12-14-2013

# Evaluation of Quantitative Polymerase Reaction and Microbial Volatile Organic Compounds to Determine Resistance to Aspergillus Flavus in Maize

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# Evaluation of quantitative polymerase reaction and microbial volatile organic compounds to determine resistance to *Aspergillus flavus* in maize

By

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A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Life Sciences
in the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology

Mississippi State, Mississippi

December 2013

Evaluation of quantitative polymerase reaction and microbial volatile organic compounds to determine resistance to *Aspergillus flavus* in maize

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Title of Study: Evaluation of quantitative polymerase reaction and microbial volatile organic compounds to determine resistance to *Aspergillus flavus* in maize

Pages in Study: 104

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Screening for resistance to *Aspergillus flavus* and aflatoxin contamination in maize is an ongoing effort by universities, state and federal agencies. We evaluated two techniques to screen for resistance; quantitative polymerase reaction (QPCR) and solid-phase microextraction (SPME). Methods were adapted to accurately detect and quantify the fungus in culture and in the vegetative stage of plant tissues. These assays can eliminate microbiological techniques. The primary objectives of the study were to utilize 1) QPCR to detect and quantify fungal biomass in maize stem tissues to evaluate resistance in maize genotypes to *A. flavus* colonization *in situ* and *in vivo* and 2) SPME to identify key MVOC's to differentiate aflatoxigenic and non-aflatoxigenic strains of *A. flavus in situ*. A novel QPCR TaqMan probe (*OMG3*) was designed to detect a region in the *aflP* gene. The *OMG3* probe detected 98.3% of the aflatoxigenic strains. The predominant MVOC's extracted from both aflatoxigenic and non-aflatoxigenic strains were alcohols, ketones and hydrocarbons. The aflatoxigenic strain produced 39 compounds and the non-aflatoxigenic strain produced 41 compounds. Dimethylsulfide

and 2-heptanol were key MVOC biomarkers produced only by the aflatoxigenic strain of *A. flavus*. Accuracy of the QPCR *OMG3* probe, *in vivo* and *in situ* procedures were developed. A toothpick inoculation method was used to artificially inoculate maize stems in the vegetative stage five (V5). Plants were harvested at V7 and sampled at predetermined sites. This method was 91% consistent for infecting maize plants. The *OMG3* probe was evaluated in *in vivo* and *in situ* studies conducted in the greenhouse, growth chamber, and field. Lesion length was greater in susceptible lines in 4 of 7 greenhouse trials. Based on inoculation data, subsequent research should focus on refining tissue-sampling methods and increasing length of plant growth time for tissue sampling post-inoculation.

# DEDICATION

I dedicate this dissertation to those of whom are capable of recognizing all that is valuable and worthy within them. I give a special feeling of gratitude to The Wonder Woman, L.P. Fye, for your authentic smile and contagious laugh. You have been at my side and are always able to "see" me. I am no longer in a corner, my trains have stopped at their stations, and I am your equal. I owe a special thanks to the lady with the cat-like reflexes, T.G. Mulvihil. You demanded and won my trust, and always offered words of reassurance. I will drive many miles to shake your hand, always.

This "Dragon" gives many, extra special thanks to Alix Huey, the best "Monkay" and friend a "Dragon" could ever have. Through building, traveling, crafting, and most importantly, whacking, you have shown me the true meaning of tolerance and patience. I dedicate this work to my sarcastic friends who continued to probe, "Have you finished that little book report yet?" I appreciate your persistence and guidance.

I give a special feeling to Bud E. Dawg, Luke S. Dawg (a.k.a Fat Dog), and to Larry. Although I love you each of you, thanks for annoying me. I am forever grateful to you, "My David", my extraordinary husband who has loved me, took care of me, stood by me, and kept me for 15 years. Without you, many things in my life including these 87 pages would not have been. I dedicate these 104 pages to "My Best Good Jethro," the boy who found me and the boy who helped me find myself. You have been my biggest fan.

ACKNOWLEDGEMENTS

I wish to express the deepest appreciation to each of my committee members who were more than generous with their precious time, expertise, and patience. Your advice and persistence throughout this process kept me focused on my goal. Firstly, I would like to give a special thanks to my major professor Dr. Richard E. Baird for instilling in me the qualities of being a good scientist and possibly one day a professor. His kindness, thoughtfulness, music selection, and collection of mushroom artifacts have been a major source of motivation throughout this journey. I am forever grateful for his persistence in finding funding for myself and for this research, without it, this dissertation would not have been possible. I would to thank the "Swiss Army Knife" Dr. Alan Henn for his valuable time, use of his antique and collectable laboratory supplies and equipment, and most importantly, his depth of knowledge. I would like to take this opportunity to thank Dr. Todd E. Mlsna for his helpful advice, valuable suggestions, and patience. I am grateful for the access and use of his laboratory and equipment and the constant availability to answer any question I may have had. I will continue to smile while I inform people how to pronounce and spell your name correctly. A special thank you to Dr. Gary L. Windham for his guidance and support. His advice throughout this process has helped me to stay focused. I appreciate the use of your equipment and the technical expertise and the valued time from your technicians in the field. I owe each of you a Moon Pie. I would like to thank Mary Scruggs for putting up with me. I owe you a baby

horse. You will get pictures and YouTube videos instead. Last but not least, a very special thank you to Dongdi Sun, the King of research. He has and will continue to be my mentor. His infectious smile and infinite zeal have been major driving forces throughout this difficult process.

I would also like to thank the Mississippi Corn Promotion Board, the Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, and the Mississippi State Office of the Graduate School for their financial support.

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# CHAPTER I

# INTRODUCTION

Screening for resistance to *Aspergillus flavus* and aflatoxin contamination in many crops is an ongoing effort by many universities, state and federal agencies. Maize is an important commodity worldwide. The versatility and uses of this crop stresses the importance of screening for resistance. In this study, we evaluated two potential techniques to screen for resistance; molecular-based and analytical-based. Methods were adapted to rapidly and accurately detect and quantify the fungus in culture and in the vegetative stage of plant tissues in compared to the standard end of season kernel sampling. These assays can eliminate the time consuming microbiological techniques including cultivation and identifying the pathogen based on morphology.

Quantitative polymerase chain reaction (QPCR), a molecular-based assay, is utilized to quantify the amount of DNA using an oligonucleotide-specific, dual-labeled florescent probe (Logan et al 2009). QPCR is an accurate, rapid and sensitive method to detect and monitor plant pathogen occurrences and/or amounts of fungal biomass or colonization in plant tissues (Criseo et al 2008; Cruz and Buttner 2008; Clemons and Stevens 2009; Degola et al 2009; Faber et al 2009; Logan et al 2009).

Analytical-based techniques, such as solid-phase microextraction (SPME) utilizing gas spectrometry and mass spectrometry (GC-MS), are used to detect and quantify microbial volatile organic volatiles (MVOC's) produced from fungi (Korpi et al

2009). MVOC's are produced by fungi during both primary metabolism (the synthesis of DNA and amino and fatty acids) and secondary metabolism (the oxidation of glucose) (Korpi et al 2009). The SPME technique is inexpensive, simple, sensitive, solventless and portable for field applications.

The primary objectives of the study were to utilize 1) QPCR to detect and quantify fungal biomass in maize stem tissues to evaluate resistant and susceptible maize genotypes to *A. flavus* colonization in *in situ* and *in vivo* applications and 2) SPME GC-MS to identify key MVOC's to differentiate aflatoxigenic and non-aflatoxigenic strains of *A. flavus* in *in situ* use.

The aflatoxin biosynthetic pathway was examined and a specifically designed dual-labeled florescent probe (*OMG3*) was designed to detect a specific region within the *aflP* gene. The *OMG3* probe detected the aflatoxigenic strains that were positive for aflatoxin production used in this study. A standard curve was developed based on known DNA concentrations, and the curve could then be used to evaluate comparing resistance and susceptible genotypes.

The predominant MVOC's extracted by SPME GC-MS from both aflatoxigenic and non-aflatoxigenic strains were alcohols, ketones and hydrocarbons. The aflatoxigenic strain produced 39 compounds and the non-aflatoxigenic strain produced 41 compounds. Dimethylsulfide and 2-heptanol were key MVOC biomarkers were produced only by the aflatoxigenic strain of *A. flavus* and distinguished the two strains.

To further ensure the accuracy of the QPCR method and the *OMG3* probe, *in vivo* and *in situ* procedures were developed and refined. The toothpick inoculation method was used to artificially inoculate maize stems in the vegetative stage. This method was

100% consistent for infecting maize plants however inoculation must occur during the fifth vegetative (V5) stage of growth to avoid mechanical tissue damage. Physiological damage occurred when inoculations were made at V3, V4, and V5 stages and inoculation was not successful (0%) due to the apical meristem being below soil line. When inoculation was initiated in stages V2, V3, and V4, the toothpick would advance and perforate the dominant leaf throughout the growth of the plant.

Following development of inoculation procedures, the *OMG3* probe (QPCR) was further evaluated to ensure aflatoxigenic strains of *A. flavus* could be used to determine maize genotype resistance *in vivo* and *in situ* studies conducted in the greenhouse, growth chamber, and field. Lesion length, 57% of the greenhouse trials, showed greater measured lengths in resistant lines than in susceptible lines. Based on this data, subsequent research focused on refining tissue-sampling methods; sampling in 0.5 cm increments in length up to 3 cm or increasing length of plant growth time for tissue sampling post-inoculation. Some slight changes in sampling procedures may provide more useful data when evaluating maize genotypes.

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CHAPTER II

# THE USE OF A NOVEL TAQMAN PROBE FOR THE DETECTION OF AFLATOXIGENIC STRAINS OF *ASPERGILLUS FLAVUS*


## Abstract

The objective of this research was to refine and utilize *Aspergillus flavus* sequence-specific oligonucleotide primer sets by quantitative polymerase chain reaction (QPCR) and their respective TaqMan probes to identify, quantify and distinguish aflatoxigenic strains from non-aflatoxigenic strains of this fungal species. A total of 43 primers were designed and evaluated. Thirteen primers (13 of 43) were designed based on the Velvet and LaeA genes involved in conidia and sclerotia formation. Thirty primers (30 of 43) were designed from four gene targets involved in the synthesis of aflatoxin including the gene *afl*P (= *omt*A) that encodes for the enzyme *O*-methylytransferaseA. For DNA quantification data, a florescent TaqMan probe (*OMG3)* was designed to amplify a 199-basepair region within the *aflP* gene. Primer specificity was tested using genomic DNA from pure cultures of 52 *A. flavus* strains isolated from diverse regions and hosts. In addition, genomic DNA from 9 isolates of other maize pathogenic fungi such as *Aspergillus niger*, *Fusarium verticilliodes* and *Penicllium* spp. were included for comparison. The *OMG3* TaqMan probe amplified 98.3% of the aflatoxigenic *A. flavus* strains and 1.7% of the non-aflatoxigenic strains or ones of other related fungal genera and species used in this study. It was uncertain why one aflatoxigenic forming strain K73

would not amplify. Furthermore, these results confirm that the *OMG3* TaqMan probe has the potential to be used to screen maize for resistance to *A. flavus* based on the amount of fungal biomass within artificially inoculated maize ears or stems which still rely on determining kernel percent infection rates *in vitro*.

## Introduction

*Aspergillus flavus* Link ex. Fries*, Aspergillus nominus* Kutzman, Horn and Hesseltime and *Aspergillus parasiticus* Speare are fungi that are known to produce aflatoxins, and as such, belong to the aflatoxigenic subgroup of the *Aspergillus flavus* grouping (Diener et al 1987). The non-aflatoxigenic subgroup contains *Aspergillus sojae* Sakag. et K.Yamada ex Murak. and *Aspergillus tamari* Kita. which are commonly used in fermented foods. Aflatoxins are secondary metabolites by the aflatoxigenic subgroup and are highly carcinogenic, mutagenic and teratogenic (Leslie et al 2008). Exposure to either the pathogen or to aflatoxin can cause significant health problems in both humans and livestock (http://www.ansci.cornell.edu/plants/toxicagents/aflatoxin/aflatoxin.html 2003). Worldwide, many countries regulate exposure to aflatoxin in crops post-harvest. For example, the European Union limit is 4 ppb and the US limit is 20 ppb (Leslie et al 2008; Munkvold et al 2009). International trade and the US economy are greatly affected by aflatoxins. Aflatoxin related expenses are estimated to cost $1 to $1.5 billion per year in the US alone (Schmale and Munkvold et al 2009). Worldwide, many countries regulate exposure to aflatoxin in crops post-harvest. For example, the European Union limit is 4 ppb and the US limit is 20 ppb (Leslie et al 2008; Munkvold et al 2009).

Control of both the fungus and aflatoxin contamination is difficult in maize (*Zea mays* subsp. *mays* L) because of the mode of infection. Chemical control is not

economical or practical at this time. The only chemical control that may have any effect would need to be systemic (Abbas et al 2009). There is some promise with biological control using an aflatoxigenic strain of *A. flavus* and *A. parasiticus* to alter the fungal population structure in the field (Dorner et al 2003; Dorner et al 2004; Abbas et al 2006; Yin et al 2008). However, the most practical approach in reducing aflatoxin accumulation in maize is through the development of host resistance to infection by *A. flavus*. While some efforts are directed towards genetic engineering, most efforts are directed to screening each new line or genotype for field resistance by inoculating maize ears. Discrimination of host resistance is done after harvest by measuring and comparing the amount of aflatoxin accumulation per line or genotype. This method of screening is labor intensive and time consuming and much land acreage must be used. Because aflatoxin accumulation is dependent on environmental stressors primarily high temperatures, high humidity, and drought, aflatoxin content will vary from year to year (Widstrom et al 1981; Payne et al 1998).

Development of preharvest aflatoxin host resistance genotypes provides growers with an economic advantage by leaving no harmful chemical or biological residue in the environment, and the lines may be well-suited to reduce costs from other measures used for various plant pathogens and pests. Plants resistant to *A. flavus* will reduce infection rates and can eliminate the need to disinfect large quantities of aflatoxin-contaminated grain (Menkir et al 2006). Therefore, maize genotypes or lines must continue to be screened for resistance to aflatoxin accumulation and fungal colonization by *A. flavus* and other pathogens.

To screen for host resistance, both the pathogen and subsequent aflatoxin contamination must be accurately and efficiently detected and quantified using methods that are less time consuming and relatively inexpensive. To date, there are highly sensitive tests used to detect and/or quantify aflatoxins using analytical-based methods; thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) (Vicam, Watertown, MA) and gas chromatography mass spectrometry (GCMS) and a rapid field-based method using enzyme linked immune-sorbent assay (ELISA) (Diagnostic Automation / Cortez Diagnostics, Inc., Calabasas, CA). Other conventional methods can identify and quantify fungal biomass or fungal colonization with plant tissue including determining ergosterol content and immunological techniques (Schnurer 1992). Molecular-based assays, such as polymerase chain reaction (PCR) or quantitative polymerase reaction (QPCR) are also used (Schaad and Frederick et al 2002; Tooley et al 2006; Chilvers et al 2007; Mackay et al 2007; Logan et al 2008; Bell et al 2009). These newer assays can eliminate the time consuming microbiological techniques including cultivation and identifying the pathogen based on morphology. In addition, because there is no sample processing post-reaction, the risk of carryover contamination is eliminated (Mackay et al 2007; Logan et al 2008). Quantitative-PCR is a rapid way to detect and monitor plant pathogens (Geiser et al 1998; Niessen et al 2007). The method has also been used to 1) detect other species of *Aspergillus* in biological samples (Ramirez et al 2008; Cruciani et al 2009; Perlin and Zhao et al 2009), 2) to detect and quantify many different plant (Suanthie et al 2009) and animal pathogens and 3) differentiate between mycotoxigenic and non-mycotoxigenic strains of fungal pathogens in human, plant

material and soil (Farber et al 2003; Sugita et al 2004; Clemons and Stevens et al 2009; Luo et al 2009).

