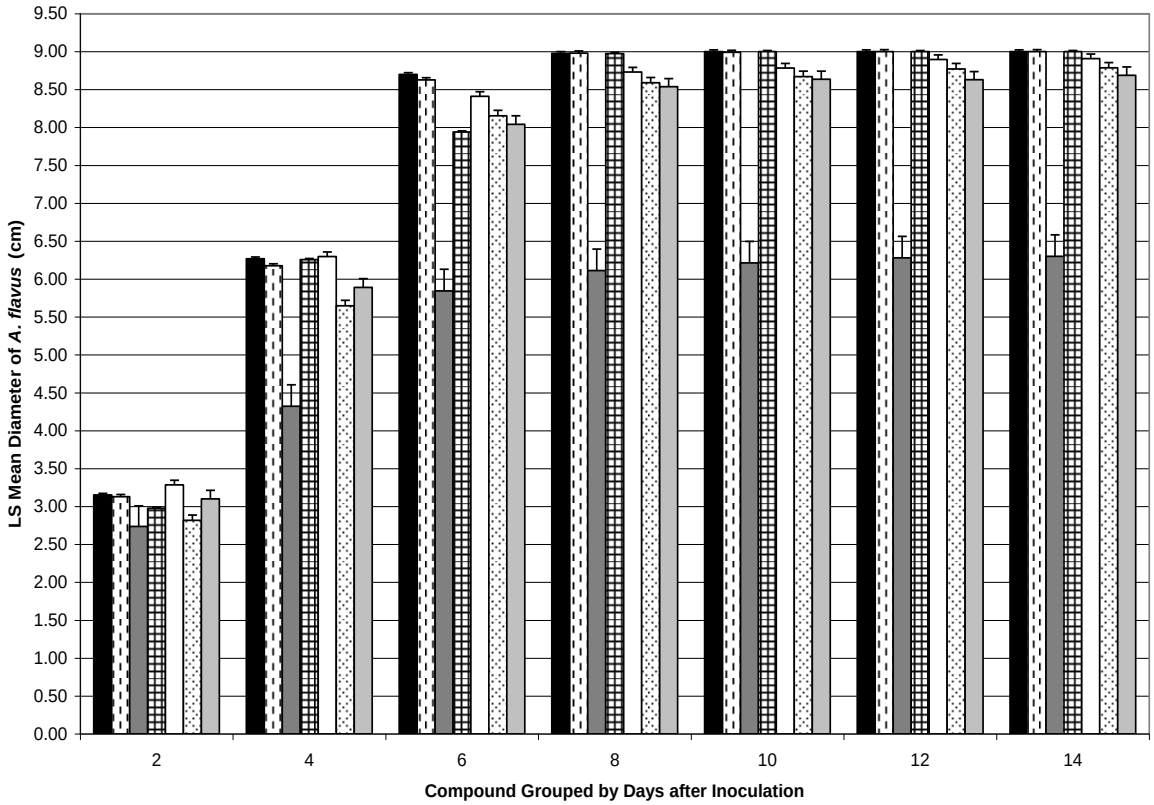


Figure 8. Mean colony diameter of *A. flavus* by day for diosmin, daidzein, daidzin, genistein, and genistin. Error bars represent the standard error of each LS Mean. Treatment are represented as followed: control (■), DMSO control (▣), diosmin (▩), daidzein (⊟), daidzin (□), genistein (⊠), and genistin (▢).

