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Monitoring Aspergillus Flavus Progression and Aflatoxin Accumulation in Inoculated Maize (Zea Mays L.) Hybrids

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By

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Aflatoxins are a secondary metabolite produced by the fungus *Aspergillus flavus*. *A. flavus* has been known to infect several crops including tree nuts, peanuts, rice, cotton and maize. Aflatoxins have been found to cause tumors with aflatoxin B1 being the most carcinogenic biologically produced substance known to man. Therefore, the FDA has restricted the amount of aflatoxin in maize for human consumption to 20 ppb (ng/g). An estimated $225 million are lost each year in the United States due to aflatoxin contamination in maize crops alone. Agriculture is a vital part of Mississippi's economy, and maize is one of its largest crops.

The purpose of this research is to track the correlations between aflatoxin accumulation and *Aspergillus flavus* fungal biomass for the first several weeks after inoculation, as well as the spreading of the fungus and the aflatoxin throughout the inoculated ear of maize. This will allow for better understanding of the pathogen-host interactions and how the fungus progresses over time. GA209 x T173 is the aflatoxin accumulation susceptible maize hybrid, GA209 x Mp313E is the susceptible and resistant hybrid, and Mp717 x Mp313E is the resistant maize hybrid to aflatoxin accumulation.

These maize hybrids were each inoculated with toxin producing Aspergillus flavus NRRL 3357 and water as a control 21 days after silk maturation. Collections of the inoculated maize cobs were made 3, 7, 14, 21, 28, 35, and 60 days after inoculation. Maize samples were collected and analyzed for aflatoxin and DNA concentration. The extracted aflatoxin was analyzed using an LC/MS. The fungal biomass was determined by performing quantitative real time polymerase chain reaction (PCR).

GA209xT173 and Mp717xMp313E showed no aflatoxin production two days after inoculation. The resistant maize hybrid lead in aflatoxin accumulation the last two years but had the least amount of fungal biomass for second and third years of the experiment The production of aflatoxin seems to begin decelerating after 21 days after inoculation. Resistance characteristics are more to prevent fungal infection. Fungal biomass was significantly higher in the susceptible hybrid GA209xT173 compared to the other hybrids. However, fungal spread was significantly higher in Mp313ExT173 and Mp717xMp313E.

Keywords: aflatoxin, *Aspergillus flavus*, maize,

DEDICATION

I would like to dedicate this dissertation to my parents Cedric and Jennifer Reid, my sister Clarissa, and my stepparents Linda Reid and Jessie Hill. Thank you for all your love and support.

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CHAPTER I

INTRODUCTION

Agriculture is an essential sector of the global economy. Approximately 40% of the world's land mass is used for agricultural purposes (Alston and Pardey, 2014a). The value of the annual global agricultural production is estimated to be just shy of $4 trillion dollars. The five most valuable crops in the world at current production rates are rice, maize, wheat, soybeans, and potatoes. These crops are also on the list of the six most produced crops in the world behind only sugar cane. Of these crops, the United States leads in global production of both maize and soybeans. In fact, maize and soybeans are the two most produced crops in the United States; combining for more than a third of the United States Agricultural GDP (FAOSTAT 2014).

Agriculture is also an important industry in the United States (US). China is the only country that spends more annually on agriculture. In Mississippi (MS), nearly 30% of the State's workforce is directly or indirectly associated with farming and forestry adding over $16.5 billion/year to the local economy. According to the Mississippi Department of Agriculture and Commerce, MS produces more catfish than any other US jurisdiction, and ranks third in pulpwood, sweet potatoes and cotton. Dollar wise, however, the top agricultural product are (in ascending order) catfish, cattle, corn/maize, cotton, soybeans, pulpwood, and poultry/eggs. The United States is the second ranked exporter of both maize and soybeans in the world with Brazil being the first (FAOSTAT

2014). Animal pests, plant pathogens (viral, fungal, or bacterial), and weeds combined destroy 37% of all potential crops. On average, plant pathogens and weeds cause two thirds of all total loss of crops due to harmful organisms (Oerke, 2006; Pimentel, 2005).

Fungi cause approximately 85% of all plant diseases (Knogge, 1996; Mendgen and Hahn, 2002). Ear rot, Pythium damping off, stalk rot, and gray leaf spot are a few of the most common maize diseases. Ear rot in maize can be caused by a variety of different fungi; however, Aspergillus ear rot is especially dangerous. This is due to the ability of *Aspergillus flavus* (*A. flavus*) to produce a carcinogenic, secondary metabolite known as aflatoxin.

Aspergillus flavus spends the majority of its life cycle growing as a saprophyte in the soil where it plays an important role in the decomposition of plant material (Klich, 2007; Mellon et al., 2007; Scheidegger and Payne, 2003). *A. flavus* is a pathogenic fungus that has been known to infect a variety of crops including rice (*Oryza sativa*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), and maize (*Zea mays*) (Dorner and Horn, 2007; Liu et al., 2006; Rajasekaran et al., 2008; Safara et al., 2010). Maize is one of the crops most harshly affected by *Aspergillus flavus.* Under environmental conditions such as high temperatures, high humidity, drought, and high levels of phytophagous insects, *A. flavus* can produce a secondary metabolite known as aflatoxin (Cardwell et al., 2000; Guo et al., 2008; Trenk and Hartman, 1970). There are four main types of aflatoxin: B1, B2, G1, and G2. Aflatoxin B1 is the most ubiquitous and carcinogenic of the aflatoxins. In 1988 the International Agency for Research on Cancer classified aflatoxin B1 as a Class 1 human carcinogen (Vainio and Wilbourn, 1992). The FDA has restricted the amount of aflatoxin allowed for human consumption

to 20 (ppb) parts per billion. It has been estimated that crop loss due to aflatoxin contamination ranges between $104.5 million to $1.68 billion dollars in the United States (Mitchell et al., 2016a; Robens and Cardwell, 2003). Certain farming techniques such as irrigation, earlier planting dates, fertilization, insect control, biological controls, proper storage, and the use of aflatoxin resistant maize lines can aid in controlling aflatoxin levels (Dorner, 2009; Pitt et al., 2013; Wiatrak et al., 2005). However, improved fungal and aflatoxin resistant maize lines are still required. There are several genes that have been identified as involved in aflatoxin resistance in maize but are difficult to transfer to increase maize's resistance (Asters et al., 2014; Chen et al., 2004; Kelley et al., 2012).

A method was developed to extract aflatoxin for a single maize kernel in order to better correlate aflatoxin concentration with the fungal biomass at the inoculation site (Reid et al., 2016). This technique is also useful in pinpointing genes that are only being activated at certain sites on the inoculated cob that may aid in aflatoxin resistance. In the field experiment, three maize hybrids GA209 x T173 (susceptible), GA209 x Mp313E (susceptible/resistant), and Mp717 x Mp313E (resistant) were used in the experiment to assess how maize hybrids respond differently to infection with *Aspergillus flavus*. The maize hybrids were allowed to self-pollinate to ensure that the ears have as many kernels as possible. 21 days after silk emergence, the top ear from each plant were inoculated with *A. flavus* isolate NRRL 3357 or water as a control in a 3 by 3 kernel grid. There were three inoculated maize plants for each row. The inoculated maize ears were then collected 3 days, 7 days, 14 days, 21 days, 28 days, and 35 days after infection as well as harvest.

Approximately two months after inoculation with *A. flavus* during harvest, ears of GA209 x T173, Mp313 x T173, and Mp717 x Mp313E maize hybrids were collected and spilt into four groups. The first group were immediately processed and used as a control for the other three groups. The samples in the second group were baked in a large wooden oven for 7 days at 40°C and then processed. This is the drying down process that is common amongst farmers. The samples in the third group were also baked for 7 days and then stored in a dry container for 8 weeks before processing. The samples in the final group were stored in a dry container for 8 weeks prior to processing. There were six samples of each of the three maize hybrids in each group. Aflatoxin concentrations were determined using an Agilent 6460 LC/MS Triple Quadruple with electrospray ionization. The fungal biomass of the inoculated maize samples was determined by running quantitative real time polymerase chain reaction (qPCR) using Roche LightCycler 480 instrument.

In global agriculture, over two thirds of all crop loss are due to plant pathogens and weeds. Fungi are the pathogen that causes the majority of plant diseases. Weeds make up 34% of the total loss of crops as a result of harmful organisms (Oerke, 2006; Pimentel, 2005). Weeds are detrimental to crops because they compete for the same resources, which can reduce crop yields. There are around 8,000 distinct species of weeds. Herbicides are widely used to combat weeds in food production. Glyphosate is the most popular herbicide used in the United States. It is a broad spectrum herbicide that inhibits the plant enzyme that is vital to the creation of aromatic amino acids. In 1996, soybeans were the first glyphosate resistant crop to be released in the United States

(Brookes and Barfoot, 2013). Since then several weeds have become tolerant to glyphosate including rigid ryegrass, horseweed, ragweed, and water hemp.

The popularity of dicamba and 2,4-D as herbicides has increased in order to address the growing issue of glyphosate resistant weeds. 2,4-Dichlorophenoxyacetic acid (2,4-D) and 3,6-dichloro-o-anisic acid (dicamba) are commonly used herbicides in agriculture. Dicamba and 2,4-D are synthetic auxins that act similar to the natural hormone indole-3-acetic acid (IAA) which is the regulator of several plant regulatory functions (Grossmann, 2000, 2009). Soybean (Glycine max) and cotton (Gossypium hirsutum) plants have 2,4-D and dicamba resistant varieties commercially available. Drift is a well-known issue particularly with the application of synthetic auxin herbicides due to their high vapor pressure. In the United States, the potential economic cost of drift is $1 billion dollars annually (Egan et al., 2014; Pimentel, 2005). Dow AgroSciences and Monsanto have taken proactive steps to address concerns of off-target movement by developing new herbicide formulations that, according to product labels and technical use guidelines for their tolerant seed products, will require new application method. Dow AgroSciences also developed a new Enlist Duo™ herbicide that contains glyphosate and a new formulation of 2,4-D-choline. This new choline formulation should provide ultra-low volatility, minimizing potential for drift. Monsanto is collaborating with BASF to address dicamba's potential to injure off-target vegetation through drift or volatilization by attaching a BAPMA group (Plume, 2016). Product Stewardship programs promote best product practices and are fundamental to an integrated pest management system. Regulatory labs routinely analyzes drift complaint samples in the spring. Most of these complaints consist of injured ornamentals or soybeans exposed to the following

herbicides: 2,4-D, atrazine, acetochlor, dicamba, glyphosate, and paraquat. The drift concerns and damage to sensitive crops is a valid concern. The lab currently uses sensitive liquid chromatographic techniques including LC-MS/MS to identify these compounds at residue levels. However, this sensitive method cannot differentiate between the amine, ester, or choline formulations. The groups (amine, ester, or choline) attached to the acid moiety are cleaved, leaving only the acid form for detection. For example 2,4-D and 2,4-D dimethylamine amine salt have the sample parent (219.1) and product ion (160.7). Therefore, we have developed an analytical method using Fourier Transform Infrared (FT-IR) in order to identify the formulation of the auxin herbicide in these cases to ensure an effective stewardship program.

Maize and soybeans are the two prominent crops produced in the United States. Potential economic loss in a year due to aflatoxin could be as high as $1.68 billion dollars in the United States (Mitchell et al., 2016a). It is imperative to study and understand how Aspergillus flavus progresses distinctively in the susceptible maize hybrids, susceptible/resistible maize hybrid, and resistant maize hybrids. Discovering more about the pathogen-host interactions between different varieties will give key insight into what genes and proteins benefit maize in aflatoxin resistance. In the same spirit, herbicide drift not only damages neighboring crops by reducing yields, but it simultaneously reduces the efficiency of the herbicide on the target. Developing an FT-IR method will potentially allow us to differentiate between the new, low volatile formulations of synthetic auxin herbicides and the older synthetic auxin herbicides in drift cases. Hopefully this method will help keep farmers in accordance with their product stewardships. The overall goal of this research is to aid in the reduction of crop loss to fungal pathogen and herbicide drift.

The main objectives of this research were:

I. Develop a single maize kernel aflatoxin extraction method.

II. Correlate and characterize the aflatoxin accumulation and fungal biomass for the several weeks after inoculation with *Aspergillus flavus*.

III. Assess if certain storage conditions can reduce the growth of *Aspergillus flavus* and additional aflatoxin accumulation of harvested maize.

IV. Develop a viable method for differentiating between formulations of synthetic auxins using Fourier Transform Infrared spectroscopy (FT-IR).

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CHAPTER II

LITERATURE REVIEW

The genus Zea contains both wild annual and perennial species native to Mexico. In 1939 George Beadle proposed that teosinte was the wild ancestor of maize which was discovered in Mexico and Central America (BucklerIV and Stevens, 2013; Hufford et al., 2012a; Piperno et al., 2015). Scientific evidence implies that maize was first domesticated approximately 10,000 years ago in the Balsas River Basin of southwestern Mexico from teosinte (Zea mays ssp. parviglumis) (Bonavia, 2013; Piperno and Flannery, 2001).

Maize

Zea divided into two sections Luxuriantes and Zea. Luxuriantes consisting of one annual or two perennial species Z. diploperemis, Z. perennies, and Z. luxurians. Zea consist of a single diploid annual species, Zea Mays (Hufford et al., 2012b; Prasanna, 2012). Teosinte and maize are different due to the fact that teosinte's kernels fall from the plant, where as the kernels in maize are enclosed and requires assisted propagation. It has been confirmed that teosinte was the wild ancestor to maize after the production of viable offspring from a teosinte and maize cross DNA analysis has highlighted the similarities and outlined the differences between the two. The difference between teosinte and maize is only about five genes. Maize is a monocotyledonous plant, has 10 chromosomes, and is a (2n=20) diploid (Hufford et al., 2012a; Piperno et al., 2015). Teosinte ears possess only

about 5 to 12 kernels each in a hard shell. Maize can have well over 500 kernels on the cob. In teosinte, the ear is a two ranked distichous while maize is polystichous (Buckler and Stevens, 2013). Maize is an excellent example of a cultigen, which is defined as a plant species that can only exist in its domesticated form.

Maize Development

A maize plant begins as a planted seed. For our research, maize is typically planted in single row plots that are 4 meters (m) long and space 0.97 m apart. The growth stages of maize are divided into two main categories vegetative and reproductive. The first stage after planting, vegetative emergence is when the coleoptile opens and the plumule emerges around seven to ten days after seeding (Bonavia, 2013; O'Keeffe et al., 2009; Verheul et al., 1996).

Vegetative Stage

V1 stage begins after the leaf collar becomes visible and the first leaf has entirely emerged. It normally occurs four to six days after vegetative emergence. V2 begins seven to ten days after emergence, once the second leaf has fully emerged. About two weeks after emergence, V3 stage is initiated. The radicle, which is the primary root, is no longer the main food source and photosynthesis takes over (Gunawardena et al., 2001; O'Keeffe et al., 2009). The tassel, leaf shoots, and ear shoots are initiated during Stages V4 and V5. The roots of the second whorl are elongated at this time. The third root whorl elongates three weeks after plant emergence in stages V6 and V7. In stages V8 and V9, the fourth whorl of nodal roots is elongated around four weeks after plant emergence. Throughout the four weeks' stages V10 through V17 progress. Soil nutrients and water are critical at

these stages to ultimately determine maize yield (Jacobs and Pearson, 1991; O'Keeffe et al., 2009; Shanahan et al., 2001). Potential number of kernel rows and ovules that developed silks are established as well as ear size (Tollenaar and Daynard, 1978a). The tips for the upper ear shoots and the tassel are visible at the top of the leaf sheaths during this time. V18 occurs only one week before silking commences. Brace roots begin developing from the above ground nodes to help support the plant as well as absorb nutrients and water from the top soil (Mollier and Pellerin, 1999). Vegetative tasseling normally occurs two to three days before silking, after the plant has reached its maximum height and pollen begins to shed from the tassel (O'Keeffe et al., 2009).

Reproductive Stage

The R1 stage begins once silks are visible outside the husk around 60 days after plant emergence. A silk grows on average about 1.25 inches per day. It only takes 24 hours for a pollen grain to grow down the silk and fertilize the ovule. Each ovule can produce an individual kernel. This is most vital period when it comes to potential yield reduction due to plant stress and nutrient (Duvick, 2005). The R2 blister stage begins around 12 days after silking. The cob is close to its max size and kernels are white and have a blister like shape. Starch is accumulating in kernels that are 85 percent moisture (Tollenaar and Daynard, 1978b). R3 is the milk stage, which occurs 21 days after silking. Kernels contain a white milky fluid but begin to yellow on the outside. The cell division of the endosperm in each seed is finished and growth continues due to starch accumulation (Tollenaar and Daynard, 1978a). The R4 dough stage is about 26 days after silking. The kernels thicken to a doughy type of consistency due to starch increasing and kernel moisture decreases. The kernels are beginning to dry at the dent while the embryo

of the seed is still growing. The kernels have about 70 percent moisture at this point (Trenk and Hartman, 1970). R5 is the dent stage occurring about 35 days after silking. Close to all kernels are dented or in the processing of denting. Kernels have about 55 percent moisture at the dent stage. The R6 stage the kernels reach maturity around 55 days after silking (Figure 2.1). Kernel moisture at the R6 stage is around 30 percent (Borras et al., 2003). Black layer formation starts when the starch line has moved to the basal of the kernel. The maize is harvested with a combine harvester.

Figure 2.1 Maize in the R6 stage.

Maize in Global Agriculture

Maize (Zea mays) is the second most produced crop in the world; sugar cane is the first (FAOSTAT 2014). Maize is predicted to become the crop with the greatest

production globally by 2050 (Alston and Pardey, 2014; Shiferaw et al., 2011). The United States is the world's largest producer of maize followed by China, Brazil, Argentina, and Ukraine. The US produced an estimated 353,699,441 metric tons in 2013 (FAOSTAT 2014). The United States is the second biggest exporter of maize behind only Brazil. The Gross Production Value of maize in the United States is higher than any other crop (FAOSTAT 2014). Japan, South Korea, Mexico, Spain, and Taiwan are the top importing countries of maize.

Maize Diseases

Although only ten percent of all known fungi infect living plants, the majority of plant diseases are caused by fungi (Knogge, 1996; Mendgen and Hahn, 2002). Fusarium stalk and ear rot, Aspergillus ear rot, Pythium damping off, and gray leaf spot are a few of the most common maize diseases. *Fusarium moniliforme* is usually the fungus responsible for Fusarium ear rot in maize plants (Munkvold, 2003; Nelson et al., 1993). Fusariums, much like *A. flavus*, not only damages the maize plant but also can produce mycotoxins. This fungal genus produces ochratoxins, trichothecenes, zearalenone, and fumonisins which can have cytotoxic and/or carcinogenic effects on animals (Abbas et al., 2002; Bruns, 2003; D'Arco et al., 2008). The fungus Cercospara zeae-maydis causes gray leaf spot. The lesions on the leaves caused by the fungus reduces the amount of area to perform photosynthesis thus resulting in a loss of yield (Ward et al., 1999). Aspergillus ear rot in maize is caused by *Aspergillus flavus*, which destroys kernels and produces the highly carcinogenic metabolite called aflatoxin (Figure 2.2).

Figure 2.2 Maize infected with *Aspergillus flavus*.

Aspergillus

In 1960 what came to be known as “turkey X disease” killed over 100,000 turkeys at poultry farms all over England. Despite the name, turkeys were not the only victims of the disease. Thousands of ducks and pheasants also died on poultry farms around the same time (Amaike and Keller, 2011; Moss, 2002). An autopsy on the deceased turkeys showed liver lesions and hemorrhages as well as swollen kidneys. At the same time, a similar outbreak occurred in the United States with hepatocellular carcinoma in rainbow trout. Postmortem examinations revealed severe liver hematoma and necrosis as well as ruled out biological agents being the cause of the disease. It was suspected that the feed, with trace amounts of fungus, was being poisoned with a known toxin. The commonality that all the incidences shared was a shipment of contaminated Brazilian peanut meal used

in the animal feed (Kensler et al., 2011; Peraica et al., 1999; Rawal et al., 2010; Rustom, 1997). The fungus was later identified as *Aspergillus flavus* and the toxin was therefore named aflatoxin. There are other fungi that produce aflatoxin including *Aspergillus parasiticus*, *Aspergillus nomius*, *Aspergillus niger*, *Aspergillus bombycis*, *Aspergillus tamari, Aspergillus ochraceroseus, and Aspergillus australis*. *Aspergillus oryzae* and *Aspergillus sojae* do not produce aflatoxin. Isolates of the Aspergillus species contain the two morphotypes: the L strain and the S strain. Isolates of the L strain morphotype produce several conidia but comparatively few large sclerotia. On the other hand, isolates of the S strain morphotype conversely make few conidia (Amaike and Keller, 2011; Gibbons and Rokas, 2013; Hesseltine et al., 1966; Scheidegger and Payne, 2003). The genus Aspergillus is one of the oldest named genera of fungi. It was classified by Italian priest Pier Antonio Micheli in 1729 and named due to its similarity in shape to a device used by the Roman Catholic clergy to sprinkle holy water during service called the "asperges"(Gibbons and Rokas, 2013). To date, there are 250 known species of Aspergilli. Aspergillus species have been identified as a pathogen to plants, insects, and animals. *Aspergillus fumigatus* is the species that can cause aspergillosis, an infection of the lungs in humans (Bertuzzi et al., 2014; Dagenais and Keller, 2009; Latgé, 2001). Citric acid was first produced commercially in England around 1826 using lemon juice. In 1919, Belgium was the first to use *Aspergillus niger* in this process. Pfizer in 1923 perfected the way citric acid was produced using *Aspergillus niger*. Citric acid is one of the best known inhibitors of glycolysis and the ability of *A. niger* to overproduce citrate by an active glycolytic pathway (Angumeenal and Venkappayya, 2013; Lotfy et al., 2007; Papagianni, 2007, 2007). *Aspergillus terrus* is used for biotechnological

applications. *Aspergillus oryzae* is use for sake and soy sauce. Soy sauce originated in China over 2500 years ago. Almost 300 volatile compounds have been identified in soy sauces including alcohols, ketones, aldehyde, and esters. The growth rate of *Aspergillus oryzae* affects koji flavors in soy sauce (Zhao et al., 2015). Soybeans and wheat are crushed and blended. Water is added and boiled until the grains are fully cooked. The mash is allowed to cool to about 80°F before *Aspergillus oryzae* is added. The mixture is allowed to mature for three days in large vats where air is circulated. The koji is transferred and fermentation tank mixed with water and salt. Lactic acid bacteria and yeast are added and allowed to ferment for several months. The raw soy sauce is separated from the liquids and then pasteurized (Feng et al., 2013; Liang et al., 2009).

Aspergillus flavus

Aspergillus flavus is considered a hemibiotrophic fungus. Hemibiotrophic fungi commonly reach a symbiotic state with its host but eventually leads to the death of the plant. The *Aspergillus flavus* genome is 36.3 Mb long and consists of eight chromosomes (Bhatnagar et al., 2006; Scheidegger and Payne, 2003). *Aspergillus flavus* reproduces by asexual spores known as conidia. The fungus can grow from temperatures ranging from 12°C to 48°C but the ideal temperature for fungal growth is 37°C (Amaike and Keller, 2011). The fungus endures harsh winter conditions in its mycelium form or due to the formation of resistant structures called sclerotia (Figure 2.3).

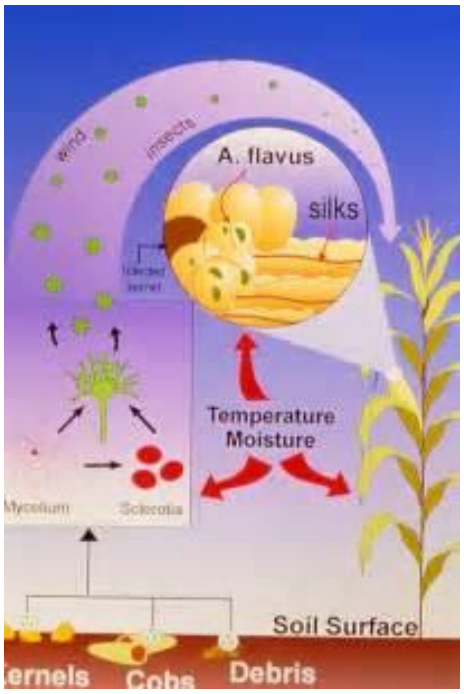

Figure 2.3 Life cycle of *Aspergillus flavus*.

Adapted from http://www.aspergillusflavus.org/aflavus

Aspergillus flavus is a common filamentous fungus that has been known to grow on a variety of crops including rice, cotton, peanuts, and corn. *A. flavus* can cause ear rot in corn, aflaroot in peanuts, and boll rot or yellow spot disease in cotton (Guchi, 2015; Smart et al., 1990; Zeringue et al., 1999). The primary mode of inoculation of *Aspergillus flavus* is due to sclerotia in the soil, releasing its conidia into the air. The fungus infiltrates through wound damage by spreading through the rachilla and the rachis. An *A. flavus* infection through the silk enters the kernel through the pedicel. Mycelium are composed of branching microscopic tubular cells called hyphae that grow and secrete enzymes that break down complex substrates into simpler compounds. Fungal adhesives are normally water-soluble glycoproteins. The sticky mucilaginous layer attaches and absorbs water in order to swell and increase its surface area. Appressoria are swollen tips

of hyphae that allow the fungus, through mechanical and enzymatic activity penetrate plant tissues (Knogge, 1996).

The first step of a phytopathogenic fungus is to adhere to the potential plant host. This is achieved through the secretion of non-water soluble glycoproteins, lipids, and polysaccharides by the fungus spores. Spores with a sticky mucilaginous layer absorb water on the plant's surface and swell. This increases their surface area, increasing the amount of contact being made with the surface of the plant (Dufresne and Osbourn, 2001). Necrotrophic and some hemibiotrophic fungi including *Aspergillus flavus* secrete hydrolytic enzymes like cutinases, cellulases, pectinases, and proteases to aid in surmounting the plant's defenses (Mellon et al., 2007). Cutinases penetrate through the cuticle, which is the waxy outer protective layer of plant cells. Cellulase breaks down cellulose in the plant cell wall. Pectinases also degrades a polysaccharide found in the cell wall known as pectin. It is generally easier for fungi to infiltrate plant tissue through cracks, wounds, and the stomata (Mendgen and Hahn, 2002). If those modes are not available, first the fungus invades plant cells by forming a germ tube. At the end of the germ tube forms a penetration organ known as an appressoria. The appressorium are penetration organs, these swollen tips of hyphae that allow the fungus to enter the epidermal cell wall of the plant. The combination of compounds and the appressoria makes infiltrating the host a much easier task. Isocitrate lyase production is heavily upregulated during the plant penetration stage but significantly decreases after the appressoria is formed (Pedras and Ahiahonu, 2005). This indicates a transition from the use of fatty acid metabolism to the use of extracted carbohydrates from the plant. This is achieved by the intake of simple carbohydrates from the plant tissue. Once the fungus is

in the plant cell a nutrient absorbing structure called haustoria is formed. The haustorium uses HXT1p sugar transporters to uptake amino acids and sugars like glucose and fructose into the fungus (Hardham, 2001; Mendgen and Hahn, 2002).

Chitin, which is present in the cell walls of fungi, induces an immune response in plants. The glycoprotein, chitin elicitor-binding protein, binds to chitin and helps enact the immune response (Huffaker et al., 2011; Kaku et al., 2006; Kanno et al., 2012). OsCERK generates reactive oxygen species to both initial immune response to upregulate defense genes and synthesize phytoalexins. Phytoalexins are a group of compounds that plants use in defense to invading pathogens. However, some fungi are able to break down phytoalexins (Ahuja et al., 2012; Grayer and Kokubun, 2001; Poloni and Schirawski, 2014). Other environmental factors such as drought stress, humidity, and heat stress also increases a plant's overall susceptibility to fungal infection.

Aflatoxins

The same environmental conditions that increase fungal susceptibility (high temperatures, high humidity, drought stress, poor crop storage and insect damage) can cause *Aspergillus flavus* to start producing aflatoxins (Thompson and Henke, 2000; Trenk and Hartman, 1970; Villers, 2014). Aflatoxins are secondary metabolites of *A. flavus* and are extremely carcinogenic. There are four major types aflatoxin B1, B2, G1, G2 (Figure 2.4). Aflatoxin B1 and B2 glow blue under UV light while aflatoxin G1 and G2 fluoresce green in the presences of UV light (Hara et al., 1974). Aflatoxin B2 is the dihydroxy form of aflatoxin B1 while aflatoxin G2 is the dihydroxy form of aflatoxin G1. The most common and toxic of these is aflatoxin B1.

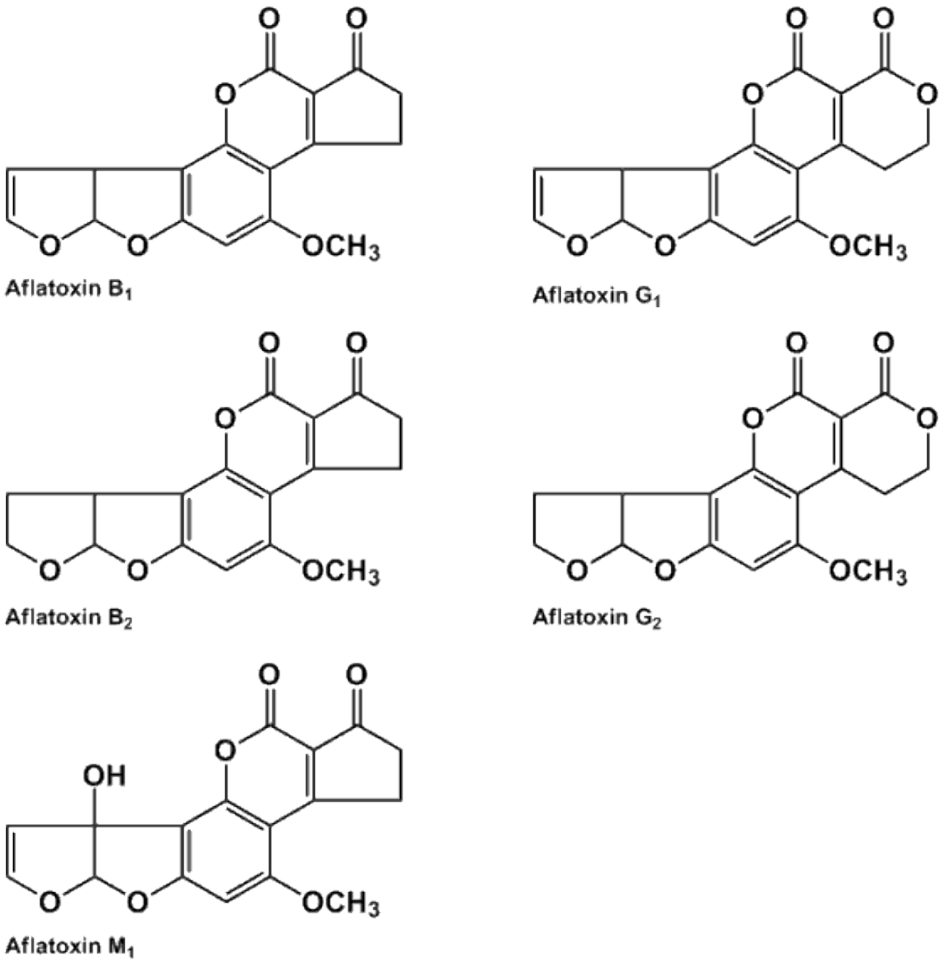

Figure 2.4 Molecular structure of aflatoxin B1, B2, G1, G2, and M1. Adapted from Walderhaug et al. (2014).

Aflatoxin Production

The mechanism for aflatoxin production in *Aspergillus flavus* starts with acetyl-CoA that was manufactured from pyruvate during the Pyruvate Dehydrogenase Complex reaction. AFlR is a regulatory gene which is required for the expression of most of genes in the aflatoxin pathway gene cluster (Amare and Keller, 2014). Acetyl-CoA is converted into malonyl-Coa by acetyl-CoA carboxylase. Fatty acid synthase transforms malonyl-

CoA into hexanoyl-CoA. Norsolorinic acid is formed when polyketide synthase modifies hexanoyl-CoA. Norsolorinic acid is converted into averantin by ketoreductase. AFlD is a structural gene whose product converts norsolorinic acid into averantin thus representing one of the first steps in the biosynthesis pathway of aflatoxin (Amare and Keller, 2014; Yu, 2012). Hydroxylase transforms averantin into 5-hydroxyaverantin. 5-oxoaverantin is formed when dehydrogenase modifies 5-hydroxyaverantin. 5-oxoaveratin is converted into averfin by cyclase. Oxidase transforms averfin into versiconal hemiacetal acetate. Versiconal is formed when acsterase versiconal hemiacetal acetate. Versiconal is converted into versicolorin B by versiconal B synthase. Versiconal B is randomly hydroxylated to Versicolorin A. DMST synthase transforms Versicolorin A into demethylsterigmatocystin and Versiconal B into Demethyldihydrosterigmatocystin (Bhatnagar et al., 2003; Hesseltine et al., 1966). Sterigmatocystin is formed when 6-O-methyltransferase modifies demethylsterigmatocystin. Sterigmatocystin is converted into O-methylsterigmatocystin by 8-O-methyltransferase. Dihydrodomethylsterigmatocystin is transformed by O-methyltransferase to dihydro-O-sterigmatocystin (Figure 2.5). Finally, P-450 monooxygenase transforms O-methylsterigmatocystin into Aflatoxin B1 or Aflatoxin G1 and dihydro-O-methylsterigmatocystin to Aflatoxin B2 and G2 (Bhatnagar et al., 2006; Trail et al., 1995). Ethylene inhibition aflatoxin biosynthesis is due to oxidative stress alleviation of fungal cells (Huang et al., 2009).