One of the most important uses of QPCR is the ability to quantitatively measure target DNA (Bustin et al 2004; Mackay et al 2007; Logan et al 2008). QPCR utilizes both PCR primers and one respective oligonucleotide probe that measures the amount of amplification product at every cycle of the reaction (Mackay et al 2007; Logan et al 2008). Oligonucleotide-specific primers are designed to bind to a specific DNA sequence that serves as a starting point for DNA synthesis and amplifies each DNA strand after each cycle. When used with QPCR, two different florescent dyes are used to measure the increase DNA PCR product of a specific to the target DNA PCR product (Logan et al 2008). When using TaqMan probes, the florescence is measured after each cycle of the PCR reaction (Bustin et al 2004; Logan et al 2008). A critical threshold (Ct) is produced only after the florescence surpasses a specific threshold (Logan et al 2008). At this point the Ct value is compared and plotted to a standard curve generated by known quantities of DNA; therefore calculating the concentration of the DNA in the unknown sample (Logan et al 2008), the pathogen DNA extracted from the host tissue.

A major drawback to PCR or QPCR is that the reaction can be adversely affected by PCR inhibitors found in the complex composition of plant material, so that the sensitivity is reduced relative to those found in pure cultures is reduced (Feng et al 2007). Primary PCR inhibitors found in plant material, including maize tissues, are lipids, acidic polysaccharides, and polyphenolic compounds. These compounds can make extraction of pathogen DNA difficult and are a major obstacle for efficient amplification in QPCR (Ma and Michailides et al 2007) and must be eliminated with the proper DNA extraction

method. There are various PCR inhibitor neutralizers used to overcome and decrease the abundance of the inhibitors in maize tissues to successfully use QPCR to detect pathogen DNA (Wilson et al 1997; Ma and Michailides et al 2007). However, further study of these methods must be conducted across additional genotypes to ensure consistent results.

To determine if a correlation exists between fungal infestation in the vegetative stage or the reproductive stage, the relationship between fungal biomass and aflatoxin accumulation must be evaluated. Several studies have examined this potential relationship between the growth of *A. flavus* (fungal biomass) and aflatoxin accumulation in kernels (Priyadarshini and Tulpule et al 1978; King and Wallin et al 1983; Windham and Williams et al 2007; Mideros et al 2009). One researcher found that the amount of toxin production is not consistent with fungal growth, suggesting that increases or decreases in growth of the fungus showed no correlation to toxin production (Priyadarshini and Tulpule et al 1978). Based on this study, the differences in the amount of toxin produced by *A. flavus* varied on selected kernels as compared to quantitative differences in fungal growth, which may be related to varying amounts of stimulatory and inhibitory factors in genotypes (Priyadarshini and Tulpule et al 1978). In a more recent study examining maize kernels, QPCR was used to amplify *A. flavus* DNA in maize kernels and found that fungal biomass was strongly correlated with aflatoxin concentration (Mideros et al 2009). Fungal biomass is a quantitative measure that may be used as an indicator of the potential for aflatoxin accumulation within maize tissues. The first PCR-based assay to detect *A. flavus* was by nested PCR in human bronchoalveolar lavages and was developed in 1993 (Tang et al 1993; Cruciani et al 2009). The primers were designed based on the gene coding for alkaline protease, not the

ability of the fungus to produce toxin. Later, PCR was used to evaluate the toxigenic capabilities and properties of *A. flavus* in plant material (Geisen et al 1996; Pater et al 1996; Niessen et al 2007). In the 2000's, the first PCR-based assays were developed for aflatoxigenic strains of *Aspergillus* (Niessen et al 2007). Both researchers designed primers based on the genes involved in the biosynthesis of aflatoxin; *afl*D (*nor*-1; norsolorinic acid), *afl*M (*ver*-1; versicolorin), and *afl*P (*omt*A; O-methyltransferase). Criseo et al (2008) used quadraplex PCR to differentiate 11 isolates of aflatoxigenic and non-aflatoxigenic of *A. flavus*, but were unable to classify all isolates. Mayer et al (2003) developed an assay to detect an aflatoxigenic strain of *A. flavus* in maize, pepper, and paprika using primers based on the *nor*-1 gene coding the norsolorinic acid reductase, the first gene in the aflatoxin biosynthetic pathway. Although Degola et al (2007) used a multiplex reverse transcription-polymerase chain reaction (RT-PCR) he was able to observe a correlation between gene expression specific gene targets and the specific enzymatic activities required for aflatoxin production. Gene targets and their respective enzymes included *afl*O and *afl*R (o-methyltransferase B), *afl*S (esterase), *afl*D (reductase) and *afl*Q (oxidoreductase) (Brown et al 1996; Degola et al 2007). Each are found to be required for aflatoxin production and are encoded in the gene cluster (Brown et al 1996; Degola et al 2007). Other potential gene targets that are being evaluated are the Velvet and LaeA genes (Calvo et al 2004; Amaike and Keller et al 2009; Georgianna et al 2010). Both genes are associated with aflatoxin production related to the formation of conidia, sclerotia and cleistothecia (Calvo et al 2004; Amaike and Keller 2009; Georgianna et al 2010). Our research evaluates the use of QPCR and a sequence specific oligonucleotide primer set and TaqMan probe to amplify a 199 bp region of within the *aflP* or *O*-

methylytransferaseA gene. Sterigmatocystin (ST) is converted to O-methylsterigmatocystin (OMST) by O-methyltransferase (*omtA* protein) and is involved in a subsequent stage of aflatoxin formation (Yu et al 1993). Research conducted by Lee et al (2002) shows that in order for the conversion from ST to OMST to be efficient, O-methyltransferase is necessary. Their data clearly shows that the conversion is needed for aflatoxin biosynthesis (Lee et al 2002). O-methyltransferase was found in both vegetative hyphae and conidiophores using immunolabeling and confocal laser scanning microscopy (Lee et al 2002). Based on previous research investigating fungal morphology and pathogenicity and subsequent aflatoxin formation and accumulation; the *aflP* gene was a good candidate for development of a specific TaqMan probe to distinguish aflatoxigenic and non-aflatoxigenic strains of *A. flavus.*

The objective of this study was to evaluate the use of QPCR to identify, quantify, and distinguish aflatoxigenic strains from non-aflatoxigenic strains of *A. flavus.* Previously designed oligonucleotide primers were evaluated and a dual-labeled florescent TaqMan probe was developed. This research has the potential to utilize molecular methods to screen for resistance to *A. flavus* based on the amount of fungal biomass or colonization within artificially inoculated plants.

## Materials and Methods

### Fungal strains

A total of 51 strains of *A. flavus,* including aflatoxigenic and non-aflatoxigenic strains and 8 different isolates of other phyla of fungi were evaluated (Table 1). In addition, other closely related species and phyla were evaluated for comparison and served as negative non-target control. All fungal strains were obtained and confirmed

aflatoxigenic and non-aflatoxigenic from the following; H.K. Abbas, United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Stoneville, MS; R.E. Baird, Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Starkville, MS; USDA-ARS Culture Collection, Peoria, IL.; and G.L.Windham, USDA-ARS-Corn Host Plant Resistance Research Unit, Starkville, MS; USDA-ARS Culture Collection, Peoria, IL, (Scott and Zummo 1988; Baird et al 2006; and Baird et al 2008). Each fungal strain was sub-cultured onto 60 x 15 mm Petri plates of Czapeks Dox agar (CZP) (Fisher Scientific, Pittsburg, PA) or Potato Dextrose agar (Fisher Scientific, Pittsburg, PA) every 14 days and stored in the dark at room temperature (20 – 24°C).

**Genomic DNA extraction**

Fungal strains from culture

Each strain listed in Table 1 was harvested after 30 days as follows; 1) 60 x 15 mm Petri plate of each strain was flooded with approximately 2 ml of 0.02% Tween (Fisher Scientific, Pittsburg, PA) solution, 2) conidia and mycelia were scraped with a sterile glass rod, 3) resulting liquid was poured and filtered through 2 layers of sterile cheesecloth placed over a 50 ml Falcon centrifuge tube (Fisher Scientific, Pittsburg, PA), and 4) the resulting filtrate (spore suspension) was diluted to 2 x $10^6$ spores / ml using a hymacytometer and used for DNA extraction previously designed Melo et al (2006). The DNA final elution was 12 µl and 2 µl was used to determine fungal DNA concentration with a NanoDrop® ND-1000 spectrophotometer (Nano-drop Technologies, Wilmington, USA). Samples were evaluated only if the quality of DNA was above 1.7 (Absorbance

$_{260}$/Absorbance $_{280}$ ratio). All DNA extractions prior to PCR evaluations were stored in -80°C freezer in 1.5 ml microfuge tubes (Fisher Scientific, Pittsburg, PA).

Maize plant tissue

For preliminary confirmation of the primers with designed probes, maize tissues were evaluated to determine if PCR inhibition would occur using the selected primers and probes for further evaluation. A total of 40 tissues pieces stored at -80°C were obtained from a previous study (Baird unpubl. data) and tested. Of the 40 stored samples, 22 inoculated pieces (verified by cultural identifications) containing *A. flavus* (NNRL 3357) and 18 control pieces were used.

The CTAB (hexadecyltrimethylammonium bromide EDTA ethylenediaminetetraacetic acid) DNA extraction method was used (Cubero et al 1988). DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nano-drop Technologies, Wilmington, USA). Samples were evaluated only if the quality of DNA was above 1.7 (Absorbance $_{260}$/Absorbance $_{280}$ ratio).

**Quantitative PCR**

All available sequences from three *A. flavus* strains listed below were obtained and compared from 1) the *Aspergillus flavus* Genome Browser Version 1.7 available through *Aspergillus flavus* Genome Browser Version 1.7 (aspergillus.org 2006) and 2) the Basic Local Alignment Search Tool algorithm (BLAST, National Center for Biotechnology Information, National Institutes of Health) (Altschul et al 1990). All sequences available from strains used in this study were 1) *A. flavus* strain NRRL 3357 (AB000532) that encodes for the enzyme *O*-methylytransferaseA (*omt*A protein) in the

aflatoxin biosynthetic pathway, 2) aflatoxigenic strain NRRL 21882 (HQ856223.1 and AY974341.1), and 3) for the *A. flavus aflP* gene (AAS90018.1 and AAS90087.1) were obtained. It is important to note that all 51 strains of *A. flavus* strains used in this study have been confirmed with internal transcribed spacer (ITS) sequences of ribosomal DNA (Wang et al 2012).

**QPCR primers and probes**

Primer testing

Preliminary screening included designing and testing 43 sequence-specific oligonucleotide primers using end-point PCR and 5 florescent oligonucleotide TaqMan probes utilizing QPCR. Out of the 43 primers designed and tested, 31 primers were designed based on the specific gene targets *afl*M *afl*O, *aflP*, *afl*R found within the aflatoxin biosynthetic pathway (Yu et al 2004; Georgianna et al 2010; Levin 2012). The remaining 12 primers were based on genes involved in the formation of sclerotia and conidia, LeA and Velvet genes (Calvo et al 2004; Amaike et al 2009). After primers *OG1* and *OG2* were evaluated by end-point PCR, 5 different oligonucleotide-specific dual-labeled florescent TaqMan probes were designed based on the 199 bp fragment amplified by primers *OG*; florophore FAM was the quencher and florophore BHQ3 was the reporter. Each dual-labeled QPCR probe was screened using the optimized amplification parameters for *OG*; annealing temperatures 61 + / - 4°C based on the melting temperature of each OG primer. The TaqMan probes were screened and were rejected if no amplification occurred.

Primer sequences

Oligonucleotide primers and probe are as follows; *OG1*: forward primer: 5' – GCC TCA AAG ATG TTG CGA GT – 3' (melting temperature or Tm) 55.7°C) and, *OG2*: reverse primer: 5' – GGT GAA GGC ACA TTC CAA CT – 3' (Tm 55.6°C) (Geiser et al 1998) and the newly designed *OMG3* TaqMan probe: 5' - FAM – TGT CCT CAC CAG GGA GAC CG – 3'BHQ-3 (Tm 61°C). The forward and reverse primer and the *OMG3* fluorescent Taqman probe were optimized using IDT (Integrated DNA Technology, Centennial, CO) Primer Quest Analysis and Design Tool (https://www.idtdna.com/pages/scitools 2013; accessed 2009).

The oligonucleotide primers and the dual labeled florescent TaqMan probe by published by (Leinberger et al 2005) were based on the 5.8 rRNA, 28S rRNA, and ITS2 target sequences. A 249-bp amplicon was amplified utilizing both end-point PCR and QPCR using the primer set and Black Hole Quencher (BHQ2) probe as follows; *Asp1S* forward primer: 5' - ATG CCT GTC CGA GCG T – 3'(Tm 57.1°C), *AflR2* reverse primer: 5' - TTA AGT TCA GCG GGT ATR CC – 3' (Tm 55.1°C) and, the *ASP2* TaqMan probe: 5' – TAM – CGC TTG CCG AAC GCA AAT CAA TCT T – 3'BHQ-2 (Tm 60.8°C).

QPCR thermocycling parameters:

Quantitative PCR parameters were optimized based on previous experience and published research (Niessen 2007; Cruz and Buttner 2008; Mideros et al 2009). Quantitative PCR thermocycling considerations were adapted and optimized using the Cephied SmartCycler system (Sunnyvale, CA). Quantitative PCR reactions of 100 µl contained 50 µl of 2X Applied Biosystems (Grand Island, NY) Universal PCR Master

Mix, 18 µl of 5 µM forward primer (Integrated DNA Technology, Centennial, CO), 18 µl of 5 µM reverse primer (Integrated DNA Technology, Centennial, CO), 13 µl of 2 µM TaqMan probe (Integrated DNA Technology, Centennial, CO), and 5 µl of DNA sample. Critical and optimized PCR cycling parameters were as follows: initial denaturation for 12 minutes at 96°C; 40 cycles of 95°C for 30 seconds, 59.5°C for 1 minute, and 72°C for 30 seconds, with a final extension of 10 minutes. Quantitative PCR products were randomly selected during each run and were separated on 1% agarose ethidium bromide gels in 1X TBE buffer confirm amplification. The 1000 bp DNA ladder (New England Biolabs, Ipswich, MA) was used as the molecular size marker.

**Standard curve**

Two standard curves were generated based on known concentrations of fungal genomic DNA. Fungal genomic DNA was extracted from pure cultures of NRRL 3357 (aflatoxigenic) and NRRL 21882 (non-aflatoxigenic) and was diluted to 10, 5, 1, 0.1 and 0.01 ng / µl. Critical threshold (Ct) values were plotted against log-transformed known amounts of DNA and linear regression equations were calculated for the standard curve. The DNA from both strains were replicate four times using the five dilutions and with each standard represented three times. Amplification efficiencies were calculated from the slopes of the standard curves (Bustin 2004; Mackay 2007; Logan et al 2008). Serial dilutions of fungal DNA's were used to define QPCR detection limits (Bustin 2004; Mackay 2007; Logan et al 2008). All Ct values and amplification statistics are reported by Cehpied Smartcycler software Version 2.01 as the mean Ct value of two replicates in all experiments conducted in this study. All 59 fungal strains were evaluated using the two florescent TaqMan probes, *ASP2* and *OMG3* separately and plotted on the two

generated standard curves; NRRL 3357 (aflatoxigenic) and NRRL 21882 (non-aflatoxigenic). Quantitative PCR runs were replicated twice.