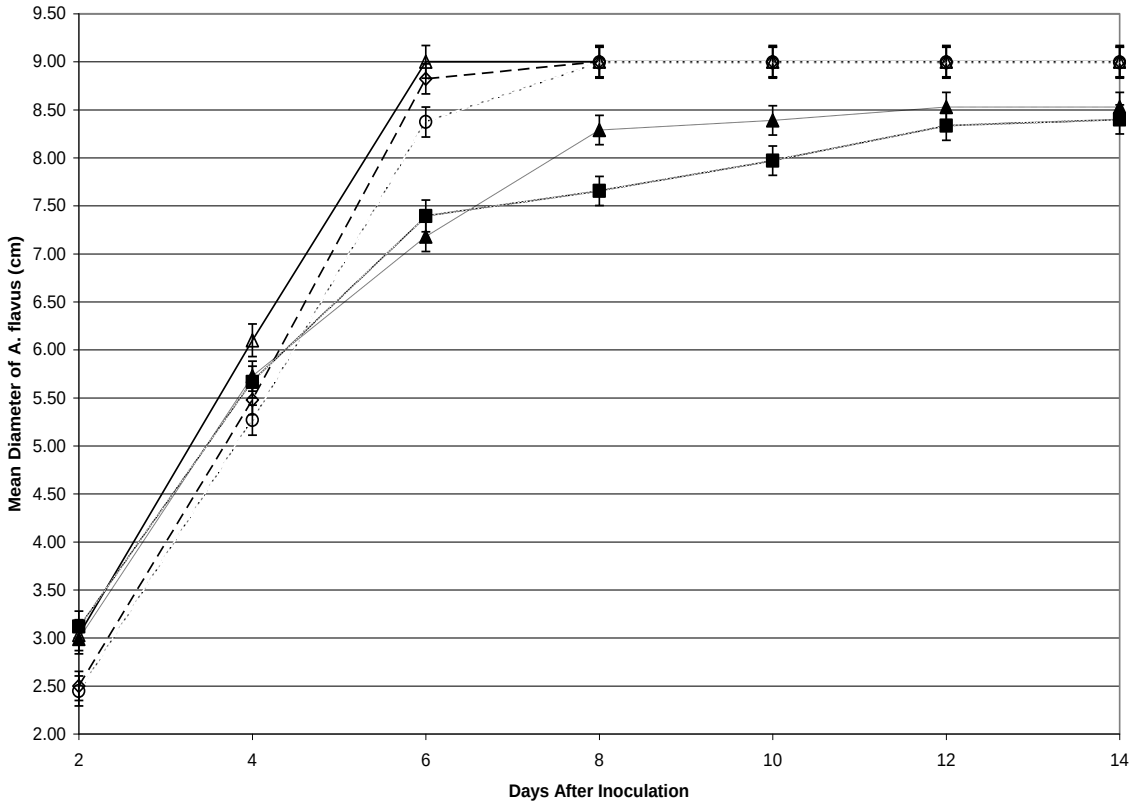


Figure 9. Mean colony diameter of *A. flavus* for genistein by concentration and day. Error bars represent the standard error of each LS Mean. Concentrations are represented as follows: 50 µM ( ), 100 µM ( ), 150 µM ( ), 200 µM ( ), 250 ( ).