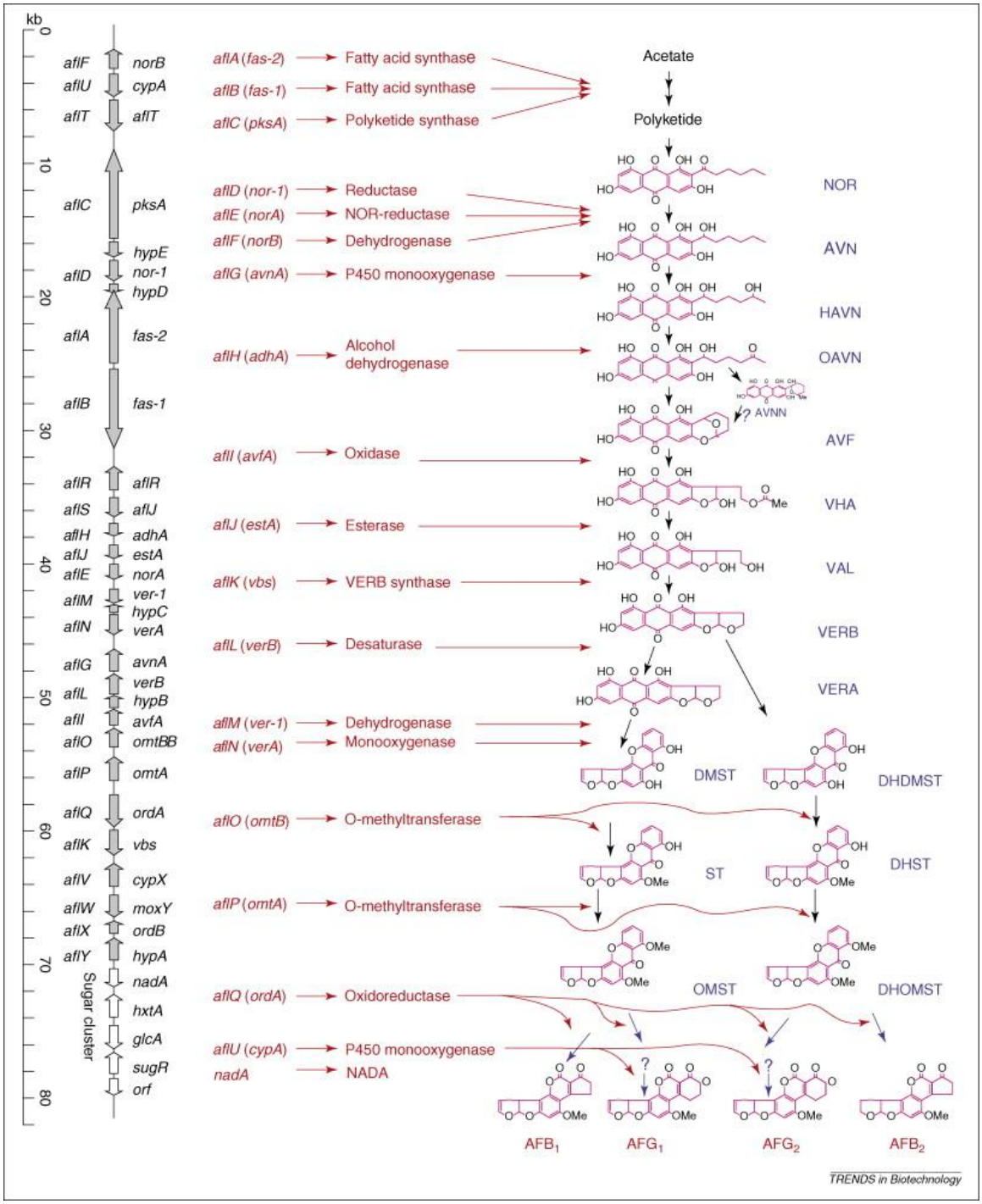

Figure 2.5 Aflatoxin synthesis pathway.

Adapted from Cleveland et al. (2009).

Aflatoxin Bioactiviation

Aflatoxin B1 is a procarcinogen because it requires enzymatic bioactiviation before it becomes carcinogenic. Aflatoxin B1 is converted in the body to AFB1-8-9-epoxide by the liver enzyme p450. Cytochrome p450 is a heme-binding enzyme that has an important role in the biotransformation of xenobiotics. Cytochrome p450 aids in the inactivation of foreign compounds however in the case of aflatoxin B_1, it metabolizes aflatoxin B_1 into AFB1-exo-8, 9-epoxide. Cyp2A4 is the p450 enzyme that activates aflatoxin B_1 in the liver to form the epoxide (Ayed-Boussema et al., 2011; Yu et al., 2000). Cyp1A2 metabolism of aflatoxin B_1 produces aflatoxin M_1 as well as the AFB1-exo-8, 9-epoxide. The AFB1-exo-8, 9-epoxide is formed by epoxidation at the 2,3 double bond. The epoxide that is formed is a highly reactive electrophile and has a high regiospecificity for the N7 position of the guanine residue in DNA (Figure 2.6). It specifically binds to the p53 encoding region to form a DNA adduct (Clewell et al., 2014; Essigmann et al., 1977; Smela et al., 2001). This results in nonfunctioning p53 proteins that are important tumor suppressors in the body.

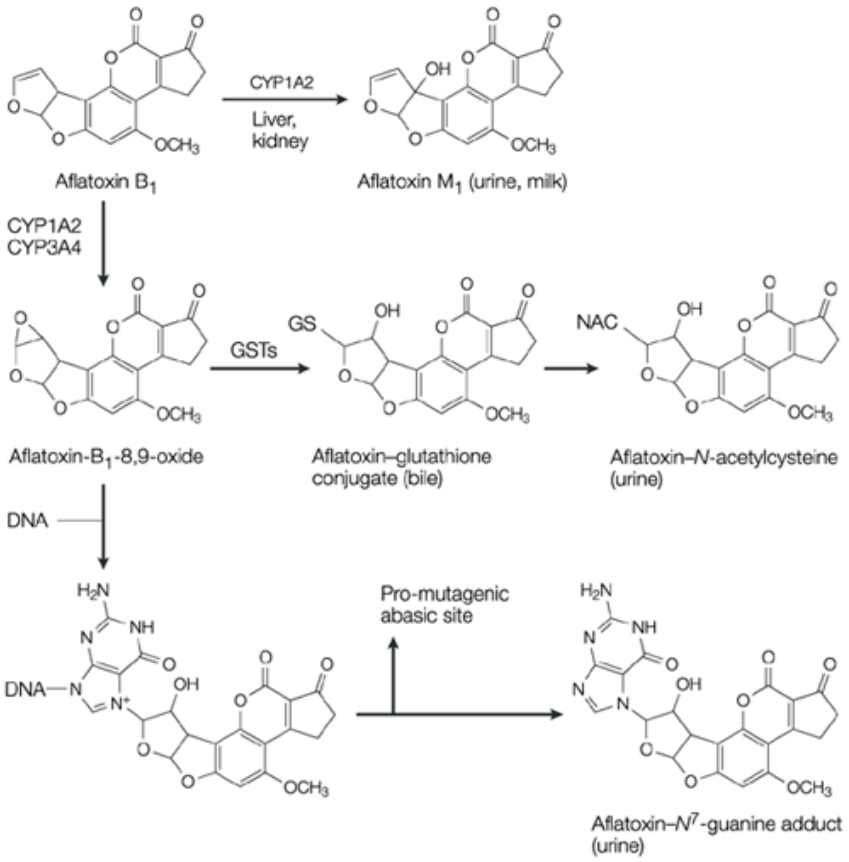

Figure 2.6 Bioactiviation of aflatoxin B1.

Adapted from Kensler et al. (2003).

The p53 controls an important cell cycle checkpoint that is responsible for maintaining the integrity of the genome. The p53 protein can bind to specific DNA sequences and activate the transcription of genes including p53 binding sites. The protein also causes the expression of the Cipl cell cycle inhibitor which induces cell growth arrest (Sablina et al., 2005; Soussi, 2000). When DNA is damaged, p53 has been shown to induce cell cycle arrest or even lysis of the cell. Some mutations to p53 eliminate this response and it results in an increased frequency of unchecked genetic mutations

(Greenblatt et al., 1994; Hollstein et al., 1991). This phenomenon increases the probability that a tumor cell escapes the normal system of checks against excessive cell growth. An aflatoxin t-RNA adduct can inhibit t-RNA binding activity of some amino acids on protein synthesis like lysine, leucine, arginine, and glycine. The glutathione S-transferase can detoxify the AFB1-8, 9-epoxide in it mediated conjugation with glutathione (Edwards et al., 2000; Hayes and Strange, 2000; Sablina et al., 2005). Aflatoxin B1 inhibits synthesis of factors II and VII involved in prothrombin synthesis and clotting mechanisms. The activity of liver UDP glucose-glycogen transglucosylase is also affected by aflatoxin B_1 (Abdollahi and Buchanan, 1981; Zhang et al., 2014). Aflatoxin B1 can also be metabolized to aflatoxin M_1, which would be found in the milk of lactating mammals. The hydroxylation of aflatoxin B_1 at C4 produces aflatoxin M_1 and at C22 produces aflatoxin Q_1. Aflatoxin P_1 results from o-demethylation of aflatoxin B_1 (Mohammadi, 2011; Yu, 2012). Aflatoxin M_1 is associated with the protein fraction of milk.

Detection of Aflatoxins

Thin Layer Chromatography

Thin layer chromatography (TLC) was one of the first analytical techniques used to separate and identify aflatoxins (Reddy et al., 1970; Shephard, 2009). In 1954 the use of silica gel coated glass plates began and a year later Stahl standardize the separation technique and popularized it for routine analysis (STAHL and KALTENBACH, 1961). The thin layer chromatography starts with a sheet of glass, aluminum, or plastic coated with a thin layer of adsorbent material silica gel. The plate preparation involves mixing silica gel, calcium sulfate, and water (Sherma, 2000). This layer of adsorbent is the

stationary phase. A small amount of the solution with the sample is added 1.5 cm from the bottom edge, and allowed to evaporate off. The TLC plate is placed in a beaker with a solvent and then the lid is closed. Before the solvent has reached the top adsorption on the surface partition on the thin layer plate, the TLC plate should be removed from the solvent (Lin et al., 1998). This allows the compounds to separate on the TLC plate. The most important thing in qualitative analysis depends on the retention factor. The retention factor is the division of the distance between starting line and the middle spot by the distance of the starting line and the solvent front. The retention is best when the values are between 0.1 and 0.8 and reproducible (Stroka and Anklam, 2000). Ultraviolet (UV) or fluorescence detectors, Fourier Transform Infrared (FT-IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy can be used in tandem with TLC to perform quantitation.

Enzyme Linked Immunosorbent Assay

ELISA stands for Enzyme Linked Immunosorbent Assay and commonly used in aflatoxin quantification (Anjaiah et al., 1989). Veratox is a direct competitive immunoassay that uses antibody-coated wells to check samples for aflatoxin. An enzyme labeled aflatoxin conjugate competes with the free aflatoxin from the sample for the antibody binding sites coating the well. Conjugate is added to the mixture and reacts with the bound aflatoxin conjugate to produce a blue color (Kolosova et al., 2006; Lee et al., 2004). This results in an inverse relationship, the bluer the color, the less aflatoxin B_1 that is concentration in the sample. The amount of aflatoxin is calculated by a micro-well reader using UV absorbance at 650 nm and by comparing that to a standard curve. The Veratox test has an accurate concentration reading between 0 ppb to 50 ppb.

Liquid Chromatography

Liquid Chromatography in tandem with a quadrupole mass spectrometer for analysis of aflatoxins is a much more sensitive and consistent than traditional aflatoxin testing methods such as ELISA (Blesa et al., 2003; Jaimez et al., 2000). Liquid Chromatography uses organic and aqueous mobile phases with the aid of a column to separate different analytes in the sample for analysis. For aflatoxins reverse phase liquid chromatography is used, which means a non-polar, typically silica based column is required (Liu et al., 2013; Spanjer et al., 2008). Methanol and acetonitrile are common organic mobile phases that are used in reverse phase chromatography while water is mostly used as the aqueous mobile phase. A gradient is required to get good separation between the different analytes. The gradient in reverse phase chromatography starts with the aqueous mobile phase and slowly transitions to the organic mobile phase throughout the five to twenty-minute run. The progression from an aqueous to an organic mobile phase will elute the compounds from the sample off the non-polar column based on the polarity of the compound itself (Stubblefield and Shotwell, 1977). The less polar the compound the longer it takes to elute off the column while the more polar analytes will come off the column more quickly. The time it takes for an analyte to elute from the column is called the retention time and is often used in the identification of compounds.

Analytical Detectors

Liquid Chromatography can be coupled to both nonspecific and specific detectors. Detectors are composed of a sensor and associated electronics. The design and performance of any detector depends heavily on the chromatographic system it is associated with. The actual detection of the analyte can be done with a variety of

instruments/detectors but the two most popular methods for aflatoxin detection are a UV detector, a florescence detector, and a mass spectrometer. Fluorescence detection is widely considered more sensitive than UV detection by liquid chromatography especially with the use of reverse phase liquid chromatography. The addition of an organic acid to the mobile phase increases the fluorescence of aflatoxin B_1 and B_2 (Jaimez et al., 2000; Manabe et al., 1978).

Mass spectrometers are a precise and efficient to quantitate aflatoxins. An ionization source, mass analyzer, and a detector are the three major components of a mass spectrometer. There are multiple ways to ionize samples using mass spectrometry including electron ionization, matrix assisted laser desorption ionization, electrospray, thermospray, inductively coupled plasma, and atmospheric pressure chemical ionization (Liu et al., 2013; Ramos Catharino et al., 2005). There are several types of mass spectrometers with various analyzers such as an ion trap, time of flight, single quadrupole, or triple quadrupole. An ion trap uses electrodes producing a magnetic field to contain ions. In time of flight detection, a uniform electromagnetic force is applied to the ions and the mass to charge ratio of the molecule is calculated by the amount of time it takes each ion to reach the detector. Smaller ions would reach the detector faster than larger mass ions. A single quadrupole filters ions by using four magnets hyperbolic in cross section with one pair having a direct current and the other an alternating current applied to it. This results in an oscillating electric field that requires a particular mass ion to resonate properly in order to reach the detector. The detector in mass spectrometry is universally an electron multiplier. For aflatoxin analysis, electrospray ionization is widely used as the ionizer and single or triple quadrupoles can be used to analyze the sample.

Aflatoxin Regulations

In 1988 the International Agency for Research on Cancer classified aflatoxin B_1 as a Class 1 human carcinogen (Vainio and Wilbourn, 1992). The FDA has restricted the amount of aflatoxin allowed for human consumption to 20 (μg/kg) parts per billion. Products with concentrations higher than 20 ppb are restricted from interstate commerce. The FDA restricted levels aflatoxin in dairy cattle feed is also 20 ppb. For beef cattle and swine, the limit for aflatoxin in feed are 300 ppb and 200 ppb, respectively (Lizárraga-Paulín et al., 2011). It was labeled a Class I carcinogen by the World Health Organization in 1993 and is considered one of the most dangerous biologically produced substances known today. In the United States, the estimated economic cost of aflatoxins annually ranges between $104.5 million to $1.68 billion dollars (Mitchell et al., 2016; Robens and Cardwell, 2003).

The United Nations Food and Agriculture Organization on international regulations for mycotoxins conducted a study to show that at least 77 countries have some sort of regulations for mycotoxins (van Egmond and Jonker, 2004). The United States is the number one exporter of maize in the world exporting 49,887,000 metric tons in 2010 to over 180 different countries. The United States is also the largest producer of maize on the planet producing 316,165,000 metric tons in 2010. China, Brazil, and Mexico are also major producers of maize and in 2010 produced over 250,000,000 metric tons. Japan, Mexico, Taiwan, and South Korea are the top importing countries of maize. The FDA limit of total aflatoxin in the United States is 20 ppb. The limit is the same in several other countries including Mexico, Japan, South Korea, Argentina, and Egypt. A few countries have a higher limit such as China and Brazil, which are 40 ppb and 30 ppb

respectively. There are several countries with a limit below 20 ppb (Egmond et al., 2007; Liu et al., 2006; Wu, 2015). All 28 countries in the European Union have a strict restriction on total aflatoxin set at 4 ppb. Canada and Taiwan both have aflatoxin limits at 15 ppb. Over 34 countries have regulations on aflatoxin M_1 at 0.05 ppb. Including the United States, there are 22 countries with limits set at 0.5 ppb (Wu and Guclu, 2012).

Aflatoxin Prevention

There are several ways of reducing *Aspergillus flavus* infection or aflatoxin accumulation. Biological controls such as non-aflatoxin producing strains of *Aspergillus flavus* can outcompete the harmful toxin producing strains. The first maize line that was released as resistant to aflatoxin accumulation was Mp313E and Mp420 in 1992 (Williams, 2006; Windham and Williams, 2002). Mp715 and Mp717 are germplasm lines that were developed in Mississippi (Williams and Windham, 2012). Due to its relatively early flowering time and resistance to aflatoxin accumulation, Mp719 is also an excellent maize line for not only aflatoxin resistance but for breeding for more resistant commercial lines of maize as well. Certain farming techniques such as irrigation, fungicides, biological controls and planting earlier would be another way of reducing the impact of *Aspergillus flavus*.

There are a few physical and chemical detoxification methods for reducing the amount of aflatoxin in food and feed products. Extremely high temperatures can decompose aflatoxins. Cooking and boiling are not effective in reducing aflatoxin because the thermal decomposition temperature of aflatoxin is 267°C. The moisture content is a determining factor in how aflatoxin is deactivated. The higher the moisture content in the feed, the higher percentage of aflatoxin degraded. Pasteurization of milk

has little to no effect on the aflatoxin M_1 concentration (Mohammadi, 2011). Solvents have been used effectively to reduce the amount of aflatoxin in foods. Ethanol, acetone, isopropanol, hexane, methanol, water, and acetonitrile have all been used in various combinations to extract aflatoxin from foods and feeds (Campone et al., 2011; Karaseva et al., 2014). The major problems that occur in this process are associated with the high cost of these solvents and the disposal of the solvents with the extracted aflatoxins. Some substances bind to aflatoxin and remove it from the solution that way. Bentonite clay at 30°C for 5 days removes 94 to 100% aflatoxin B1 from the solution. Hydrated sodium calcium aluminosilicate (HSCAS) has been shown to remove 80% of aflatoxin B_1 from solution because it has such a high affinity (Diaz et al., 2003; PHILLIPS et al., 1988). Calcium bentonite, esterified glucomannan, and activated carbon have also been effect in binding aflatoxin (Diaz et al., 2004; Phillips et al., 2008). Radiation is another technique that can be used to decompose aflatoxin. Aflatoxin B_1 absorbs ultraviolet light optimally at 362 nm. AFB_1 in peanut oil exposed to UV light for two hours eliminated 45% of the aflatoxins (Diao et al., 2015). Contaminated milk exposed to UV light for 10 minutes with 1% hydrogen peroxide added after completely inactivated 100% of the aflatoxin M_1. Gamma radiation has been used to successfully reduce aflatoxin B_1 concentration in peanut meal by 75% after being exposed to gamma rays at a dose of 1 kilogray (kGy). 100% of the aflatoxin has been detoxified by a 10kGy dose of gamma rays. A dose of gamma radiation greater than 10kGy has been shown to inhibit seed germination and increase the peroxide value of the peanut oil (Diao et al., 2015; Iqbal et al., 2012; Van Dyck et al., 1982). Ammonia is a popular chemical means to treat aflatoxin contaminated feeds. The reaction of ammonia and aflatoxin irreversibly alters the molecular formula of

aflatoxin B_1 if it is exposed long enough (Allameh et al., 2005; Bagley, 1979). Formaldehyde at just 0.5% in contaminated milk reduced aflatoxin M_1 concentration by 95%.

Health Impact of Aflatoxins

Due the bioactiviation of aflatoxin B_1 and how it inhibits cytochrome p53 in liver cells from properly preventing tumor cell growth, the chance of developing liver cancer in an individual who has consumed aflatoxins increases dramatically. The rate of people who develop liver cancer due to aflatoxin exposure increases by 60 times if they have Hepatitis B (Wild and Montesano, 2009; Wu et al., 2009). Early symptoms of liver damage hepatotoxicity from aflatoxicosis can present as anorexia, malaise, and with a high fever. Other symptoms include vomiting, abdominal pain jaundice, hypertension, hepatitis, and death (Dhanasekaran et al., 2011). Kenya has had multiple documented aflatoxin outbreaks dating all the way back to 1981. In 2004, there was a serious outbreak of aflatoxin poisoning from infected maize crops in Kenya that killed over 100 people (Gieseker et al., 2004; Yard et al., 2013). In March 2013, Germany found high levels of aflatoxin contaminated animal feed was sent to over 4,000 farms. The contaminated feed originated from a shipment 40,000 tons of maize from Serbia. This led to German authorities banning milk deliveries from hundreds of dairy farms while they test the milk for aflatoxin M_1. The test showed that the aflatoxin M_1 concentration in the milk below the national limit and therefore was safe for consumption.

Soybeans

The genus Glycine is divided into two subgenera: Soja and Max. Glycine Soja is the wild ancestor of the domesticated modern soybean, Glycine Max. G. Soja was discovered over 6,000 years ago in Southeast Asia (Kim et al., 2012). Soybeans are diploids with 20 chromosomes (2n=40). G. Soja shares 97.65% of the genomic sequence with G. Max. 425 genes in Glycine Max are not present in Glycine Soja (Joshi et al., 2013; Li et al., 2010). G. Soja has tiny, black seeds while G. Max has large yellow seeds. The first documented occurrence of soybean use in agriculture was in Northeastern China during the Shang Dynasty between 1700 to 1100 B.C.E. Soybeans first came to the United States at the end of the 18th Century (Lockeretz, 1988). Soybeans are the sixth most produced crop in the world with 307 million tonnes made in 2014. Soybeans are behind only maize as the highest produced crop in the United States. Brazil recently has topped the US by being the top exporter of soybeans in 2014 (FAOSTAT 2014). China, Germany, Mexico, Spain, and the Netherlands are the top five global importers of soybeans.

Soybean Development

Soybean growth stages are broken up into vegetative and reproductive. The soybean seed should be planted between one and two inches deep. Emergence begins between five and ten days after planting. The primary root, shortly followed by the stem, emerges from the seed and the stem makes its way to the surface. Once the unifoliate leaves have fully expanded, the cotyledon stage has begun. For these ten days, the cotyledons are the main source of the plant's nutrients.

Vegetative Stage

The first vegetative stage (V1) is characterized by the emergence of the first trifoliolate. The number of trifoliolate on the main stem determines the vegetative stage. The soybean plant switches from using the cotyledons for its nutrients to photosynthesis. Between V1 and V2 is when At V2, the plants are about eight inches tall with three nodes and two unfolded leaflets and nitrogen fixation has begun (Chen and Wiatrak, 2010). New nodes will appear every 5 days until V5 stage. Between V3 and V5, the soybean plant grows from nine to around twelve inches tall with six nodes. During V5, the plant develops buds in the top stem that will soon develop into clusters of flowers. At the final vegetative growth stage (V6), the soybean has reached a high of 14 inches tall with seven nodes with unfolded leaflets.

Reproductive Stage

The first phase of the reproductive stage (R1) occurs when the first flower on the soybean plant has bloomed. This normally happens 6-8 weeks after emergence and around the third to sixth node on the main stem on the plant. During this time, the plant's height has increased to about 18 inches. Once an open flower has made its way to one of the top nodes of the main stem, R2 is in full bloom. The R3 stage is characterized by the formation of a pod on the upper nodes of the soybean plant. During this stage, the soybean plant can get as tall as 32 inches. Full pod development is shown during R4. The importance of R4 for potential seed yield cannot be understated (Egli and Bruening, 1992). The R5 and R6 stages begin with initial seed development and ends when pod weight peaks out. The plant also reaches its maximum height during this period. Leaf yellowing begins until all the leaves have fallen. In the final two reproductive stages (R7)

and (R8), the soybean plant reaches full maturity when most of the pods mature, and the dry weight starts to peak.

Herbicides

Weeds in global agriculture make up 34% of the total loss of crops as a result of harmful organisms (bacteria, viruses, fungi, and insects). The Crop Life Foundation and the Weed Science Society of America estimate that without pesticide use US crop production would drop 20% with a loss in value of $16 billion (Gianessi and Reigner, 2007; Savary et al., 2012). Herbicides are widely used to combat weeds in agriculture. Glyphosate is the most popular herbicide used in the United States.

Glyphosate

Glyphosate (N-(phosphonomethyl)glycine) is a non-selective herbicide. When applied to plants, 5-enylpyruvylshikimate-3-phosphate synthase (EPSPS) is inhibited by glyphosate (Steinrucken and Amrhein, 1980). It catalyzes the transfer of the enolpyruvyl moiety of phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P). This is a key in the synthesis of aromatic amino acids for hormones and plant metabolites. The active site of the EPSPS enzyme in higher plants is highly conserved (Gao et al., 2014). Glyphosate is competitive with respect to PEP binding to EPSPS but uncompetitive with respect to S3P and the resulting S3P complex is very stable (Figure 2.7). Phenylalanine, tyrosine, and tryptophan are the aromatic amino acids that are synthesized from this pathway (Duke and Powles, 2008). In 1996 soybeans were the first glyphosate resistant crop to be released in the United States (Barrows et al., 2014; Brookes and Barfoot, 2013; Green, 2014; Green and Owen, 2011).

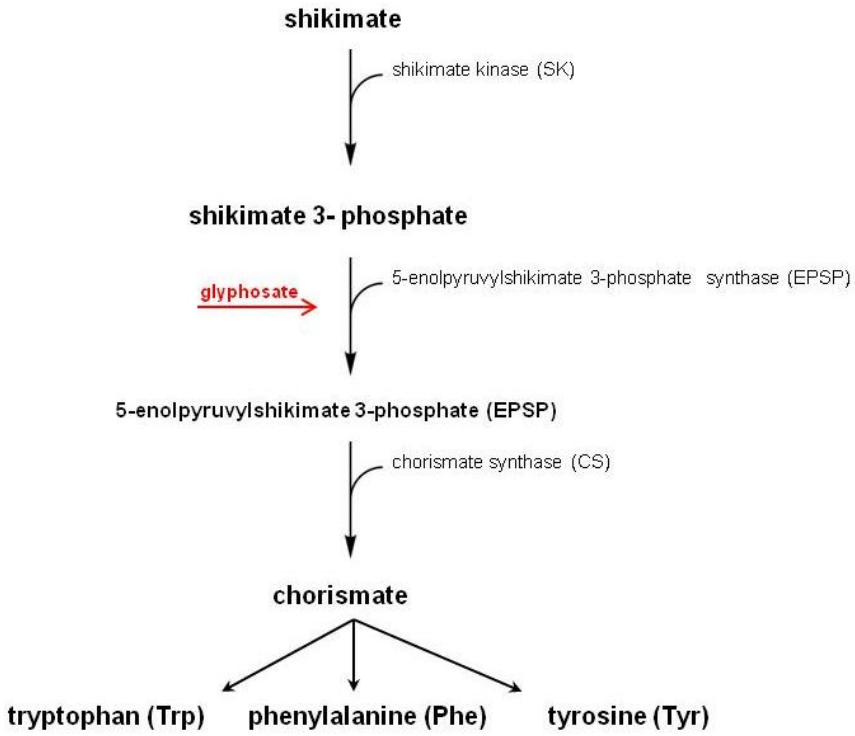

Figure 2.7 Inhibition of 5-enylpyruvylshikimate-3-phosphate synthase (EPSPS) by glyphosate.

Adapted from Azania et al. (2013).

Glyphosate Resistant Weeds

Glyphosate resistant crops overcome this generally by the expression of CP4 a type of EPSPS protein that is insensitive to glyphosate. Over time, weeds have become more tolerant of glyphosate leading to glyphosate resistant weeds (Gao et al., 2014; Wiersma et al., 2015). In glyphosate resistant weeds, glyphosate is transported in the phloem. It is sequestered in the vacuole from the cytosol. This reduces the amount of glyphosate available to enter the chloroplast and inhibit EPSPS. Glyphosate can be released into the cell at a nontoxic rate or potentially stay in the vacuole indefinitely (Ge et al., 2010; González-Torralva et al., 2012). A mutation of the EPSPS Prot106 codon or an increase in the production of EPSPS are other ways that weeds can become glyphosate

resistant (Délye et al., 2013). As of 2011, 21 weed species have developed resistance to glyphosate including rigid ryegrass, horseweed, ragweed, and water hemp (Christoffoleti et al., 2015; Sammons and Gaines, 2014).

Auxin Herbicides

Broadleaf (dicot) plants are damaged and killed by synthetic auxins such as dicamba and 2,4-D. 2,4-D and dicamba have been used for weed control since their discovery during World War II (Gianessi, 2013). Both 2,4-D and dicamba mimic the plant hormone, indole-3-acetic acid. Dicamba and 2,4-D act as the natural hormone indole-3-acetic acid (IAA) regulator of several plant regulatory functions.

Auxins activate Auxin Binding Protein 1 on the plasma membrane, which alters the cytoskeleton and reduces the peroxisomes. Auxin Binding Protein 1 induces proton pump hyperactivity due to the decreases in pH because of the accumulation of protons outside the extracellular membrane (Christoffoleti et al., 2015; Mano and Nemoto, 2012). The hydrogen ion concentration outside cell opens potassium channel and pumps potassium inside the cell. As a result, water influxes into the cell through aquaporins. The acidic condition outside the cell breaks noncovalent bonds between cellulose and hemicellulose that loosens the cell wall and allows more water into the cell. Calcium increases inside the cell and activates phosphatidylinositol-3-phosphate, which phosphorylates NADPH oxidase and produces reactive oxygen species (Grossmann, 2009). The Auxin Binding Protein 1 at the plasma membrane also activates RAC/ROP GTPase. G proteins have a key role in signal transduction in eukaryotic cells as well as an important role in cytoskeleton organization modeling the structure and arrangement of actin filaments and microtubules. Peroxisomes travel on actin so it affects the mobility of

the peroxisomes to remove reactive oxygen species. The abscisic acid (ABA) and ethylene induces the cause of death in plant tissues (Grossmann, 2000; Kelley and Riechers, 2007; Woodward, 2005). Due to the loss of cell wall structure, reactive oxygen species are able to penetrate into the plasma membrane where they can interact with phospholipids, promoting unsaturation of plasma membrane lipids, and leakage of the cytosol leads to cell death.

Application Issues

The ester formulations of 2,4-D have a higher vapor pressure so are generally more volatile than the amine formulations. However, the esters are also more soluble in the plants cuticle due to lipid solubility than the amines. Amine formulations can precipitate out of the solution so they are overall less effective in weed control than ester formulations (Sosnoskie et al., 2015). The dimethylamine (DMA) and diglycolamine (DGA) formulations of dicamba are more volatile than the BAPMA dicamba formulation (Cojocaru et al., 2013). The movement of spray droplets that land off-target causes spray drift. The smaller the droplet and the longer it remains in the air, the higher the chance for drift. Vapor drift occurs when applied herbicide evaporates from the target plant and aerosolizes to an unintended location. Crops affected by drift at the late vegetation or early reproduction stage show the greatest yield reduction. Soybean (Glycine max) and cotton (Gossypium hirsutum) plants have 2,4-D and dicamba resistant varieties commercially available (Green, 2014; Green and Owen, 2011). According to the Missouri Department of Agriculture (MDA), for the 2015 fiscal year (July 1, 2014-June 30, 2015) only three out of the 90 drift complaints were dicamba related. Compared to 2016 fiscal year (July 1, 2015-June 30, 2016) where they had 97 cases where 27 were

dicamba related. Within an eight-month span of the 2017 fiscal year (July 1, 2016-Feb 27, 2017), 164 complaints were dicamba related out of 181. (Office of Enforcement and Compliance Assurance, 2016). Companies such as Monsanto, Dow Agro, and BASF have recently released low volatile versions of dicamba (Engenia and XtendiMax with Vapor Grip) and 2,4-D (Enlist Duo) in hopes to reduce the amount of drift that occurs. Reuters reports that over one million acres of Xtend soybeans were planted in the United States in 2016. Monsanto predicts 15 million acres of Xtend soybeans to be planted in 2017 in the US and up to 55 million acres of Xtend soybeans will be planted in 2019 (Plume, 2016).

Symptoms of Drift

Symptoms for 2,4-D drift on cotton can appear two days after exposure. Bent stem and horizontal leaf petioles are common signs around this time. Four days after exposure, petioles start twisting and new leaves begin curling downwards. After a week red to dark brown patches start forming (Figure 2.8). One month after exposure, new leaves have parallel venation and have finger like projections. Chlorosis and severe reddening of petioles are common six weeks after cotton exposure to 2,4-D (Colquhoun et al., 2014; Egan et al., 2014; Kelley et al., 2005).

Figure 2.8 2,4-D damaged cotton.

Symptoms for dicamba drift on cotton can appear two days after exposure. Two days after treatment leaf petioles begin curving. One week later, new leaves begin to yellow and the blister at the leaf veins. Around 10 days after exposure, petioles are severely curved. After a month, other leaves are chlorotic and the meristem has been aborted (Egan et al., 2014; Everitt and Keeling, 2009).