## Results

Results were inconsistent and amplification was unpredictable following evaluation of the 41 primers. However, the 51 *A. flavus* and the *A. niger* strain were amplified by two of the *aflR* gene-specific primer pairs. Results amplifying sequences within the Velvet and the LaeA genes were inconsistent and amplified 10 to 12 aflatoxigenic *A. flavus* strains and 7 to 12 strains of non-aflatoxigenic strains at any given PCR run. The toxigenic *Penicillium* strain was amplified once with the LaeA primers. The VeA primers did not amplify any of the other fungi from other groups listed in Table 1. Furthermore, maize tissue sampling, from stored tissues (Baird unpubl. data) inoculated with *A. flavus* strains, showed that 100% of *A. flavus* toxigenic isolates were positive using QPCR.

Two primers (*OG1* and *OG2*) out of the total forty-three were successful and consistently amplified 98.3% of the aflatoxigenic strains of *A. flavus.* These were used in this study and were designed by Geiser et al (1998). The dual-labeled florescent TaqMan probe designed (*OMG3*) was based on the oligonucleotide primers designed by Geiser et al (1998) and the ASP2 TaqMan probe (Cruz and Butner 2008 from Leinberger et al 2005) were evaluated. Amplification of both the *OMG3* - 199 bp only occurred by using an increase of both time and temperature in the initial denaturation (12 min and 96°C), an extension of 72°C for 10 min, and most importantly, the exact annealing temperature of 59.5°C in all strains and in artificially inoculated plant tissues. Using the *OMG3* probe and these precise parameters, the QPCR reaction was successful and amplified 98.3% of

the aflatoxigenic strains and 1.7% of the non-aflatoxigenic strains of *A. flavus,* excluding strain K73, or other closely related fungi listed in Table 1. The QPCR results showed that the range of Ct values from the *OMG3* probe (aflatoxigenic strains) were between 18.13 and 24.2. Gel electrophoresis confirmed amplification of a 199 bp fragment for randomly chosen samples.

The *ASP2* TaqMan probe was evaluated. All 51 strains of *A. flavus* (both aflatoxigenic and non-aflatoxigenic) were detected with the *ASP2* TaqMan probe. *Aspergillus candidus* Link., *Aspergillus niger* van Tieghem, *Aspergillus parasiticus* Speare*, Fusarium verticillioides* (Sacc.) Nirenberg (= *Fusarium moniliforme* Sheldon)*,* and the *Penicillium* strains were not detected with the *ASP2* probe. The *ASP2* probe detected 89% (2743 of 3082 samples) of the artificially inoculated plant tissues and 27% (865 of 3205 samples) from the control non-inoculated tissues. The QPCR Ct values were between 34.25 and 38.18 for all 51 *Aspergillus* strains. Ct values for inoculated plant tissues were slightly higher, 35.54 and 38.49. Gel electrophoresis confirmed amplification of a 249 bp fragment for randomly chosen samples. However strain K73 showed a predictable Ct value range between 27.94 and 30.26; above the Ct value range for aflatoxigenic-positive amplification (18.13 to 24.2) and below the Ct value for negative amplification (34.25). All QPCR amplification positive or negative results are listed in Table 1 and are referred to as *OMG3* and *ASP2*.

**Standard curves**

Two standard curves were generated; the *OMG3* probe generated from the aflatoxigenic *A. flavus* strain NRRL 3357 and the *ASP2* probe generated from the non-aflatoxigenic strain NRRL 21882. The QPCR data indicated that the log-transformed Ct

values correlated with the five known DNA standards (10, 5, 1, 0.1 and 0.01 ng) (Figure 1). The linear regression models are 1) y = -3.3226 x + 33.6144, $R^2$ = 0.9832 for the *OMG3* probe and 2) y = -3.4828 x + 33.8095, $R^2$ = 0.9926 for the *ASP2* probe.

## Discussion

Aflatoxins are the most predominant mycotoxins produced by the *A. flavus* group. To date, there are as many as 30 genes, over 15 intermediate precursors and approximately 20 corresponding enzymatic reactions involved in the synthesis of aflatoxins (Levin 2012). The combination of these represents the entire aflatoxin biosynthetic pathway (Yu et al 2004). Of these, the *aflP* or *O*-methylytransferaseA gene has been studied extensively (Yu et al 2004). The *omtA* gene is involved in converting sterigmatocystin (ST) to O-methylsterigmatocystin (OMST) and occurs during in the later stage of aflatoxin formation (Yu et al 1993). This gene was of interest because in a study by Yu et al (1993), OMST was expressed in *Escherichia coli* when ST was converted to OMST when testing different feeding substrates. Furthermore, other data clearly shows that the ST to OMST conversion is needed for aflatoxin biosynthesis (Brown et al 1996; Lee et al 2002). The latter study provides evidence that the *O*-methylytransferaseA enzyme may migrate with the cytoplasm and organelles from the older to younger cells (Lee et al 2002). The *omt*A protein was found within both vegetative hyphae and conidiophores therefore it is plausible the protein is present within the cytoplasm (Lee et al 2002).

Because aflatoxin production remains a variable trait, distinguishing a clear difference between aflatoxin-producing and non-producing isolates or strains of *A. flavus* can be a challenge (Geiser et al 1998). It is likely that exposure to plant fungal pathogens

may result in low or high levels of gene expression, including the *aflP* gene (Georgianna et al 2010). The gene expression, either low or high, will most likely have a role in the response of the host and / or the fungal pathogen (Georgianna et al 2010). In this work, a highly specific molecular protocol utilizing QPCR was optimized to distinguish the two *A. flavus* strain-types based on the expression of *omt*A and the production of OMST. Optimization appeared to be dependent on the annealing temperature of 59.5ºC when using the newly developed *OMG3* TaqMan probe.

Various thermocycling parameters were tested using the *OMG3* probe, specifically annealing temperature. Amplification of the 199 bp amplicon (*OMG3*) would only occur for 98.3% of the aflatoxigenic strains of *A. flavus* when using an annealing temperature of 59.5°C. Primer or probe mismatch occurred when either increasing or decreasing the stringency of the annealing temperature, even by 0.5°C. It is plausible that with this temperature specificity, the slight mutations or mismatches were avoided (Bustin 2004; Mackay 2007; Logan et al 2008).

In regards to other fungal species, the toxigenic-producing *Pencillium* strain was not detected with either *OMG3* or *ASP3* probe. This particular *Penicillium* strain and some aflatoxigenic strains of *A. flavus* produce the mycotoxin cyclopiazonic acid (CPA). CPA production occurs in a variety of environmental conditions and is at times associated with aflatoxin synthesis (Georgianna 2010). These results provide further evidence that the *OMG3* probe is specific to aflatoxigenic strains of *A. flavus.* Interestingly, the Ct value range for the aflatoxigenic *A. flavus* strain K73 (Baird et al 2012) using the *OMG3* probe was between 28 and 30 which was between Ct value range for aflatoxigenic-positive amplification (Ct 18 to 24) and below the Ct value for

aflatoxigenic-negative amplification (Ct 34). Data generated by unweighted pair group method with arithmetic mean (UPGMA) cluster analysis shows that strain K73 isolated from maize tissue in Mississippi is clustered with both aflatoxigenic and non-aflatoxigenic strains (Wang et al 2012). Low or high levels of *aflP* gene expression may explain the predictable amplification and Ct range.

It is important to note that the entire aflatoxin biosynthetic gene cluster is present within the aflatoxigenic strain NRRL 3357 and is not present within the non-aflatoxigenic strain NRRL 21882. NRRL 3357 is used for all inoculations and the standard curve for the *OG* primer set and *OMG3* probe are based on this strain. Since the gene cluster is not present in the NRRL 21882 strain, it was used to provide evidence that non-producing aflatoxin strains and other fungal taxa representing different phyla could be differentiated from the aflatoxin-producing strains. K73 may have a section of the *alfP* gene missing, or expression was low, therefore the strain could be amplified by *OMG3* probe, but the Ct value was between the positive and negative range.

Since the *aflP* gene is a precursor to aflatoxin synthesis, the gene was a suitable candidate for the work and for distinguishing the two strain-types. Furthermore, when examining the parameters acquired from both standard curves, the high $R^2$ coefficients indicate that QPCR assays are efficient, reproducible and robust. Adaptation of this technique, especially utilizing the *OMG3* primer and probe set outlined in this study, would provide rapid results and minimize the risk of contamination when analyzing artificially inoculated plants, *in vivo* or *in situ.*

## Conclusion

In summary, a highly specific protocol was developed to detect and quantify aflatoxigenic strains of *A. flavus* in culture and plant tissue. In this study, 51 strains of *A. flavus* and 8 other fungal taxa representing different phyla, were screened with two sets of oligonucleotide primers and their respective florescent TaqMan probes; 1) oligonucleotide primer set (*OG1* and *OG2*) based on the *aflP* gene that encodes for the enzyme *O*-methylytransferaseA (*omt*A) (Geiser et al 1998) and a novel sequence-specific, dual-labeled florescent TaqMan probe (*OMG3*), and 2) tested oligonucleotide primer set (*Asp*) based on the 5.8 rRNA, 28S rRNA, and ITS2 target sequences (Leinberger et al 2005) and the sequence-specific, dual-labeled florescent TaqMan probe (*ASP2*) to confirm amplification of *Aspergillus* spp., both aflatoxigenic and non-aflatoxigenic. The *OMG3* TaqMan probe was specific and amplified 98.3% of the aflatoxigenic *A. flavus* strains, 100% of inoculated plant tissues (Baird unpubl. data) and 1.7% of the non-aflatoxigenic strains or other closely related fungi. The *ASP2* TaqMan probe successfully amplified the 249 bp fragment of all *Aspergillus* strains, both aflatoxigenic and non-aflatoxigenic and in plant tissues. Results from this study may lead to the application of a rapid and sensitive QPCR protocol to use in further *in situ* and *in vivo* studies in maize resistance evaluations. These data demonstrate that QPCR is an effective tool for detecting and quantifying fungal biomass in both culture and plant tissue; therefore these results would be useful to study the relative resistance of maize varieties to the colonization of aflatoxigenic strains of *A. flavus*.

Table 2.1 Fungal strains screened with specific primer and probe sets.

| STRAIN | SPECIES | HOST / SUBSTRATE | ORIGIN | MYCOTOXIN PRODUCTION[u] | Probe[w] ASP2 | Probe[x] OMG3 |
|---|---|---|---|---|---|---|
| 66 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 67 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 97 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 105 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 132 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 199 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 203 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 213 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 228 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 233 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 312 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 318 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 342 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 352 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 355 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 356 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |

Table 2.1 (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| 359 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 364 | *Aspergillus flavus* | Soil | Sunflower County, MS | - | + | - |
| 508 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 509 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 519 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 528 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 531 | *Aspergillus flavus* | Soil | Sunflower County, MS | + | + | + |
| 604 | *Aspergillus flavus* | Soil | Washington County, MS | - | + | - |
| NRRL 3357[y] | *Aspergillus flavus* | Peanut | Washington County, MS | + | + | + |
| 5565 | *Aspergillus flavus* | Muddy Turkey Foot | Washington County, MS | - | + | - |
| 5941 | *Aspergillus flavus* | Peanut | College Station, TX | - | + | - |
| NRRL 21882[z] | *Aspergillus flavus* | Unknown | Unknown | - | + | - |
| CR1 | *Aspergillus flavus* | Soil | Washington County, MS | + | + | + |
| CR26 | *Aspergillus flavus* | Soil | Washington County, MS | - | + | - |
| CR29 | *Aspergillus flavus* | Soil | Washington County, MS | - | + | - |
| CR3 | *Aspergillus flavus* | Soil | Washington County, MS | + | + | + |
| CR5 | *Aspergillus flavus* | Soil | Washington County, MS | + | + | + |
| K100 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |

Table 2.1 (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| K115 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K116 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K133 | *Aspergillus flavus* | Maize | Unkown | - | + | - |
| K134 | *Aspergillus flavus* | Maize | Sunflower County, MS | - | + | - |
| K17 | *Aspergillus flavus* | Maize | Sunflower County, MS | - | + | - |
| K21 | *Aspergillus flavus* | Maize | Unkown | - | + | - |
| K32 | *Aspergillus flavus* | Maize | Sunflower County, MS | - | + | - |
| K54 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K55 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K61 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K63 | *Aspergillus flavus* | Maize | Sunflower County, MS | - | + | - |
| K66 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K72 | *Aspergillus flavus* | Maize | Unkown | + | + | + |
| K73 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | +/-* |
| K88 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K89 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| K94 | *Aspergillus flavus* | Maize | Sunflower County, MS | + | + | + |
| 2129 | *Aspergillus candidus* | Unkown | CSIRO Coll. FRR 1224 | - | - | - |

Table 2.1 (continued)

| | | | | | | |
|---|---|---|---|---|---|---|
| 2378 | *Aspergillus niger* | Unkown | Neotype IMI 50566 | NA | - | - |
| 143-A | *Aspergillus parasiticus* | Peanut | Uganda (SU-1) | + | - | - |
| F13 | *Fusarium verticillioides* | Unkown | Mississippi | + | - | - |
| NRRL 20956 | *Fusarium verticillioides* | Unkown | Unkown | + | - | - |
| NRRL 22050 | *Fusarium verticillioides* | Unkown | Unkown | - | - | - |
| PEN | *Penicillium* sp. | Soil | Unkown | - | - | - |
| 213-A | *Penicillium* sp. | Unkown | Unkown | + | - | - |

[U]Aflatoxin, fumonisin (*Fusarium verticillioides*) or CPA (produced by *Penicillium* sp. in this report) are toxins produced by fungal species represented in study. [W]Specific Probe for species of *Aspergillus flavus* 249 bp fragment). [X]Specific Probe for production of *OmtA* and aflatoxin production (199 bp fragment). + Indicates positive amplification and – indicates negative amplification based on previous studies or QPCR amplication. [Y]A new sub-culture of 3357 was obtained each year from G.L. Windham, USDA-ARS-CHPRRU Mississippi State University, Mississippi State, MS and is used in resistance studies by USDA personnel (Scott and Zummo 1988). [Z]NRRL 21882 strain was obtained from G.L. Windham, USDA-ARS-CHPRRU, Mississippi State University, Mississippi *Penicillicium* sp (PEN) was collected by R.E. Baird, Dept BCH/EPP, Mississippi State University, Starkville, MS. Remaining isolates or strains were obtained from USDA-ARS Culture Collection, Peoria, IL. *Strain K73 Ct value range was between 26.94 and 28.26, above the Ct value range for positive amplification and below the Ct value for negative amplification.