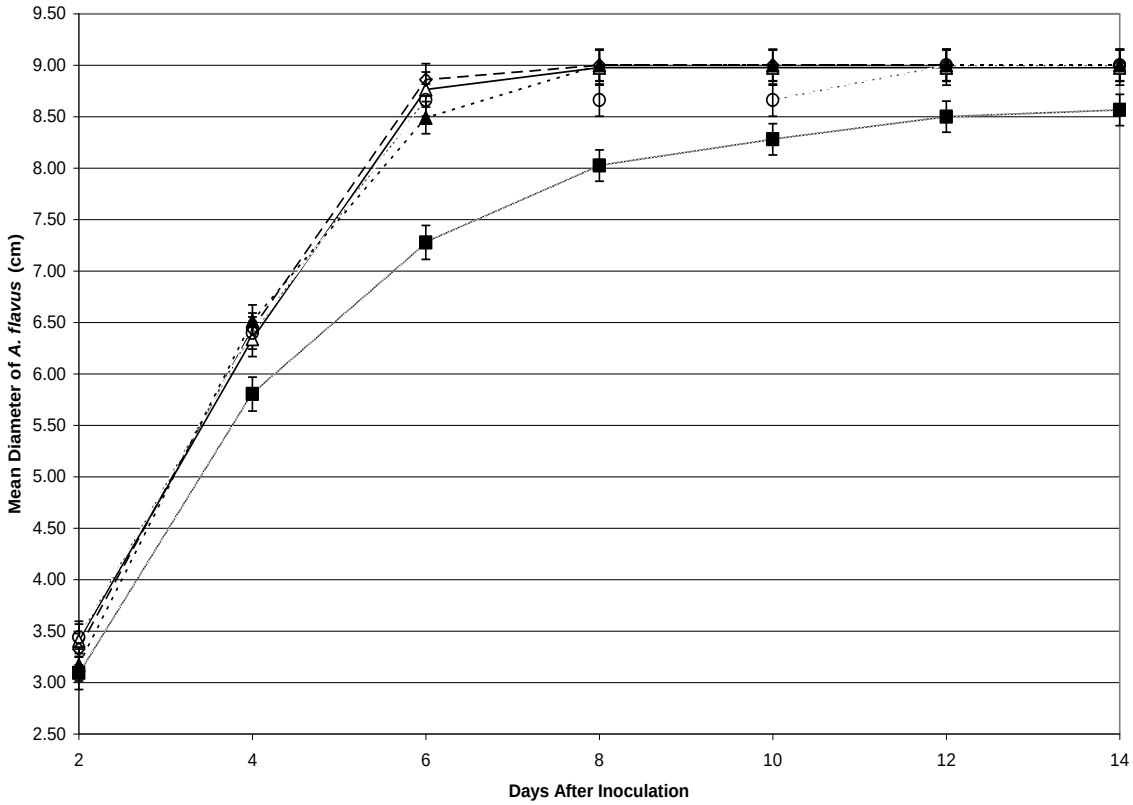


Figure 10. Mean colony diameter of *A. flavus* for daidzein by concentration and day. Error bars represent the standard error of each LS Mean. Concentrations are represented as follows: 50 µM ( ), 100 µM ( ), 150 µM ( ), 200 µM ( ), 250 ( ).