Herbicide Detection

LC-MS

There are methods available to detect both 2,4-D and dicamba in affected crops using liquid chromatography and mass spectroscopy (Voyksner et al., 1984; Xu and Armstrong, 2013). The issue with this analysis is that the extraction required cleaves the salt groups from the auxin herbicides. This is due to the rise of pH after the addition of

sodium hydroxide that is required in the sample preparation for the extraction. So although it is possible to distinguish between 2,4-D and dicamba using LC/MS, it is difficult to differentiate the DMA and DGA formulations of these herbicides using this method (Guo et al., 2016).

FT-IR

Fourier Transform Infrared spectroscopy (FT-IR) is a fast, accurate, and usually non-destructive and requires little to no sample preparation. This would potentially make it ideal for analyzing soybean and cotton samples affected by herbicide drift from the different formulations of synthetic auxins. An infrared spectrometer analyzes a compound by passing infrared radiation, over a range of different frequencies, through a sample and measuring the absorptions made by each type of bond in the compound. This produces a spectrum, normally a 'plot' of % transmittance against wavenumber (Coates, 2000). Analyzing the infrared spectrum of a sample can give abundant structural information of the sample molecules. Fourier transformation is a mathematical process that expresses a waveform as a weighted sum of sines and cosines (Welch, 1967).

FT-IR uses a Helium Neon (He-Ne) laser. The laser serves as an internal wavenumber standard which all the infrared wavenumbers are compared against it. Michelson Interferometer splits the infrared light from the laser into two beams of light. One beam goes to a stationary mirror and the other goes to an adjustable mirror. The two beams of light recombine with one another at the beamsplitter once they are reflected back by the mirrors. The beamsplitter is to split the light beam in two. Some of the light reflects off the moving mirror and some of the light reflects off the fixed mirror. Potassium bromide (KBr) is almost universally used as a substrate material in FT-IR

beamsplitter (Jaggi and Vij, 2006). A thin amount of coating of germanium is sandwiched between two pieces of KBr. The germanium coating is what actually splits the beam. The KBr acts as a substrate for the beamsplitter coating and to protect it from the environment. The beamsplitters are usable from 4,000 to 400 cm-1 that covers the mid-infrared range. The resulting signal is an interferogram which is a function of the distance of the moving mirror position (Griffiths and de Haseth, 2007). KBr can fog over due to high humidity so the FT-IR spectrometer is purged with dry nitrogen to maintain desiccation.

The Deuterated Triglycine Sulfate (DTGS) detector is a pyroelectric bolometer which changes in the amount of infrared radiation striking the detector causes the temperature of the DTGS element to charge. This change in capacitance with temperature results in a measurable voltage across the detector(Griffiths and de Haseth, 2007; Kupper et al., 2001). The second major detector used in the mid infrared is the Mercury Cadmium Telluride (MCT) detector. The detector element absorbs infrared photons and it results in electrons being promoted from the valence band to the conduction band. Once these electrons are in the conduction band, they can create an electrical current when a voltage is applied. The electrical current that is generated measures the number of electrons in the conduction band and is directly proportional to the number of infrared photons hitting the detector. Although more sensitive than DTGS detectors, MCT detectors are required to be cooled with liquid nitrogen (Chan and Kazarian, 2006; Jaggi and Vij, 2006).

A classification model for the applied synthetic auxin herbicides can then be created from the spectral data using Principal Component Analysis (PCA) and Linear Discriminant Analysis (LDA). PCA is an unsupervised classification method that

condenses data into a limited number of components that account for the maximum amount of variance (Martínez and Kak, 2001; Saed-Moucheshi et al., 2013). LDA is a supervised classification method that takes within group variance and the between group variance to construct analysis rules for pre-specified classes. The model can then be used to identify unknown samples by their most probable class (Juwei Lu et al., 2003; Martínez and Kak, 2001).

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CHAPTER III

SINGLE CORN KERNEL AFLATOXIN B1 EXTRACTION AND ANALYSIS METHOD

Introduction

Aspergillus flavus (*A. flavus*) is a fungus that commonly grows in the soil and is known to infect a variety of crops including cotton, peanuts, and corn. *A. flavus* can cause ear rot in maize, aflaroot in peanuts, and yellow spot disease in cotton (Guchi, 2015; Smart et al., 1990; Zeringue et al., 1999). Environmental conditions such as high temperatures, high humidity, drought stress, and poor crop storage can cause *A. flavus* to start producing aflatoxins (Trenk and Hartman, 1970). Aflatoxins are secondary metabolites of the fungus and are extremely carcinogenic. The four major types of aflatoxins are aflatoxin B1, B2, G1, and G2. Aflatoxin B1 and B2 fluoresce blue under UV light while aflatoxin G1 and G2 fluoresce green in the presence of UV light. Aflatoxin B1 can also be metabolized to aflatoxin M1, which would be found in the milk of lactating mammals. Aflatoxin B1 is the most common and carcinogenic of these compounds (Figure 3.1). Aflatoxin B1 is converted in vivo to an aflatoxin B1-exo-8, 9-epoxide by the liver enzyme cytochrome p450 oxidase (Smela et al., 2001). The epoxide that is formed is a highly reactive electrophile and has an extremely high regiospecificity for the N7 position of the guanine residue in DNA (Greenblatt et al., 1994). It specifically

binds to the p53 encoding region to form a DNA adduct (Clewell et al., 2014). This results in nonfunctioning p53 proteins that are important tumor suppressors in humans.

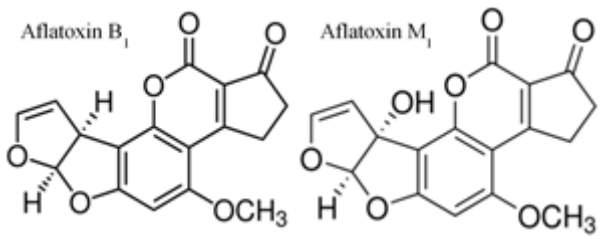

Figure 3.1 Structures of aflatoxin B_1 and aflatoxin M_1.

In 1988 the International Agency for Research on Cancer classified aflatoxin B1 as a Class 1 human carcinogen (Vainio and Wilbourn, 1992). Therefore, the U.S. Food and Drug Administration (FDA) has restricted the amount of aflatoxin in food for human consumption to 20 parts per billion (ppb) in the United States. The European Commission (EC) limits aflatoxins in food for human consumption to 4 ppb in the European Union (Otsuki et al., 2001; Wu and Guclu, 2012). The Council of Agricultural Science and Technology has estimated that the US has an annual loss of almost a billion dollars due to crop damage from mycotoxins. An estimated $225 million of that are due to aflatoxin contamination in maize crops (Robens and Cardwell, 2003). Study of the maize genome has increased in recent years in hopes to discover genes that are involved with aflatoxin resistance in corn. The DNA from a single corn kernel can be used to analyze the gene expression that occurs in *Aspergillus flavus* inoculated maize. In order to complement this procedure, aflatoxin extraction should be equally versatile. Aflatoxin extraction methods normally require several (10-250) grams of ground maize, which may not be

feasible in determining aflatoxin accumulation for a smaller region of the *A. flavus* infected ear. A single kernel extraction method is needed in order to accurately track aflatoxin concentration and maize gene expression at specific *A. flavus* infected sites on the cob.

Materials and Methods

All solvents and reagents were Optima LC/MS grade. Methanol, water, formic acid, and ammonium acetate were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Purified aflatoxin B1 and M1 standards were acquired from Sigma Aldrich (St. Louis, MO, USA).

Maize kernels were flash frozen in liquid nitrogen. The kernels were ground into a fine powder with a mortar and pestle. 200 mg of each ground sample was placed into a 1.5 mL micro-centrifuge tube. Aflatoxin free ground maize was spiked with aflatoxin B1 to yield two levels of spiked samples (4 ppb and 20 ppb) of aflatoxin B1 for recovery. These concentrations were chosen because they are the limit for aflatoxin in food for human consumption in the European Union and the United States, respectively.

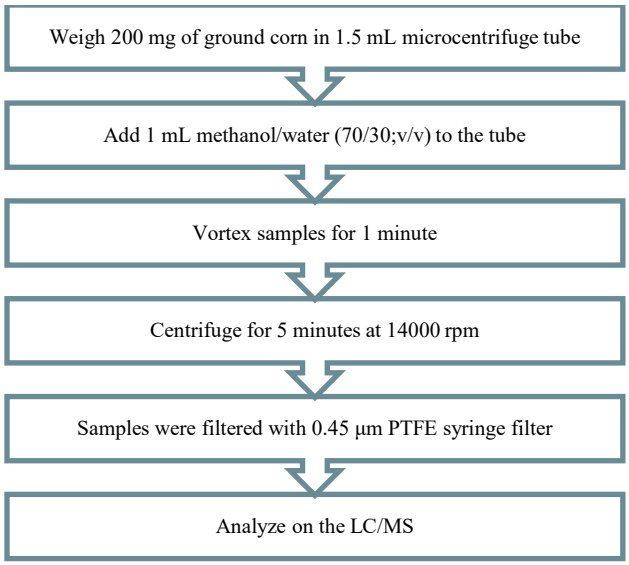

Figure 3.2 Sample preparation workflow detailing single kernel aflatoxin extraction.

A solution of 1 mL (70/30, v/v) methanol/water was added to each 1.5 mL microcentrifuge tube. The samples were mixed for 1 minute and then centrifuged for 5 minutes at 14,000 rpm. Polytetrafluoroethylene (PTFE) syringe filters (0.45 µm) were used to remove any large particles from the samples after centrifugation. The liquid extracts were transferred to auto-sampler vials and analyzed using an Agilent 6460 LC/MS/MS Triple Quadrupole with electrospray ionization (Figure 3.2). Aflatoxin M1 was added as an internal standard at a concentration of 10 ppb. The calibration curve was matrix- matched with extracted aflatoxin-free maize solution.

Experimental

The samples were analyzed using an Agilent 6460 LC/MS/MS Triple Quadrupole Mass Spectrometer with ESI, using an Agilent 1200 Series High Performance Liquid Chromatography (HPLC). The HPLC system consists of a binary pump (G1312B), infinity high performance degasser (G1379B), high performance autosampler (G1367E), thermostatted column compartment (G1316B), sampler thermostat (G1330B), and MassHunter data software. Aflatoxin B1 and M1 were optimized using the Agilent Optimization software. Aflatoxin M1 was used as an internal standard (ISTD) at 10 ppb.

The Agilent Optimization software produces the ideal fragmentor voltage and collision energy for each MRM transition of aflatoxin B1 and M1 (Table 3.2).

Table 3.2 MRM transitions for aflatoxin B_1 and aflatoxin M_1.

Compound (Aflatoxin)	Retention Time (min)	Fragmentor Voltage (V)	Collision Energy (eV)	Precursor Ion (m/z)	Product Ion (m/z)
B_1	3.4	166	21	313.1	285.1
B_1	3.4	166	33	313.1	269.1
B_1	3.4	166	41	313.1	241.1
M_1 (ISTD)	3.0	131	25	329.1	273.1
M_1 (ISTD)	3.0	131	41	329.1	229.1

Instrumentation

An Agilent 6460 LC/MS Triple Quadrupole Mass Spectrometer equipped with electrospray ionization (ESI) was used to analyze the aflatoxin concentration of the samples. The autosampler temperature was set to 4°C. The HPLC used a Zorbax Eclipse Plus-C18 Narrow Bore 2.1 x 50mm, 5µm column with a temperature of 50°C. The mobile phase consisted of 5mM ammonium acetate with 0.1% formic acid in HPLC-grade water and 5mM ammonium acetate with 0.1% formic acid in methanol. The flow rate of the mobile phase during the analysis was consistently 0.6 mL/min. The mobile phase gradient was transitioned from 95% water to 100% methanol during the six minutes of the analysis time. Then for the final three minutes of the run, the mobile phase re-versed from 100% methanol back to 95% water. The total run time of the method was nine minutes that includes a six-minute analysis time and an additional three minutes for the system to get back to equilibrium. The calibration curve was matrix-matched in order

to reduce matrix effects. The six-point calibration curve consisted of the concentrations 1 ppb, 5 ppb, 10 ppb, 25 ppb, 50 ppb, and 100 ppb (Figure 3.3).

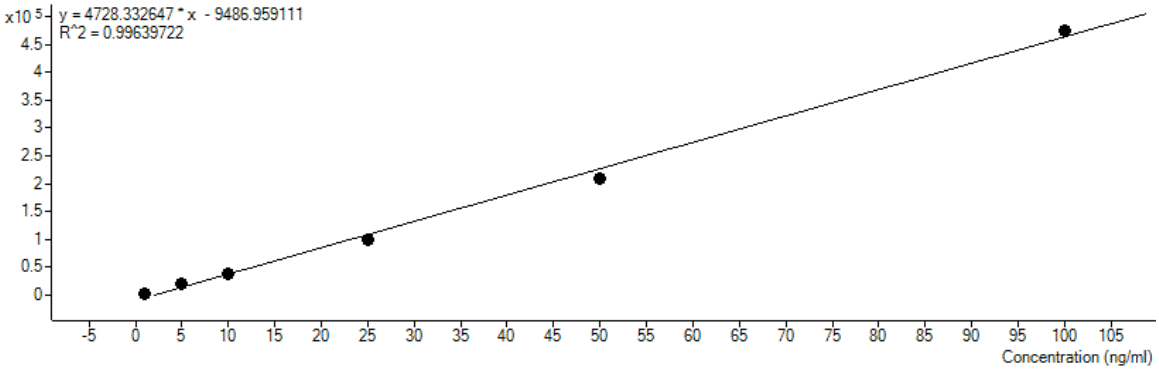

Figure 3.3 Matrix matched calibration curve for aflatoxin B_1 from concentration of 1.0 ng/mL to 100 ng/mL.

The parameters for the mass spectrometer were the following. The Agilent 6460 Triple Quadrupole Mass Spectrometer (MS/MS) system coupled to an electrospray analyzed the samples while in positive mode. The drying gas temperature was 325°C while the gas flow was set to 10 liters per minute. The nebulizer gas pressure was set to 50 psi and the capillary voltage was 4000V. The Sheath Gas Flow had an output of 11 liters per minute and the sheath gas temperature reached temperatures of 350°C. The delta electron multiplier voltage (EMV) was 800V and the dwell time lasted for 200msec. The precursor ion for aflatoxin B1 was 313.1 m/z and 329.1 m/z for aflatoxin M1. While in multiple reaction monitoring (MRM) mode, the mass spectrometer was set to look for the daughter ions after the precursor ion entered the collision cell. The transitions for aflatoxin B1 included 313.1 > 285.1 m/z with a collision energy of 20kEV, 313.1 > 269.1

m/z with a collision energy of 25kEV, and 313.1 > 241.1 m/z with a collision energy of 35kEV. The fragmentor value was 166 for aflatoxin B1 and for aflatoxin M1 was 131. The cell accelerator (7) values were the same for both aflatoxin transitions. The two transitions used for identifying aflatoxin M1 are 329.1>273.1 m/z and 329.1>229.1 m/z. The retention time of aflatoxin M1 was 3.0 minutes and 3.4 minutes for aflatoxin B1 (Figure 3.4). Agilent MassHunter Quantitative Analysis Workstation Software v. B.04.0.225.19 was used to analyze the quantitative data obtained from the samples and the calibration curve.

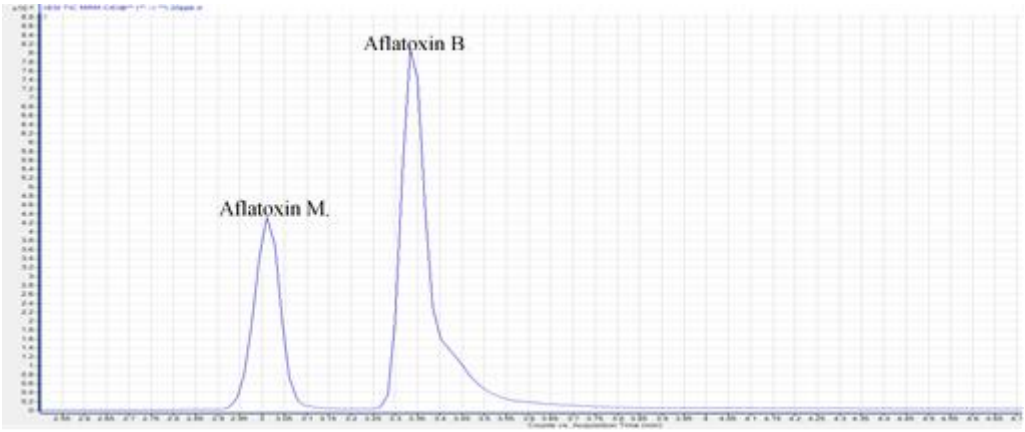

Figure 3.4 Chromatogram of aflatoxin B_1 standard at 25 ppb with aflatoxin M_1 as the internal standard at 10 ppb.

Results and Discussion

Table 2 demonstrates the average percent recoveries and relative standard deviation (RSD) values obtained from the spiked corn samples after performing the single maize kernel aflatoxin extraction. The analysis was performed in replicates of five at each of the two levels. The average percent recoveries for the 4 ppb and the 20 ppb

aflatoxin B1 spiked maize were 90.83% with a RSD of 4.11% and 90.72% with a RSD value of 14.45% respectively. Calibration standards were matrix matched with a range from 1 ppb to 100 ppb with a linear correlation (R2) of 0.996 as shown in Figure 3.3. In The limit of detection (LOD) and limit of quantification (LOQ) were estimated from the concentration of aflatoxin B1 required to give a signal to noise ratio of 3:1 and 10:1 respectively. The LOD was determined to be 0.344 ng/ml and the LOQ was calculated to be 1.042 ng/ml (Table 3.3). The internal standard, aflatoxin M1, injected at 10 ppb accounts for possible instrument variation. Aflatoxin M1 was chosen to be the internal standard because it has a structure similar to aflatoxin B1 but is not produced by *A. flavus*. Aflatoxin M1 is also less expensive and more readily available commercially than the radiolabeled form of aflatoxin B1. Methanol was the preferred solvent for aflatoxin extraction due to its compatibility with the ELISA testing and the cost effectiveness over other solvents like acetone and acetonitrile (Bertuzzi et al., 2011; Spanjer et al., 2008). This simplified aflatoxin extraction method is analogous to other extractions methods in terms of aflatoxin recovery without the need for solid phase extraction or clean up columns (Khayoon et al., 2010). The single maize kernel extraction method was needed in order to more accurately determine the changes in aflatoxin production, gene transcription, and protein production between inoculated and control maize kernels.

Table 3.3 Average percent recoveries and RSD values obtained from spike corn samples.

	4 ppb spiked maize	**20 ppb spiked maize**
Aflatoxin B_1	**% Recovery**	**% Recovery**
Replicate 1	91.65	93.23
Replicate 2	86.7	113
Replicate 3	97.38	72.384
Replicate 4	87.82	87.087
Replicate 5	90.59	87.88
Avg % Recovery	90.828	90.716
RSD (n=5)	4.11	14.45
LOD (ppb)	0.344	0.344
LOQ (ppb)	1.042	1.042

Conclusion

This process demonstrates a fast, simple, and effective analytical method for determining aflatoxin concentrations in a single maize kernel using an Agilent 6460 Triple Quadruple Mass Spectrometer. The detection levels for aflatoxin B1 were below both the limit set by the FDA in the United States and the limit set by the EC in the European Union. The recovery percentages for aflatoxin B1 were 90.83% for 4 ppb and 90.72% for 20 ppb with a satisfactory average RSD less than 15%. The single kernel extraction method will be a useful technique in determining how aflatoxin producing *Aspergillus flavus* affects infected maize.

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CHAPTER IV

MONITORING ASPERGILLUS FLAVUS PROGESSION AND AFLATOXIN ACCUMULATION IN MAIZE HYBRIDS

Introduction

The fungus, *Aspergillus flavus* (*A. flavus*), spends most of its life growing as a saprophyte in the soil where it plays a major role in the decomposition of plant material (Klich, 2007; Mellon et al., 2007; Scheidegger and Payne, 2003). The fungus can develop at temperatures ranging from 12°C to 48°C but the ideal temperature for fungal growth is 37°C (Amaike and Keller, 2011). *Aspergillus flavus* reproduces by asexual spores known as conidia. It can endure harsh winter conditions in its mycelium form due to the formation of resistant structures called sclerotia (Wicklow, 1983). *Aspergillus flavus* is a common filamentous fungus that has been known to infect a variety of crops including rice, cotton, peanuts, and corn. *A. flavus* can cause ear rot in corn, aflaroot in peanuts, and boll rot or yellow spot disease in cotton (Liu et al., 2006; Rajasekaran et al., 2008; Zeringue et al., 1999). Maize kernels are most susceptible to infection in the early reproductive stage (R3) approximately three weeks after mid silk has occurred (Jones et al., 1980; Zuber and Lillehoj, 1979). Environmental conditions such as high temperatures, high humidity, drought stress as well as poor crop storage and insect damage can cause *A. flavus* to start producing aflatoxins (Cardwell et al., 2000; Guo et al., 2008; Hell et al., 2003; Trenk and Hartman, 1970). Aflatoxins are secondary

metabolites of *Aspergillus flavus* and are extremely carcinogenic. There are four major types of aflatoxins: B1, B2, G1, and G2. Aflatoxins B1 and B2 glow blue under UV light while aflatoxins G1 and G2 fluoresce green in the presences of UV light (Hara et al., 1974). Aflatoxin B2 is the dihydroxy form of aflatoxin B1 while aflatoxin G2 is the dihydroxy form of aflatoxin G1.

Aflatoxin B1 is a procarcinogen because it requires enzymatic bioactiviation before it becomes carcinogenic. Aflatoxin B1 is converted in the body to AFB1-8-9-epoxide by the liver enzyme p450. Cytochrome p450 is a heme-binding enzyme that has an important role in the biotransformation of xenobiotics. Cytochrome p450 aids in the inactivation of foreign compounds. However in the case of aflatoxin B_1, it metabolizes aflatoxin B_1 into AFB1-exo-8, 9-epoxide (Ayed-Boussema et al., 2011; Yu et al., 2000). The AFB1-exo-8, 9-epoxide is formed by epoxidation at the 2,3 double bond. The epoxide that is formed is a highly reactive electrophile and has a high regiospecificity for the N7 position of the guanine residue in DNA. It specifically binds to the p53 encoding region to form a DNA adduct (Clewell et al., 2014; Essigmann et al., 1977; Smela et al., 2001). This results in nonfunctioning p53 proteins, which are important tumor suppressors in the body. The p53 controls an important cell cycle checkpoint that is responsible for maintaining the integrity of the genome. The p53 protein can bind to specific DNA sequences and activate the transcription of genes including p53 binding sites. When DNA is damaged, p53 has been shown to induce cell cycle arrest or even lysis of the cell. Some mutations to p53 eliminate this response and it results in an increased frequency of unchecked genetic mutations (Greenblatt et al., 1994; Hollstein et

al., 1991). This phenomenon increases the probably that a tumor cell escapes the normal system of checks against excessive cell growth.

In 1988, the International Agency for Research on Cancer classified aflatoxin B_1 as a Class 1 human carcinogen (Vainio and Wilbourn, 1992). The FDA has restricted the amount of aflatoxin allowed for human consumption to 20 (µg/kg) parts per billion. Products with concentrations higher than 20 ppb are restricted from interstate commerce. The FDA restricted levels aflatoxin in dairy cattle feed is also 20 ppb. For beef cattle and swine, the limit for aflatoxin in feed are 300 ppb and 200 ppb, respectively (Lizárraga-Paulín et al., 2011). More than 30 percent of the maize harvested in the US is used for ethanol production. If maize contains aflatoxins, it is not cost effective to produce ethanol from it (Hertel et al., 2010) as the aflatoxins concentrate in the byproducts. A major byproduct of ethanol production is Dried Distillers Grains (DDGS), which are sold to farmers to use as feed. During ethanol production, the aflatoxin from the maize is transferred into the DDGS.

In the United States, the estimated economic cost of aflatoxins annually ranges between $104.5 million to $1.68 billion dollars (Mitchell et al., 2016; Robens and Cardwell, 2003). The United Nations Food and Agriculture Organization on international regulations for mycotoxins conducted a study to show that at least 77 countries have some sort of regulations for mycotoxins (van Egmond and Jonker, 2004; Wu and Guclu, 2012). The United States is also the largest producer of maize on the planet producing 351,000,000 metric tons in 2013. The United States is the second exporter of maize in the world shipping 24,178,452 metric tons in 2013 to over 180 different countries.

There are a few physical and chemical detoxification methods for reducing the amount of aflatoxin in food and feed products. A major problem with chemical detoxifications of aflatoxin is the unknown toxicity of the byproducts. Pre-harvest measures that can be taken to help prevent aflatoxin contamination are irrigation, earlier planting dates, fertilization, insect control, and biological controls such as non-toxigenic *A.flavus* strains like Afla-Guard.

One of the most effective ways of reducing aflatoxins in corn is by breeding resistant lines. The first maize line that was released as resistant to aflatoxin accumulation was Mp313E and Mp420 in 1992 (Scott and Zummo, 1992). Mp717 is germplasm line that were developed in Mississippi from a cross between Mp420 and Tx601 (Williams and Windham, 2006). Due to its relatively early flowering time and resistance to aflatoxin accumulation, Mp719 is also an excellent maize line for not only aflatoxin resistance but for breeding for more resistant commercial lines of maize as well (Williams and Windham, 2012). Maize lines that are resistant to aflatoxin accumulation are still the best option for combating this problem yet increased resistance in maize hybrids are required. Moreover, there are several proteins Pr10 proteins, 14KDA trypsin-inhibitor proteins, α-amylase, and PER1 (Peroxidredoxin antioxidant) whose gene expression increased in aflatoxin resistant maize but are difficult to transfer to offspring in order to make more resistance lines (Chen et al., 2010; Tripathi et al., 2009; Yan et al., 2015). There is still a great deal to learn about influential genes and proteins as well as how they interact to contribute to aflatoxin resistance. The purpose of this experiment is to track the correlations between aflatoxin accumulation and *A. flavus* fungal biomass for the first several weeks after inoculation, as well as the spreading of the fungus and the

aflatoxin throughout the inoculated ear of corn. This will allow for better understanding of the pathogen/host interactions and how the fungus progresses over time.

Methods and Materials

First Year

GA209 x T173 and Mp313E x Mp717 maize hybrids were planted and allowed to self-pollinate. Hybrids were assigned to single row plots that were 4 m long and spaced 0.97 m apart with irrigation. GA209 x T173 is the aflatoxin accumulation susceptible maize hybrid while Mp717 x Mp313E is the resistant maize hybrid to aflatoxin accumulation (Table 4.1). The average temperature was 24.8°C (76.6°F) with a total rainfall of 28.52cm (11.23in). Two and three weeks after pollination two ranges of GA209 x T173 hybrid and two ranges of Mp313E x Mp717 maize hybrid were each inoculated with toxin producing *Aspergillus flavus* NRRL 3357.

Table 4.1 Type of maize hybrid used in the experiments each year.

Aflatoxin Susceptibility	**Maize Hybrids (First Year)**	**Maize Hybrids (Second Year)**	**Maize Hybrids (Three Year)**
Susceptible Hybrid	GA209 x T173	GA209 x T173	GA209 x T173
Susceptible and Resistant Hybrid	N/A	Mp313E x T173	GA209 x Mp313E
Resistant Hybrid	Mp717 x Mp313E	Mp717 x Mp313E	Mp717 x Mp313E

A device was created using sewing needles and a mold to inoculate the corn in a three by three-kernel grid similar to the pinbar technique (Zummo and Scott, 1989). The needles were then dipped into a suspension containing $3x10^8$ *A. flavus* conidia and used

to infect the maize through the husk (Figure 4.1). Collections of the inoculated maize cobs were made 2, 3, 7, 14, 21, 28, and 35 days after inoculation. A randomized complete block design with n=72 was used. The main plot was the maize genotype with three reps. The treatment was the number of days after pollination. The experimental unit was the number of days after inoculation. Maize samples were collected and stored at -80°C until the aflatoxin and DNA extractions could be performed.

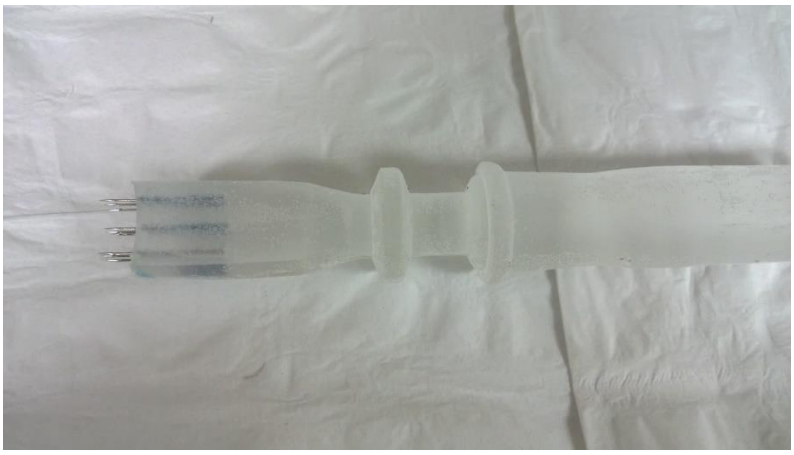

Figure 4.1 Inoculation device used to inject *A. flavus* into maize hybrids.

The single maize kernel aflatoxin extraction method was used to extract and quantify aflatoxin (Reid et al., 2016). Maize kernels were flash frozen in liquid nitrogen. The corn kernels (200 mg) was ground with a mortar and pestle into a fine powder and extracted with 1 mL of a 70% methanol/water solution was added to the tube. The samples were shaken for 2 minute and then centrifuged for 5 minutes at 3000. The extract was filtered through a 0.45 µm PTFE syringe filters. An Agilent 6460 LC/MS Triple Quadruple, which uses electrospray ionization (ESI), was used to analyze the aflatoxin

concentration of the samples. The autosampler temperature was set to 4°C. A Zorbax Eclipse Plus-C18 Narrow Bore 2.1 x 50mm, 5µm column was used with a temperature of 50°C. The mobile phase consisted of 5mM ammonium acetate with 0.1% formic acid in HPLC-grade water and 5mM ammonium acetate with 0.1% formic acid in methanol. The flow rate of the mobile phase during the analysis was consistently 0.6 mL/min. The mobile phase gradient was transitioned from 95% water to 100% methanol during the six minutes of the analysis time. For the final three minutes of the run, the mobile phase reversed from 100% methanol back to 95% water. The total run time of the method was nine minutes, which includes a six-minute analysis time and an additional three minutes for equilibration.

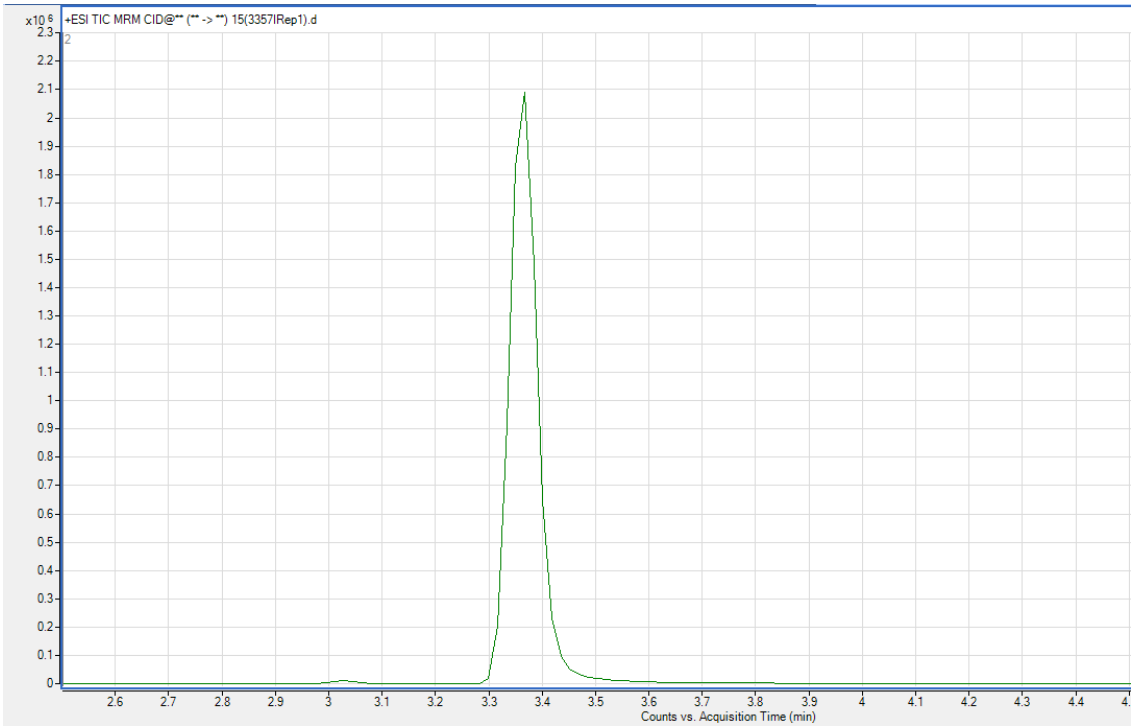

Figure 4.2 Chromatogram of aflatoxin B_1 sample with aflatoxin M_1 as the internal standard at 10 ppb

The calibration curve was matrix-matched in order to reduce matrix effects. The six-point calibration curve consisted of the concentrations 1ppb, 5 ppb, 10 ppb, 20 ppb, 50 ppb, and 100 ppb. The Agilent 6460 Triple Quadrupole Mass Spectrometer (MS/MS) system coupled to an electrospray analyzed the samples while in positive mode. The drying gas temperature was 325°C while the gas flow was set to 10 liters per minute. The nebulizer gas pressure was set to 50 psi and the capillary voltage was 4000V. The sheath gas flow had an output of 11 liters per minute and the sheath gas temperature reached temperatures of 350°C. The delta electron multiplier voltage (EMV) was 800V and the dwell time lasted for 200msec. The retention time of aflatoxin M_1 was 3.0 minutes and 3.4 minutes for aflatoxin B1 (Figure 4.2). The precursor ion for aflatoxin B_1 was 313.1 and 329.1 for aflatoxin M_1. While in multiple reaction monitoring (MRM) mode, the mass spectrometer was set to look for certain daughter ions after the precursor ion entered the collision cell. The transitions for aflatoxin B_1 included 313.1 > 285.1 with a collision energy of 20kEV, 313.1 > 269.1 with a collision energy of 25kEV, and 313.1 > 241.1 with a collision energy of 35kEV. The fragmentor value was 166 for aflatoxin B_1 and 131 for aflatoxin M_1. The cell accelerator (7) values were the same for both aflatoxin transitions. The two transitions used for identifying aflatoxin M_1 are 329.1>273.1 and 329.1>229.1 (Table 4.2). Agilent MassHunter Quantitative Analysis Workstation Software v. B.04.0.225.19 was used to analyze the quantitative data obtained from the samples and the calibration curve. This method can also be used to check for the presence of other types of aflatoxins being produced by *A. flavus*.