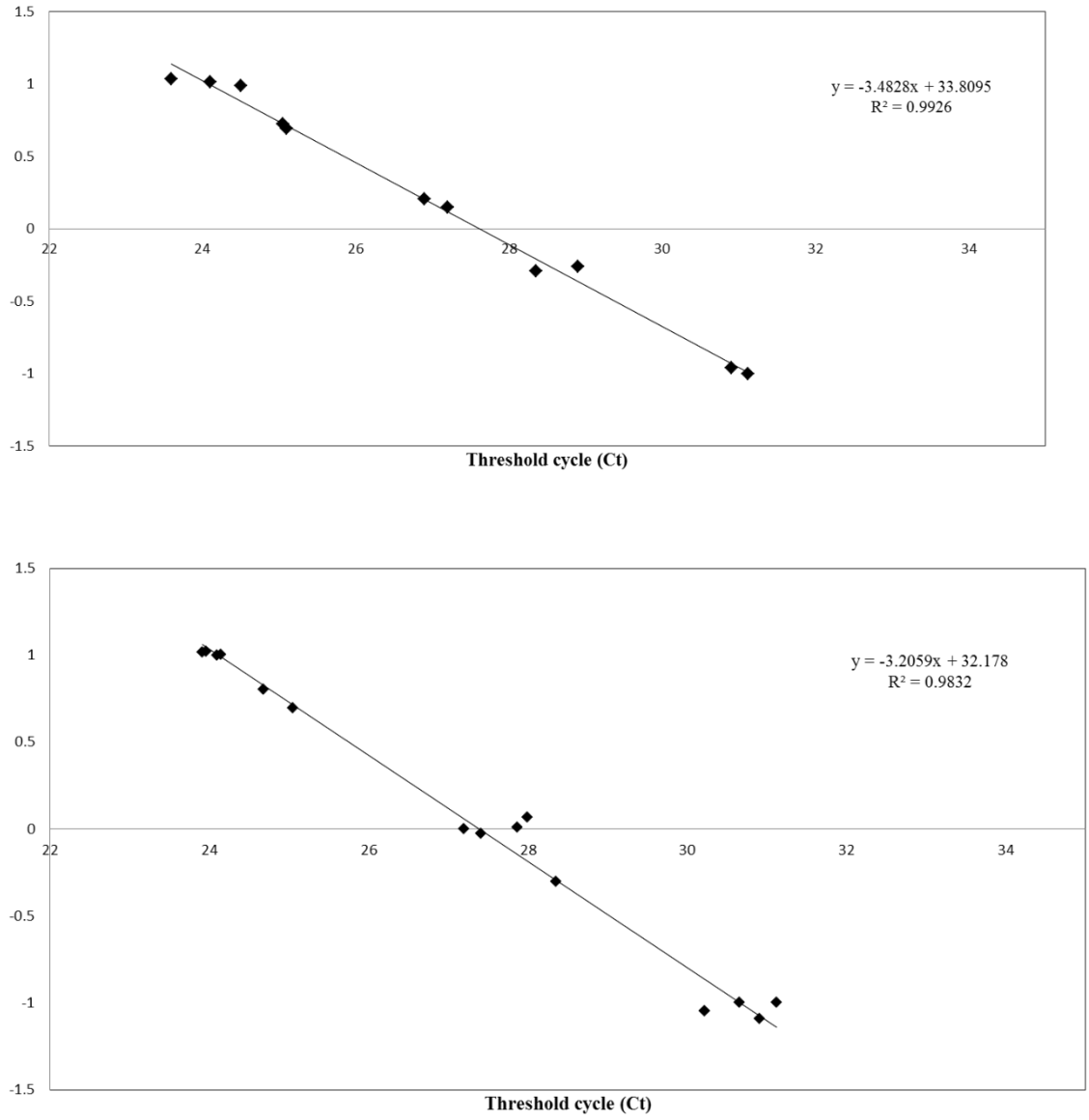


Figure 2.1 Standard curves derived from QPCR.

Standard curves were derived from QPCR for (a) aflatoxigenic-specific using the aflatoxigenic strain NRRL 3357 and the *OMG3* probe and, (b) *A. flavus* DNA using the non-aflatoxigenic strain NRRL 21882 and the *ASP2* probe. The Ct values were plotted against log-transformed known amounts of DNA and linear regression equations were calculated for the standard curve. Both standard curves were run four times with each standard represented three times. The linear regression model for *A. flavus*; *aflP* gene (*OMG3* probe); y = -3.4828x + 33.8095, $R^2$ = 0.9926 and the linear regression model for *ASP2* probe; y = -3.2059x + 32.178, $R^2$ = 0.9832.

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# CHAPTER III

# DISCRIMINATION OF AFLATOXIGENIC AND NON-AFLATOXIGENIC STRAINS OF *ASPERGILLUS FLAVUS* BASED ON VOLATILE METABOLIC PROFILES USING SPME GCMS AND MULTIVARIATE ANALYSIS


## Abstract

Fungi produce a variety of microbial volatile organic compounds (MVOCs), during primary and secondary metabolism. The fungus, *Aspergillus flavus*, is a human, animal and plant pathogen, and more importantly, produces aflatoxin, one of the most carcinogenic substances known. Specific MVOCs identified using solid phase microextraction (SPME) combined with GCMS, may serve as biomarkers to distinguish between strains of *A. flavus*. In this study, MVOCs were extracted from two genetically different *A. flavus* strains. The aflatoxigenic, NRRL 3357, and non-aflatoxigenic, NRRL 21882 strains using a PDMS/CAR SPME fiber over 30 days to observe any significant variation of MVOCs over time. Fisher's linear discriminant analysis, a multivariate analysis method, was successfully used to compare the two strains with MVOC functional group data. This study underscores the potential feasibility of using SPME GCMS coupled with multivariate analysis for early aflatoxigenic and non-aflatoxigenic fungi discrimination prior to the onset of significant aflatoxin production.

## Introduction

Aflatoxins are polyketide-derived, secondary fungal metabolites and only three *Aspergillus* species, *Aspergillus flavus* (Diener et al 1987), *Aspergillus nominus* (Kurtzman et al 1987) and *Aspergillus parasiticus* (Yu et al 1995), are known to produce these naturally carcinogenic compounds (Gourama and Bullerman 1995). The economic impact is immense because mycotoxin contamination is estimated to affect one quarter of the world's food crops (CAST 2003) including maize, cotton and peanuts (Gourama and Bullerman 1995). Crop losses are estimated to cost between $1 and $1.5 billion/year in the United States (Wu and Guclu 2012). These losses do not account for livestock losses or the impact on human health or healthcare costs from exposure to the fungi or to the toxins. The FDA has set limits of 20 ppb total aflatoxins for interstate commerce of food and 0.5 ppb of aflatoxin $M_1$ for milk (Bhatnagar et al 2006). Today many countries regulate acceptable aflatoxin levels in order to protect human and livestock populations. Many methods have been proposed and are in development for the detection of aflatoxins or *A. flavus* including those that identify the presence of the toxin and those that identify the fungus.

Conventional analytical methods being used for aflatoxin detection are high-performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GCMS), enzyme linked immune-sorbent assay (ELISA) and multiplex polymerase chain reaction (multiplex PCR) (Turner et al 2009). These methods can be sensitive, inexpensive and give both qualitative and quantitative measurement of aflatoxins, however, initial enrichment, interference or inhibitor removal is generally required for detection and quantification.

Common molecular identification methods for fungi include fluorescence *in situ* hybridization, DNA array hybridization, and multiplex tandem PCR (Tsui et al 2011). However, to our knowledge, there are no known aflatoxigenic-specific PCR primers that are able to successfully differentiate aflatoxigenic and non-aflatoxigenic strains. This is an obvious inconvenience in many industrial applications, particularly in the field of maintaining food safety in crops destined for livestock and human consumption. Thus there is an urgent need for a practical, rapid and cost-effective strategy to identify the presence of aflatoxin-producing fungi.

The method described here focuses on identification and quantification of microbial volatile organic compounds fungal (MVOCs). The major source of MVOCs produced by organisms such as fungi and bacteria, are from primary (synthesis of DNA, amino and fatty acids) and secondary (oxidation of glucose) metabolism (Korpi et al 2009). For several years, there has been a continuous interest in identifying characteristic "fingerprints" of MVOCs produced by fungal and bacteria species that can be used as unique identifiers. Some MVOCs, such as 3-methyl-1-butanol, 1-octen-3-ol, 3-octanone and sesquiterpenes have been proposed as indicators for most fungal species (Keshri et al 1998; Schnürer et al 1999; Sunesson et al 1995). Nilsson et al (1996) reported some unique biomarkers (1-octen-3-ol, 3-octanol and several sesquiterpenes) emitted from *Penicillium* spp.. *Aspergillus flavus* is known to produce strain-specific volatiles such as 3-methylbutanol, 2-methyl-1-propanol, hexanol, *trans*-caryophyllene, nonanal and naphthalene (Jurjevic et al 2009). Moreover, several studies have demonstrated that fungal species produce a unique pattern or grouping of MVOCs that can also be used for species identification (Schleibinger et al 2005). Cluster analysis (CA), principle

component analysis (PCA) in 2 or 3 dimensional space, and linear discriminant analysis (LDA) have utilized MVOC data to discriminate bacteria at either the species or strain level (Bianchi et al 2009; Thorn et al 2011). This is known as chemotaxonomy, which is applied to classify and identify organisms based on distinguishable biochemical composition of microorganisms (Polizzi et al 2012b). This field is becoming more and more important for discrimination and identification of fungal species (Frisvad et al 2011).

Techniques that involve solid phase vapor collection followed by thermal desorption are widely applied techniques used in MVOC analysis. Thermal desorption tubes have been used for field sample collection followed by transportation to a lab for analysis (Jurjevic et al 2009; Larsen and Frisvad 1995). Solid phase microextraction (SPME) has recently been used to collect and concentrate MVOCs from fungi and bacteria (Demyttenaere et al 2003). This technique has the potential to be part of an efficient method for field applications due to its portability and simplicity. The application of SPME in conjunction with GCMS has been successfully applied to the detection of indoor mold (Lavine et al 2012; Vishwanath et al 2011), fungal species identification (Drew et al 2012; Gioacchini et al 2005), and the diagnosis of foodborne pathogen infection (Siripatrawan 2008; Siripatrawan and Harte 2007).

The focus of this study was 1) to identify individual or groups of MVOC biomarkers that can be applied to differentiate the aflatoxigenic and non-aflatoxigenic strains of *A. flavus*, 2) to monitor fungal volatile profiles over time (30 days), and 3) to develop a method for discriminating aflatoxigenic and non-aflatoxigenic *A.flavus*. To our knowledge, multivariate analysis has not been used to differentiate between aflatoxigenic

or non-aflatoxigenic strains of *A. flavus*. The general methods represented in this study can be applied to identify other strains and species of fungi using headspace solid phase microextraction GCMS (HS-SPME-GCMS).

## Materials and standards

### Chemical standards

Twenty-six reference chemical standards were purchased from several suppliers: 2-heptanone (99%), 2-heptanol (98%), hexanal (≥ 97%), 2-methyl-1-butanol (≥ 99%), 3-methyl-1-butanol (98%), 2-nonanone (≥ 99%), 2-pentanol (98%), isovaleraldehyde (97%), 3-octanone (≥ 98%), 2-pentylfuran (≥ 97%), 2-undecanone (98%), 2-nonanol (99%), 1-octen-3-ol (98%), 2-methylbutyric acid (98%), methyl isobutyrate (99%), 1,2,4,5- tetramethylbenzene (98%), 2-octanone (98%), ethyl acetate (HPLC grade ≥99.7%), 2-heptanone (99%), octane (98%) and ethyl isobutyrate (99%), Fluka Analytical standards, ethyl isovalerate, ethyl butyrate and ethyl proionate were from Sigma-Aldrich (St. Louis, MO). Pentane (98%) was obtained from Alfa Aesar (Ward Hill, MA).

### Fungal sample preparation

The aflatoxigenic strain NRRL 3357 (L-strain; http://www.aspergillusflavus.org/) and NRRL 21882 were provided by the United States Department of Agriculture-Agricultural Research Service, Corn Host Plant Resistance Research Unit, Mississippi State University, Starkville, MS (USDA-ARS-CHPRRU), Mississippi State University, MS. Both fungal strains were cultured onto potato dextrose agar (Difco, Sparks, MD), which was prepared by dissolving 39 g of the powder in 1L of purified water and

autoclaving at 121°C for 15 minutes. The fungal spores were extracted using 0.02% Tween 20 solution and diluted with distilled water to $2\times10^6$ spores/ml for inoculation. 2% corn media were obtained through mixing 0.6 g corn grit (Martha White Yellow Corn Meal, Jackson, Tennessee) with 28 ml distilled water and stored in sterile 40-ml glass headspace vials covered with a polypropylene screw cap and PTFE/silicone septum (Sigma-Aldrich, St. Louis, MO). This basal medium was chosen based on preliminary studies performed in this laboratory and studies performed by Demain et al (1986). Growth took placed in 30 ml 2% corn grit liquid media in sterile 40-ml glass headspace vials covered with a polypropylene screw cap and PTFE/silicone septum (Sigma-Aldrich, St. Louis, MO). The corn media were autoclaved for 1 hour to avoid contamination, and then the inoculations were performed by adding 10 ul spore suspension of each strain on the cooled 2% corn media in the headspace vials. The aflatoxigenic and non-aflatoxigenic *A.flavus* cultures were prepared in five replicates each and four replicates of non-inoculated corn grit liquid media were used as control. Each vial were incubated in the absence of light at 30°C followed by MVOC analysis after 1, 3, 6, 10, 20, 24 and 30 days.

**SPME fibers comparison and MVOCs analysis**

A SPME fiber comparison study was done in order to optimize MVOC collection. Standard solutions of known fungal MVOCs (1-heptanol, 1-hexanol, 1-octen-3-ol, 2-heptanone, 2-methyl-1-butanol, 2-octanone, 3-methyl-1-butanol, 3-octanone, ethyl acetate, ethyl butyrate, ethyl isobutyrate, ethyl isovalerate, ethyl propionate, hexanal, methyl isobutyrate, and styrene) were mixed and diluted with dichloromethane to mixture concentrations between 300 ppm to 10,000 ppm. Final concentrations of hydrocarbons (5

ppb), alcohols (300 ppb), ketones (20 ppb), aldehydes (20 ppb), esters (20 ppb) and organic acids (20 ppb) were achieved when 1 *u*l of the standard solutions were injected with a 1 *u*l syringe into 30ml of deionized water in 40ml septa equipped vials. SPME fibers with the following materials and thickness were tested: 100µm Polydimethylsiloxane (PDMS), 85 µm Carboxen/ PDMS (CAR/PDMS), 65 µm Divinylbenzene/PDMS (DVB/PDMS), 85 µm Polyacrylate (PA) and Carboxen/Divinylbenzene/PDMS (CAR/DVB/PDMS) fibers (Supelco Inc., Bellefonte, PA, USA). The standard volatiles were extracted in triplicate for each type of SPME fiber for one hour at 30℃.

The CAR/PDMS fiber was selected for headspace extraction of the fungal isolates and non-inoculated corn control for one hour at 30℃. After 1 hour of exposure the fiber was pulled into the needle sheath, the SPME device was removed from the vial and then inserted into the injection port of GC system for thermal desorption. In order to monitor the changes in VOC profiles from fungal species over time, the VOC metabolites were collected and analyzed after 1, 3, 6, 10, 20, 24 and 30 days.

**GCMS conditions**

All GCMS analysis was performed on an Agilent 5975C Inert XL MSD coupled with 7890A Gas Chromatography system. SPME fibers were desorbed at 250 °C in a split/splitless injection port, equipped with a 78.5 mm × 6.5 mm × 0.75 mm SPME inlet liner (Supelco Inc., Bellefonte, PA, USA) while working in the splitless mode. The GC system was equipped with a DB-1 capillary column (60 m × 320 µm × 1 µm). Helium was used as a carrier gas with a flow velocity of 1.2 ml $min^{-1}$. The oven temperature program was as follows: 45°C held for 9 min, 10°C $min^{-1}$ ramp to 85°C followed by a 3

min hold; ramp to 120°C at 3°C $min^{-1}$ followed by a 3 min hold, then a final ramp at 10°C $min^{-1}$ to 270°C. The MS analysis was carried out in full scan mode (scan range from 35-350 amu) with ionization energy of 70 eV. Ion source and quadrupole temperatures were 230°C and 150°C, respectively. Fungal metabolites were identified by comparing the retention time of chromatographic peaks with standards analyzed under the same conditions and by mass spectrum database search using the NIST 08 spectral database.

**Multivariate analysis**

Linear discriminant analysis (LDA) was employed to visualize resultant clustering of fungal culture samples based on MVOC profiles and to examine the relationship between toxigenic and non-toxigenic *A. flavus* isolates. Prior to analysis, peak area data were standardized to mean zero and unit variance. The signal zero mean was calculated by removing the average and the unit variance by dividing by the standard deviation. Fisher's linear discriminant analysis was performed using statistic software IBM SPSS statistics 21 (International Business Machines Corp.).