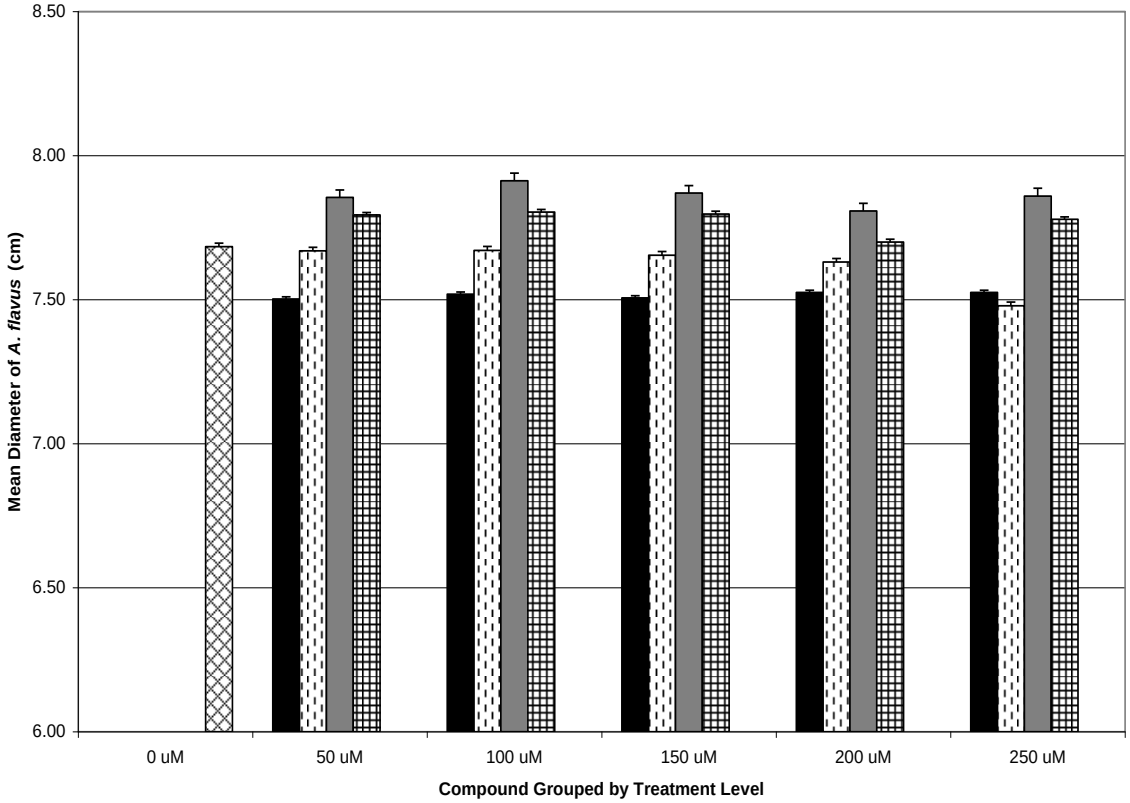


Figure 11. Mean colony diameter of *A. flavus* with hesperetin, naringenin, quercitin, and rutin at five concentrations. Error bars represent the standard error of each LS Mean. Treatments are represented as followed: control ( ), hesperetin ( ), naringenin ( ), quercetin ( ), rutin ( ).

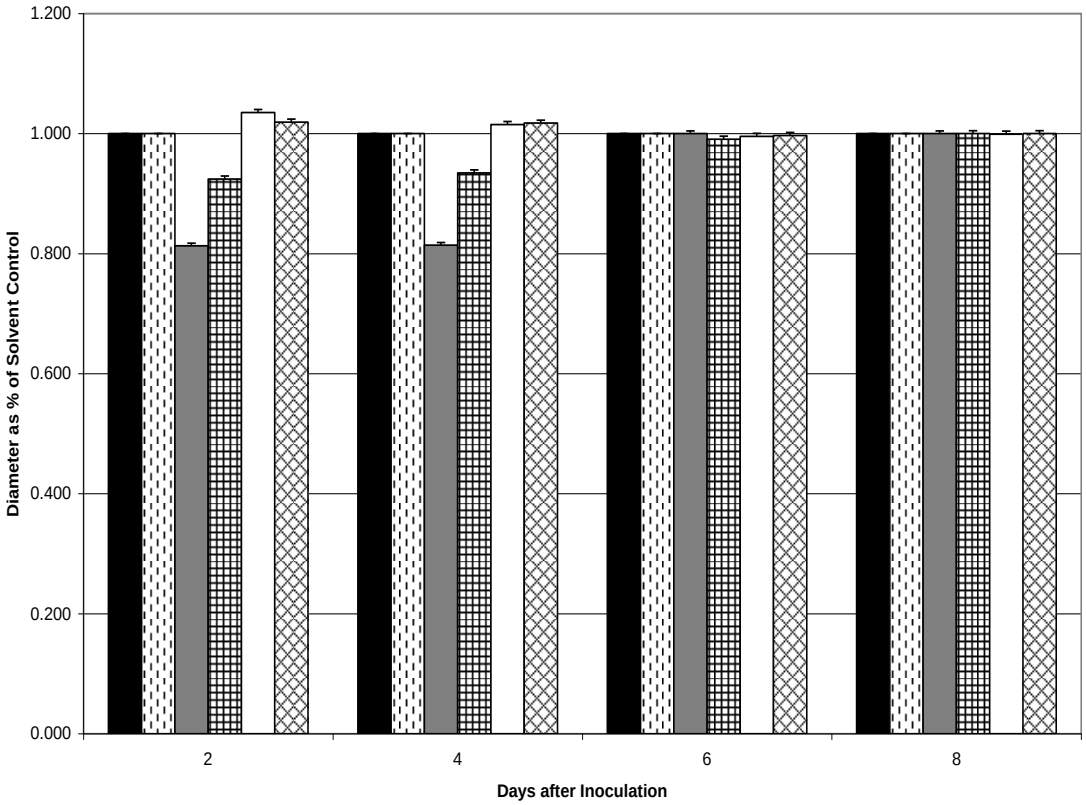


Figure 12. Mean colony diameter of *A. flavus* by day for control, ethanol control, DMSO control, hesperetin, naringenin, quercetin, and rutin. Error bars represent the standard error of each LS Mean. Treatments are represented as followed: ethanol control ( ), DMSO control ( ), hesperetin ( ), naringenin ( ), quercetin ( ), and rutin ( ).