Table 4.2 MRM transitions for aflatoxin B_1 and aflatoxin M_1.

Compound (Aflatoxin)	Retention Time (min)	Fragmentor Voltage (V)	Collision Energy (eV)	Precursor Ion (m/z)	Product Ion (m/z)
B_1	3.4	166	20	313.1	285.1
B_1	3.4	166	25	313.1	269.1
B_1	3.4	166	35	313.1	241.1
M_1 (ISTD)	3.0	131	25	329.1	273.1
M_1 (ISTD)	3.0	131	41	329.1	229.1

The maize and fungal DNA was extracted from 100 milligrams of the ground corn kernel using a modified CTAB method (Cetyl Trimethyl Ammonium Bromide) for plant tissue (Cota-Sánchez et al., 2006). Two 250 µL aliquots of the buffer was added to the 100 mg ground corn kernel samples. The samples were then placed into a 60°C water bath for 15 minutes. Two aliquots of 200 µL of the chloroform/octanol solution was added to each sample. The samples were then inverted 50 times in order to thoroughly mixed. Once that was completed, the samples were centrifuged at 3000 rpm for 15 minutes. The supernatant was then transferred into clean micro centrifuge tubes. 300 µL of isopropanol was added to the tubes. The samples were inverted 15 times to properly mix the solution. The samples were centrifuged at 3000 rpm for 15 minutes at 4°C, decanted, and 300 µL of 90% ethanol was pipetted into the tubes. Then the samples were centrifuged again at 3000 rpm for 5 minutes at 4°C. Finally, the samples were decanted and the pellets were allowed to air dry. The pellets were suspended in 100 µL of TE buffer. The CTAB method is ideal for DNA extraction because of its ability to remove

the high number of polysaccharides and phenolic compounds that are found in plant tissue, which could interact irreversibly with nucleic acid. A Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE) was used to determine the quality and quantity of DNA. The fungal biomass of the inoculated maize samples was determined by using quantitative real time polymerase chain reaction (qPCR).

The fungal biomass of the inoculated maize samples was determined by using quantitative real time polymerase chain reaction (qPCR). The forward primer and reverse primer for the fungal quantification Af2 (forward primer: 5 - ATCATTACCGAGTGTAGGGTTCCT-3; reverse primer: * 5- GCCGAAGCAACTAAGGTACAGTAAA-3; amplicon 73 bp) designed in the internal transcribed spacer 1 (ITS1) sequence (Mideros et al., 2009). The forward and reverse primers for the maize quantification Zmt3 (forward primer: 5 - TCCTGCTCGACAATGAGGC-3; reverse primer: 5 -TTGGGCGCTCAATGTCAA-3; amplicon 63 bp) were used for amplifying maize α-tubulin. 5 µL of Power SYBR green PCR Master Mix, 0.5 µL of the forward primer, 0.5 µL of the reverse primer, 2.0 µL of DNA-free water, and 2.0 µL of sample DNA at a concentration of 10 ng/µL were combined to make up the 10 µL reaction volume. The Roche LightCycler 480 instrument (Roche Diagnostics Corporation, Indianapolis, IN) was used to determine the fungal biomass. The temperature profile for denaturation, melting curve, gradual heating, and cooling step conditions for the qPCR were as follows: denature at 95°C for 10 min, 45 cycles at 95°C for 10s, 60°C for 5s, 72°C for 10s, 95°C for 10s, anneal at 65°C for 1 min, 97°C for 5s, and cool at 40°C for 10s. Two standard curves for both maize and fungal

DNA at concentrations of 10, 1.0, 0.1, 0.01, 0.001, and 0.0001 ng/µL were both ran with the DNA samples. Statistical analysis was done using SAS® (SAS Institute In.; Cary, NC) 9.3 software.

Second Year

The three different maize hybrids that were used in the experiment were GA209 x T173 (susceptible cross), Mp313E x T173 (resistant and susceptible cross), and Mp313E x Mp717 (resistant cross). The maize hybrids were allowed to self-pollinate to ensure that the ears have as many kernels as possible. The total rainfall measured at 33.15cm (13.05in) while the average temperature was 24.6°C (76.3°F). The top ear from each plant was inoculated with *A. flavus* isolate NRRL 3357 or water as a control in a 3 by 3-kernel grid. Each row contained three inoculated maize plants. The inoculated maize was then be collected 3, 7, 14, 21, 28, and 35 days after infection. This experiment was carried out using a split plot design with n=162. The block was the plot. The main plot unit (MPU) is the range, the main plot factor (MPF) are the different maize varieties, the simple plot unit (SPU) is the row, and the simple plot factor is the number of days after inoculation. Each of the 7 days were randomly assigned to a row. There were ten rows within a range. The maize varieties were also randomly assigned to each range. There were three plots with each plot containing three ranges. Significance was tested at α=0.05. Statistical analysis was done using SAS® (SAS Institute In.; Cary, NC) 9.4 software. The twelve-point calibration curve consisted of the concentrations 1, 5, 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ppb (Figure 4.3). The aflatoxin concentration using

LC/MS and the DNA quantification using real time PCR were carried out the same way as year one.

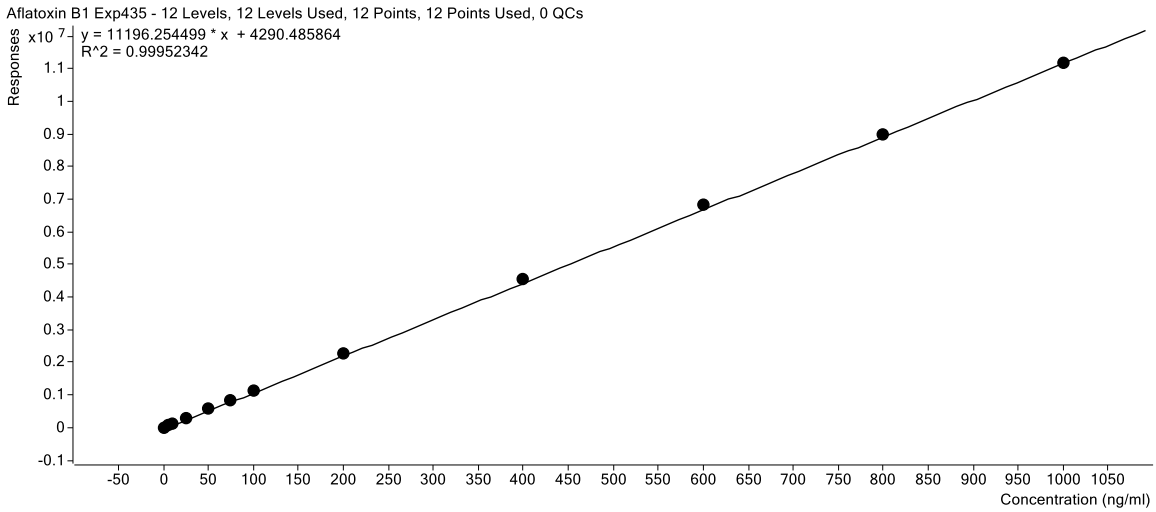

Figure 4.3 Matrix matched calibration curve for aflatoxin B_1 from concentration of 1.0 ppb to 1000 ppb.

Third Year

The three maize hybrids used in this experiment are GA209 x T173 (susceptible cross), GA209 x Mp313E (resistant and susceptible cross), and Mp313E x Mp717 (resistant cross). The maize hybrids were self-pollinated. The mean temperature was 25.7°C (78.3°F) with total rainfall of 24.05cm (9.47 in). The top ear from each plant were inoculated with *Aspergillus flavus* isolate NRRL 3357 or water as a control in a 3 by 3-kernel grid 21 days after silk maturation.

There were three inoculated maize plants for each row. The inoculated maize were then collected 3, 7, 10, 14, 21, 28, and 35 days after infection. The extractions for both aflatoxin and DNA in the infected maize were performed the same way as in year

one and two. The aflatoxin and DNA quantification using LC/MS and real time PCR respectively were performed the same way as in year two. The statistical analysis was done the same as year two with n=189.

Results

First Year

The inoculated resistant maize line Mp717 x Mp313E had less fungal biomass overall then susceptible line GA209 x T173 but there was no significant difference (P=0.2741). The fungus spread farther form the point of inoculation in the infected GA209 x T173 maize than the Mp717 x Mp313E inoculated maize. The aflatoxin accumulation was significantly higher in GA209 x T173 maize lines than the Mp717 x Mp313E inoculated maize, and it was significantly different (P<0.0001). There was no aflatoxin detected in the maize hybrids 2 days after inoculation with *A. flavus* (Table 4.3).

Table 4.3 Mean aflatoxin concentration in ppb of GA209xT173 and Mp717 x Mp313E.

	Day 2	Day 3	Day 7	Day 14	Day 21	Day 28
GA209 x T173 (n=36)	0.0 ± 0.0	31.01 ± 46.17	147.11 ± 173.28	333.42 ± 130.86	275.3 ± 196.44	163.81 ± 174.81
Mp717 x Mp313E (n=36)	0.0 ± 0.0	0.0 ± 0.0	83.26 ± 59.94	172.26 ± 202.37	184.75 ± 202.32	145.48 ± 124.76

The maize inoculated 21 days after silk maturation had a linear correlation between fungal biomass and aflatoxin accumulation for both the resistance and susceptible lines. The maize inoculated 14 days after silk emergence had significantly

less aflatoxin (P<0.0001). GA209 x T173 started producing aflatoxin as early as three days after inoculation while Mp717 x Mp313E does not. The average aflatoxin concentration in maize was significantly different between 14 days, 21 days, and 28 days after inoculation and 2 days and 3 days after inoculation (P<0.0001). On average, maize inoculated 21 days after silk maturation had more aflatoxin accumulation regardless of resistance. The mean fungal biomass for the maize hybrid, Mp717 x Mp313E, is very similar on 7, 14, and 21 days after inoculation (Table 4.4).

Table 4.4 Average fungal biomass of GA209xT173 and Mp717 x Mp313E.

	Day 2	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
GA209xT173	0.00004 ±	0.001 ±	0.00273 ±	0.00667 ±	0.0102 ±	0.00266 ±
(n=36)	0.00003	0.00093	0.00255	0.0099	0.01724	0.00368
Mp717xMp313E	0.00004 ±	0.00009 ±	0.00339 ±	0.00353 ±	0.00318 ±	0.001 ±
(n=36)	0.00007	0.00013	0.00311	0.00598	0.00224	0.0013

Second Year

The mean aflatoxin concentrations of the *Aspergillus flavus* infected maize collected three days after inoculation were 579.12 ± 436.17 ppb for Mp313E x Mp717, 1234.76 ± 1818.1 ppb for Mp313E x T173, and 2.39 ± 3.72 ppb for GA209 x T173. It was significantly higher for Mp313E x T173 when compared to the other hybrids (P<0.0001). For the samples collected seven days after inoculation, the aflatoxin concentrations were 1030.84 ± 676.38 ppb for Mp313E x T173, 1552.18 ± 1736.95 ppb for Mp313E x Mp717, and 2415.54 ± 3017.98 ppb for GA209 x T173. The susceptible

hybrid contained more aflatoxin on average, there was a significant difference ($P<0.0001$). The aflatoxin concentrations of samples collected 14 days after inoculation were 3936.07 ± 1951.41 ppb for GA209 x T173, 3692.54 ± 1471.96 ppb for Mp717 x Mp313E, and 2927.56 ± 2672.19 ppb for Mp313E x T173. There was no significant difference between susceptible maize hybrid and the other hybrids. Infected maize collected 21 days after inoculation had aflatoxin concentrations of 5852.07± 3137.22 ppb for GA209 x T173, 4935.66 ± 1280.93 ppb for Mp313E x Mp717, and 3069.88 ± 1276.14 ppb for Mp313E x T173. There was a significant difference between all the maize hybrids with the susceptible hybrid having more aflatoxin concentration. 4137.19 ± 1253.09 ppb for GA209 x T173, 5774.67 ± 4221.02 ppb for Mp313E x Mp717, and 3936.23 ± 2107.39 ppb for Mp313E x T173 were the aflatoxin concentrations for the collected maize 28 days after inoculation (Table 4.5). There was a significant difference between Mp313E x Mp717 and the other maize hybrids ($P<0.0001$).

Table 4.5 Mean aflatoxin concentration of maize hybrids comparing days after inoculation (DAI).

	3 DAI	**7 DAI**	**14 DAI**	**21 DAI**	**28 DAI**	**35 DAI**
GA209 x T173 (n=54)	2.39 ± 3.72	2415.54 ± 3017.98	3936.07 ± 1951.41	5852.07± 3137.22	4137.19 ± 1253.09	4565.48 ± 3166.08
Mp313E x T173 (n=54)	1234.76 ± 1818.1	1030.84 ± 676.38	2927.56 ± 2672.19	3069.88 ± 1276.14	3936.23 ± 2107.39	3842.37 ± 1991.37
Mp313E x Mp717 (n=54)	579.12 ± 436.17	1552.18 ± 1736.95	3692.54 ± 1471.96	4935.66 ± 1280.93	5774.67 ± 4221.02	5080.66 ± 2764.39

The aflatoxin concentrations of samples collected 35 days after inoculation were 4565.48 ± 3166.08 ppb for GA209 x T173, 5080.66 ± 2764.39 ppb for Mp313E x Mp717, and 3842.37 ± 1991.37 ppb for Mp313E x T173. There was only a significant difference between the susceptible maize hybrid and the susceptible/resistant maize cross ($P<0.0001$). Maize collected 21 days, 28 days, 35 days, and 14 days after inoculation was significantly higher in aflatoxin concentration than maize collected 3 and 7 days after inoculation ($P<0.0001$). There was no difference between samples collected after 3 and 7 days of inoculation. There was an insignificant difference between all the three technical reps preformed for each maize sample in the experiment. Overall, there was a significant difference between the aflatoxin concertation in Mp717 x Mp313E and Mp313E x T173 with the resistant hybrid containing more aflatoxin ($P<0.0001$).

There was no significant difference between the number of days after inoculation in fungal biomass ($P=0.6450$). The only significant different for the mean biomass was for the maize collected 14 days after inoculation were Mp313E x Mp717 with higher biomass when compared to the other hybrids. Mp717 x Mp313E had the least amount of average fungal biomass at 0.01138 ± 0.0166 0.01663ng/µL, followed by Mp313E x T173 at 0.01591 ± 0.00233 ng/µL, and with the most was GA209 x T173 at 0.01791± 0.03719ng/µL (Table 4.6).

Table 4.6 Average *A. flavus* biomass of maize hybrids at the inoculation site comparing days after inoculation.

	3 DAI	**7 DAI**	**14 DAI**	**21 DAI**	**28 DAI**	**35 DAI**
GA209 x T173 (n=18)	0.00062 ± 0.00101	0.00384 ± 0.00566	0.01793 ± 0.02175487	0.00734 ± 0.00935	0.05729 ± 0.0867	0.01989 ± 0.0205
Mp313E x T173 (n=18)	0.008332 ± 0.001237	0.018885 ± 0.0253	0.006016 ± 0.00502	0.012800 ± 0.0024	0.04175 ± 0.0487	0.004868 ±0.0049
Mp313E x Mp717 (n=18)	0.00429 ± 0.003179	0.001176 ± 0.003609	0.01323 ± 0.00473	0.00756 ± 0.01133	0.02639 ± 0.0407	0.007868 ± 0.0082

The spread of *Aspergillus flavus* biomass at the inoculation site and first row was significantly greater than at the second and third row ($P<0.0001$). There was significant more fungal spread between 35 DAI and 28 DAI when compared to the 21 DAI, 14 DAI, 7 DAI, and 3 DAI maize samples ($P<0.0001$). *A. flavus* spread farther from the inoculation site in Mp313E x T173 when compared to the other maize hybrids but it was not significant. There was no significant difference overall between the maize genotypes when it came to the mean fungal biomass (Figure 4.4).

Figure 4.4 Mp313E x T173, resistant/susceptible hybrid 28 days after *A. flavus* inoculation.

Third Year

The mean aflatoxin concentrations of the *Aspergillus flavus* infected maize collected three days after inoculation were 8.48 ± 9.59 ppb for Mp313E x Mp717, 3.30 ± 3.89ppb for GA209 x Mp313E, and 32.50 ± 29.28 ppb for GA209 x T173. It was significantly higher for the susceptible maize hybrids when compared to the other hybrids ($P<0.0001$). For the samples collected seven days after inoculation, the aflatoxin concentrations were 9.45 ± 8.21 ppb for GA209 x Mp313E, 98.51 ± 76.69 ppb for Mp313E x Mp717, and 101.81 ± 56.81 ppb for GA209 x T173. There was significant difference between the other maize hybrids and GA209 x Mp313E which contained less aflatoxin on average ($P<0.0001$). 319.65 ± 183.80 ppb for Mp717 x Mp313E, 350.73 ± 288.92 ppb for GA209 x T173, and 71.64 ± 55.07 ppb were the aflatoxin concentrations ten days after inoculation with *A. flavus*. The aflatoxin concentrations of samples collected 14 days after inoculation were 750.21 ± 416.83 ppb for GA209 x T173, 896.36

± 504.82 ppb for Mp717 x Mp313E, and 339.80 ± 229.34 ppb for GA209 x Mp313E. Infected maize collected 21 days after inoculation had aflatoxin concentrations of 1638.60 ± 768.27 ppb for GA209 x T173, 2634.92 ± 1148.85 ppb for Mp313E x Mp717, and 726.37 ± 191.89 ppb for GA209 x Mp313E (Table 4.7). There was significantly less aflatoxin contamination in the susceptible/resistant maize hybrid when compared to the two other maize hybrids ($P<0.0001$).

Table 4.7 Mean aflatoxin concentration of maize hybrids comparing days after inoculation (DAI).

	3 DAI	**7 DAI**	**10 DAI**	**14 DAI**	**21 DAI**	**28 DAI**	**35 DAI**
GA209 x T173 (n=63)	32.50 ± 29.28	101.81 ± 56.81	350.73 ± 288.92	750.21 ± 416.83	1638.60 ± 768.27	2224.05 ± 384.01	3756.99 ± 814.62
GA209 x Mp313E (n=63)	3.30 ± 3.89	9.45 ± 8.21	71.64 ± 55.07	339.80 ± 229.34	726.37 ± 191.89	1737.59 ± 911.55	2577.69 ±1029.69
Mp717 x Mp313E (n=63)	8.48 ± 9.59	98.51 ± 76.69	319.65 ± 183.80	896.36 ± 504.82	2634.92 ± 1148.85	3427.53 ± 1349.88	3729.34 ± 1544.98

There was a significant difference between all the maize hybrids. 2224.05 ± 384.01 ppb for GA209 x T173, 3427.53 ± 1349.88 ppb for Mp313E x Mp717, and 1737.59 ± 911.55 ppb for GA209 x Mp313E were the aflatoxin concentrations for the collected maize 28 days after inoculation. There was no significant difference. The aflatoxin concentrations of samples collected 35 days after inoculation were 3756.99 ± 814.62 ppb for GA209 x T173, 3729.34 ± 1544.98 ppb for Mp313E x Mp717, and 2577.69 ± 1029.69 ppb for GA209 x Mp313E. Overall GA209 x Mp313E contained significantly less mean aflatoxin accumulation at 780.74± 1064.65 ppb when compared

to the two other maize lines; although Mp313E x Mp717 did contain more average aflatoxin concentration at 1587.8 ± 1747 ppb than GA209 x T173 at 1233.98 ± 1338.47 ppb (P<0.0001). Maize collected 35 days after inoculated was significantly higher in aflatoxin concentration then all other collection days (P<0.0001). The aflatoxin concentration was also significantly higher in 28, 21, and 14 after inoculation than maize collected 3, 7, and 10 days after inoculation (P=<0.0001). There are no significant difference between samples collected three, seven, and ten days after inoculation.

Table 4.8 Average *A. flavus* biomass of maize hybrids at the inoculation site comparing days after inoculation.

	3 DAI	**7 DAI**	**10 DAI**	**14 DAI**	**21 DAI**	**28 DAI**	**35 DAI**
GA209 x T173 (n=21)	0.00867 ± 0.00913	0.00777 ± 0.00345	0.01668 ± 0.011	0.03517 ± 0.0316	0.07066 ± 0.1	0.0138 ± 0.0119	0.14276 ± 0.1296
GA209 x Mp313E (n=21)	0.00921 ± 0.0108	0.01088 ± 0.0066	0.08251 ± 0.01162	0.0512 ± 0.061520	0.08591 ± 0.1255	0.01525 ± 0.01596	0.013135 ± 0.1328
Mp717 x Mp313E (n=21)	0.00813 ± 0.0076	0.004657 ± 0.0005	0.01561 ± 0.0088	0.01982 ± 0.0148	0.05541 ± 0.0751	0.01236 ± 0.0118	0.12694 ± 0.1389

The fungal biomass of the maize collected 35 days after inoculation was higher from the maize gathered at all the other dates after inoculation (DAI) with *A. flavus*. GA209 x Mp313E had the greatest amount of average fungal biomass at 0.05908 ± 0.0883 ng/µL, followed by GA209 x T173 at 0.0423 ± 0.071 ng/µL, and Mp717 x Mp313E with the least at 0.0347 ± 0.065 ng/µL (Table 4.8). There was no significant difference overall between the maize genotypes when it came to the mean fungal biomass

although GA209xMp313E had the most on average. The spread of *Aspergillus flavus* biomass at the inoculation site and first row was significantly greater than at the second and third row ($P<0.0341$). There was a significant difference in fungal spread between 28 DAI when compared to the 21 DAI, 14 DAI, 10 DAI, 7 DAI, and 3 DAI maize sample (Figure 4.5). There was no significant difference in fungal spread from the inoculation site between the maize hybrids.

Figure 4.5 GA209 x T173, susceptible hybrid 28 days after *A. flavus* inoculation.

Discussion

First Year

It was questioned if *A. flavus* started producing aflatoxin in traceable amounts as early as 2 days after inoculation. The level of detection (LOD) for aflatoxin B1 was 0.344 ppb. According to our data, we were not able to detect any aflatoxin B1 within the corn

samples that were tested two days after inoculation with *A. flavus*. However, there was consistent data to suggest that aflatoxin accumulation can begin as soon as 3 days after inoculation. The inoculated resistant maize line Mp717 x Mp313E had less fungal biomass overall then susceptible line GA209 x T173. The aflatoxin accumulation was higher in GA209 x T173 maize lines than the Mp717 x Mp313E inoculated maize. There was a significantly different (P=0.0001). GA209 x T173 started producing aflatoxin as early as three days after inoculation while Mp717 x Mp313E did not. On average, maize inoculated 21 days after silk maturation had more aflatoxin accumulation regardless of resistance.

Second Year

The susceptible/resistant maize hybrid, Mp313E x T173, had a significantly higher aflatoxin concentration three days after inoculation when compared to the other two maize hybrids in the experiment. The susceptible maize hybrid, GA209 x T173, contained higher amounts of aflatoxin accumulation for 7, 14, and 21 days after inoculation. Overall aflatoxin contamination was greater in the resistant maize hybrid, the susceptible maize hybrid possessing the second most amount of aflatoxin, and the susceptible/resistant maize cross, Mp313E x T173, containing the least. The overall fungal biomass of *A. flavus* in the inoculated maize did not significantly differ between the different genotypes or the days after inoculation. The spread of the *A.flavus* from the inoculation site was not significantly different comparing the maize lines. The maize samples collected 28 days after inoculation had more fungal biomass when compared to the rest of the samples. The susceptible maize hybrid, GA209 x T173, had the most aflatoxin concentration and *A. flavus* biomass while Mp313E x T173 had the least. The

susceptible/resistant maize cross Mp313E x T173 often had less *A. flavus* biomass and mean aflatoxin accumulation when compared to the rest of the maize hybrids. There was not significant difference between the genotypes in the spread of the fungus however, the fungus did travel father in the inoculated Mp313E x T173 maize.

Third Year

The susceptible maize hybrid, GA209 x Mp313E, had significantly less aflatoxin 21 days after inoculation when compared to the other two maize hybrids in the experiment. GA209 x Mp313E consistently showed less aflatoxin production for every collection date when compared to Mp717 x Mp313E and GA209 x T173. GA209 x T173 contained higher amounts of aflatoxin accumulation 3, 10, and 35 days after inoculation but not significantly so. Aflatoxin was higher in Mp717 x Mp313E for 7, 14, 21, and 28 days after inoculation. Overall aflatoxin contamination was greater in the resistant maize hybrid, the susceptible hybrid possessing the second most amount of aflatoxin, and the susceptible/resistant maize cross, Mp313E x T173, containing the least. The fungal biomass of *Aspergillus flavus* in the inoculated maize did not significantly differ between the different genotypes. The fungal biomass was higher 35 days after inoculation when compared to the other inoculation dates however; the fungal biomass did not differ significantly. The spread of the *A. flavus* from the inoculation site was not significantly different when comparing maize lines. The maize samples collected 28 and 35 days after inoculation had significantly more fungal biomass farther out when compared to the rest of the samples.

Conclusion

From the results of this study, aflatoxin accumulation does not seem to be directly correlated with the amount of fungal biomass of *A. flavus*. With the exception of the first year, the resistant maize hybrid lead overall in aflatoxin accumulation but had the least amount of fungal biomass in all three years of the experiment. The pinbar technique for inoculation was used as oppose to the side needle, which is commonly used for breeding new resistant lines of maize. Wounding techniques that simulate insect damage may bypass the resistance of the pericarp layers to natural infections resulting in higher aflatoxin concentrations (Scott and Zummo, 1992; Williams et al., 2013; Zummo and Scott, 1989). The aflatoxin resistant lines have been noted to express higher concentrations of stress related and/or antifungal proteins due to having a higher number of these genes (Warburton et al., 2015; Williams et al., 2015). Peroxidases, chitnases, trypsin inhibitors, and α-amylase are proteins known to either impede the progression of *A. flavus*, slow the production of aflatoxin by *A. flavus*, or cause the degradation of aflatoxin (Chen et al., 2005; Dowd and Johnson, 2016; Hawkins et al., 2015). The production of aflatoxin seems to begin decelerating after 21 days after inoculation. This could be due to *A. flavus* not getting as much nutrients from the kernels or natural plant defenses eventually overcoming the infection.

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CHAPTER V

STORAGE CONDITIONS EFFECT ON *ASPERGILLUS FLAVUS* BIOMASS AND AFLATOXIN CONCENTRATION IN INOCULATED MAIZE

Introduction

Maize (Zea mays) is the second most produced crop in the world. By 2050, maize is estimated to become the number one crop globally (Alston and Pardey, 2014). Although maize is used for human consumption and the production of biofuels, the majority of maize is used as animal feed. Crop storage is an essential part of global agriculture and food security. There are several established storage systems for maize including bags, bulk, cribs, and metal silos (Tefera et al., 2011; Tubbs et al., 2016; Williams et al., 2017). According to Food and Agriculture Organization, between 5-25% annually of all food grains are lost during storage (FAOSTAT 2014). To avoid postharvest losses from storage pests and pathogens, local farmers are forced to sell what they produce almost immediately after harvest.

Aspergillus flavus is a fungus that causes ear rot in maize. The majority of *A. flavus* infections occur post-harvest during storage. It also under certain conditions produces a hazardous byproduct known as aflatoxin. Aflatoxin B_1 was classified as a Group I carcinogen by the International Agency for Research on Cancer (IARC) (Tomatis, 1988; Vainio and Wilbourn, 1992). Group I carcinogens are substances that have been proven to cause cancer or tumors in humans. The FDA imposed a limit of 20

ppb for aflatoxin in food for human consumption. The potential annual economic cost of aflatoxins in the United States is between $104.5 million and $1.68 billion dollars (Mitchell et al., 2016; Robens and Cardwell, 2003).

Temperature, humidity, moisture content, and insects are the main factors in postharvest *A. flavus* infection. *A. flavus* prefers to grow at 37°C but has been known to develop at temperatures as low as 12°C and as high as 48°C (Paraginski et al., 2014; Schindler et al., 1967; Xu et al., 2007). It also favors a moisture content of at least 15%, and between 70-90% humidity (Niaz et al., 2011; Trenk and Hartman, 1970). The purpose of this experiment is to assess if certain storage conditions can reduce the growth of *Aspergillus flavus* and aflatoxin accumulation of harvested maize.

Methods and Materials

The three different maize hybrids that were used in the experiment were GA209 x T173 (susceptible cross), Mp313E x T173 (resistant and susceptible cross), and Mp313E x Mp717 (resistant cross). GA209 x T173 (susceptible cross), GA209 x Mp313E (resistant and susceptible cross), and Mp313E x Mp717 (resistant cross) were the three maize hybrids used the following year (Table 5.1).

Table 5.1 Maize hybrid used in both years of maize storage experiment.

Aflatoxin Susceptibility of Maize	First Year	Second Year
Susceptible Maize Hybrid	GA209 x T173	GA209 x T173
Resistant and Susceptible Maize Hybrid	Mp313E x T173	GA209 x Mp313E
Resistant Maize Hybrid	Mp313E x Mp717	Mp313E x Mp717

The maize hybrids were self-pollinated to guarantee that the ears have as many kernels as possible. The field was irrigated. Approximately 21 days after silk emergence, the top ear from each plant was inoculated with *Aspergillus flavus* isolate NRRL 3357 or water as a control in a 3 by 3-kernel grid. There will be three inoculated maize plants for each row. Approximately two months after inoculation with *Aspergillus flavus* during harvest, GA209 x T173, Mp313 x T173, and Mp717 x Mp313E maize hybrids were collected and split into four groups. The first group was immediately analyzed and used as a control for the other three groups. The samples in the second group was baked in a large wooden oven for seven days at 40°C and then processed. This drying down process is common amongst farmers. The samples in the third group would also be baked for seven days and then stored in a dry container for eight weeks before processing. The samples in the final group were stored in a dry container for eight weeks prior to processing. There were six samples of each of the three maize hybrids in each group. Maize hybrids inoculated with water acted as a control. The experiment was completed using a randomized complete block design with n=72. The block was the maize genotype. The treatment was the storage option. The experimental unit was the row of maize. Significance was tested at $\alpha=0.05$. Statistical analysis was done using SAS® (SAS Institute In.; Cary, NC) 9.4 software.

Maize kernels were flash frozen in liquid nitrogen. The corn kernels were then ground with a mortar and pestle into a fine powder. 200 milligrams of each ground kernel was placed into a 1.5 mL micro-centrifuge tubes. Then 1 mL of a 70% methanol/water solution was added to the tube. The samples were shaken for two minute and then centrifuged for five minutes at 3000. 0.45 μm PTFE syringe filters were used to purify

the samples after centrifugation. An Agilent 6460 LC/MS Triple Quadruple, which uses electrospray ionization (ESI), was used to analyze the aflatoxin concentration of the samples. The autosampler temperature was set to 4°C. The HPLC used a Zorbax Eclipse Plus-C18 Narrow Bore 2.1 x 50mm, 5µm column with a temperature of 50°C. The mobile phase consisted of 5mM ammonium acetate with 0.1% formic acid in HPLC-grade water and 5mM ammonium acetate with 0.1% formic acid in methanol. The flow rate of the mobile phase during the analysis was consistently 0.6 mL/min. The mobile phase gradient was transitioned from 95% water to 100% methanol during the six minutes of the analysis time. Then for the final three minutes of the run, the mobile phase reversed from 100% methanol back to 95% water. The total run time of the method was nine minutes, which includes a six-minute analysis time and an additional three minutes for the system to get back to equilibrium. The calibration curve was matrix-matched in order to reduce matrix effects. The twelve-point calibration curve consisted of the following concentrations: 1 ppb, 5 ppb, 10 ppb, 20 ppb, 50 ppb, 100 ppb, 200 ppb, 400 ppb, 600 ppb, 800 ppb, and 1000 ppb. The Agilent 6460 Triple Quadrupole Mass Spectrometer (MS/MS) system coupled to an electrospray analyzed the samples while in positive mode. The drying gas temperature was 325°C while the gas flow was set to 10 liters per minute. The nebulizer gas pressure was set to 50 psi and the capillary voltage was 4000V. The Sheath Gas Flow had an output of 11 liters per minute and the sheath gas temperature reached temperatures of 350°C. The delta electron multiplier voltage (EMV) was 800V and the dwell time lasted for 200msec. The precursor ion for aflatoxin B_1 was 313.1 and 329.1 for aflatoxin M_1. While in multiple reaction monitoring (MRM) mode, the mass spectrometer was set to look for certain daughter ions after the precursor

ion entered the collision cell. The transitions for aflatoxin B_1 included 313.1 > 285.1 with a collision energy of 20kEV, 313.1 > 269.1 with a collision energy of 25kEV, and 313.1 > 241.1 with a collision energy of 35kEV. The fragmentor value was 166 for aflatoxin B_1 and 131 for aflatoxin M_1. The cell accelerator (7) values were the same for both aflatoxin transitions. The two transitions used for identifying aflatoxin M_1 are 329.1>273.1 and 329.1>229.1. The retention time of aflatoxin M_1 was 3.0 minutes and 3.4 minutes for aflatoxin B_1. Agilent MassHunter Quantitative Analysis Workstation Software v. B.04.0.225.19 was used to analyze the quantitative data obtained from the samples and the calibration curve. This method can also be used to check for the presence of other types of aflatoxins being produced by *A. flavus*.