## Results and Discussion

**HS-SPME extraction method optimization**

To investigate the extraction efficiency for the MVOCs, the following specific fibers were evaluated: 100µm PDMS, 85 µm CAR/PDMS, 65 µm PDMSDVB, 85 µm PA and 50/30 µm DVB/CAR/PDMS. Figure 3.1 shows the resulting TIC chromatograms for the 17 standard VOC mixture after one hour headspace extraction at 30 °C. The data is displayed on the same scale to emphasize the difference in extraction efficiencies.

PDMS and PA fibers were determined to be not suitable because of relatively low collection amounts when compared to the other fiber types. CAR/PDMS, PDMS/DVB and DVB/CAR/PDMS fibers show similar TIC chromatograms. For further investigation, the peak areas of the 17 standard VOCs obtained by the three types of fibers were compared as shown in Figure 3.2. The average relative standard deviations of the 17 standard VOCs for these fibers are 18.4% (CAR/PDMS), 13.1% (PDMS/DVB) and 14.9% (DVB/CAR/PDMS). Although DVB coated fibers extracted larger amount of high molecular weight alcohols and ketones (1-octen-3-ol, 2-octanone and 3-octanone), they have less affinity to esters (ethyl butyrate, ethyl isobutyrate and methyl isobutyrate) and low molecular weight alcohols (3-methyl-1-butanol and 2-methyl-1-butanol). Furthermore, insufficient amounts of 2-methyl-1-propanol and ethyl acetate were collected using DVB coated fibers to permit detection; therefore, CAR/PDMS fiber was subsequently used in the subsequent fungus MVOC studies. A culture media volume of 30 mL and 10 mL headspace volume provided sufficient amounts of VOCs during a 1 hour collection period at 30 °C.

**Identification of volatiles produced by *A. flavus***

The volatile MVOC profiles produced by aflatoxigenic and non-aflatoxigenic *A. flavus* were monitored over 30 days. The resulting chromatograms obtained from the headspace analysis of the emitted MVOCs after incubation for 6 days are shown in Figure 3.3 for the control (growth media only), toxic (aflatoxigenic *A. flavus*) and nontoxic *(*non-aflatoxigenic *A.flavus)* samples. A very clear difference in MVOCs abundance was observed where the toxic strain produce significantly less amounts of MVOCs compared to nontoxic strain. MVOC's produced by the fungal strains and

control were identified by comparing with the standards and the NIST 08 library. Ethanol was produced in significantly large amounts in all fungal cultures, we found that this chemical did not aid in discrimination and was therefore removed from consideration when looking for identifying MVOC patterns. The most significant signals (detected in all replicates) with high abundance (TIC peak area > $1\times10^4$ units) are listed in Table 3.1 (excluding ethanol). This table contains the retention time, standard deviation of this retention, compound name, the days it was detected in the samples and the relative composition.

The relative composite percentage of each compound is the average peak area percentage of the listed MVOCs during the 30 days (samples collected on day 1, 3, 6, 10, 20, 24 and 30) of incubation. The detected MVOCs were further clustered by functional groups including alcohols, aldehydes, esters, furans, hydrocarbons, ketones, and organic acids. In total, 57 different volatile compounds were identified in all samples (fungus and control). Twenty-seven compounds were detected in non-aflatoxigenic strain, and 25 compounds were detected in aflatoxigenic strain. The predominant MVOCs were alcohols (ethanol, 2-methyl-1-propanol, 3-methyl-1-butanol, and 2-methyl-1-butanol), aldehydes (3-methylbutanal, 2-methylbutanal), esters (ethyl isobutyrate, methyl isovalerate), hydrocarbons (toluene, α-pinene, and styrene), ketones (2, 3-butanedione, 3-octanone) and organic acids (acetic acid, 2-methylpropanoic acid).

Ethanol and carbon dioxide were both formed as the side-products in the metabolic oxidation of glucose during the primary and the secondary metabolism of non-aflatoxigenic and aflatoxigenic *A. flavus* cultures (Korpi et al 2009). It was also observed by Jurjevic et al (2009) in the headspace gases produced by the aflatoxigenic and non-

aflatoxigenic strains grown on the corn substrate for 25 days incubation. Several observations can be made from Table 1 data. Specific prominent and common MVOC chemicals were found in our study including 3-methyl-1-butanol, 2-methyl-1-butanol, 2-methyl-1-propanol and 3-octanone that are in agreement with the literature (Schnurer et al 1999). Many hydrocarbons were produced by the corn control; however some hydrocarbons (toluene, styrene and α-pinene) were only emitted by the non-aflatoxigenic strain. A relatively high percentage of 2-heptanol (2.23%) consistently appeared in volatiles produced by the aflatoxigenic strain however this compound was not found in the non-aflatoxigenic strain. In addition, a low percentage of furans (2-methylfuran, 2-ethylfuran, and 2,4-dimethylfuran) were detected at day 6 and dimethyl sulfide was detected at day 3 only in aflatoxigenic strain.

In a related previous study (Zeringue et al 1993), $C_{15}H_{24}$ volatile compounds (alpha-gurjunene, *trans*-caryophyllene, and cadinene) were detected using a purge and trap technique and were considered to be unique "fingerprints" for aflatoxigenic strains of *A. flavus*. In addition, dimethyl disulfide and nonanal were reported to be associated only with the aflatoxigenic *A.flavus*, while hexanal, 1-hexanol, 1-octen-3-ol, and 2-pentyl furan were only associated with non-toxigenic *A.flavus* (Jurjevic et al 2009). Possible explanations for variations in profiles are: 1) different instrument and sampling protocols; 2) variations in growth conditions (PH, growing substrate, humidity and temperature); and 3) variations in colony age when samples were collected. Variable results from variable techniques emphasize the complexity of the issue and the need for a consistent general method that can be used for fungus identification.

As an example, Ewen et al (2004) compared four volatile collection techniques including solvent extraction, SPME, and thermal desorption and showed substantial qualitative differences of volatile profiles obtained from the fungi. It was reported that substrate, humidity and temperature had tremendous effect on MVOCs classification and their emitted quantities (Polizzi et al 2012a). Our results demonstrate that there are numerous qualitative and quantitative fluctuations in MVOCs profiles during different days. According to Borjesson et al (1992) and Jurjevic et al (2009), the appearance of special MVOCs strongly depends on the stage of fungi growth. In order to use this complex data for fungus discrimination we have applied chemotaxonomy techniques to reveal potential species-specific MVOC patterns. Multivariate analysis was performed by utilizing the standardized data for each identified compound produced by the control and the fungal strains to discriminate aflatoxigenic and non-aflatoxigenic strains.

**Investigation of the fungal VOC profile overtime**

To investigate the fungal MVOCs profile overtime, cultures were evaluated over 30 days. Each fungal strain was grown in 5 replicates for each day of testing and were incubated at 30 °C. After a certain time period (1, 3, 6, 10, 20, 24 and 30 days) 5 of the sample cultures were analyzed using HS-SPME-GCMS. The variation of MVOCs over time were determined using peak area percentage. The total amounts of MVOCs from aflatoxigenic and non-aflatoxigenic strains were investigated during 30 days incubation as shown in Figure 4. The total peak areas for each day were calculated by summing the peak areas of all detected MVOCs in a sample (excluding ethanol). The results show that the amount of MVOCs significantly increases after day 6 due primarily to increasing amounts of alcohols and esters being produced. It is interesting to note that, after 10 days

the quantity of MVOCs begins to decrease, possibly because the lack of nutrients retards the biosynthetic process of fungi. The results given in Fig.4 demonstrate one significant difference in the lifecycles of these aflatoxigenic and non-aflatoxigenic isolates, where their maximum MVOCs abundances are reached at day 24 and 10, respectively. It is difficult to give a definite explanation for the results obtained within the scope of the present study. However, we hypothesize that the difference in amount of MVOCs production are caused by the following reasons: 1) Aflatoxin biosynthesis is induced by simple carbohydrates, such as glucose and sucrose (Payne and Brown 1998), therefore aflatoxin production reduces nutrients available for fungi growth. 2) The non-toxigenic isolate has a characteristic gene for rapid growth compared to toxigenic isolate. 3) The presence of aflatoxin inhibits some biological pathways that produce MVOCs.

**Multivariate analysis of MVOC profile**

Due to the large number of peaks present in the chromatograms, multivariate analysis is required to recognize patterns in the data and to discriminate the different fungal strains. To evaluate the capability of this HS-SPME-GCMS method for distinguishing aflatoxigenic and non-aflatoxigenic *A. flavus*, the GCMS data (day 1, 3, 6, 10, 20, 24, 30) from fungi and control samples were collected and analyzed using Fisher's linear discriminant analysis (LDA) model. LDA builds up a predictive modelwhich is composed of a discriminant function based on linear combinations of predictor variables. It can be used to discard variables that are little related to group distinctions and to maximally separate the groups. LDA was applied to calculate the discrimination functions for classification of aflatoxigenic, non-aflatoxigenic *A.flavus* and control in clusters, which minimizes the variance within the classes and maximizes the

variance among the classes. LDA provides a number of discriminant functions equal to the number of categories of grouping variables minus one. Since three categories were considered including toxic, nontoxic and control, two discriminant functions were obtained in which the first function maximizes the difference between the values of the dependent variables, and the second function maximizes the difference between the values of the dependent variable while controlling the first function.

Two discriminant functions were calculated, with the first accounting for 97.6% of the variance. In summary, the low Wilks' lambda values of function 1 (0.00) and function 2 (0.028) indicate the ideal discriminatory ability of the functions. The standardized discriminant function coefficients indicate the relative importance of the independent variables in predicting the dependence, where coefficients with large absolute values correspond to variables with greater discriminating ability. A stepwise method was performed by automatically selecting the best MVOCs to use in this model. Using this approach the 21 MVOCs (Table 3.2) were considered with 1-octen-3-ol, butanal, 3-methylbutanal, 2-heptanal, hexanal, hexane, decane, 2-methylbutanoic acid being the most significant compounds for group classification. Fig. 6 shows the plot of discriminant scores of the analyzed samples. The three classified groups (toxic, nontoxic, control) were satisfactorily separated, proving that this method can be used to discriminate these strains of aflatoxigenic and non-aflatoxigenic *A. flavus* during the fungi growing process. All of the group cases were correctly classified by the discriminant functions built by Fisher's linear discriminant model, thus achieving perfect discrimination (Table 3.3). The "leave-one-out" cross-validation were performed in order to determine the accuracy of predictive model, where each identity tested is removed one-

at-a-time from the initial matrix of data; then the classification model is rebuilt and the case removed is classified in this new model. The discriminant analysis model based on MVOCs of inoculated samples correctly classified 100% of the observations based on cross-validation. The result obtained from LDA can be considered very satisfactory for detection of aflatoxin producing *A.flavus* growing in corn media.

## Conclusions

In conclusion, based on VOC absorption data, the CAR/PDMS SPME fiber is considered to be the best fiber for *A. flavus* MVOC profiling. The time course experiments (carried out over 30 days) revealed that MVOC production is time-dependent and that aflatoxigenic and non-aflatoxigenic strain had significantly different MVOC expression patterns. HS-SPME-GCMS was applied successfully to detect and differentiate two *A.flavus* strains (aflatoxigenic and non-aflatoxigenic strains). An LDA plot achieved satisfactory performance in classifying *A.flavus* strains and control based on quantitative MVOCs data even though different isolates produce similar MVOCs. Results indicate that it is possible to build a database for chemotaxonomic application by performing MVOC monitoring at specific growth conditions (temperature, humidity and substrate). Future studies will be done to expand the number of fungal strains that can be discriminated using MVOCs and HS-SPME-GCMS in concert with multivariate analysis in order to build up a fungal screening database.

## Acknowledgements

The authors thank the Mississippi Corn Promotion Board for partial funding of this research and Mary Scruggs for excellent technical assistance. This article reports the

results of research only. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by Mississippi State University.

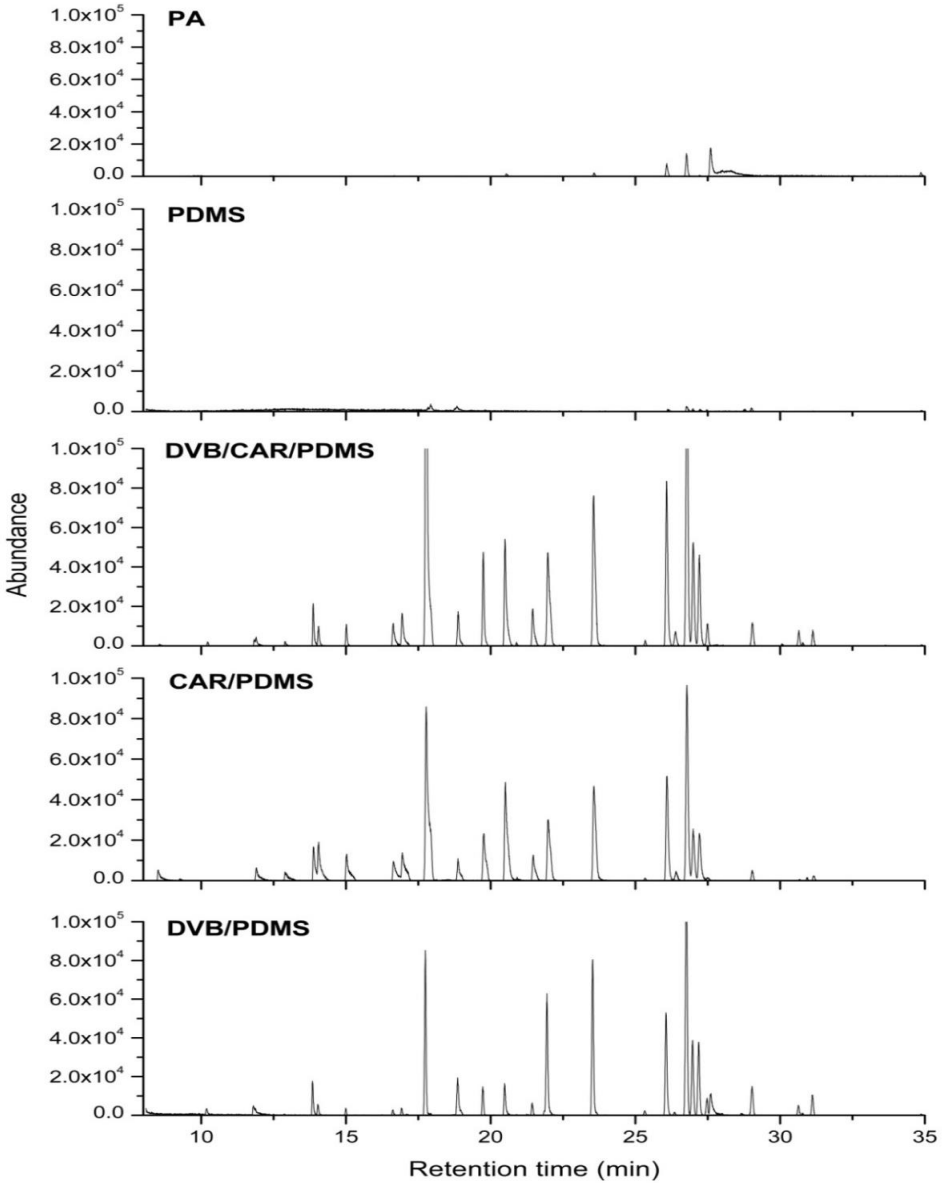


Figure 3.1 Comparison of TIC chromatograms from varied SPME extraction of 17 standard VOCs followed by GCMS analysis.