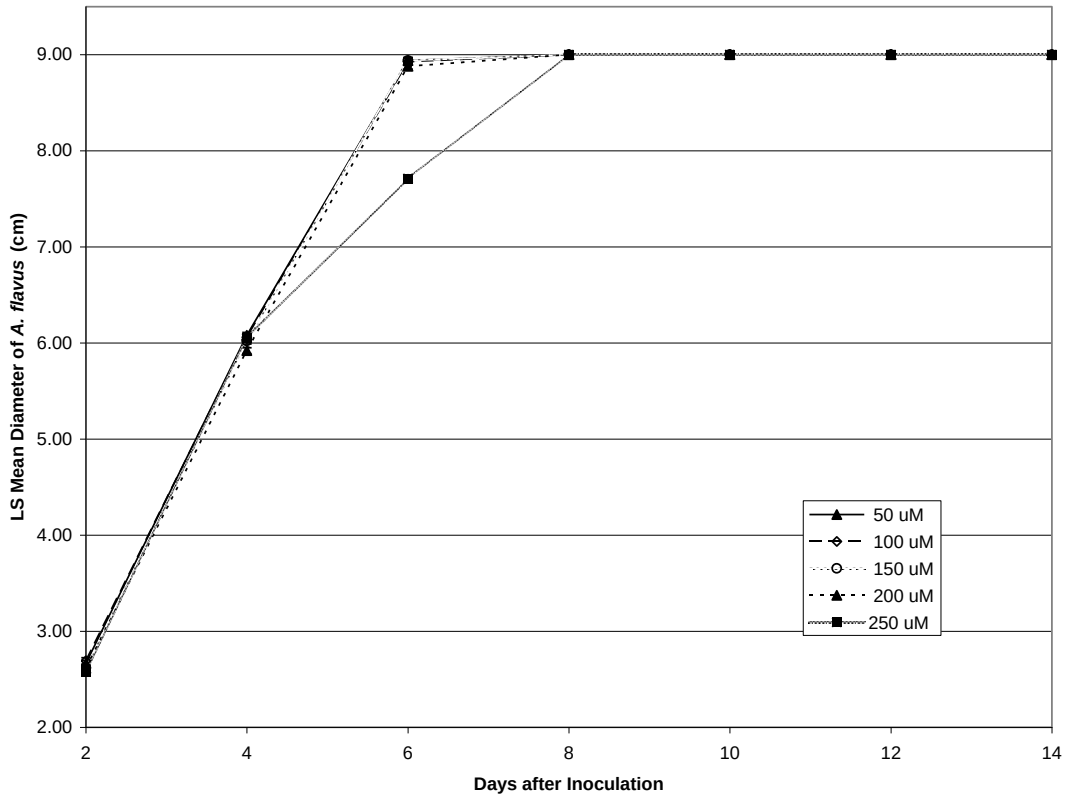


Figure 13. Mean colony diameter of *A. flavus* for each concentration on each day for naringenin. Error bars represent the standard error of each LS Mean but error bars are smaller than graph symbols. Concentrations are represented as followed: 50 µM ( ), 100 µM ( ), 150 µM ( ), 200 µM ( ), 250 ( ).