The maize DNA were extracted from 100 milligrams of the ground corn kernel using the CTAB method (Cetyl Trimethyl Ammonium bromide) for plant tissue. 250 µL of the buffer was added twice to the 100 milligrams ground corn kernel samples in tubes for a final volume of 500 µL. The samples were then placed into a 60°C water bath for 15 minutes. 200 µL of the chloroform/octanol mixture was added to each sample twice. The samples were then inverted 50 times in order to thoroughly mix the solution. Once that was completed, the samples were centrifuged at 3000 rpm for 15 minutes. The supernatant was then transferred into clean micro centrifuge tubes. 300 µL of isopropanol was added to the tubes. The samples were inverted 15 times to properly mix the solution. Then the samples were set in the freezer overnight. The samples were centrifuged at 3000 rpm for 15 minutes at 4°C. Once the samples were decanted, 300 µL of 90% ethanol was pipetted into the tubes. Then the samples were centrifuged again at 3000 rpm for 5 minutes at 4°C. Finally, the samples were decanted and the pellets were allowed to air

dry. The pellets were suspended in 100 µL of TE buffer. The CTAB method is ideal for DNA extraction because of its ability to remove the high number of polysaccharides and phenolic compounds that are found in plant tissue, which could interact irreversibly with nucleic acid. A Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE) was used to determine the quality and quantity of DNA. The fungal biomass of the inoculated maize samples was determined by using quantitative real time polymerase chain reaction (qPCR).

The forward primer and reverse primer for the fungal quantification Af2 (forward primer: 5 -ATCATTACCGAGTGTAGGGTTCCT-3; reverse primer: * 5-GCCGAAGCAACTAAGGTACAGTAAA-3; amplicon 73 bp) designed in the internal transcribed spacer 1 (ITS1) sequence. The forward and reverse primers for the maize quantification Zmt3 (forward primer: 5 -TCCTGCTCGACAATGAGGC-3; reverse primer: 5 -TTGGGCGCTCAATGTCAA-3; amplicon 63 bp) amplifying maize α-tubulin. 5 µL of Power SYBR green PCR Master Mix, 0.5 µL of the forward primer, 0.5 µL of the reverse primer, 2.0 µL of DNA-free water, and 2.0 µL of sample DNA at a concentration of 10 ng/µL were combined to make up the 10 µL reaction volume. The Roche LightCycler 480 instrument (Roche Diagnostics Corporation, Indianapolis, IN) was used to determine the fungal biomass. The temperature profile for denaturation, melting curve, gradual heating, and cooling step conditions for the qPCR were as follows: denature at 95°C for 10 min, 45 cycles at 95°C for 10s, 60°C for 5s, 72°C for 10s, 95°C for 10s, anneal at 65°C for 1 min, 97°C for 5s, and cool at 40°C for 10s. Two standard curves for both maize and fungal DNA at concentrations of 10, 1.0, 0.1, 0.01, 0.001, and 0.0001 ng/µL were both ran with the DNA samples.

Results and Discussion

The collect and process method had a mean fungal biomass of 0.00958 ± 0.058 ng/µL and an average aflatoxin concentration of 5403.145 ± 2619.835 ppb. The mean aflatoxin accumulation and fungal biomass of the bake and process method were 3119.135 ± 1935.912 ppb and 0.07281 ± 0.176 ng/µL, respectively. The bake and store method had a mean aflatoxin concentration of 3975.518 ± 1389.85 ppb and an average fungal biomass of 0.00889 ± 0.016 ng/µL. The average fungal biomass and aflatoxin accumulation of the store and process method were 0.00825 ± 0.010 ng/µL and 3326.083 ± 1781.933 ppb respectively (Figure 5.1).

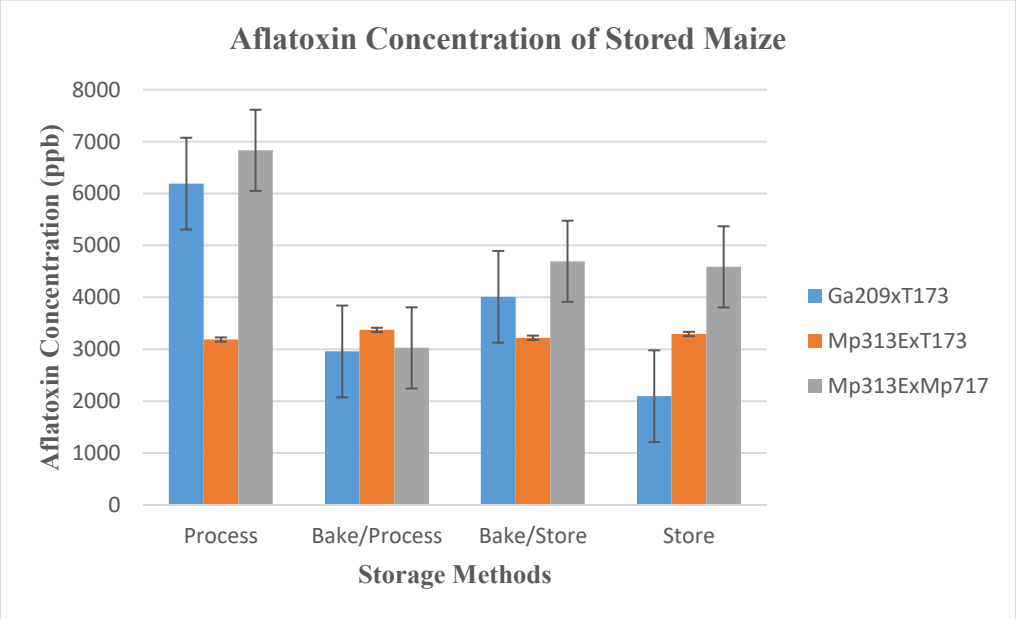

Figure 5.1 Average aflatoxin concentration of inoculated maize stored using different methods (n=72).

There was a significant difference in the aflatoxin levels between the collect and process method and the other storage methods (P=0.0033). The fungal biomass was higher in the maize that underwent the bake and process method although it was not significant (P=0.111). The susceptible x resistant maize hybrids had the highest amount of *A. flavus* biomass while the resistant maize hybrids had greatest amount of aflatoxin. The eight-week storage method had the least amount of fungal biomass (Table 5.2). The bake and process method had the least amount of aflatoxin accumulation when compared to the other methods.

Table 5.2 Mean *A. flavus* biomass concentration (ng/μL) of inoculated maize stored using different methods with p.

Method	**GA209xT173**	**Mp313ExT173 GA209xT173**	**Mp313ExMp717**
Process (n=18)	0.0215 ± 0.0195	0.0019 ± 0.0021	0.0054 ± 0.0062
Bake/Process (n=18)	0.0014 ± 0.0011	0.2160 ± 0.2626	0.0011 ± 0.0006
Bake/Store (n=18)	0.0047 ± 0.0044	0.0006 ± 0.0002	0.0214 ± 0.0243
Store (n=18)	0.0140 ± 0.0126	0.0084 ± 0.0095	0.0023 ± 0.0009

The maize inoculated with water was not often infected with *Aspergillus flavus* and the aflatoxin concentration in those that were did not exceed one parts per billion. The storage method had significantly higher fungal biomass and aflatoxin concentration (P=0.00258). The resistant hybrid, Mp717 x Mp313E, had significantly lower aflatoxin accumulation when compared to the other hybrids (P=0.0004).

Conclusion

The resistant maize hybrid, Mp717 x Mp313E, in the experiments contained the highest levels of aflatoxin compared to the other maize hybrids but the lowest fungal biomass. The average aflatoxin concentration was lowest in the susceptible/resistant maize hybrids. Although the fungal biomass was higher in the bake and process method; it was not significantly worst when compared to the other methods. In terms of aflatoxin concentration, the collect and process method was significantly worst for aflatoxin concentration. This experiment suggests that the storage method of maize has little effect on the amount of fungal biomass and aflatoxin in maize kernels that were previously infected with *Aspergillus flavus*.

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CHAPTER VI

IDENTIFYING AUXIN HERBICIDIES FORMULATIONS USING FT-IR

Introduction

Weeds are undesirable plants that grow competitively with other plants around them. There are around 8,000 distinct species of weeds. They are usually characterized by having ample seed production, swift population establishment, and vegetative reproductive structures (Heap, 2014; Pimentel et al., 1997). These properties allow weeds to produce potentially thousands of seeds per plant while most crops produce a few hundred seeds per plant. Weeds are detrimental to crops because they compete for the same resources (water, sunlight, nutrients etc.). The speed of their population establishment makes it easier for weeds to outcompete crops (Mullin, 2009). This results in potential crop yield loss. Harmful organisms such as bacteria, virus, fungi, insects, and weeds cause 34% of the total potential crop loss in global agriculture (Pimentel, 2005). The lower the available soil moisture, the higher the potential yield loss due to weeds. Before the use of herbicides, weed management was labor-intensive involving removal by hand weeding or tillage. Herbicide use in earnest began in the United States after World War II. Herbicides were often used either prior to seeding or before plant emergence to avoid damaging the crop. Over 95% of maize (Zea mays), soybeans (Glycine max), and cotton (Gossypium hirsutum) crops in the United States have herbicides applied to them. More than a third (35%) of the United States Agricultural

GDP is composed of maize, soybean, and cotton crops (FAOSTAT 2014). There are several types of herbicides that can be sorted into two main categories: selective and nonselective. Selective herbicides use mechanisms that exploit physiological variance that only effect specific plant species. Acetolactate synthase (ALS) inhibitors and synthetic auxins are widely used example of selective herbicide (Green, 2014). Nonselective herbicides effect most plant species focusing on biological pathways that are widely conserved. 5-enylpyruvylshikimate-3-phosphate synthase (EPSPS) inhibitors such as glyphosate are a popular example of a nonselective herbicide.

Glyphosate

Monsanto first released Glyphosate (Roundup) in 1974. Glyphosate is currently the most popular herbicide used in the United States (Figure 6.1). It is unique because it was the first modern non-selective herbicide that inactivated in the soil. When applied to plants, EPSPS is inhibited by glyphosate (Steinrucken and Amrhein, 1980). It catalyzes the transfer of the enolpyruvyl moiety of phosphoenol pyruvate (PEP) to shikimate-3-phosphate (S3P). This is a key in the synthesis of aromatic amino acids for hormones and plant metabolites. The active site of the EPSPS enzyme in higher plants is highly conserved (Gao et al., 2014). Glyphosate is competitive with respect to PEP binding to EPSPS but uncompetitive with respect to S3P and the resulting S3P complex is very stable. Phenylalanine, tyrosine, and tryptophan are the aromatic amino acids that are synthesized from this pathway (Duke and Powles, 2008).

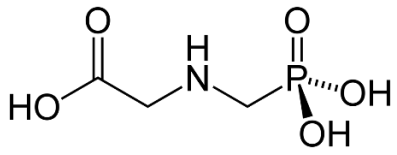

Figure 6.1 Molecular structure of glyphosate.

Developed in the 1990s, glyphosate resistant crops express CP4, which is a type of EPSPS protein that is insensitive to glyphosate. Soybeans were the first glyphosate-resistant crop to be released in 1996 (Barrows et al., 2014; Brookes and Barfoot, 2013; Green, 2014; Green and Owen, 2011). Two years later, both corn and cotton had glyphosate tolerant lines available. The United States is the leading producer of both maize and soybeans in the world. It is third in global production of cotton behind China and India. Since the release of glyphosate resistant crops, only a modest increase as been seen in the yield of these crops (Pimentel, 2005). However, there is an economic benefit. For selective herbicides to be as effective, a combination of them used at a higher volume is required. With the release of glyphosate resistant crops, farmers went from purchasing up to 11 unique herbicides to apply to their fields to just glyphosate. Glyphosate resistant crops have save U.S. farmers a calculated $1.2 billion dollars annually due to the reduction in herbicide purchases and application (Carpenter and Gianessi, 1999; Gianessi, 2005). This has led to 93% of all soybean, 85% of all corn, and 80% of all cotton crops grown in the United States now being glyphosate resistant. The increased application of glyphosate as the primary herbicide over several years has led to a rise in glyphosate resistant weeds (Foresman and Glasgow, 2008). Rigid ryegrass, horseweed, ragweed,

Palmer Amaranth, Italian ryegrass, water hemp, and goosegrass have all become tolerant to glyphosate (Christoffoleti et al., 2015; Sammons and Gaines, 2014).

Glyphosate resistance in weeds is achieved in a variety of diverse ways. Glyphosate can be transported in the phloem where it is sequestered in the vacuole from the cytosol. This reduces the amount of glyphosate available to enter the chloroplast and inhibit EPSPS. Glyphosate can be released into the cell at a nontoxic rate or potentially stay in the vacuole indefinitely (Ge et al., 2010; González-Torralva et al., 2012). A mutation of the EPSPS Prot106 codon or an increase in the amount of EPSPS produced are other ways that weeds can become glyphosate resistant (Délye et al., 2013). Worldwide, 24 species of weeds have developed glyphosate resistance (Edwards et al., 2014).

Synthetic Auxins

The use of synthetic auxins as additional herbicides has increased to combat the rise of glyphosate tolerate weeds. Broadleaf (dicot) plants are damaged and killed by synthetic auxins such as dicamba and 2,4-dichlorophenoxyactic acid (2,4-D). 2,4-D and dicamba have been used for weed control since their discovery during World War II (Gianessi, 2013). Dicamba and 2,4-D act as the natural hormone indole-3-acetic acid (IAA) regulator of several plant regulatory functions (Figure 6.2).

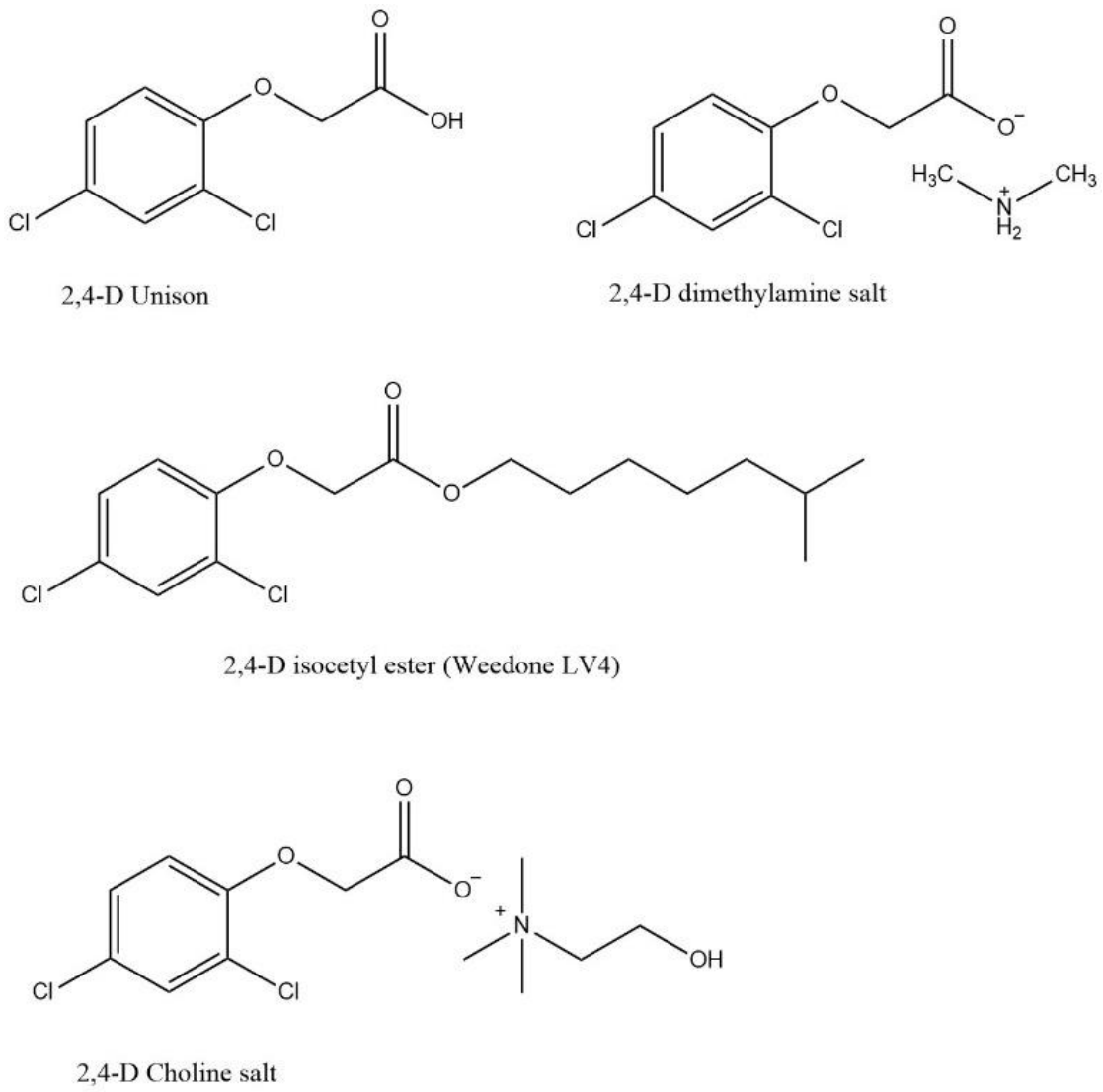

Figure 6.2 Different formulations of the herbicide 2,4-D.

Auxins attaching to the Auxin Binding Protein 1 induces proton pump hyperactivity due to the decreases in pH because of the accumulation of protons outside the extracellular membrane (Christoffoleti et al., 2015; Mano and Nemoto, 2012). The hydrogen ion concentration outside the cell causes the opening of the potassium channel to move potassium inside the cell. As a result, water influxes into the cell through

aquaporins. The acidic condition outside the cell breaks noncovalent bonds between cellulose and hemicellulose, which loosens the cell wall and allows more water into the cell. Calcium increases inside the cell and activates phosphatidylinositol-3-phosphate, which phosphorylates NADPH oxidase and produces reactive oxygen species (Grossmann, 2000, 2009). The Auxin Binding Protein 1 at the plasma membrane also activates RAC/ROP GTPase. G proteins have a key role in signal transduction in eukaryotic cells as well as a vital role in cytoskeleton organization modeling the structure and arrangement of actin filaments and microtubules. Peroxisomes travel on actin so it affects the mobility of the peroxisomes to remove reactive oxygen species. The abscisic acid (ABA) and ethylene induces the death of plant tissues (Kelley and Riechers, 2007; Woodward, 2005). The loss of cell wall structure allows reactive oxygen species to penetrate the plasma membrane where they can interact with phospholipids, promoting unsaturation of plasma membrane lipids, reactive oxygen species, and leakage of the cytosol is what leads to cell death. Soybean and cotton plants have 2,4-D and dicamba resistant varieties commercially available like Xtend soybeans from Monsanto and Enlist cotton from Dow AgroSciences.

The main issue with the surging popularity of synthetic auxins is their tendency to drift after application due to their volatility. The movement of spray droplets that land off-target causes spray drift. The smaller the droplet and the longer it remains in the air, the higher the chance for drift. Vapor drift occurs when applied herbicide evaporates from the target plant and aerosolizes to an unintended location. Crops affected by drift at the late vegetation or early reproduction stage show the greatest reduction of yield. Companies such as Monsanto, Dow Agro, and BASF have recently released low volatile

versions of dicamba (Engenia) and 2,4-D (Enlist Duo) in hopes to reduce the amount of drift that occurs when used with commercially resistant variety. The dimethylamine (DMA) and diglycolamine (DGA) formulations of dicamba are more volatile than the newly released BAPMA dicamba formulation. Reuters reports that over one million acres of, the dicamba resistant variety, Xtend soybeans were planted in the United States in 2016. Monsanto predicts 15 million acres of Xtend soybeans to be planted in 2017 in the US and up to 55 million acres of Xtend soybeans will be planted in 2019 (Plume, 2016).

A common analytical method used to detect both 2,4-D and dicamba in affected crops is liquid chromatography coupled to mass spectroscopy. The issue with this analysis is that the extraction cleaves the salt groups from the auxin herbicides. This is due to the rise of pH after the addition of sodium hydroxide that is required in the sample preparation for the extraction. So, although it is possible to distinguish between 2,4-D and dicamba using LC/MS, it is difficult to differentiate the DMA and DGA formulations of these herbicides using this method. Fourier Transform Infrared spectroscopy (FT-IR) is a fast, accurate, and usually non-destructive and requires little to no sample preparation (Figure 6.3). This would potentially make it ideal for analyzing soybean and cotton samples affected by herbicide drift from the different formulations of synthetic auxins.

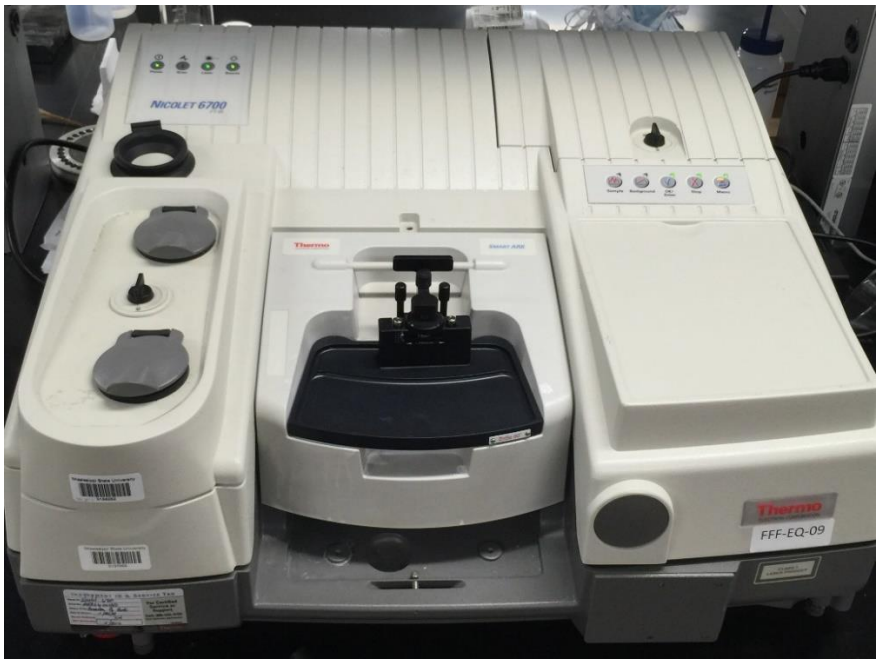

Figure 6.3 Thermo Nicolet 6700 FT-IR Spectrometer.

Materials and Methods

To simulate drift, soybean plants in the R3 stage were sprayed at a rate of (1/64X) with Banvel (dimethylamine salt of dicamba), Clarity (diglycolamine salt of dicamba), MON76980 (diglycolamine salt of dicamba), Engenia (BAMPA salt of dicamba), and Roundup with untreated soybeans used as a control. Cotton plants in the reproductive stage were sprayed at a rate of (1/128X) to with Unison (2,4-D), Weedar64 (2,4-D amine salt), Weedone LV4 (2,4-D ester salt), 2,4-D Choline, and Roundup with untreated cotton plants used as a control. The rates were normalized to equal amounts of acid equivalence. The plants were collected immediately, 3, 7, 14, and 28 days after application and placed in a -80°C. The samples were ground with a mortar and pestle using liquid nitrogen and analyzed using a Thermo Nicolet 6700 FT-IR spectrometer with a liquid nitrogen-cooled

MCT High-D detector, a KBr beam splitter, and the Smart ARK accessory. Approximately 1 gram of sample was placed onto a ZnSe horizontal attenuated total reflectance crystal for each analysis. The ZnSe horizontal attenuated total reflectance crystal had an angle of incidence of 60° that allows for 10 reflections of infrared light to pass through the crystal for each scan with 64 scans per spectra. The spectra were collected from 4000 to 650 cm^{-1}. Spectra were baseline corrected, normalized to the area under the curve, and converted to the first derivative using the Savitzky-Golay algorithm using OMNIC 7.3 and the Unscrambler X 10.3 software. Derivation of the spectra was done in order to remove linear baseline effect as well as potentially reveal hidden spectral feature in overlapping peaks. For statistical analysis, the spectra region of 1800-800 cm^{-1} was used because the only other significant peak was a broad OH from the water in the plant (Figure 6.4).

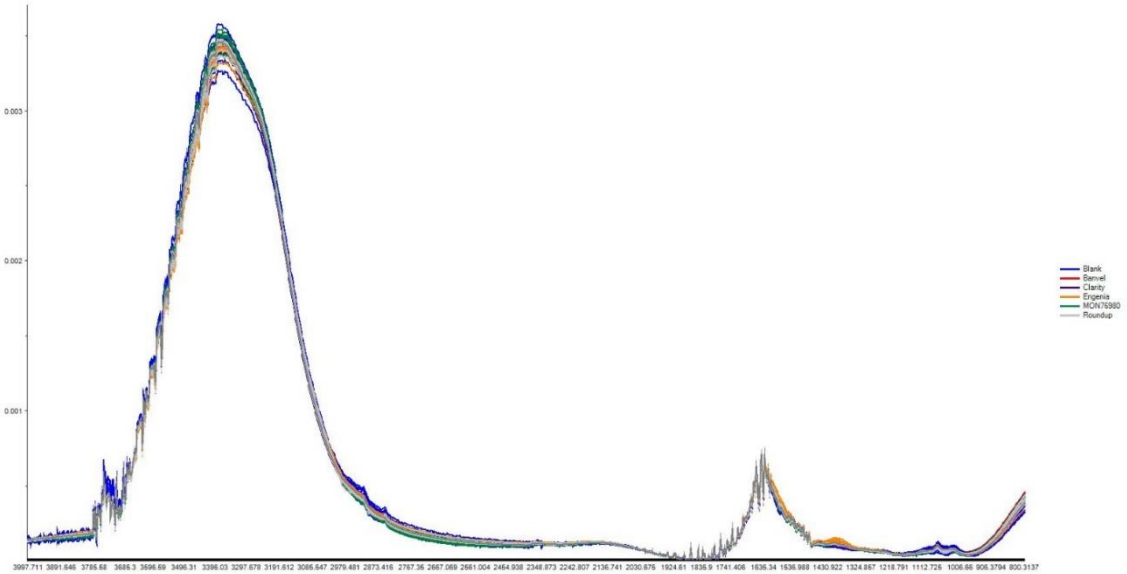

Figure 6.4 Infrared spectra of soybean plants 28 days after herbicide application.

Results and Discussion

This first step to creating a model was first analyzing the spectra using Principal Component Analysis. Principal Component Analysis (PCA) involves a mathematical modeling procedure that clarifies the relationships between samples and their variables. It take the original information and condenses it into a number of variables called principal components, which explains various amounts of the original dataset. The first principal component accounts for the greatest amount of variance while the second principal component is the second most variance. Explained variance is a measure of the amount of variation considered by the model. For the soybean samples collected 28 days after the application of herbicides, there was 96% total explained variance with initial seven principal components. The first three principal components accounted for 86% of the explained variance. Distinct clustering of the five sample types can be observed using a 3D PCA graph of the initial three principal components (Figure 6.5). The explained variance from the principal component analysis for the soybean samples collected 14 day, 7 day, 3 day, and immediately after herbicide application were 92%, 92%, 91%, and 96% respectively. The loading plot illustrates the importance of the wavelengths 1687-1560 cm^{-1} for distinguishing between the herbicide applied samples. Peaks between 1687 and 1560 cm^{-1} are more than likely from the aromatic ring of the dicamba as well as the primary or secondary amine from the salts attached to the dicamba formulations.

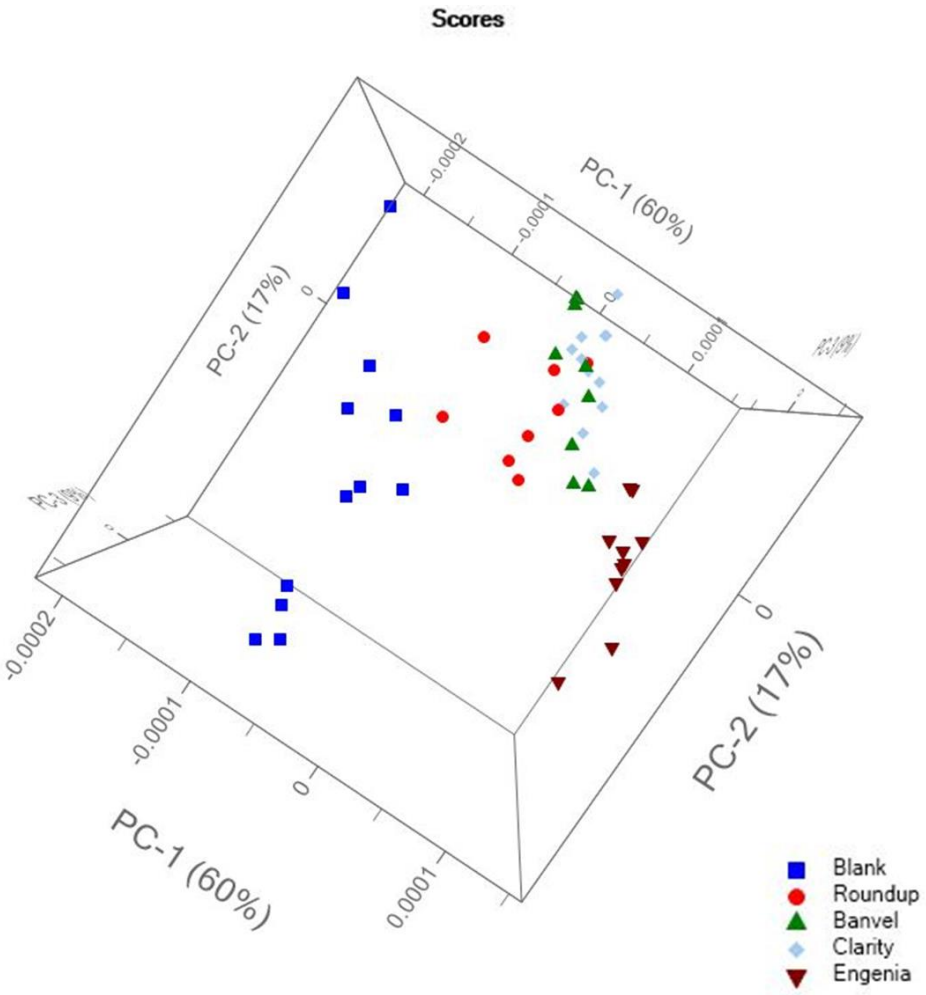

Figure 6.5 Principal component analysis of soybean 28 days after herbicide application.

The cotton samples retrieved 28 days after herbicide application had 92% of the total explained variance. 91% of the total variance was explained with only the first three principal components. A 3D PCA was constructed using the original three principal components. Separation between the six clusters are easily distinguishable. The cotton plants analyzed 14 day, 7 day, 3 day, and immediately after herbicide application had an explained variance from the principal component analysis of 83%, 91%, 91%, and 95%

respectively. The loading plot shows that peaks 1633-1556 cm^{-1} and 1395-1350 cm^{-1} are important wavelengths when detecting variation between the samples. The peaks between 1633 and 1556 cm^{-1} are possibly from the aromatic ring of the auxin herbicides and the primary or secondary amines from the salts attached to 2,4-D herbicides. The peaks from 1395-1350 cm^{-1} are indicative of a carboxylic acid group, which is present in the majority of the 2,4-D formulations.

Linear Discriminant Analysis (LDA) is a supervised, classification method to identify unknown samples. LDA uses the parameters of the samples from the PCA to consider the within group and between group variance. A classification model was created for each of the soybean and cotton plant collection dates. The model accuracy for the soybean samples analyzed initially, 3 days, 7 days, and 14 days after herbicide application was 89%, 92%, 84%, and 91% respectively. Soybean plants collected 28 days after application had an accuracy of 92.6%. It successfully classified all the groups using except for mistakenly classifying Clarity as MON76980. This is most likely because both Clarity and MON76980 are both the formulation of dicamba with the diglycolamine salt attached. When the model was adjusted by categorizing MON76980 and Clarity the same, the accuracy of the model increased to 98.04% (Figure 6.6). All unknowns samples ran in the model were then correctly identified.