Best results were obtained using DVB/CAR/PDMS, DVB/PDMS and CAR/PDMS.

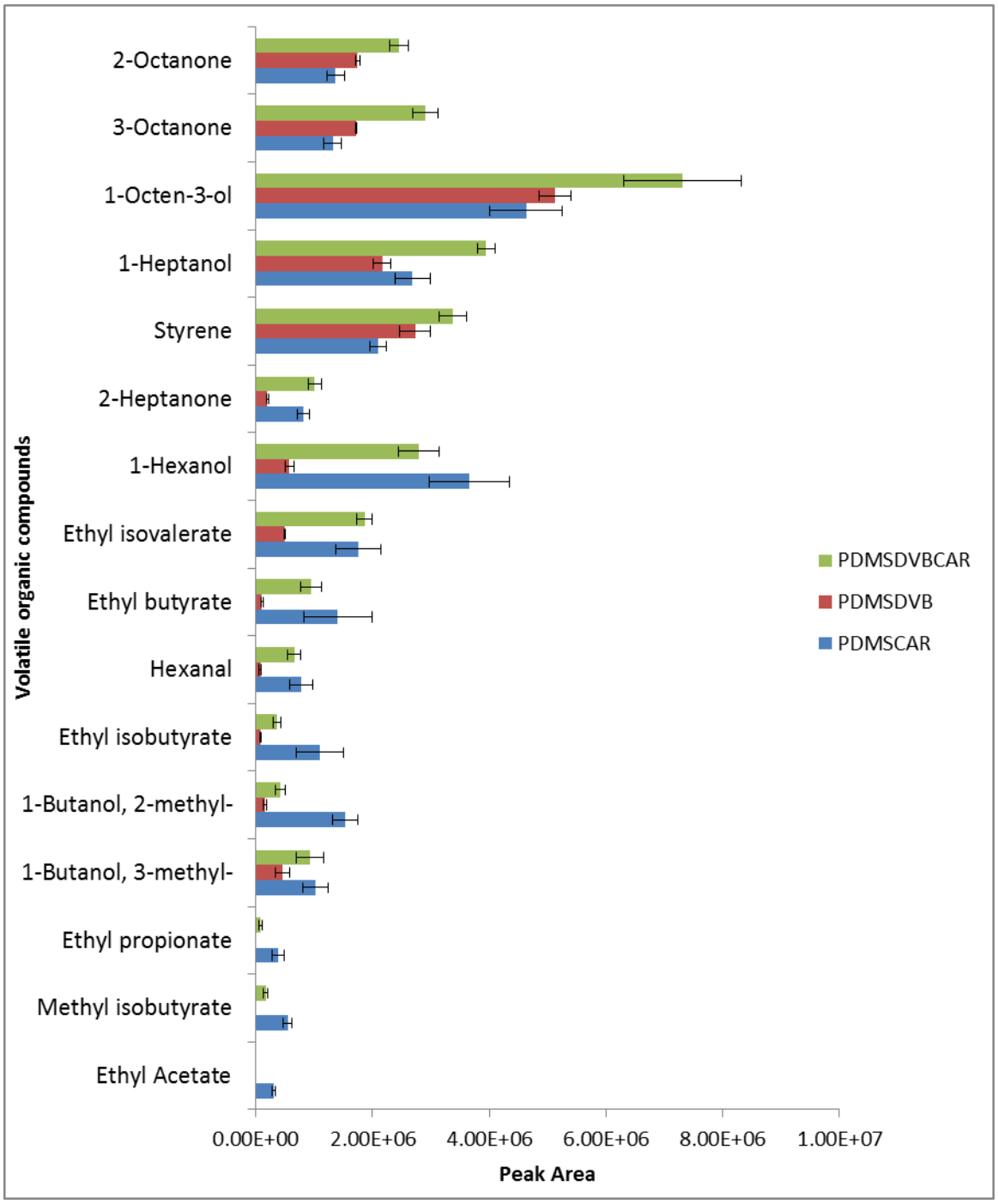


Figure 3.2 Comparison of peak areas.

Peak areas showing 17 standard VOCs after HS-SPME-GCMS analysis using different SPME fiber coating, including PDMSDVBCAR, PDMSDVB and PDMSCAR Each fiber was tested in triplicate.

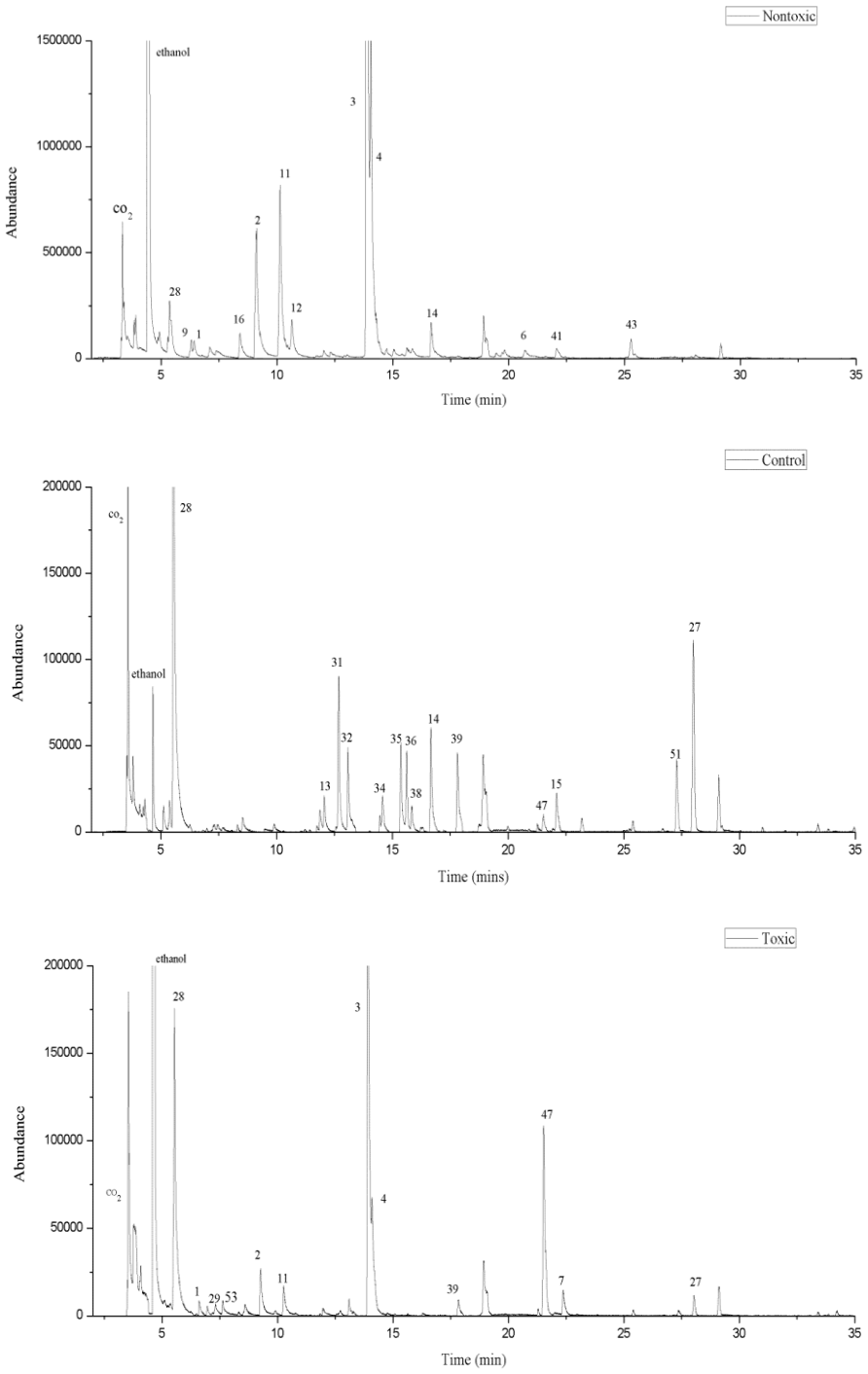


Figure 3.3 HS-SPME-GCMS total ion current (TIC) chromatogram.

VOCs identified from the fungal strains and non-inoculated media at day 6 for the control (upper), toxigenic *A.flavus* (center), and non-toxigenic *A. flavus* (lower). Peak numbers refer to the volatiles listed in Table 3.1. (Ethanol and carbon dioxide was detected in all samples).

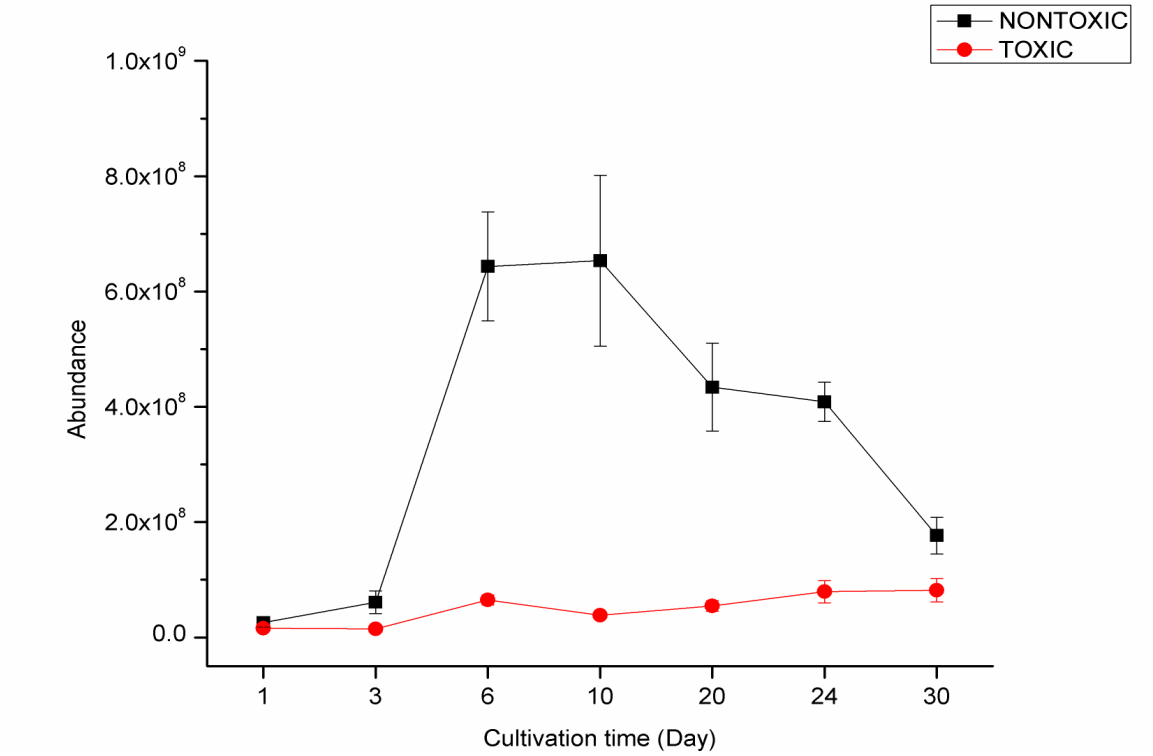


Figure 3.4 Comparison of total amount of MVOCs between aflatoxigenic and non-aflatoxigenic *A.flavus* during a cultivation period of 30 days

The abundance is the total peak area of all compounds detected from both aflatoxigenic and non-toxigenic *A.flavus*.

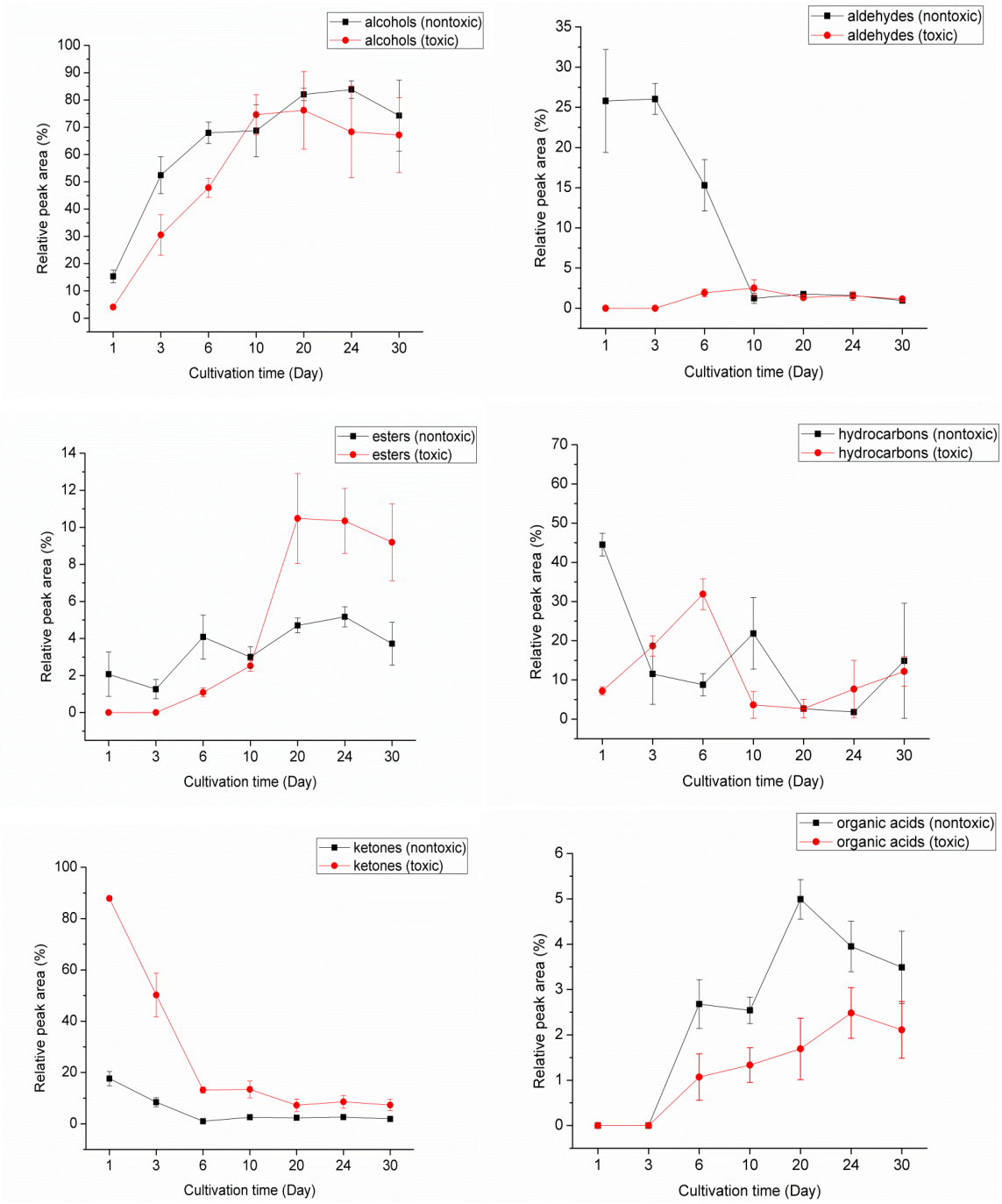


Figure 3.5 Variation of MVOCs expression patterns of aflatoxigenic and non-aflatoxigenic *A.flavus* during a cultivation period of 30 days for selected volatiles from classified compounds

A) alcohols, B) aldehydes, C) esters, D) hydrocarbons, E) ketones, and F) organic acids.