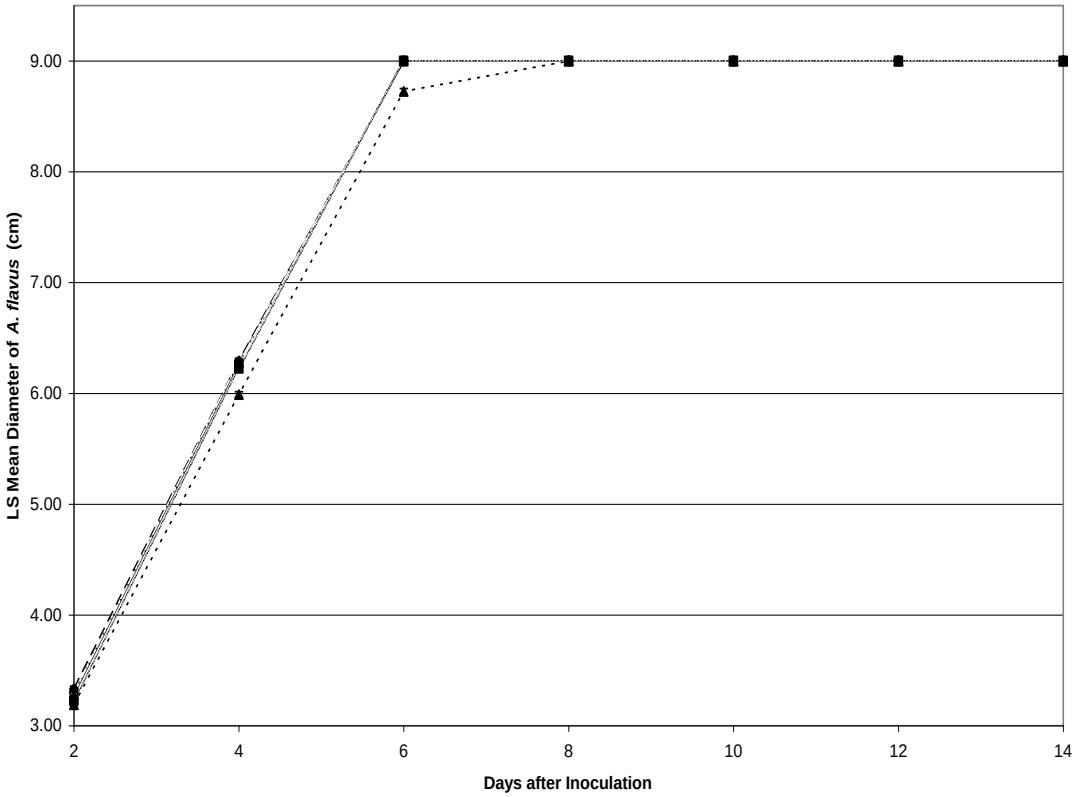


Figure 14 Mean colony diameter of *A. flavus* for rutin by concentration and day. Error bars represent the standard error of each LS Mean but error bars are smaller than graph symbols. Concentrations are represented as followed: 50 µM ( ), 100 µM ( ), 150 µM ( ), 200 µM ( ), 250 ( ).

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VITA

Dana Lynn Woodruff Bush was born in Kirksville, Missouri and grew up on a farm near Kahoka, Missouri. Her parents, Terry and Karen Woodruff, fostered her love of reading and the outdoors. Dana graduated from Clark County R-1 High School in 1999 with a college prep diploma. She earned an associate of science degree in liberal studies from Indian Hills Community College in 2000. During her time at Indian Hills Community College, Dana met her future husband Broderick Bush from Shelbyville, MO. In the fall of 2000, Dana began her undergraduate career at the University of Missouri. Shortly after starting school, she began working in the Davis lab under the direction of Georgia Davis. Dana accepted a competitive summer Plant Genomics Internship at Mizzou.

After graduating in 2003, Dana married her community college sweetheart Broderick Bush. Dana began graduate school in the fall of 2003. Poncho Villa Bush, a devoted chocolate lab, was added to the family in 2005. On October 26, 2007, Dana and Broderick brought their daughter Merrick Lynn Bush into the world.

Dana enjoys hunting, NASCAR, cooking, scrapbooking, sewing, spending time with her daughter, and garage sales. One of her most memorable moments in graduate school was going to Puerto Rico for a winter nursery and discovering delicious flan.