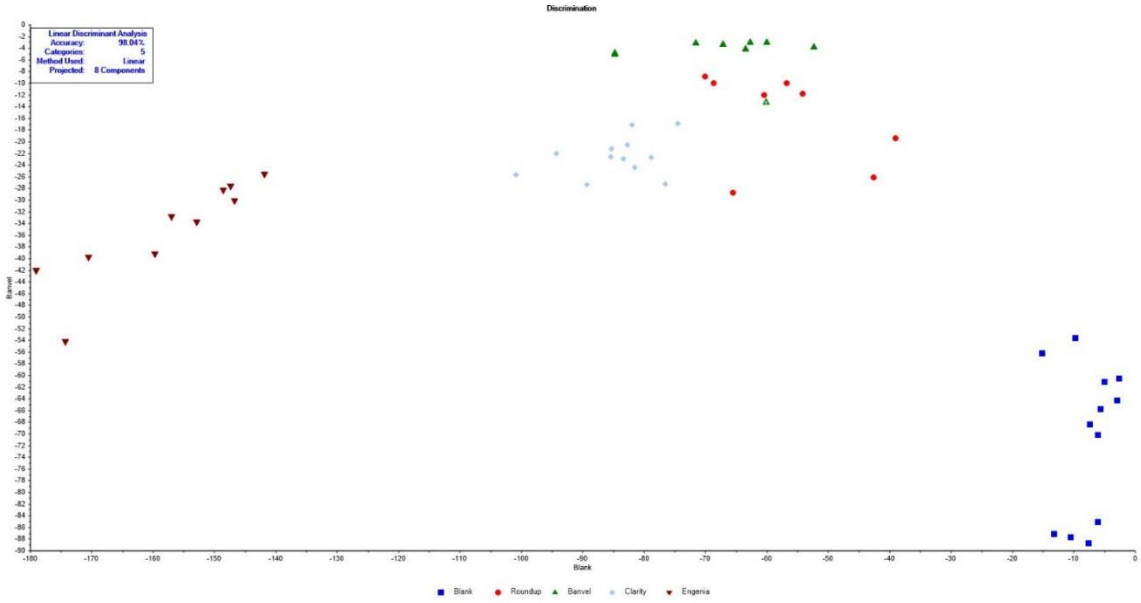

Figure 6.6 Linear discriminant analysis of soybean 28 days after herbicide application.

The cotton plants analyzed 14 days, 7 days, 3 days, and immediately after herbicide application had a model accuracy from the linear discriminant analysis of 84%, 90%, 87%, and 90% respectively. The accuracy of the classification model for the cotton plants collected 28 days after herbicide application was 88.1%. Unknowns put through the model for validation were all properly recognized except for Unison being mistaken for Roundup.

Conclusion

Using these statistical techniques, the various herbicides were successfully differentiated up to 28 days after application. Promising models have been developed that can determine which type of 2,4-D and dicamba were applied to soybean or cotton plants up to 28 days after application. In the future, more samples will analyzed in order to increase the precision of the model, and more unknowns will need to be run to increase the robustness of the model. These models will hopefully allow us to determine unknown herbicide applications due to drift in crops to help solve and ultimately diminish drift cases.

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(2014) < http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor>

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APPENDIX A

CHAPTER IV SUPPLEMENTERY MATERIAL

```
OPTIONS PS=55 LS=85 NODATE;
DATA AFLATOXIN;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 329\aflatoxin.dat';
INPUT DAP MAIZE DAI AFLATOXIN;
RUN;
PROC MEANS SUM MEAN MAXDEC=2 FW=10;
VAR AFLATOXIN;
CLASS DAP MAIZE DAI;
TYPES DAP*MAIZE DAP MAIZE*DAI MAIZE DAI;
RUN;
PROC GLM;
CLASS DAP MAIZE DAI;
MEANS DAI / LSD LINES;
MEANS MAIZE / LSD LINES E = MAIZE*DAP;
PROC PRINT;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : AFLATOXIN

DAI	N Obs	Sum	Mean	Std Dev
1	**12**	0.00	0.00	0.00
2	**12**	186.06	15.50	35.09
3	**12**	1382.23	115.19	128.03
4	**12**	3034.06	252.84	182.98
5	**12**	2760.36	230.03	195.91
6	**12**	1855.72	154.64	153.84

Analysis Variable : AFLATOXIN

MAIZE	N Obs	Sum	Mean	Std Dev
1	**36**	5703.90	158.44	177.83
2	**36**	3514.53	97.63	145.63

Analysis Variable : AFLATOXIN

PLOT	N Obs	Sum	Mean	Std Dev
1	**36**	2838.40	78.84	135.61
2	**36**	6380.02	177.22	177.10

The GLM Procedure

Class Level Information

Class	Levels	Values
PLOT	2	1 2
MAIZE	2	1 2
DAI	6	1 2 3 4 5 6

Number of Observations Read 72

Number of Observations Used 72

The SAS System

The GLM Procedure

Dependent Variable: AFLATOXIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	12	963743.748	80311.979	4.98	<.0001
Error	59	951843.254	16132.937		
Corrected Total	71	1915587.002			

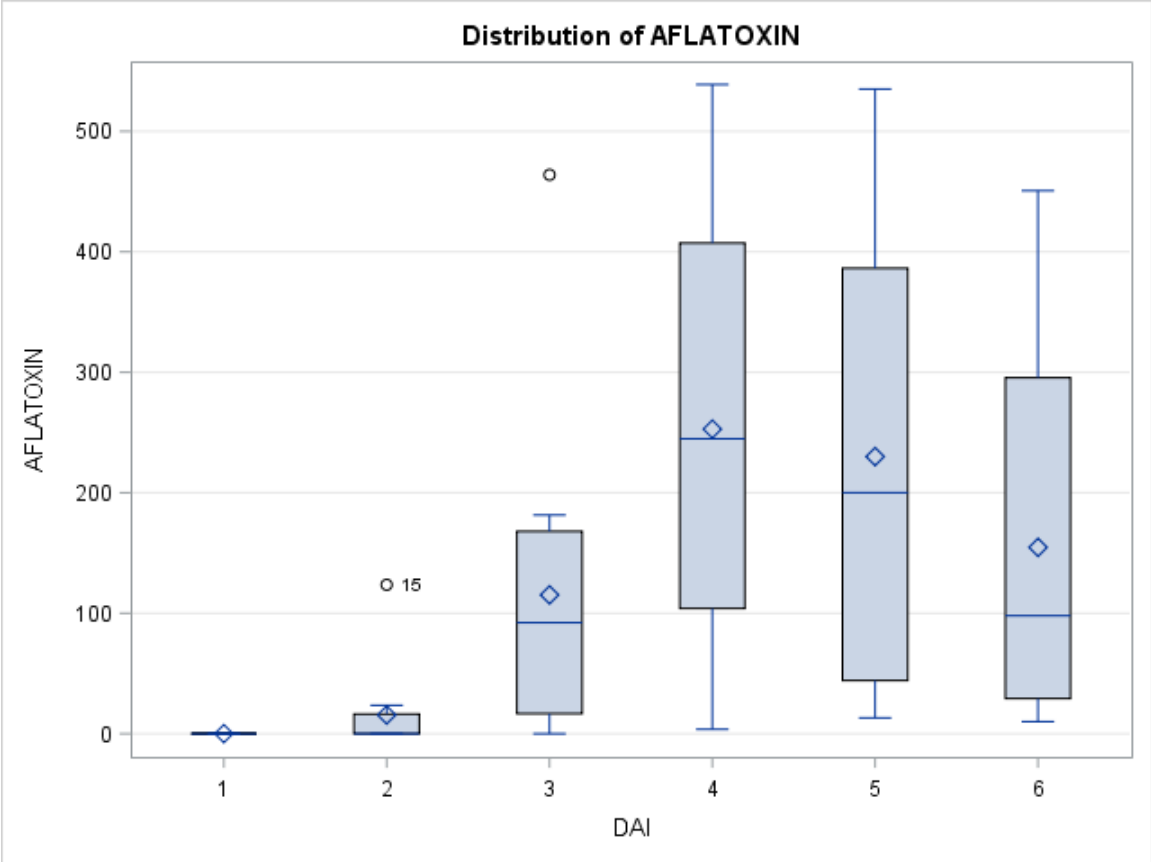
R-Square	Coeff Var	Root MSE	AFLATOXIN Mean
0.503106	99.20476	127.0155	128.0337

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PLOT	1	174209.2755	174209.2755	10.80	0.0017
MAIZE	1	66573.9024	66573.9024	4.13	0.0467
DAI	5	670895.1131	134179.0226	8.32	<.0001
MAIZE*DAI	5	52065.4570	10413.0914	0.65	0.6660

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLOT	1	174209.2755	174209.2755	10.80	0.0017
MAIZE	1	66573.9024	66573.9024	4.13	0.0467
DAI	5	670895.1131	134179.0226	8.32	<.0001
MAIZE*DAI	5	52065.4570	10413.0914	0.65	0.6660

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	59
Error Mean Square	16132.94
Critical Value of t	2.00100
Least Significant Difference	103.76

Means with the same letter

are not significantly different.

t Grouping	Mean	N	DAI
A	252.84	12	4
A			
A	230.03	12	5

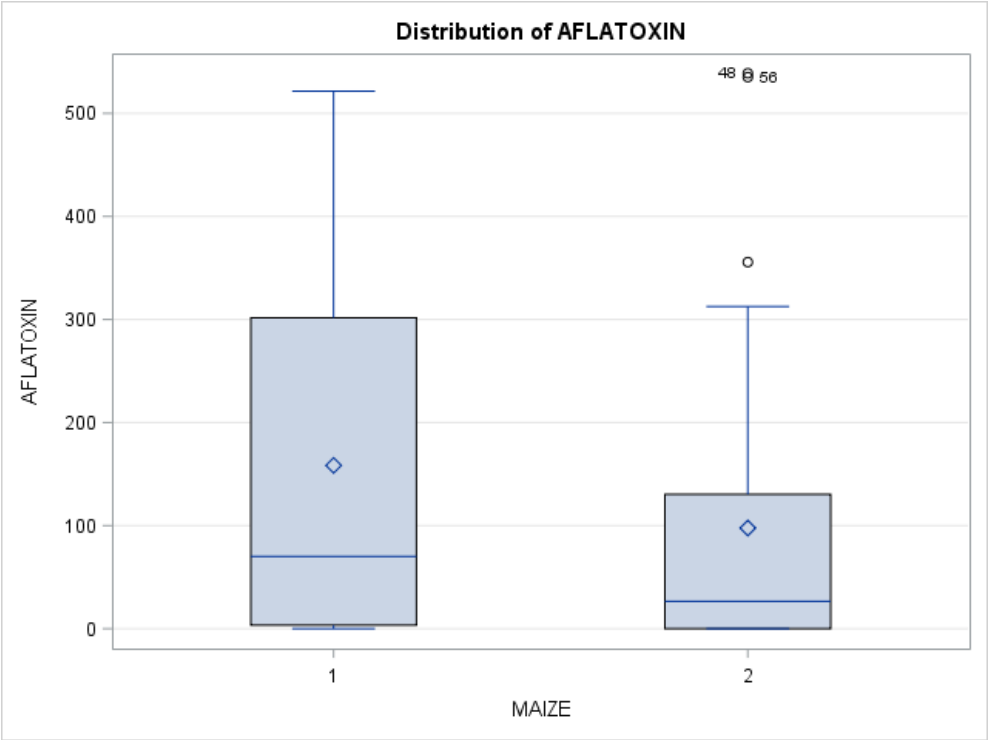
Means with the same letter

are not significantly different.

t Grouping		Mean	N	DAI
	A			
B	A	154.64	12	6
B				
B	C	115.19	12	3
	C			
D	C	15.50	12	2
D				
D		0.00	12	1

The SAS System

The GLM Procedure



The SAS System

The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	59
Error Mean Square	16132.94
Critical Value of t	2.00100
Least Significant Difference	59.905

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	158.44	36	1
B	97.63	36	2

Figure A.1 Year one aflatoxin data.

```
OPTIONS PS=55 LS=85 NODATE;
DATA BIOMASSFINAL;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 329\fungalbiomass.dat';
INPUT DAP MAIZE DAI REP $ BIOMASS;
RUN;
PROC MEANS SUM MEAN MAXDEC=2 FW=10;
VAR BIOMASS;
CLASS DAP MAIZE DAI REP;
WAYS 1;
RUN;
PROC GLM;
CLASS DAP MAIZE DAI REP;
MODEL BIOMASS = DAP MAIZE DAI MAIZE*DAI;
MEANS DAI / LSD LINES;
MEANS MAIZE / LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : BIOMASS

DAI	N Obs	Sum	Mean	Std Dev
1	**12**	0.00	0.00	0.00
2	**12**	0.01	0.00	0.00
3	**12**	0.04	0.00	0.00
4	**12**	0.06	0.01	0.01
5	**12**	0.08	0.01	0.01
6	**12**	0.02	0.00	0.00

Analysis Variable : BIOMASS

MAIZE	N Obs	Sum	Mean	Std Dev
1	**36**	0.14	0.00	0.01
2	**36**	0.07	0.00	0.00

Analysis Variable : BIOMASS

PLOT	N Obs	Sum	Mean	Std Dev
1	**36**	0.10	0.00	0.01
2	**36**	0.10	0.00	0.01

The GLM Procedure

Class Level Information

Class	Levels	Values
PLOT	2	1 2
MAIZE	2	1 2
DAI	6	1 2 3 4 5 6

Number of Observations Read 72

Number of Observations Used 72

The SAS System

The GLM Procedure

Dependent Variable: BIOMASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	12	0.00059561	0.00004963	1.25	0.2741
Error	59	0.00234731	0.00003978		
Corrected Total	71	0.00294292			

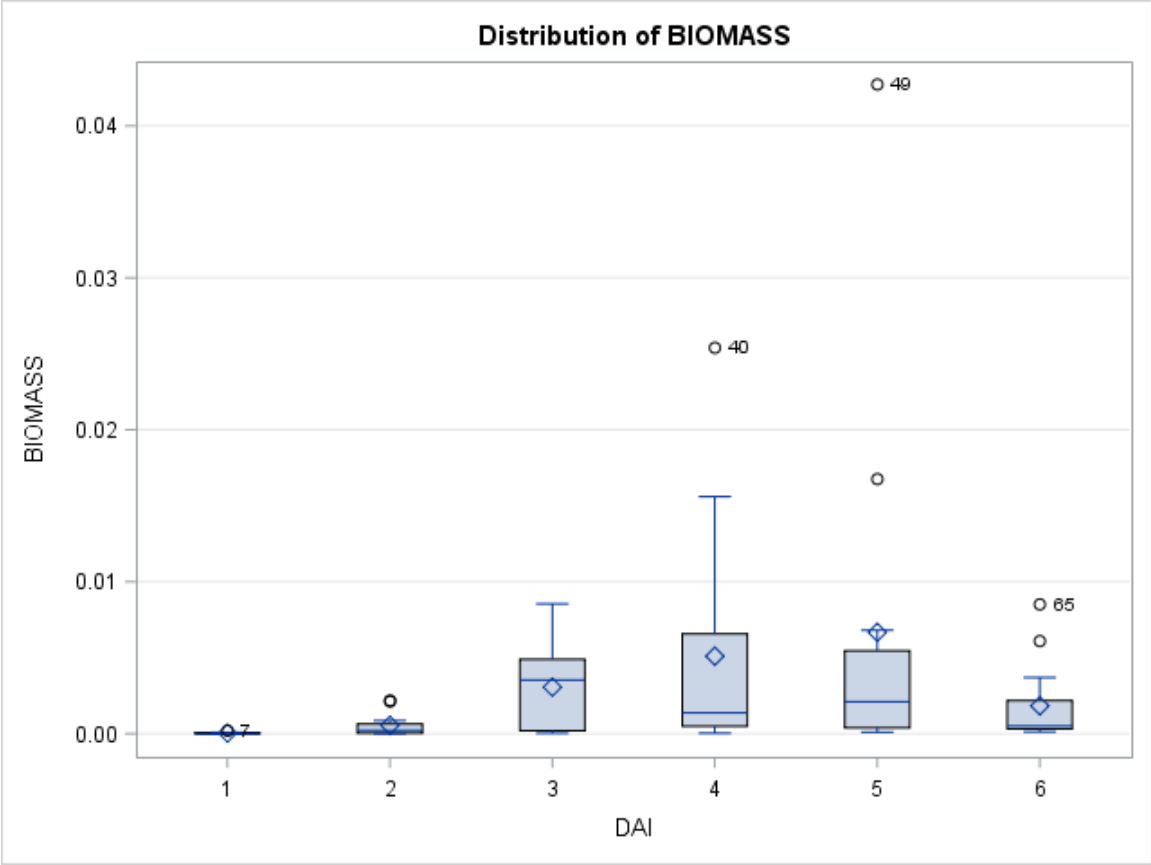
R-Square	Coeff Var	Root MSE	BIOMASS Mean
0.202387	219.4019	0.006308	0.002875

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PLOT	1	0.00000012	0.00000012	0.00	0.9559
MAIZE	1	0.00007223	0.00007223	1.82	0.1830
DAI	5	0.00040742	0.00008148	2.05	0.0849
MAIZE*DAI	5	0.00011584	0.00002317	0.58	0.7134

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLOT	1	0.00000012	0.00000012	0.00	0.9559
MAIZE	1	0.00007223	0.00007223	1.82	0.1830
DAI	5	0.00040742	0.00008148	2.05	0.0849
MAIZE*DAI	5	0.00011584	0.00002317	0.58	0.7134

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	59
Error Mean Square	0.00004
Critical Value of t	2.00100
Least Significant Difference	0.0052

Means with the same letter

are not significantly different.

t Grouping		Mean	N	DAI
	A	0.006672	12	5
	A			
B	A	0.005099	12	4

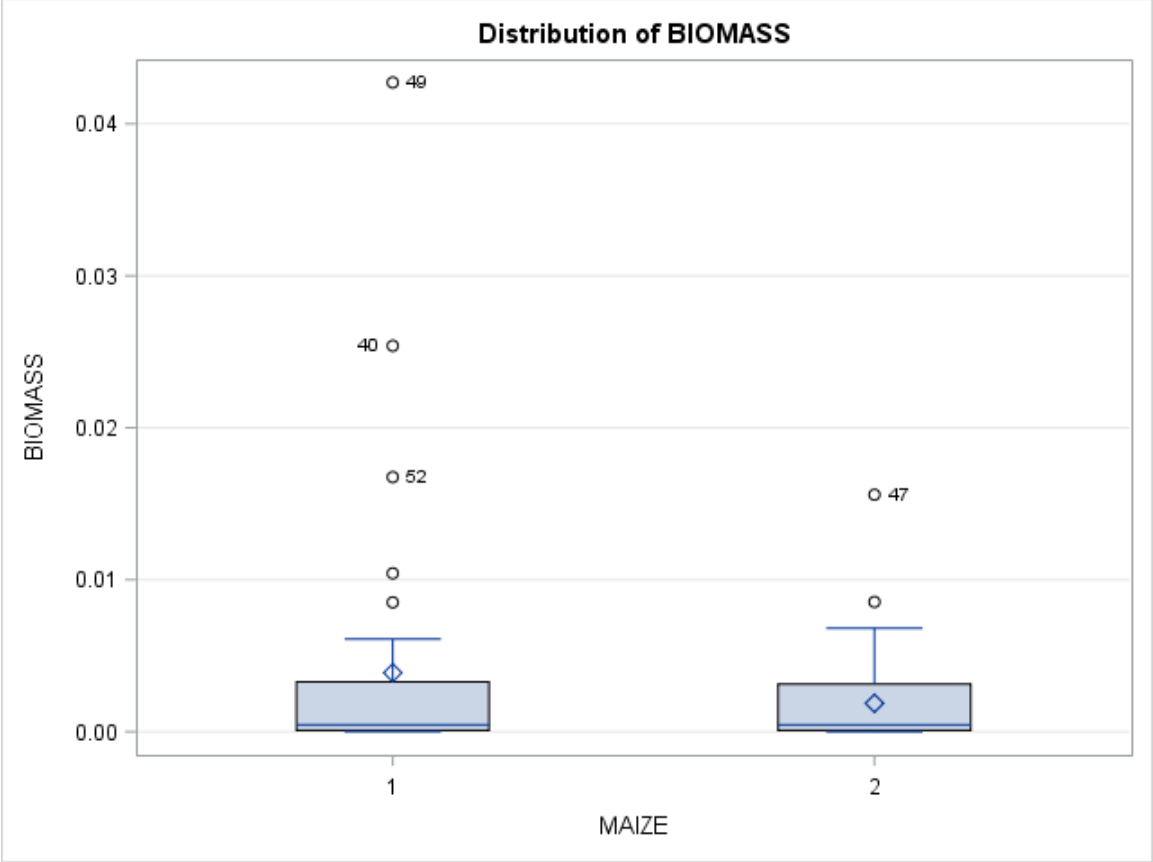
Means with the same letter

are not significantly different.

t Grouping		Mean	N	DAI
B	A			
B	A	0.003062	12	3
B	A			
B	A	0.001830	12	6
B				
B		0.000546	12	2
B				
B		0.000040	12	1

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	59
Error Mean Square	0.00004
Critical Value of t	2.00100
Least Significant Difference	0.003

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	0.003876	36	1
A			
A	0.001873	36	2

Figure A.2 First year *A. flavus* biomass data.

```
OPTIONS PS=55 LS=85 NODATE;
DATA AFLATOXIN;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 435\aflatoxin.dat';
INPUT PLOT MAIZE DAI REP $ AFLATOXIN;
RUN;
PROC MEANS SUM MEAN MAXDEC=2 FW=10;
VAR AFLATOXIN;
CLASS PLOT MAIZE DAI;
WAYS 1;
RUN;
PROC GLM;
CLASS PLOT MAIZE DAI;
MODEL AFLATOXIN = PLOT MAIZE DAI MAIZE*DAI;
MEANS DAI / LSD LINES;
MEANS MAIZE / LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : AFLATOXIN

DAI	N Obs	Sum	Mean	Std Dev
1	**27**	16346.42	605.42	1157.08
2	**27**	44987.07	1666.19	2051.88
3	**27**	95005.51	3518.72	2056.01
4	**27**	124718.47	4619.20	2329.38
5	**27**	124632.76	4616.03	2834.75
6	**27**	121396.63	4496.17	2631.30

Analysis Variable : AFLATOXIN

MAIZE	N Obs	Sum	Mean	Std Dev
1	**54**	188178.64	3484.79	2948.83
2	**54**	144374.73	2673.61	2123.84
3	**54**	194533.49	3602.47	2939.93

Analysis Variable : AFLATOXIN

PLOT	N Obs	Sum	Mean	Std Dev
1	**54**	145841.24	2700.76	2435.19
2	**54**	161481.26	2990.39	2446.59
3	**54**	219764.36	4069.71	3064.98

The GLM Procedure

Class Level Information

Class	Levels	Values
PLOT	3	1 2 3
MAIZE	3	1 2 3
DAI	6	1 2 3 4 5 6

Number of Observations Read 162

Number of Observations Used 162

The SAS System

The GLM Procedure

Dependent Variable: AFLATOXIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	19	539735086	28407110	6.25	<.0001
Error	142	645908981	4548655		
Corrected Total	161	1185644066			

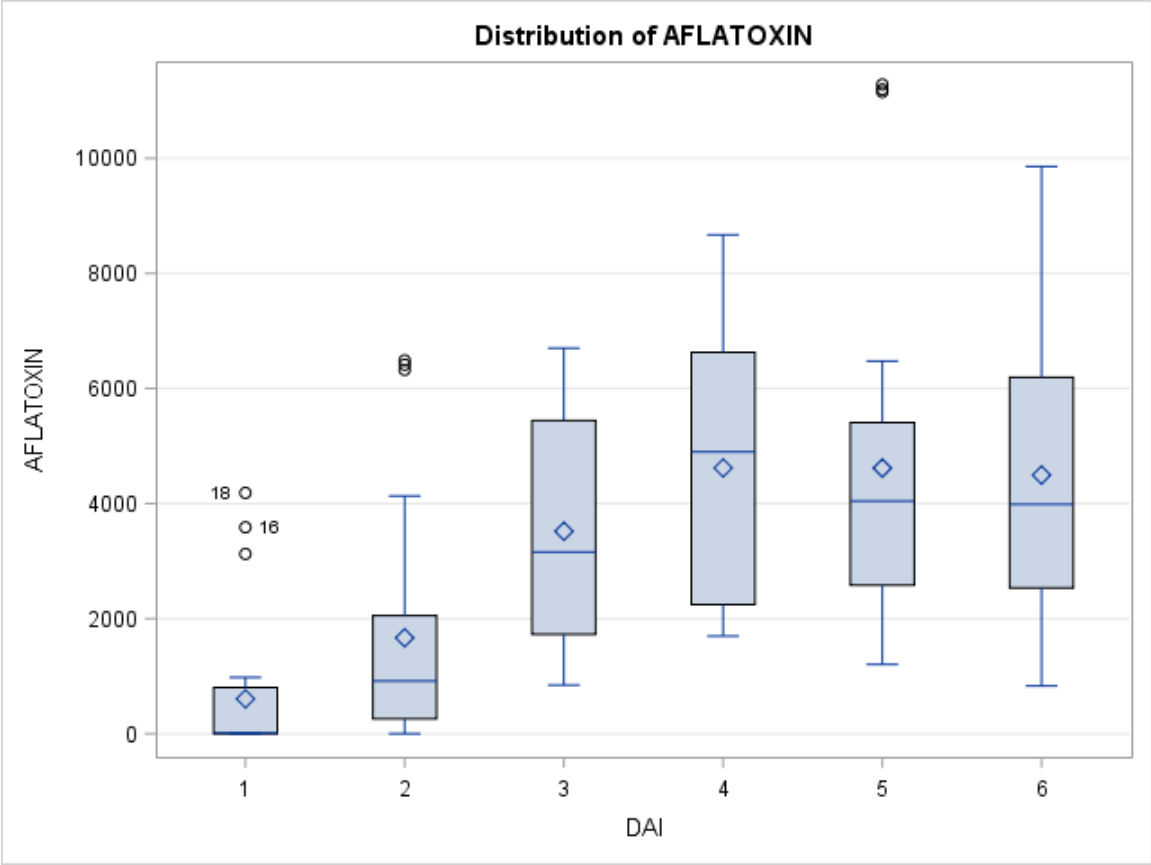
R-Square	Coeff Var	Root MSE	AFLATOXIN Mean
0.455225	65.55024	2132.758	3253.623

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PLOT	2	56210846.7	28105423.4	6.18	0.0027
MAIZE	2	27623880.0	13811940.0	3.04	0.0511
DAI	5	401438003.0	80287600.6	17.65	<.0001
MAIZE*DAI	10	54462355.9	5446235.6	1.20	0.2977

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLOT	2	56210846.7	28105423.4	6.18	0.0027
MAIZE	2	27623880.0	13811940.0	3.04	0.0511
DAI	5	401438003.0	80287600.6	17.65	<.0001
MAIZE*DAI	10	54462355.9	5446235.6	1.20	0.2977

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	142
Error Mean Square	4548655
Critical Value of t	1.97681
Least Significant Difference	1147.5

Means with the same letter are not significantly different.

t Grouping	Mean	N	DAI
A	4619.2	27	4
A			
A	4616.0	27	5

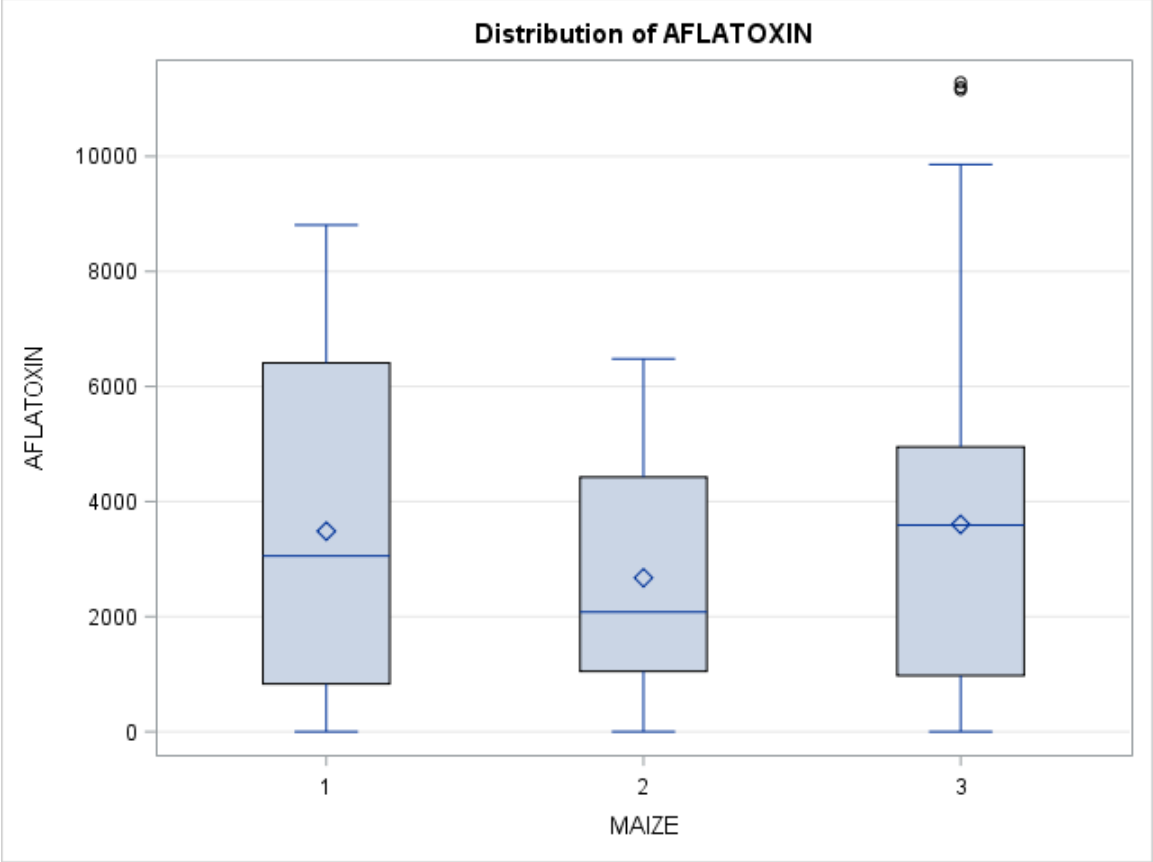
Means with the same letter

are not significantly different.

t Grouping	Mean	N	DAI
A			
A	4496.2	27	6
A			
A	3518.7	27	3
B	1666.2	27	2
B			
B	605.4	27	1

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	142
Error Mean Square	4548655
Critical Value of t	1.97681
Least Significant Difference	811.38

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	3602.5	54	3
A			
B A	3484.8	54	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
B			
B	2673.6	54	2

Figure A.3 Second year aflatoxin data.