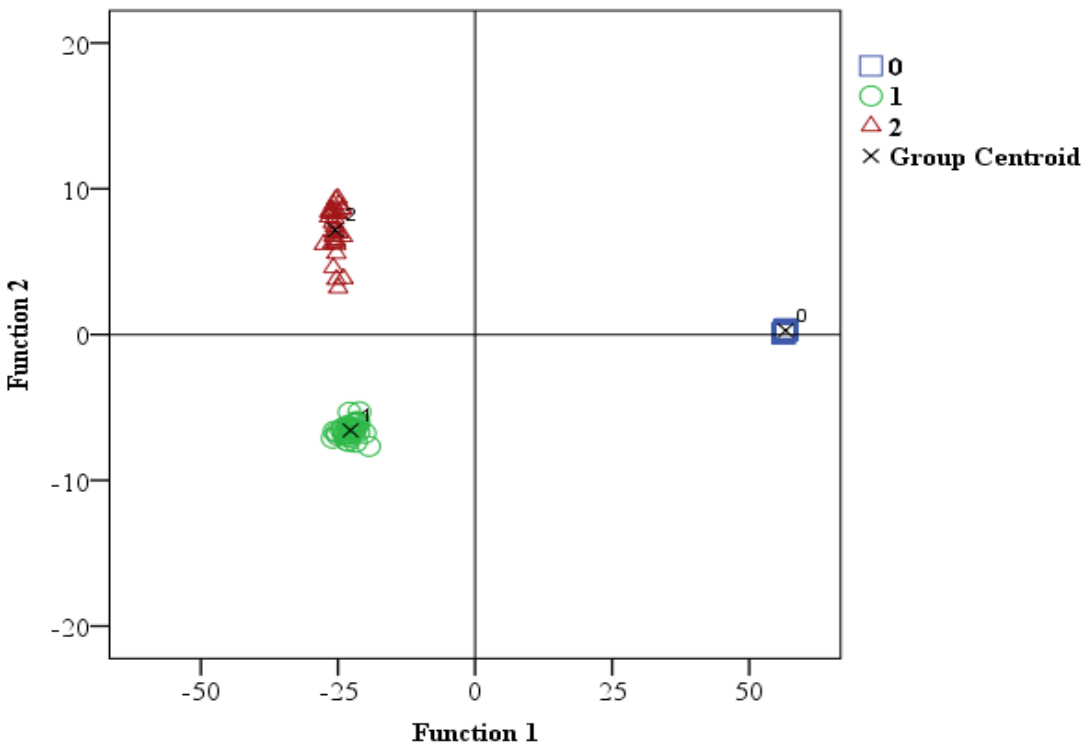


Figure 3.6 Discriminant score plot.

MVOCs analyzed by HS-SPME-GCMS were grouped by chemical classes of toxigenic and non-toxigenic isolates and non-inoculated control during 30 days incubation (0: non-inoculated control, 1: non-aflatoxigenic strain culture, 2: aflatoxigenic strain culture).

Table 3.1 Headspace SPME-GCMS analysis of 52 microbial volatile metabolites from both aflatoxigenic and non-aflatoxigenic strains of *Aspergillus flavus*.

| no. | R.T. | STD[d] | Compound Name[e] | Days detected | | | Relative composition % [a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Nontoxic | Toxic | Control | Nontoxic | Toxic | Control |
| *Alcohols* | | | | | | | | | |
| 1 | 6.560 | 0.035 | 1-propanol | 3,6,10,20,24,30 | 6,10,20,24,30 | n.d[b]. | 0.91 | 0.66 | -- |
| 2 | 9.300 | 0.016 | 2-methyl-1-propanol | a.d[c]. | 3,6,10,20,24,30 | n.d. | 7.89 | 2.77 | -- |
| 3 | 13.917 | 0.009 | 3-methyl-1-butanol | a.d. | a.d. | n.d. | 39.50 | 38.24 | -- |
| 4 | 14.105 | 0.006 | 2-methyl-1-butanol | a.d. | a.d. | n.d. | 15.54 | 10.42 | -- |
| 5 | 15.468 | 0.015 | 1-pentanol | n.d. | 6 | n.d. | 0.04 | -- | -- |
| 6 | 20.712 | 0.024 | 1-hexanol | 6,10 | 30 | n.d. | 0.21 | -- | -- |
| 7* | 22.310 | 0.009 | 2-heptanol | n.d. | a.d. | n.d. | -- | 2.23 | -- |
| 8 | 27.004 | 0.028 | 1-octen-3-ol | 6 | n.d. | n.d. | 0.01 | -- | -- |
| *Aldehyde* | | | | | | | | | |
| 9 | 6.464 | 0.015 | 2-methyl-propanal | 1,3,6 | n.d. | n.d. | 0.86 | -- | -- |
| 10 | 7.492 | 0.032 | butanal | n.d. | n.d. | a.d. | -- | -- | 0.45 |
| 11 | 10.247 | 0.015 | 3-methylbutanal | a.d. | 6,10,20,24,30 | n.d. | 6.93 | 1.22 | -- |
| 12 | 10.774 | 0.018 | 2-methylbutanal | a.d. | 10,24,30 | n.d. | 1.16 | 0.10 | -- |
| 13 | 12.039 | 0.080 | pentanal | 6 | n.d. | a.d. | 0.07 | -- | 2.06 |
| 14 | 16.684 | 0.015 | hexanal | 6,10,20 | n.d. | a.d. | 0.39 | -- | 4.71 |

Table 3.1 (continued)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 15 | 25.082 | 0.014 | 2-heptenal | 6 | n.d. | a.d. | 0.01 | -- | 3.37 |
| *Esters* | | | | | | | | | |
| 16 | 8.557 | 0.013 | ethyl acetate | a.d. | 6,10,20,24,30 | n.d. | 1.68 | 3.69 | -- |
| 17 | 12.964 | 0.023 | propanoic acid, ethyl ester | 20,24,30 | n.d. | n.d. | 0.09 | -- | -- |
| 18 | 15.178 | 0.283 | ethyl isobutyrate | 6,10,20,24,30 | 20,24,30 | n.d. | 0.56 | 0.22 | -- |
| 19 | 15.946 | 0.010 | ethyl butyrate | 6,10,20,24,30 | 20,24,30 | n.d. | 0.02 | 0.27 | -- |
| 20 | 16.029 | 0.390 | methyl isovalerate | n.d. | 20,24 | n.d. | 0.38 | 0.13 | -- |
| 21 | 19.736 | 0.041 | ethyl 2-methylbutyrate | 6,10,20,24,30 | 20,24,30 | n.d. | 0.19 | 0.20 | -- |
| 22 | 19.840 | 0.037 | ethyl 3-methylbutyrate | 6,10,20,24,30 | 20,24,30 | n.d. | 0.42 | 0.24 | -- |
| *Furan-related compounds* | | | | | | | | | |
| 23 | 5.374 | 0.013 | furan | 1,20,24,30 | 1,3,6,10,24,30 | a.d. | 0.48 | 0.73 | 1.29 |
| 24* | 8.329 | 0.022 | 2-methylfuran | n.d. | 6 | n.d. | -- | 0.04 | -- |
| 25* | 12.729 | 0.010 | 2-ethylfuran | n.d. | 6 | n.d. | -- | 0.08 | -- |
| 26* | 13.287 | 0.009 | 2,4-dimethylfuran | n.d. | 6 | n.d. | -- | 0.06 | -- |
| 27 | 28.025 | 0.012 | 2-pentylfuran | 1,3,6,10 | 6,10,20,24,30 | a.d. | 0.29 | 0.43 | 10.25 |
| *Hydrocarbons* | | | | | | | | | |
| 28 | 5.535 | 0.011 | pentane | a.d. | a.d. | a.d. | 6.94 | 11.45 | 36.51 |
| 29 | 7.321 | 0.015 | 2-methylpentane | n.d. | 6,10 | n.d. | -- | 0.26 | 0.52 |
| 30 | 8.544 | 0.015 | hexane | n.d. | n.d. | a.d. | -- | -- | 1.05 |

Table 3.1 (continued)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 31 | 12.684 | 0.007 | 2,2,3,3-tetramethylbutane | 1,3 | 1,3 | a.d. | 1.78 | 0.41 | 8.61 |
| 32 | 13.061 | 0.060 | heptane | 6,24 | 6 | a.d. | 0.08 | 0.12 | 3.87 |
| 33 | 14.463 | 0.010 | 2,5-dimethylhexane | 1,3 | n.d. | a.d. | 0.19 | -- | 0.73 |
| 34 | 14.583 | 0.012 | 2,4-dimethylhexane | 1,3 | n.d. | a.d. | 0.61 | -- | 2.49 |
| 35 | 15.371 | 0.007 | 2,3,4-trimethylPentane | 1,3 | 1,3 | a.d. | 1.48 | 0.43 | 5.06 |
| 36 | 15.609 | 0.008 | 2,3,3-trimethylPentane | 1,3 | 1,3 | a.d. | 1.80 | 0.68 | 4.01 |
| 37 | 15.681 | 0.138 | toluene | 6,10,20,24 | n.d. | n.d. | 0.33 | -- | -- |
| 38 | 15.843 | 0.005 | 2,3-dimethylhexane | 1,3 | 3 | a.d. | 0.42 | 0.09 | 1.62 |
| 39 | 17.826 | 0.009 | octane | 6,30 | 6,24 | a.d. | 0.06 | 0.22 | 4.64 |
| 40 | 21.284 | 0.024 | 2,3,4-trimethylhexane | n.d. | n.d. | a.d. | 0.05 | 0.05 | 0.48 |
| 41 | 22.085 | 0.027 | styrene | 6,10 | n.d. | n.d. | 0.14 | -- | -- |
| 42 | 22.367 | 0.007 | p-xylene | 3,20,24 | n.d. | n.d. | 0.09 | -- | -- |
| 43 | 25.295 | 0.016 | α-pinene | 6,10,20,24,30 | n.d. | n.d. | 0.25 | -- | -- |
| 44 | 35.812 | 0.007 | decane | 3 | n.d. | n.d. | 0.20 | -- | 0.20 |
| *Ketones* | | | | | | | | | |
| 45 | 5.126 | 0.017 | acetone | a.d. | a.d. | a.d. | 1.19 | 1.24 | 1.22 |
| 46 | 7.263 | 0.041 | 2,3-butanedione | 3,6,10,14,20,24 | 20,24,30 | n.d. | 0.58 | 0.11 | -- |
| 47 | 11.758 | 0.012 | 2-pentanone | 1,3,10,20,24,30 | 1,3,24,30 | n.d. | 0.39 | 0.45 | -- |
| 48 | 12.375 | 0.022 | 3-hydroxy-2-butanone | 3,6,10,20,24,30 | n.d. | n.d. | 0.37 | -- | -- |

Table 3.1 (continued)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 49 | 21.486 | 0.006 | 2-heptanone | 1,3 | a.d. | a.d. | 2.36 | 17.67 | 1.21 |
| 50 | 27.147 | 0.005 | 3-octanone | 6 | 1,20,24,30 | n.d. | 0.01 | 0.16 | -- |
| 51 | 27.341 | 0.014 | 2-octanone | 1 | 1,6,10,20,24,30 | a.d. | 0.13 | 0.36 | 0.24 |
| 52 | 34.108 | 0.005 | 2-nonanone | 1 | 1,3,6 | n.d. | 0.30 | 3.28 | -- |
| *Organic acids* | | | | | | | | | |
| 53 | 7.463 | 0.179 | acetic acid | 10,20,24,30 | 6,10,20,24,30 | n.d. | 1.52 | 1.08 | -- |
| 54 | 14.828 | 0.172 | 2-methylpropanoic acid | 6,10,20,24,30 | 30 | n.d. | 0.72 | 0.16 | -- |
| 55 | 19.575 | 0.169 | 2-methylbutanoic acid | 6,10,20,24,30 | n.d. | n.d. | 0.36 | -- | -- |
| *Sulfur containing compounds* | | | | | | | | | |
| 56* | 5.740 | 0.004 | dimethyl sulfide | n.d. | 3 | n.d. | -- | 0.07 | -- |

[a] Relative composition % is the average peak area percentage of each compound collected on the 7 sampled days (1,3,6,10,20,24,30), and the peak area of each compound for days not detected was counted as zero.
[b] n.d. : not detected in the culture samples which were analyzed by GCMS
[c] a.d. : detected in all days sampled (1,3,6,10,20,24,30)
[d] STD: standard deviation of each compound retention time in five replicates
[e] Identification based on the comparison of retention time and mass spectra with standards under the same conditions
[f] * : VOCs detected in aflatoxigenic *A.flavus* only
[g] Ethanol and carbon dioxide was detected in all samples; it is not listed due to large amount of VOC production interference the other peak area% result.

Table 3.2 Standardized canonical discriminant function coefficients for HS-SPME-GC-MS data from samples analyzed during 30 days culture incubation.

| Standardized Canonical Discriminant Function Coefficients [a] | | |
|---|---|---|
| Variable | Discriminant Function[b] | |
| | 1 | 2 |
| 1-hexanol | -0.717 | -0.496 |
| 2-heptanol | 0.271 | -1.732 |
| 1-octen-3-ol | 9.397 | 1.532 |
| Butanal | -6.703 | 0.952 |
| 3-methylbutanal | 5.334 | 0.776 |
| Hexanal | -4.622 | -0.565 |
| 2-heptenal | 13.024 | 1.875 |
| methyl isovalerate | -1.260 | -0.129 |
| ethyl 3-methylbutyrate | -2.625 | -0.892 |
| Furan | -1.228 | -0.486 |
| Heptane | -2.532 | 0.090 |
| Hexane | 3.226 | 0.644 |
| Octane | 1.421 | 1.014 |
| 2,3,4-trimethylhexane | 0.336 | 1.271 |
| Decane | 5.721 | -1.366 |
| 2-pentanone | 1.082 | 0.256 |
| 3-hydroxy-2-butanone | -1.978 | -0.270 |
| 3-octanone | 0.561 | -0.230 |
| 2-octanone | 1.443 | -2.041 |
| 2-nonanone | 0.171 | 1.320 |
| 2-methylbutanoic acid | 3.951 | 1.052 |

[a] Fisher's discriminant analysis was performed using standardized GC-MS data from aflatoxigenic, non-aflatoxignic *A.flavus* and control samples analyzed in day 1, 3, 6, 10, 20, 24, 30.

[b] Discriminant function 1 and 2 were used as linear combinations of independent variables for 3-group discriminant analysis

Table 3.3 Classification and cross-validation results using HS-SPME-GC-MS data from samples analyzed during 30 days culture incubation

| | | Classification Results [a,c] | | | |
|---|---|---|---|---|---|
| | | Predicted Group Membership[d] | | | Total |
| | ID | 0 | 1 | 2 | |
| Original | 0 | 100% | 0 | 0 | 100% |
| | 1 | 0 | 100% | 0 | 100% |
| | 2 | 0 | 0 | 100% | 100% |
| Cross-validated[b] | 0 | 100% | 0 | 0 | 100% |
| | 1 | 0 | 100% | 0 | 100% |
| | 2 | 0 | 0 | 100% | 100% |

[a] 100.0% of original grouped cases correctly classified.
[b] Cross validation is done only for those cases in the analysis. In cross validation, each case is classified by the functions derived from all cases other than that case.
[c] 100.0% of cross-validated grouped cases correctly classified.
[d] Predicted group membership includes 0: non-inoculated control, 1: non-aflatoxigenic strain culture, 2: aflatoxigenic strain culture.