```
OPTIONS PS=55 LS=85 NODATE;
DATA BIOMASS;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 435\fungalbiomass.dat';
INPUT PLOT MAIZE DAI $ BIOMASS;
RUN;
PROC MEANS SUM MEAN STD MAXDEC=2 FW=10;
VAR BIOMASS;
CLASS PLOT MAIZE DAI;
WAYS 1;
RUN;
PROC GLM;
CLASS PLOT MAIZE DAI;
MODEL BIOMASS = PLOT MAIZE DAI MAIZE*DAI;
MEANS DAI / LSD LINES;
MEANS MAIZE / LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : BIOMASS

DAI	N Obs	Sum	Mean	Std Dev
1	**9**	0.05	0.01	0.01
2	**9**	0.10	0.01	0.01
3	**9**	0.11	0.01	0.01
4	**9**	0.08	0.01	0.01
5	**9**	0.38	0.04	0.06
6	**9**	0.10	0.01	0.01

Analysis Variable : BIOMASS

MAIZE	N Obs	Sum	Mean	Std Dev
1	**18**	0.32	0.02	0.04
2	**18**	0.29	0.02	0.02
3	**18**	0.21	0.01	0.02

Analysis Variable : BIOMASS

PLOT	N Obs	Sum	Mean	Std Dev
1	**18**	0.19	0.01	0.01
2	**18**	0.22	0.01	0.02
3	**18**	0.41	0.02	0.04

The GLM Procedure

Class Level Information

Class	Levels	Values
PLOT	3	1 2 3
MAIZE	3	1 2 3
DAI	6	1 2 3 4 5 6

Number of Observations Read 54

Number of Observations Used 54

The SAS System

The GLM Procedure

Dependent Variable: BIOMASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	19	0.01211811	0.00063780	0.84	0.6450
Error	34	0.02568475	0.00075543		
Corrected Total	53	0.03780286			

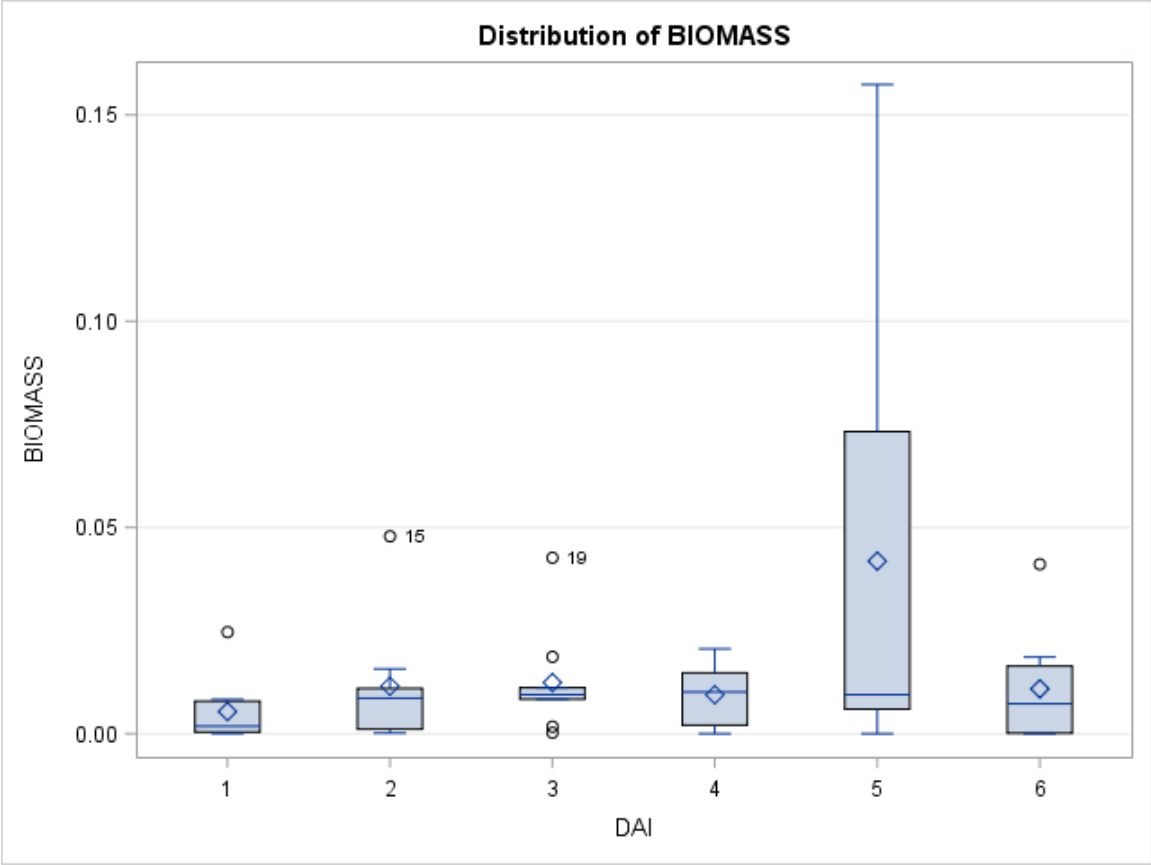
R-Square	Coeff Var	Root MSE	BIOMASS Mean
0.320561	180.5113	0.027485	0.015226

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PLOT	2	0.00161256	0.00080628	1.07	0.3552
MAIZE	2	0.00034380	0.00017190	0.23	0.7977
DAI	5	0.00791718	0.00158344	2.10	0.0899
MAIZE*DAI	10	0.00224457	0.00022446	0.30	0.9771

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLOT	2	0.00161256	0.00080628	1.07	0.3552
MAIZE	2	0.00034380	0.00017190	0.23	0.7977
DAI	5	0.00791718	0.00158344	2.10	0.0899
MAIZE*DAI	10	0.00224457	0.00022446	0.30	0.9771

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	34
Error Mean Square	0.000755
Critical Value of t	2.03224
Least Significant Difference	0.0263

Means with the same letter are not significantly different.

t Grouping	Mean	N	DAI
A	0.04182	9	5
B	0.01239	9	3

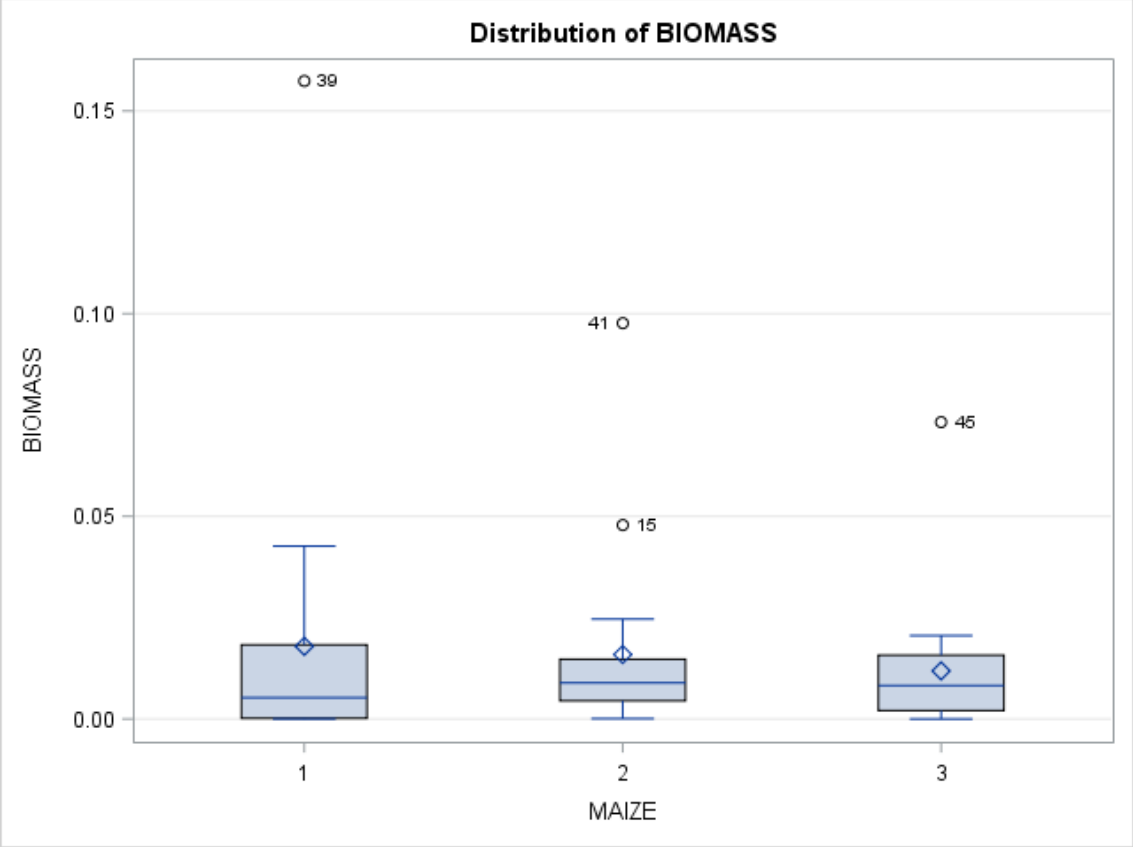
Means with the same letter

are not significantly different.

t Grouping	Mean	N	DAI
B			
B	0.01150	9	2
B			
B	0.01088	9	6
B			
B	0.00943	9	4
B			
B	0.00534	9	1

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	34
Error Mean Square	0.000755
Critical Value of t	2.03224
Least Significant Difference	0.0186

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	0.017917	18	1
A			
A	0.015911	18	2

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A			
A	0.011851	18	3

Figure A.4 Second year *A. flavus* biomass data.

```
OPTIONS PS=55 LS=85 NODATE;
DATA AFLATOXIN;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 537\aflatoxin.dat';
INPUT PLOT MAIZE DAI REP $ AFLATOXIN;
RUN;
PROC MEANS SUM MEAN MAXDEC=2 FW=10;
VAR AFLATOXIN;
CLASS PLOT MAIZE DAI;
WAYS 1;
RUN;
PROC GLM;
CLASS PLOT MAIZE DAI;
MODEL AFLATOXIN = PLOT MAIZE DAI MAIZE*DAI;
MEANS DAI / LSD LINES;
MEANS MAIZE / LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : AFLATOXIN

DAI	N Obs	Sum	Mean	Std Dev
1	**27**	398.50	14.76	21.56
2	**27**	1887.93	69.92	68.73
3	**27**	6687.17	247.67	231.11
4	**27**	17880.42	662.24	453.60
5	**27**	44990.00	1666.30	1109.30
6	**27**	64549.54	2390.72	1196.85
7	**27**	90576.19	3354.67	1256.37

Analysis Variable : AFLATOXIN

MAIZE	N Obs	Sum	Mean	Std Dev
1	**63**	77756.08	1234.22	1338.47
2	**63**	49177.55	780.60	1064.65
3	**63**	100036.11	1587.87	1747.70

Analysis Variable : AFLATOXIN

PLOT	N Obs	Sum	Mean	Std Dev
1	**63**	59639.63	946.66	1105.91
2	**63**	100756.14	1599.30	1855.83
3	**63**	66573.98	1056.73	1181.96

The GLM Procedure

Class Level Information

Class	Levels	Values
PLOT	3	1 2 3
MAIZE	3	1 2 3
DAI	7	1 2 3 4 5 6 7

Number of Observations Read 189

Number of Observations Used 189

The SAS System

The GLM Procedure

Dependent Variable: AFLATOXIN

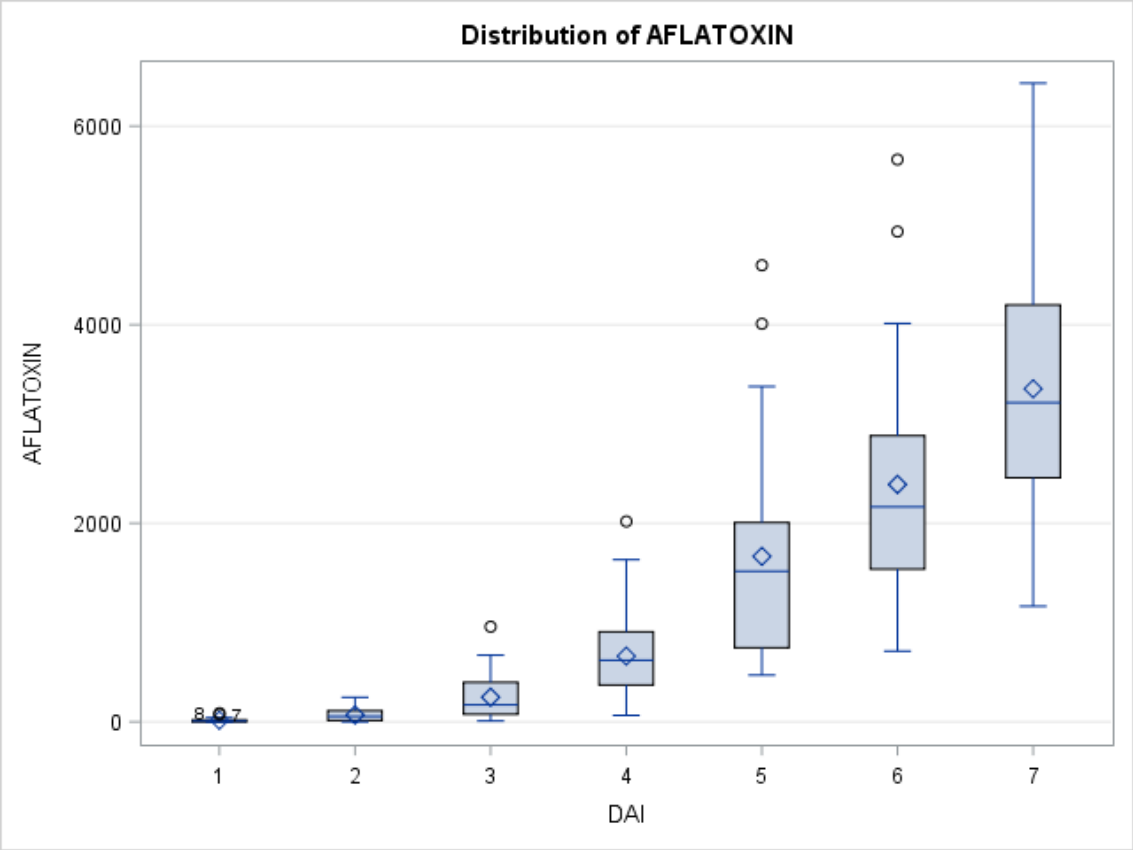
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	330980798.0	15044581.7	41.36	<.0001
Error	166	60378189.7	363724.0		
Corrected Total	188	391358987.7			

R-Square	Coeff Var	Root MSE	AFLATOXIN Mean
0.845722	50.22036	603.0954	1200.898

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PLOT	2	15381334.7	7690667.4	21.14	<.0001
MAIZE	2	20633466.0	10316733.0	28.36	<.0001
DAI	6	274207987.4	45701331.2	125.65	<.0001
MAIZE*DAI	12	20758009.9	1729834.2	4.76	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLOT	2	15381334.7	7690667.4	21.14	<.0001
MAIZE	2	20633466.0	10316733.0	28.36	<.0001
DAI	6	274207987.4	45701331.2	125.65	<.0001
MAIZE*DAI	12	20758009.9	1729834.2	4.76	<.0001

The GLM Procedure



The SAS System

The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	166
Error Mean Square	363724
Critical Value of t	1.97436
Least Significant Difference	324.07

Means with the same letter

are not significantly different.

t Grouping	Mean	N	DAI
A	3354.7	27	7
B	2390.7	27	6

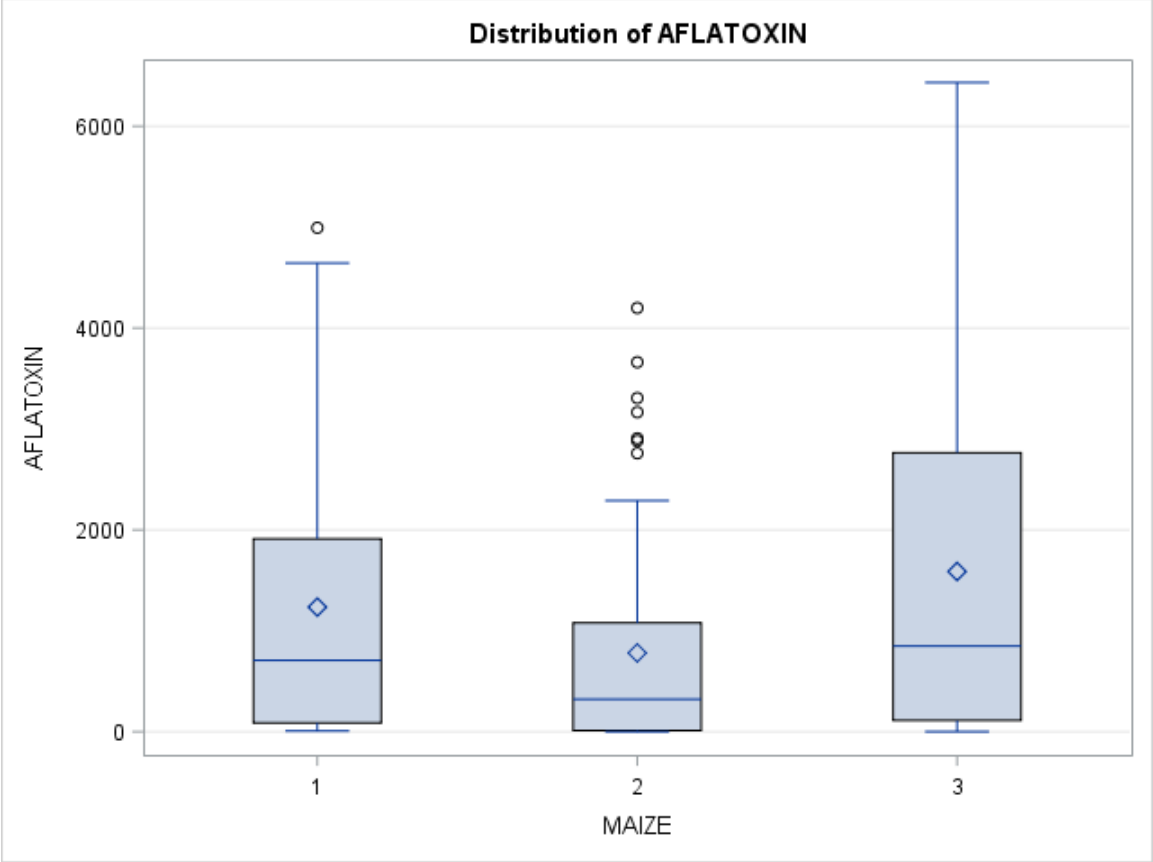
Means with the same letter

are not significantly different.

t Grouping	Mean	N	DAI
C	1666.3	27	5
D	662.2	27	4
E	247.7	27	3
E			
E	69.9	27	2
E			
E	14.8	27	1

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	166
Error Mean Square	363724
Critical Value of t	1.97436
Least Significant Difference	212.16

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	1587.9	63	3
B	1234.2	63	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
C	780.6	63	2

Figure A.5 Third year aflatoxin data.

```
OPTIONS PS=55 LS=85 NODATE;
DATA BIOMASS;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 537\fungalbiomass.dat';
INPUT PLOT MAIZE DAI $ BIOMASS;
RUN;
PROC MEANS SUM MEAN STD MAXDEC=2 FW=10;
VAR BIOMASS;
CLASS PLOT MAIZE DAI;
WAYS 1;
RUN;
PROC GLM;
CLASS PLOT MAIZE DAI;
MODEL BIOMASS = PLOT MAIZE DAI MAIZE*DAI;
MEANS DAI / LSD LINES;
MEANS MAIZE / LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : BIOMASS

DAI	N Obs	Sum	Mean	Std Dev
1	**9**	0.08	0.01	0.01
2	**9**	0.07	0.01	0.00
3	**9**	0.34	0.04	0.07
4	**9**	0.32	0.04	0.04
5	**9**	0.64	0.07	0.09
6	**9**	0.12	0.01	0.01

Analysis Variable : BIOMASS

DAI	N Obs	Sum	Mean	Std Dev
7	**9**	1.28	0.14	0.12

Analysis Variable : BIOMASS

MAIZE	N Obs	Sum	Mean	Std Dev
1	**21**	0.89	0.04	0.07
2	**21**	1.24	0.06	0.09
3	**21**	0.73	0.03	0.07

Analysis Variable : BIOMASS

PLOT	N Obs	Sum	Mean	Std Dev
1	**21**	1.08	0.05	0.09
2	**21**	0.76	0.04	0.07
3	**21**	1.02	0.05	0.07

The GLM Procedure

Class Level Information

Class	Levels	Values
PLOT	3	1 2 3
MAIZE	3	1 2 3
DAI	7	1 2 3 4 5 6 7

Number of Observations Read 63

Number of Observations Used 63

The SAS System

The GLM Procedure

Dependent Variable: BIOMASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	22	0.14222492	0.00646477	1.25	0.2635
Error	40	0.20680925	0.00517023		
Corrected Total	62	0.34903418			

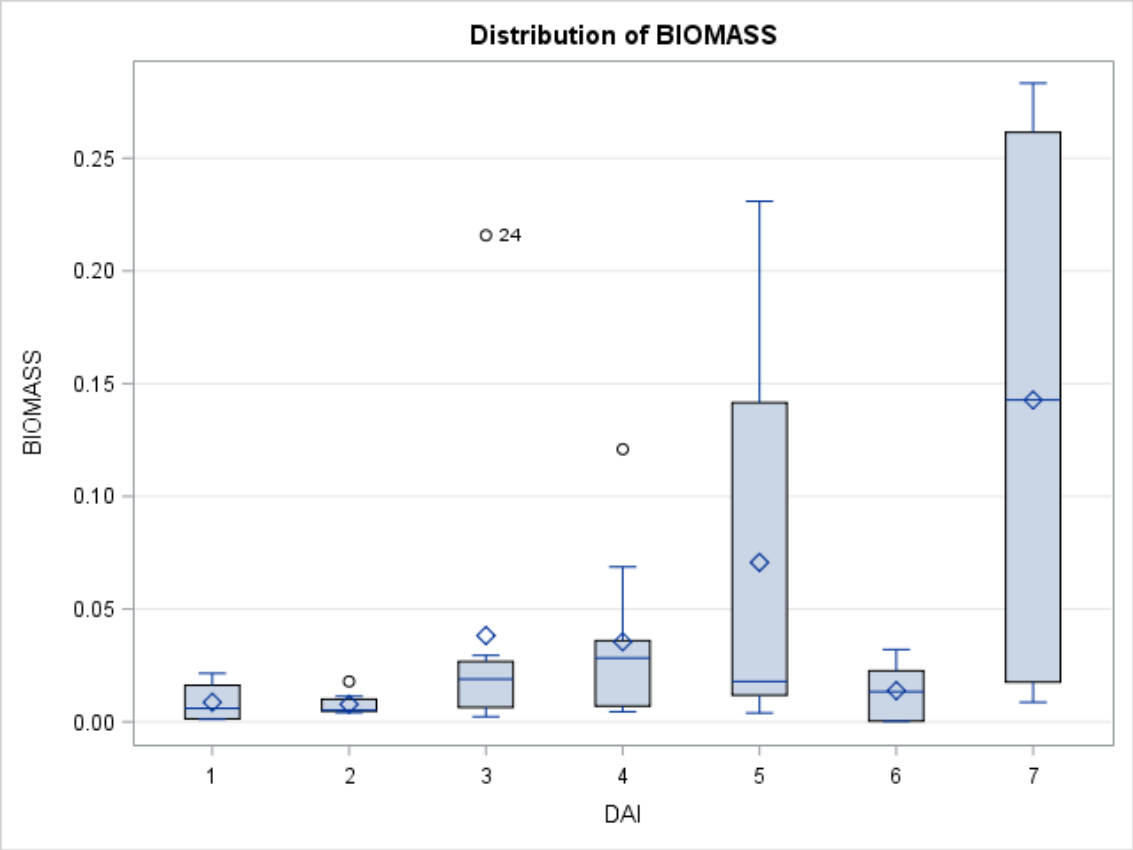
R-Square	Coeff Var	Root MSE	BIOMASS Mean
0.407481	158.5538	0.071904	0.045350

Source	DF	Type I SS	Mean Square	F Value	Pr > F
PLOT	2	0.00270766	0.00135383	0.26	0.7709
MAIZE	2	0.00653881	0.00326941	0.63	0.5366
DAI	6	0.12625968	0.02104328	4.07	0.0028
MAIZE*DAI	12	0.00671878	0.00055990	0.11	0.9999

Source	DF	Type III SS	Mean Square	F Value	Pr > F
PLOT	2	0.00270766	0.00135383	0.26	0.7709
MAIZE	2	0.00653881	0.00326941	0.63	0.5366
DAI	6	0.12625968	0.02104328	4.07	0.0028
MAIZE*DAI	12	0.00671878	0.00055990	0.11	0.9999

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	40
Error Mean Square	0.00517
Critical Value of t	2.02108
Least Significant Difference	0.0685

Means with the same letter are not significantly different.

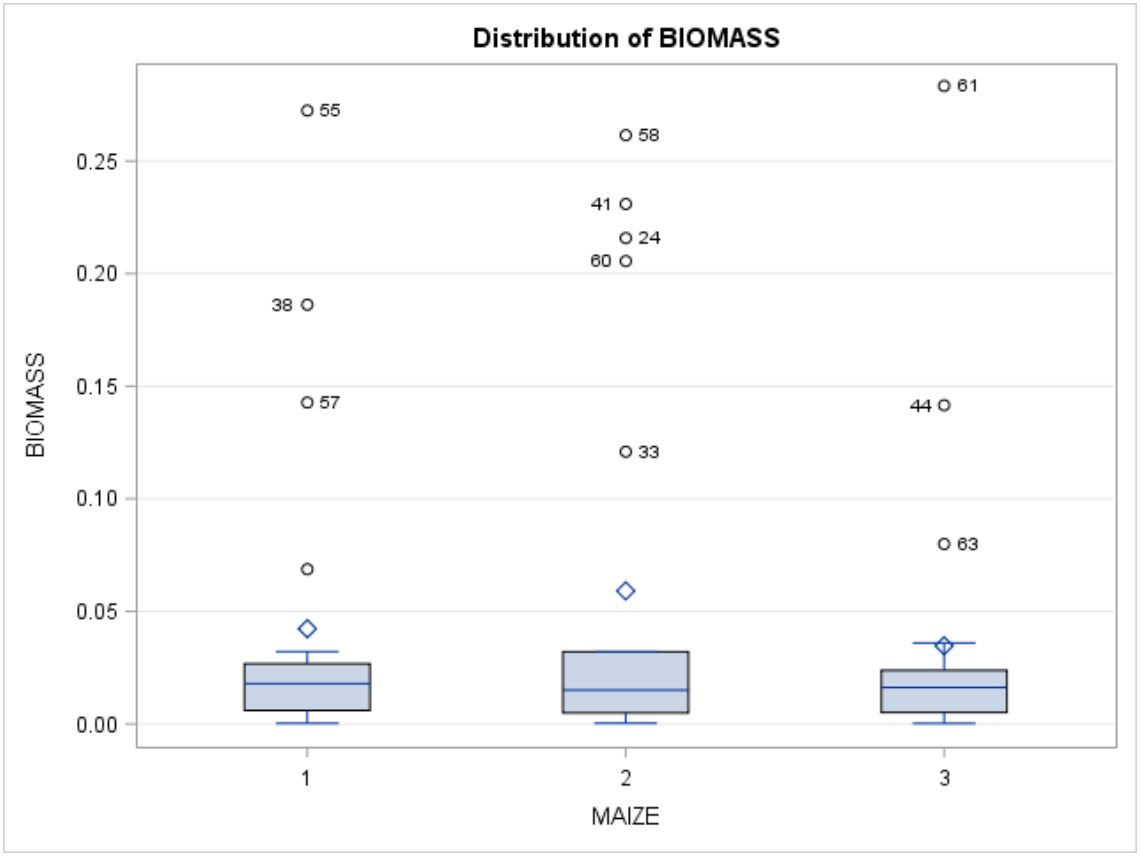
t Grouping	Mean	N	DAI
A	0.14276	9	7
B	0.07066	9	5

Means with the same letter

are not significantly different.

t Grouping	Mean	N	DAI
B			
B	0.03827	9	3
B			
B	0.03552	9	4
B			
B	0.01380	9	6
B			
B	0.00867	9	1
B			
B	0.00777	9	2

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	40
Error Mean Square	0.00517
Critical Value of t	2.02108
Least Significant Difference	0.0448

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	0.05908	21	2
A			
A	0.04227	21	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A			
A	0.03470	21	3

Figure A.6 Third year *A. flavus* biomass data.

APPENDIX B

CHAPTER V SUPPLEMENTAL MATERIAL

```
Data Harvest;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 435\harvest.dat';
INPUT MAIZE METHOD REP $ AFLATOXIN;
RUN;
PROC MEANS SUM MEAN MAXDEC=3 FW=12;
VAR AFLATOXIN;
CLASS MAIZE METHOD REP;
WAYS 1;
RUN;
PROC GLM;
CLASS MAIZE METHOD REP;
MODEL AFLATOXIN = MAIZE METHOD REP;
MEANS METHOD/LSD LINES;
MEANS MAIZE/LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : AFLATOXIN

REP	N Obs	Sum	Mean
1	**24**	87988.522	3666.188
2	**24**	96319.304	4013.304
3	**24**	100522.038	4188.418

Analysis Variable : AFLATOXIN

METHOD	N Obs	Sum	Mean
1	**18**	97256.612	5403.145
2	**18**	56144.433	3119.135
3	**18**	71559.320	3975.518

Analysis Variable : AFLATOXIN

METHOD	N Obs	Sum	Mean
4	**18**	59869.498	3326.083

Analysis Variable : AFLATOXIN

MAIZE	N Obs	Sum	Mean
1	**24**	91529.225	3813.718
2	**24**	78460.419	3269.184
3	**24**	114840.220	4785.009

The GLM Procedure

Class Level Information

Class	Levels	Values
MAIZE	3	1 2 3
METHOD	4	1 2 3 4
REP	3	1 2 3

Number of Observations Read 72

Number of Observations Used 72

The GLM Procedure

Dependent Variable: AFLATOXIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	89143683.9	12734812.0	3.46	0.0033
Error	64	235518233.0	3679972.4		
Corrected Total	71	324661917.0			

R-Square	Coeff Var	Root MSE	AFLATOXIN Mean
0.274574	48.49191	1918.325	3955.970

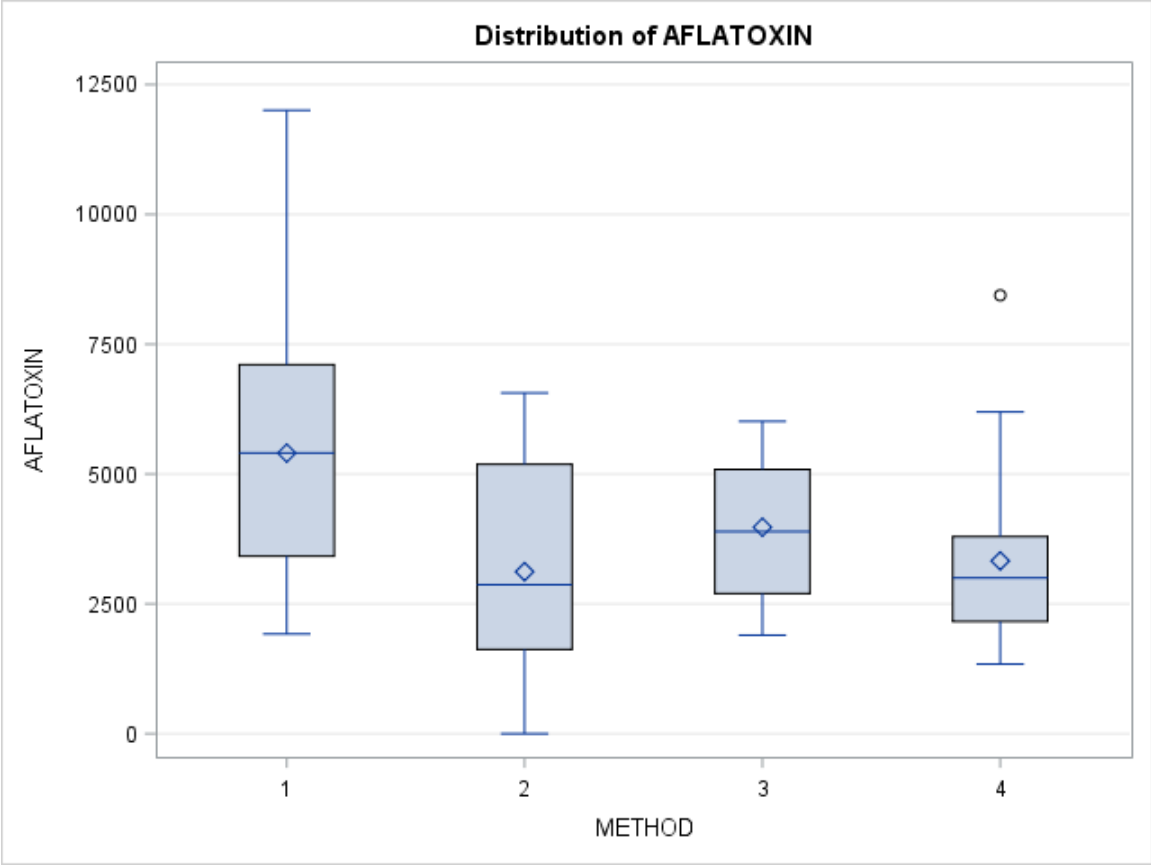
Source	DF	Type I SS	Mean Square	F Value	Pr > F
MAIZE	2	28301195.40	14150597.70	3.85	0.0265
METHOD	3	57451462.01	19150487.34	5.20	0.0028
REP	2	3391026.52	1695513.26	0.46	0.6329

Source	DF	Type III SS	Mean Square	F Value	Pr > F
MAIZE	2	28301195.40	14150597.70	3.85	0.0265

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	3	57451462.01	19150487.34	5.20	0.0028
REP	2	3391026.52	1695513.26	0.46	0.6329

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	64
Error Mean Square	3679972
Critical Value of t	1.99773
Least Significant Difference	1277.4

Means with the same letter

are not significantly different.

t Grouping	Mean	N	METHOD
A	5403.1	18	1
B	3975.5	18	3

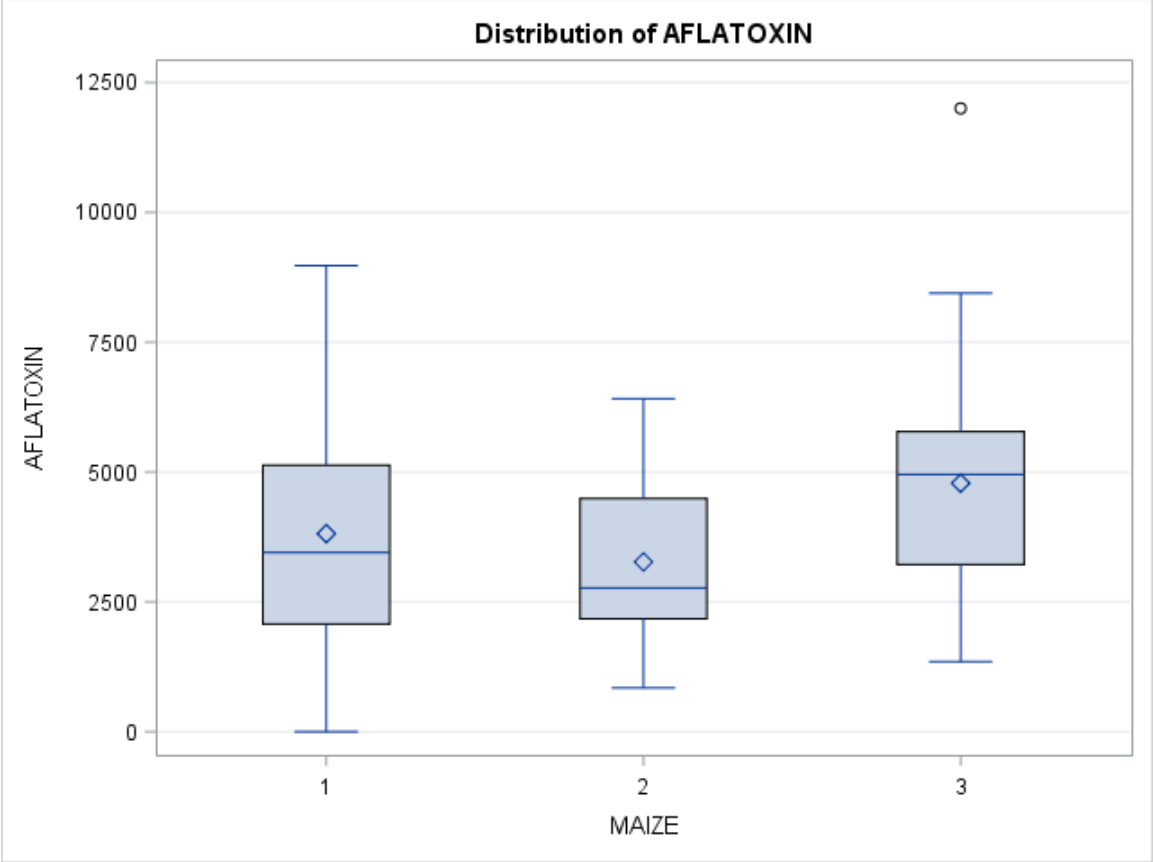
Means with the same letter

are not significantly different.

t Grouping	Mean	N	METHOD
B			
B	3326.1	18	4
B			
B	3119.1	18	2

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	64
Error Mean Square	3679972
Critical Value of t	1.99773
Least Significant Difference	1106.3

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	4785.0	24	3
A			
B A	3813.7	24	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
B			
B	3269.2	24	2

Figure B.1 Aflatoxin data for harvest samples for both years.

```
Data Harvests;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 435\harvests.dat';
INPUT MAIZE METHOD REP $ BIOMASS;
RUN;
PROC MEANS SUM MEAN MAXDEC=3 FW=12;
VAR BIOMASS;
CLASS MAIZE METHOD REP;
WAYS 1;
RUN;
PROC GLM;
CLASS MAIZE METHOD REP;
MODEL BIOMASS = MAIZE METHOD REP;
MEANS METHOD/LSD LINES;
MEANS MAIZE/LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : BIOMASS

REP	N Obs	Sum	Mean	Std Dev
1	**24**	0.404	0.017	0.058
2	**24**	0.543	0.023	0.076
3	**24**	0.843	0.035	0.129

Analysis Variable : BIOMASS

METHOD	N Obs	Sum	Mean	Std Dev
1	**18**	0.172	0.010	0.014
2	**18**	1.310	0.073	0.176
3	**18**	0.160	0.009	0.016

Analysis Variable : BIOMASS

METHOD	N Obs	Sum	Mean	Std Dev
4	**18**	0.148	0.008	0.010

Analysis Variable : BIOMASS

MAIZE	N Obs	Sum	Mean	Std Dev
1	**24**	0.249	0.010	0.014
2	**24**	1.361	0.057	0.154
3	**24**	0.181	0.008	0.014

The GLM Procedure

Class Level Information

Class	Levels	Values
MAIZE	3	1 2 3
METHOD	4	1 2 3 4
REP	3	1 2 3

Number of Observations Read 72

Number of Observations Used 72

The SAS System

The GLM Procedure

Dependent Variable: BIOMASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.09592665	0.01370381	1.76	0.1111
Error	64	0.49838604	0.00778728		
Corrected Total	71	0.59431269			

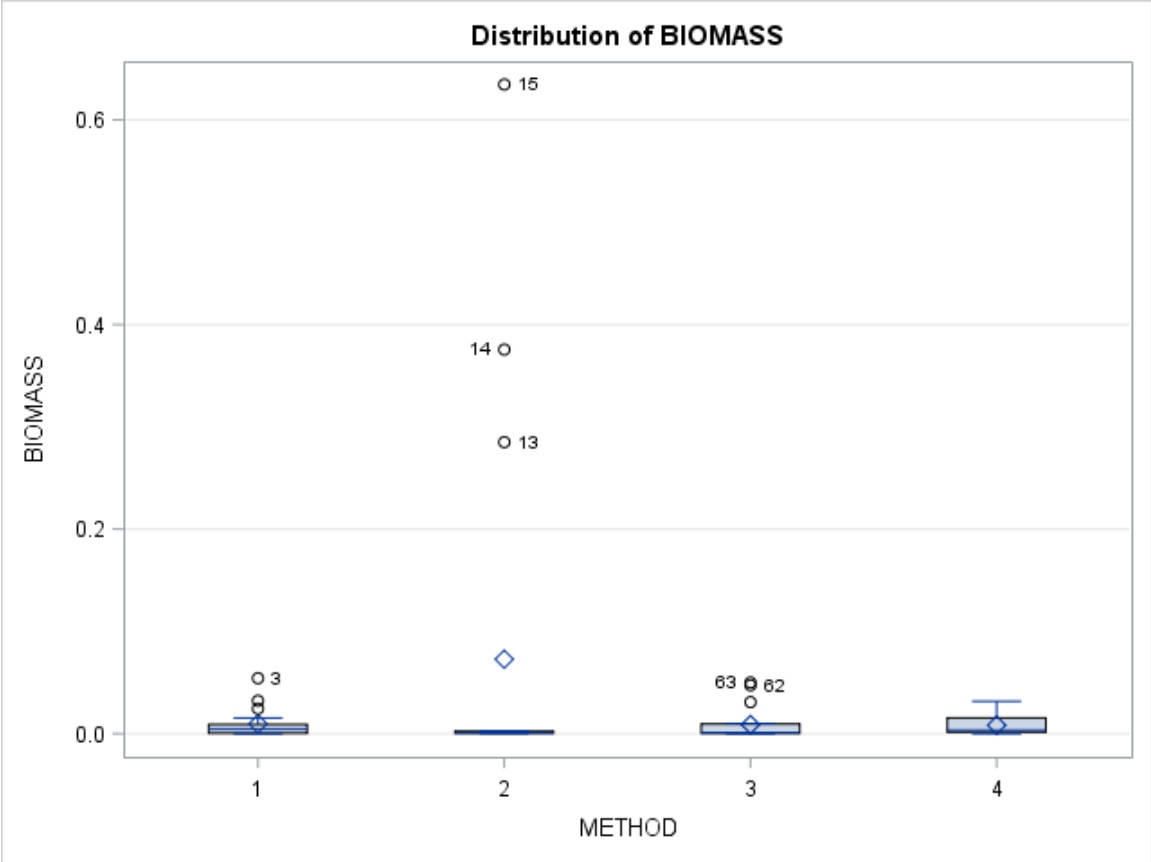
R-Square	Coeff Var	Root MSE	BIOMASS Mean
0.161408	354.7051	0.088246	0.024879

Source	DF	Type I SS	Mean Square	F Value	Pr > F
MAIZE	2	0.03658756	0.01829378	2.35	0.1036
METHOD	3	0.05514375	0.01838125	2.36	0.0797
REP	2	0.00419534	0.00209767	0.27	0.7647

Source	DF	Type III SS	Mean Square	F Value	Pr > F
MAIZE	2	0.03658756	0.01829378	2.35	0.1036

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	3	0.05514375	0.01838125	2.36	0.0797
REP	2	0.00419534	0.00209767	0.27	0.7647

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	64
Error Mean Square	0.007787
Critical Value of t	1.99773
Least Significant Difference	0.0588

Means with the same letter

are not significantly different.

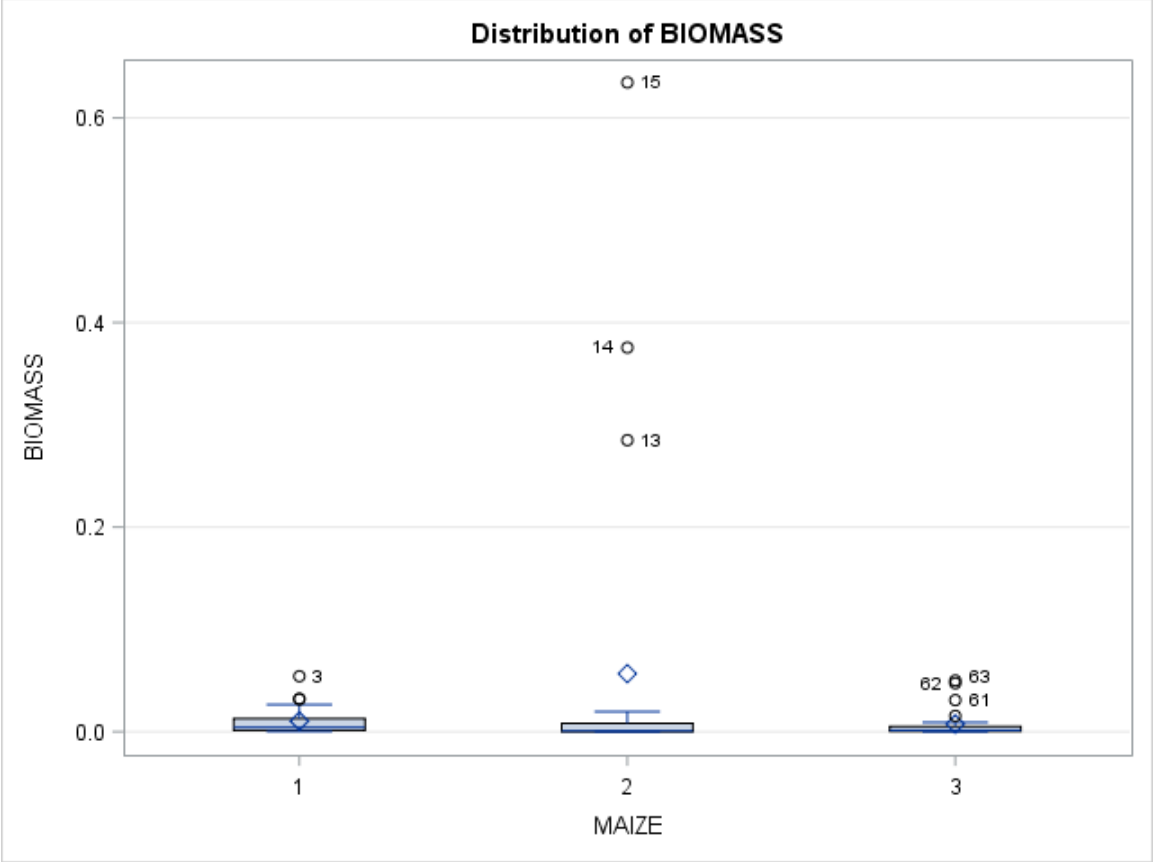
t Grouping	Mean	N	METHOD
A	0.07281	18	2
B	0.00958	18	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	METHOD
B			
B	0.00889	18	3
B			
B	0.00825	18	4

The GLM Procedure



The SAS System

The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	64
Error Mean Square	0.007787
Critical Value of t	1.99773
Least Significant Difference	0.0509

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	0.05672	24	2
A			
A	0.01039	24	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A			
A	0.00753	24	3

Figure B.2 *A. flavus* biomass data for harvest samples in both years.

```
Data Water;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 435\waters.dat';
INPUT MAIZE METHOD REP $ BIOMASS;
RUN;
PROC MEANS SUM MEAN MAXDEC=3 FW=12;
VAR BIOMASS;
CLASS MAIZE METHOD REP;
WAYS 1;
RUN;
PROC GLM;
CLASS MAIZE METHOD REP;
MODEL BIOMASS = MAIZE METHOD REP;
MEANS METHOD/LSD LINES;
MEANS MAIZE/LSD LINES;
RUN;
Data Water;
INFILE 'C:\Users\cxr1\OneDrive\Statistics\Exp 435\water.dat';
INPUT MAIZE METHOD REP $ AFLATOXIN;
RUN;
PROC MEANS SUM MEAN MAXDEC=3 FW=12;
VAR AFLATOXIN;
CLASS MAIZE METHOD REP;
WAYS 1;
RUN;
PROC GLM;
CLASS MAIZE METHOD REP;
MODEL AFLATOXIN = MAIZE METHOD REP;
MEANS METHOD/LSD LINES;
MEANS MAIZE/LSD LINES;
RUN;
```

The SAS System

The MEANS Procedure

Analysis Variable : BIOMASS

REP	N Obs	Sum	Mean	Std Dev
1	**12**	0.00321193	0.00026766	0.00087531
2	**12**	0.00467739	0.00038978	0.00129739
3	**12**	0.00174647	0.00014554	0.00045330

Analysis Variable : BIOMASS

METHOD	N Obs	Sum	Mean	Std Dev
1	**9**	0.00007379	0.00000820	0.00000690
2	**9**	0.00015891	0.00001766	0.00001234
3	**9**	0.00005981	0.00000665	0.00000522
4	**9**	0.00934328	0.00103814	0.00167463

Analysis Variable : BIOMASS

MAIZE	N Obs	Sum	Mean	Std Dev
1	**12**	0.00019892	0.00001658	0.00001442
2	**12**	0.00016479	0.00001373	0.00001101
3	**12**	0.00927208	0.00077267	0.00150656

The GLM Procedure

Class Level Information

Class	Levels	Values
MAIZE	3	1 2 3
METHOD	4	1 2 3 4
REP	3	1 2 3

Number of Observations Read 36

Number of Observations Used 36

The SAS System

The GLM Procedure

Dependent Variable: BIOMASS

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	0.00001207	0.00000172	2.76	0.0258
Error	28	0.00001749	0.00000062		
Corrected Total	35	0.00002956			

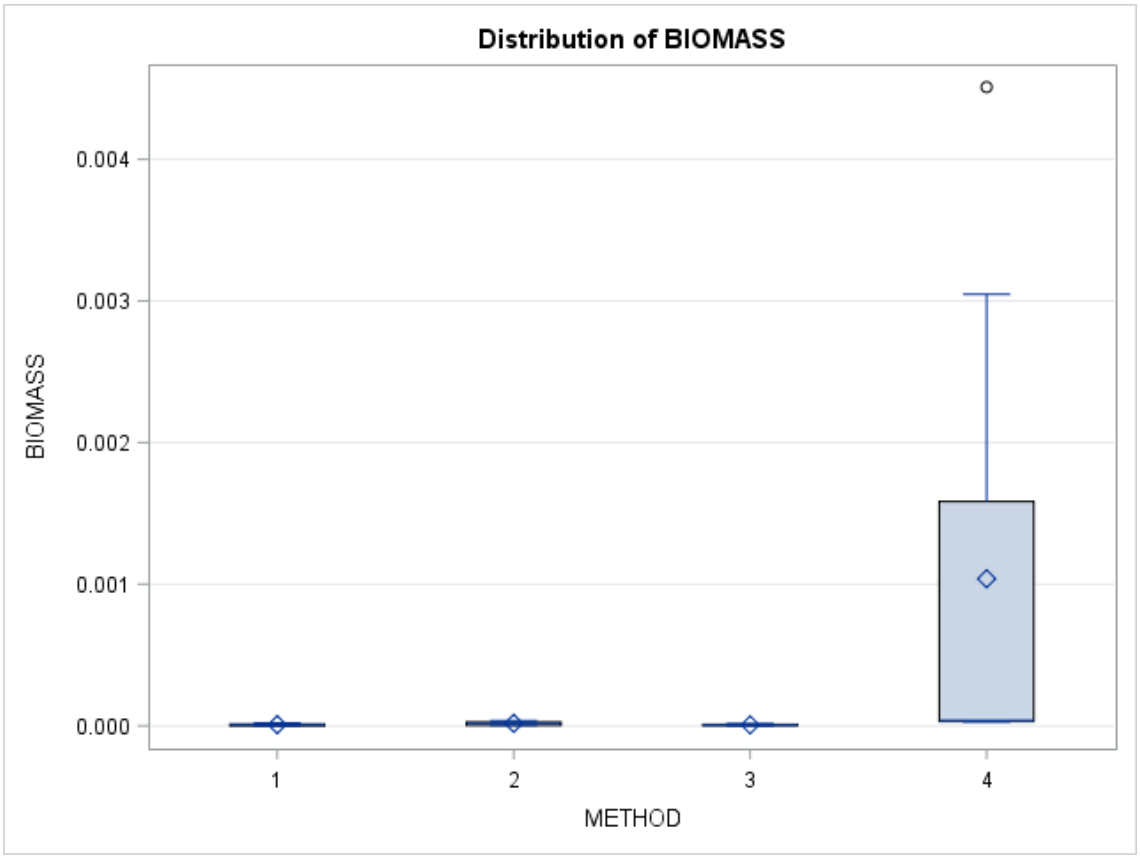
R-Square	Coeff Var	Root MSE	BIOMASS Mean
0.408405	295.2642	0.000790	0.000268

Source	DF	Type I SS	Mean Square	F Value	Pr > F
MAIZE	2	4.5907287E-6	2.2953643E-6	3.68	0.0383
METHOD	3	7.1243441E-6	2.3747814E-6	3.80	0.0210
REP	2	3.5792681E-7	1.789634E-7	0.29	0.7530

Source	DF	Type III SS	Mean Square	F Value	Pr > F
MAIZE	2	4.5907287E-6	2.2953643E-6	3.68	0.0383

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	3	7.1243441E-6	2.3747814E-6	3.80	0.0210
REP	2	3.5792681E-7	1.789634E-7	0.29	0.7530

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	6.246E-7
Critical Value of t	2.04841
Least Significant Difference	0.0008

Means with the same letter

are not significantly different.

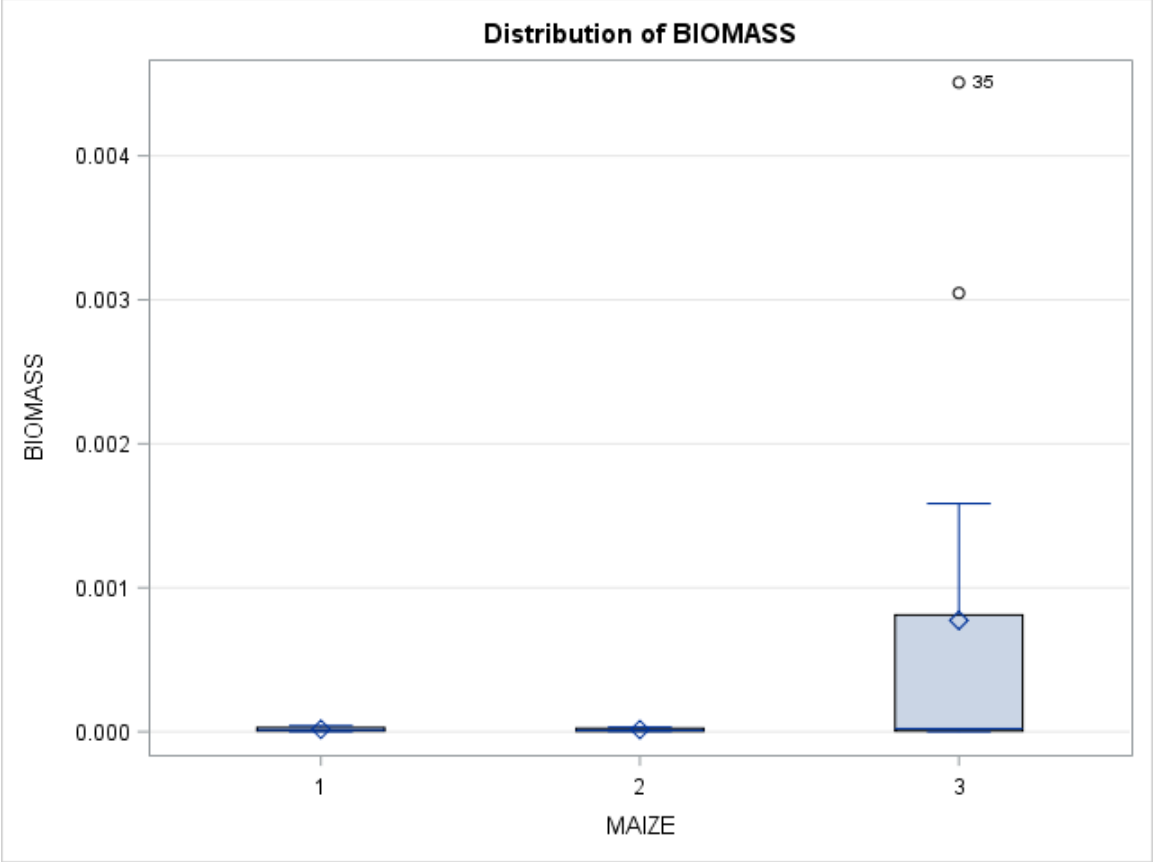
t Grouping	Mean	N	METHOD
A	0.0010381	9	4
B	0.0000177	9	2

Means with the same letter

are not significantly different.

t Grouping	Mean	N	METHOD
B			
B	0.0000082	9	1
B			
B	0.0000066	9	3

The GLM Procedure



The GLM Procedure

t Tests (LSD) for BIOMASS

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	6.246E-7
Critical Value of t	2.04841
Least Significant Difference	0.0007

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	0.0007727	12	3
B	0.0000166	12	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
B			
B	0.0000137	12	2

The MEANS Procedure

Analysis Variable : AFLATOXIN

REP	N Obs	Sum	Mean	Std Dev
1	**12**	1.87054300	0.15587858	0.33060541
2	**12**	1.55569500	0.12964125	0.19540523
3	**12**	1.98358560	0.16529880	0.27967507

Analysis Variable : AFLATOXIN

METHOD	N Obs	Sum	Mean	Std Dev
1	**9**	0.52973700	0.05885967	0.09577273
2	**9**	0.67296400	0.07477378	0.12244201
3	**9**	0.18911860	0.02101318	0.03421051
4	**9**	4.01800400	0.44644489	0.39081456

Analysis Variable : AFLATOXIN

MAIZE	N Obs	Sum	Mean	Std Dev
1	**12**	2.46258260	0.20521522	0.23982685

Analysis Variable : AFLATOXIN

MAIZE	N Obs	Sum	Mean	Std Dev
2	**12**	2.88083700	0.24006975	0.36588224
3	**12**	0.06640400	0.00553367	0.01076830

The GLM Procedure

Class Level Information

Class	Levels	Values
MAIZE	3	1 2 3
METHOD	4	1 2 3 4
REP	3	1 2 3

Number of Observations Read 36

Number of Observations Used 36

The SAS System

The GLM Procedure

Dependent Variable: AFLATOXIN

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	7	1.45891511	0.20841644	5.65	0.0004
Error	28	1.03199342	0.03685691		
Corrected Total	35	2.49090853			

R-Square	Coeff Var	Root MSE	AFLATOXIN Mean
0.585696	127.7553	0.191982	0.150273

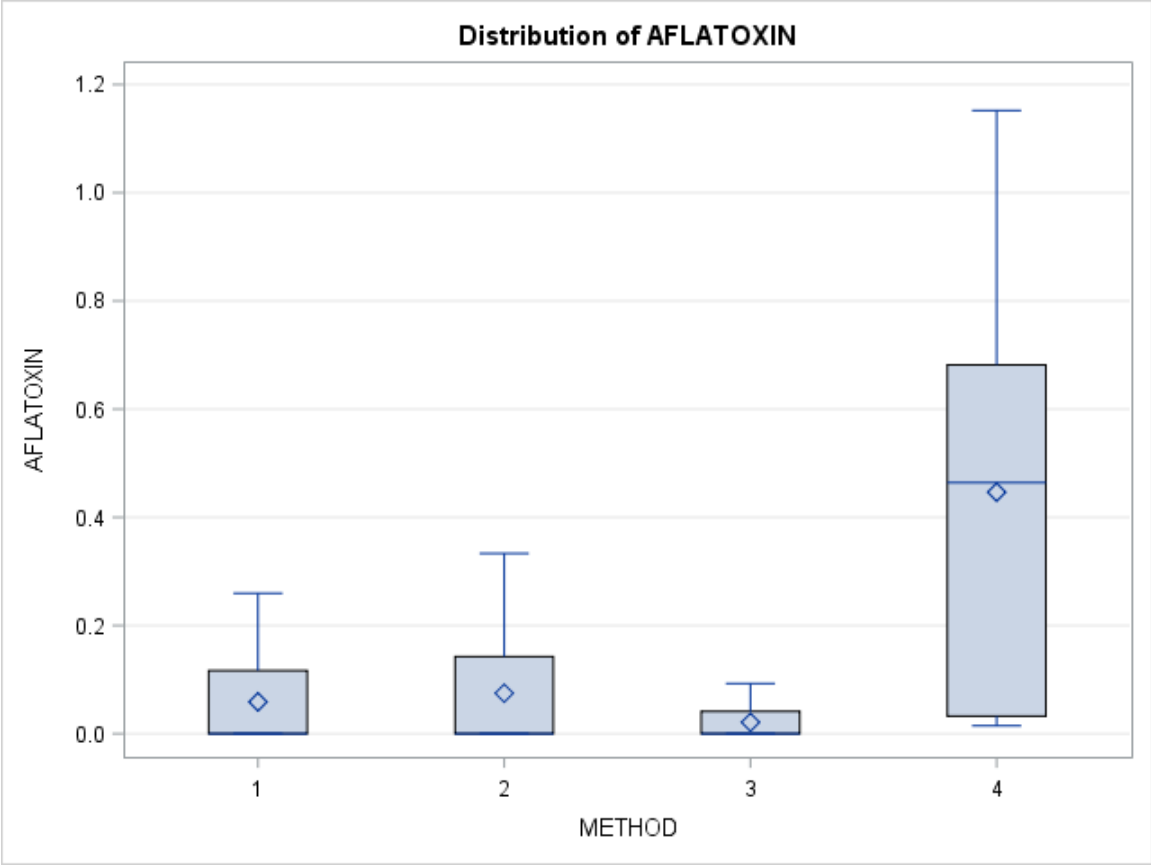
Source	DF	Type I SS	Mean Square	F Value	Pr > F
MAIZE	2	0.38437894	0.19218947	5.21	0.0119
METHOD	3	1.06634178	0.35544726	9.64	0.0002
REP	2	0.00819440	0.00409720	0.11	0.8952

Source	DF	Type III SS	Mean Square	F Value	Pr > F
MAIZE	2	0.38437894	0.19218947	5.21	0.0119

Source	DF	Type III SS	Mean Square	F Value	Pr > F
METHOD	3	1.06634178	0.35544726	9.64	0.0002
REP	2	0.00819440	0.00409720	0.11	0.8952

The SAS System

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	0.036857
Critical Value of t	2.04841
Least Significant Difference	0.1854

Means with the same letter

are not significantly different.

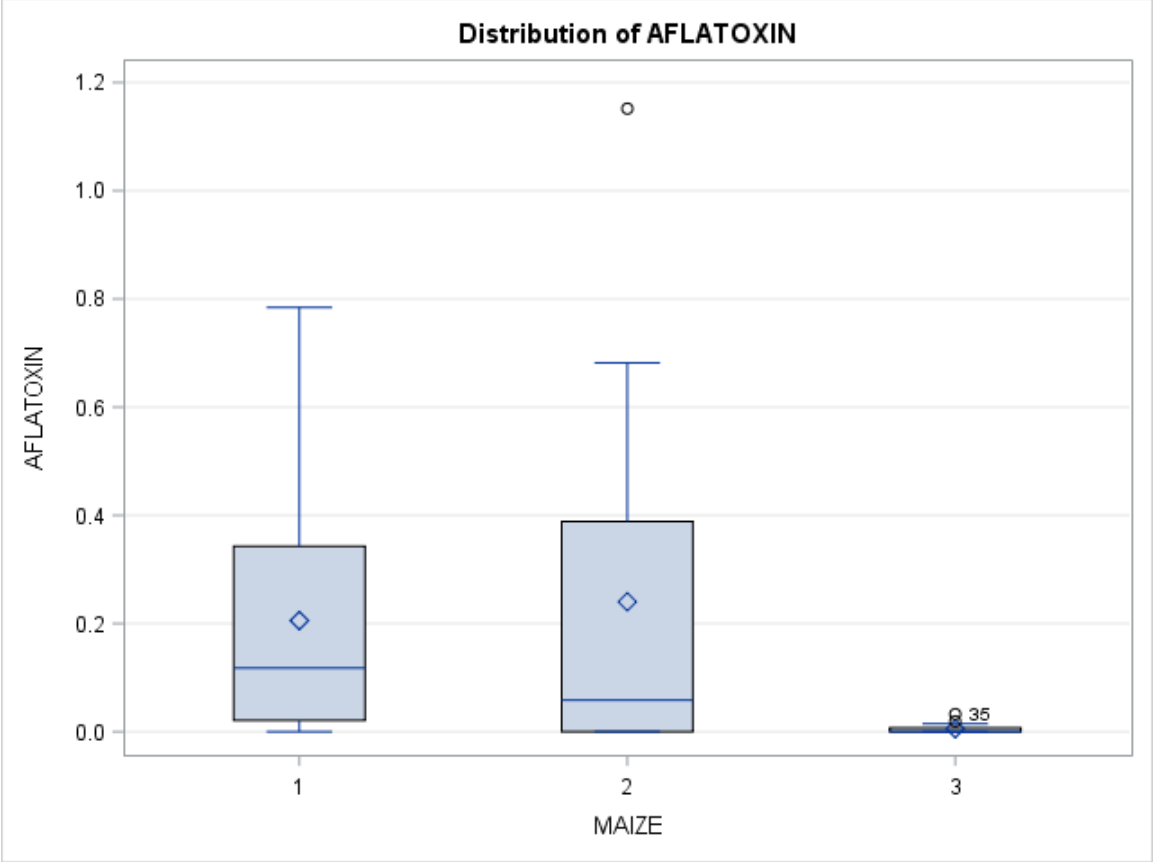
t Grouping	Mean	N	METHOD
A	0.44644	9	4
B	0.07477	9	2

Means with the same letter

are not significantly different.

t Grouping	Mean	N	METHOD
B			
B	0.05886	9	1
B			
B	0.02101	9	3

The GLM Procedure



The GLM Procedure

t Tests (LSD) for AFLATOXIN

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	0.036857
Critical Value of t	2.04841
Least Significant Difference	0.1605

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
A	0.24007	12	2
A			
A	0.20522	12	1

Means with the same letter

are not significantly different.

t Grouping	Mean	N	MAIZE
B	0.00553	12	3

Figure B.3 *A. flavus* biomass and aflatoxin data from water inoculated maize.

APPENDIX C

CHAPTER VI SUPPLEMENTAL MATERIAL

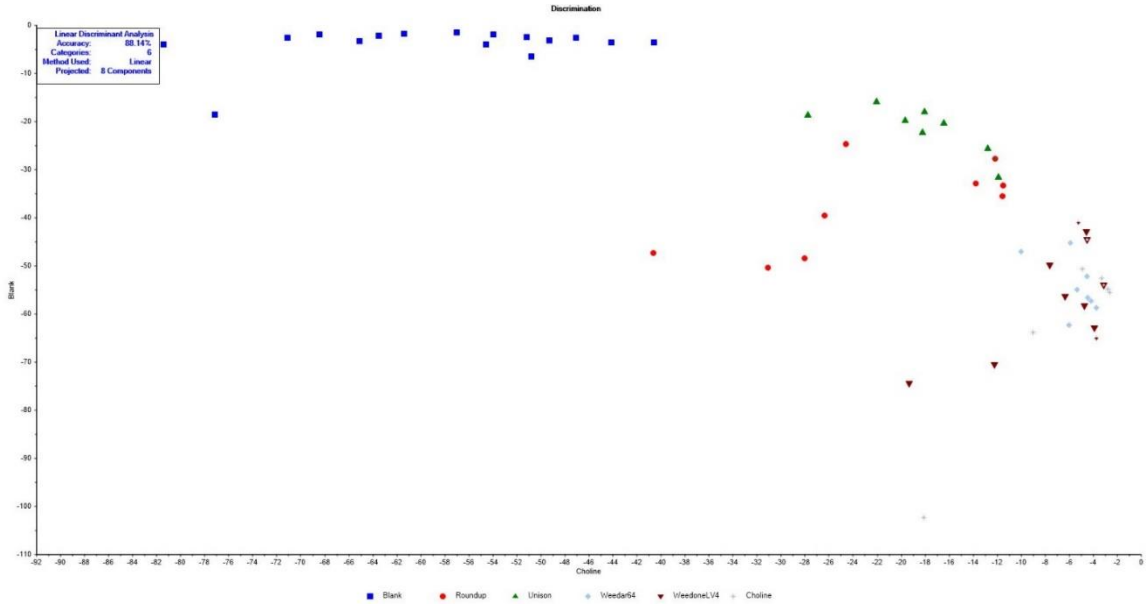


Figure C.1 Linea discriminant analysis for cotton 28 days after 2,4-D application.

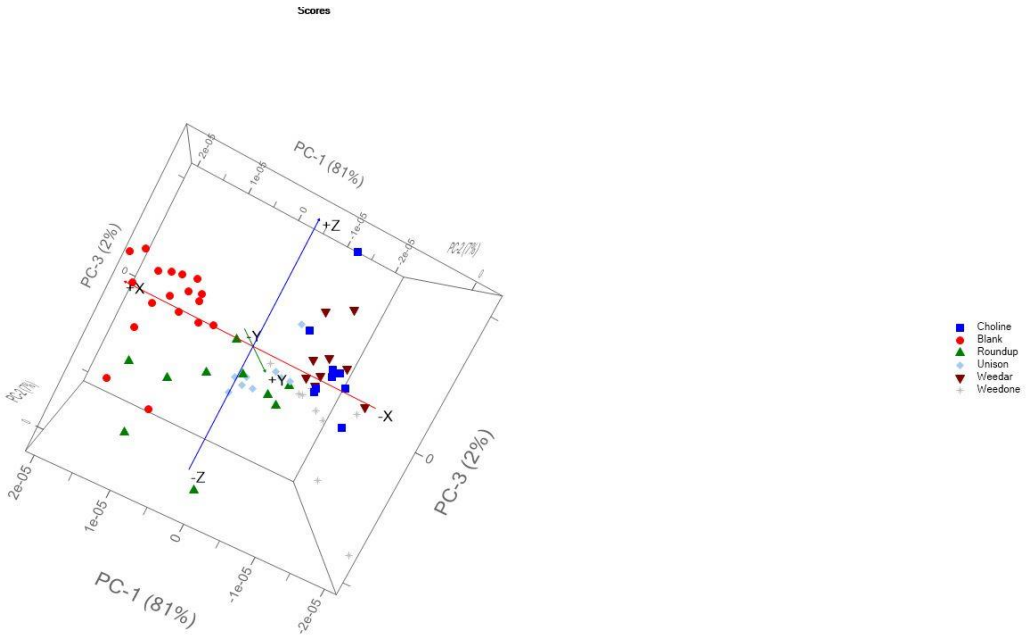


Figure C.2 Principal component analysis of cotton 28 days after 2,4-D application.