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CHAPTER IV

# DETERMINATION OF RESISTANCE TO *ASPERGILLUS FLAVUS* IN MAIZE GENOTYPES USING STEM INOCULATIONS AND QUANTITATIVE PCR


## Abstract

A study was conducted to evaluate maize seedlings inoculated with *Aspergillus flavus* at vegetative stage V5, to screen for resistance using cultural and DNA biomass data with previously developed TaqMan probes by quantitative PCR (QPCR). All cultural and molecular data were compared to visible lesion lengths. Using a refined toothpick inoculation method, maize seedlings were evaluated at V7 from seven greenhouse (GH), four environmental growth chamber (EGC), and two field (FD) trials. At V7, replicate stems were sectioned for visible tissue necrosis at three different sampling points 0, 3 cm and 6 cm). The inoculation method at V5 stage was a reliable and successful technique for initiating stem infections based on visible tissue necrosis without plant mechanical injury. There were no significant differences in lesion lengths in FD or EGC trials when comparing each setting separately. Fifty-seven percent of the GH trials (four of seven trials) displayed significantly greater lesion lengths in susceptible (S) genotypes. Overall, there were no significant trends in lesion lengths across the three experimental settings (GH, EGC, or FD), across all GH, EGC, and FD trials, or by genotype (two susceptible (S); GA209 and SC212M and two resistant (R); Mp313E and Mp717E). A QPCR *OMG3* TaqMan probe developed previously was successfully

employed to detect and quantify *A. flavus* aflatoxigenic strain 3357 in this study. However results varied with no consistent significant trends within the maize tissues for; 1) inoculated and control plants, 2) each sampling site (0, 3 and 6 cm), and 3) all settings (GH, EGC, and FD). Traditional isolations and QPCR was used to verify the presence of the fungus within inoculated tissues. Overall, both methodologies detected *A. flavus* in 91% of inoculated stem pieces. The inconclusive results from this research did provide insight for future studies to evaluate genotypes *in vivo* and *in situ*.

## Introduction

Maize (*Zea mays* L.) germplasm has been developed for resistance against microorganisms, primarily *Aspergillus flavus* Link : Fr. and *Aspergillus parasiticus* Speare, that cause plant disease and subsequent mycotoxin accumulation (Scott and Zummo 1988; Windham and Williams 2002). *Aspergillus flavus* is of particular importance since aflatoxins pose great risk to humans and livestock if these toxins entered into the food chain. Aflatoxins are secondary metabolites that are highly carcinogenic, mutagenic and teratogenic (Diener et al 1987). Worldwide, many countries regulate exposure to aflatoxin in crops post-harvest. For example, the European Union limit is 4 ppb and the US limit is 20 ppb (Leslie et al 2008; Munkvold et al 2009).

Control of both the fungus and aflatoxin contamination is difficult in maize (*Zea mays subsp. mays* L.) because of the mode of infection. Chemical control is not economical or practical at this time. The only chemical control that may have any effect would need to be systemic (Abbas et al 2009). There is some promise with biological control using non-aflatoxigenic strains of *A. flavus* and *A. parasiticus* to adjust the fungal population structure in the field (Dorner et al 2003; Dorner 2004; Abbas et al 2006; Yin

et al 2008). However, the most practical approach in reducing aflatoxin accumulation in maize is through the development of host resistance to infection of *A. flavus*, primarily by genetic engineering; screening each new line or genotype for resistance primarily in the field by inoculating maize ears (Barug et al 2004; Yu and Cleveland 2007). Preharvest host resistance is economical for growers, leaves no harmful chemical or biological residue in the environment, and can also eliminate the need to disinfect large quantities of aflatoxin-contaminated grain (Menkir et al 2006).

In the 1990's, several maize germplasms were shown to be resistant to infection to *A. flavus* but mean aflatoxin levels of parental crosses using these germplasms were drastically higher than the 20 ppb limit (Scott and Zummo 1988; Scott and Zummo 1990; Windham and Williams 1998; Windham and Williams 1999). Later, several additional hybrids appeared to be resistant to aflatoxin accumulation in field inoculation trials. Resistance is determined by the amount of aflatoxin accumulation and traditional isolations to compare fungal growth per line or genotype (Windham and Williams 1998; Williams 2006). This method of screening is labor intensive and time consuming and much land acreage must be used. Because aflatoxin accumulation and fungal growth is dependent on environmental parameters primarily high temperatures, high humidity, and drought, aflatoxin content will vary from year to year (Widstrom et al 1981; Payne 1998; Windham and Williams 2007) results can be sporadic and long-term studies must be conducted for verification.

Breeding efforts include both molecular marker-assisted selection and conventional approaches. Research is directed toward developing resistant germplasms by identifying genes, quantitative trait loci, and proteins associated with resistance to

infection by *A. flavus*, aflatoxin accumulation, and insect damage in maize hybrids (Brooks et al 2005; Wisser et al 2006). Molecular markers play an important role in analyzing the genome of a plant. In maize, randomly amplified DNA (RAPD) (da Silva et al 2000), simple sequence repeats (SSR) (Zhang et al 2002), and amplified fragment length polymorphism *polymerase chain reaction* (AFLP) (Cai et al 2003; Zhang et al 2002) markers have been utilized to build genetic linkage maps to identify important resistance traits. Some sources of resistances have been identified and work is continuing in this area (Kang et al 1990; Li et al 2002; Scott and Zummo 1988; Zhang et al 1997). Despite these findings, the genetics of resistance to *A. flavus* and aflatoxin accumulation is still poorly understood (Kang et al 1990; White et al 1997; Li et al 2002; Li and Kang 2005). Additional molecular work is being conducted to determine the relationships between the fungal structures responsible for reproduction to the process of infestation within a host (Amaike and Keller 2009; Georgianna et al 2010).

Because aflatoxin is the primary concern, the mode of infection of *A. flavus* in the reproductive stage of maize has been studied. The fungus will readily colonize maize external silk tissue (moribund styles) (Jones et al 1980; Wilson et al 1988) that are in the yellow-brown stage of senescence (Marsh and Payne 1984; Smart et al 1990) and grow rapidly down the silks to colonize glumes, and kernel surfaces (Wilson et al 1988; Wilson and Payne 1994). Conidia become lodged in pistillate inflorescences (ears), germinate, and rapidly colonize developing kernels. Germination can also occur first near the pollen grains, and the hyphae spread rapidly across the silk, producing widespread lateral branching (Marsh and Payne 1984). Kernel colonization will occur when the moisture content is approximately 32% and penetration will occur in the pedicel region (Wilson et

al 1988; Payne 1992; Wilson and Payne 1994). The exact mode of entry is still unknown; however, it seems that *A. flavus* prefers to grow through the barrier that is easiest to penetrate, such as a wound or crack in the kernel or the husk (Diener et al 1987; Payne 1992; Burow et al 1997; Payne 1998). Smart et al (1990) described the histology of fungal development in maize ears wound inoculated with *A. flavus* using (Smart et al 1990). Interestingly, the host cell died, and even collapsed prior to fungal colonization, but no other structural alterations were seen. In conclusion, aflatoxins may serve as the chemical compound responsible for the perthotrophic behavior of *A. flavus* since changes in cellular structure of host cells precede cellular deterioration (Smart et al 1990; Burow et al 1997).

To date, few researchers have studied *A. flavus* in the vegetative stage, primarily systemic infection within the stalk in maize genotypes and consequent aflatoxin accumulation (Kelley 1984; Windham and Williams 2007). For example, systemic infection occurred in seedlings from *A. flavus*-inoculated kernels and infection was observed in the vegetative parts of the plant during the early whorl stage of growth (Kelley 1984). Windham et al (2007) showed that a mutant of *A. parasiticus* could move readily through inoculated maize stalks to the ear and that even though kernel infection levels were low, systemic infection could be another route to kernel infection (Windham and Williams 2007). Therefore, systemic infection and subsequent quantitative differences in fungal biomass in maize seedlings may serve as a good indicator of host resistance to the fungus in maize. Other types of artificial stem inoculation techniques have been studied using a variety of pathogens including *Sclerotinia* spp. on soybean (Pratt 1991) and *Macrophomina phaseolina* (Tassi) Goid. on soybean (Twizeyimana et al

2012). The *M. phaseolina* study demonstrated that using their stem inoculation technique, the soybean genotypes could be successfully screened for resistance (Twizeyimana et al 2012). Pratt (1991) observed high variability using the stem inoculation on alfalfa and soybean throughout their experiments (Pratt 1991). In summary, results from these studies show that stem inoculations have the potential to evaluate resistance in field crops (Pratt 1991; Twizeyimana et al 2012), but more research is needed in this area.

In addition to artificial inoculation techniques, accurate quantitative techniques are necessary to monitor occurrence of *Aspergillus* spp. (naturally and artificially inoculated) in maize. To date, conventional methods can identify and quantify fungal biomass or fungal colonization within plant tissue; ergosterol content, immunological techniques and molecular-based assays, such as polymerase chain reaction (PCR) or quantitative polymerase reaction (QPCR). Quantitative PCR can eliminate the time consuming microbiological techniques including traditional isolations and identifying the pathogen based on morphology. Quantitative-PCR is a rapid way to detect, monitor and quantitatively measure target plant pathogen genomic DNA (Schaad and Frederick 2002; Niessen 2007). Oligonucleotide-specific primers are designed to bind to a specific DNA sequence that serves as a starting point for DNA synthesis and amplifies each DNA strand after each cycle. When used with QPCR, two different florescent dyes are used to measure the increase of a specific to the target DNA PCR product (Logan et al 2008). In this study, we used previously designed TaqMan oligonucleotide-specific probes (Wood-Jones et al *unpublished*). When using TaqMan probes, the florescence is measured after each cycle of the PCR reaction (Logan et al 2008). A critical threshold (Ct) is produced only after the florescence surpasses a specific threshold (Logan et al 2008). At this point

the Ct is compared and plotted to a standard curve generated by known quantities of DNA; therefore calculating the concentration of the DNA in the unknown sample (Logan et al 2008), the pathogen DNA extracted from the host tissue.

A major drawback to PCR or QPCR is that the reaction can be adversely affected by PCR inhibitors found in the complex composition of plant material such as maize, so that the sensitivity found in pure cultures is reduced (Feng 2007). Primary PCR inhibitors found in plant material, especially maize tissues, are lipids, acidic polysaccharides, and polyphenolic compounds. These compounds can make extraction of pathogen DNA difficult and are a major obstacle for efficient amplification in QPCR (Ma and Michailides 2007) and must be eliminated with the proper DNA extraction method and the addition of neutrilizers (Wilson 1997; Ma and Michailides 2007).

To determine if a correlation exists between fungal infestation in the vegetative stage and the reproductive stage, the relationship between fungal biomass and aflatoxin accumulation must be evaluated. Several studies have examined this potential relationship between the growth of *A. flavus* (fungal biomass) and aflatoxin accumulation in kernels (King and Wallin 1983; Priyadarshini and Tulpule 1978; Mideros et al 2009;). One researcher found that the amount of toxin production is not consistent with fungal growth, suggesting that increases or decreases in growth of the fungus showed no correlation to toxin production (Priyadarshini and Tulpule 1978). Based on this study, the differences in the amount of toxin produced by *A. flavus* varied on selected kernels as compared to quantitative differences in fungal growth, which may be related to varying amounts of stimulatory and inhibitory factors in genotypes (Priyadarshini and Tulpule 1978). In a more recent study examining maize kernels, QPCR was used to amplify *A.*

*flavus* DNA in maize kernels and found that fungal biomass was strongly correlated with aflatoxin concentration (Mideros et al 2009).

The purpose of this study was to 1) develop and refine a novel, effective and efficient maize stem inoculation procedure during the vegetative stage of growth using the aflatoxigenic strains of *A. flavus* to rapidly screen for resistance to the pathogen and 2) evaluate designed or previously developed real-time quantitative PCR (QPCR) oligonucleotide primers and florescent TaqMan probes to quantify the fungal biomass in artificially inoculated maize using a newly developed stem inoculation method, 3) determine if observed maize stem necrosis from artificial inoculation can be directly correlated to *A. flavus* fungal biomass QPCR data and 4) from above results determine if *A. flavus* biomass data can be used to distinguish susceptible from resistant genotypes during the vegetative growth of maize.

## Materials and Methods

### Fungal strain and plant material

The aflatoxigenic fungal strain NRRL 3357 was obtained from G.L.Windham, USDA-ARS-Corn Host Plant Resistance Research Unit, Starkville, MS. The strain was sub-cultured onto 60 x 15 mm Petri plates of Czapeks Dox agar (CZP) (Fisher Scientific, Pittsburg, PA) every 14 days and stored at room temperature (20 - 24 °C) and was used throughout the study for all fungal stem inoculations. Seed from four genotypes was obtained from G.L.Windham, USDA-ARS-CHPRRU, Starkville, MS; susceptible (S) genotypes, GA209 (S) and SC212M (S) (Windham and Williams 2002) and the putative resistant (R) genotypes, Mp313E (R) (Scott and Zummo 1990) and Mp717E (R) (Windham and Williams 2002).

**Greenhouse conditions for all tests**

Greenhouse temperatures and light intensity

For all GH tests, temperatures were monitored using six WatchDog B-Series Button 3619WD-2K Temperature Loggers (Spectrum Technologies, Inc., Aurora, IL). Temperature loggers were interspersed throughout the greenhouse. Data from each logger consisted of high and low temperatures collected every 120 min and the mean hourly, daily, and weekly temperatures. All data from each logger and the six loggers combined were reported and analyzed by SpecWare 9 Pro software. Data from each logger were downloaded once per week to monitor GH conditions. For all GH tests, PAR (photosynthetically active radiation measured in μmol $m^{-2}s^{-1}$) (light intensity) was measured at whorl level at between 10 am and 11 am Eastern Standard Time (EST) once per week utilizing the Spectrum Field Scout Quantum Meter (Spectrum Technologies, Inc., Aurora, IL).

Maize growth parameters

Three seeds from each of the four genotypes were chosen at random and planted (*ca.* 1 cm deep) in a 3.79 L black plastic nursery pot (15.24 cm diameter x 17.78 cm depth) filled with sterilized (autoclaved for 2 hours on 2 consecutive days at 121°C) 100% baked calcined clay growth media with grain size between 2.5 and 3.5 mm with a of pH 6.2 (Turface MVP, Buffalo Grove, IL). Calcined clay growth media was used in this study to standardize fertilization rates due to the substrates high nutrient retention, ability to maintain damp but not waterlogged media and to prevent growth of algae and fungus gnats, and capability to provide good aeration for roots and stems of plants within each treatment (Eddy et al 2010).

At the vegetative V2 (2 nodes or collars) stage, plants were culled to one seedling per pot. All pots were watered with 200 ml twice per day via a timed drip irrigation system (Claber 8454 Aquauo Video 2-Cycle Water Timer, Clabor, Elk Grove Village, IL; Drip Irrigation Kit for Container Gardening, Irrigation Direct, Livermore, CA). Each pot was hand-fertilized twice per week with a 50 ml mix of nitrogen at a rate of 9.1 kg per ha (33% ammonium nitrate) and potassium phosphate at a rate of 9.1 kg per ha (28.7% potassium; 22.8% phosphorus) until sampling. Tissue was sampled at vegetative stage V10 for the EXPERIMENT 1 – stem inoculation method evaluation and at V7 for EXPERIMENT 2 - fungal biomass using QPCR evaluation.

## Experiment 1 – stem inoculation method evaluation

### Experimental Design

Tests were conducted from July 2009 until October 2009. Three seeds were planted at random within one pot from two genotypes; GA209 (S) and Mp313E (R) (refer to Maize growth parameters discussed above). At the V2 stage, plants were thinned to one seedling per pot. Two tests were performed utilizing a randomized complete block design. Seedlings (one seedling per pot) of each of the two genotypes were subjected to three different treatments; inoculation with the syringe, slit and toothpick using the *A. flavus* aflatoxigenic strain NRRL 3357 and the non-inoculated controls used for comparison. Two trials were evaluated. Trial-1 had four replicates per treatment and Trial-2 had eight replicates per treatment.

Slit inoculation

Inoculum from *A. flavus* strain NRRL 3357 was prepared with a 2 cm CZP agar plug from an actively growing culture and inoculated onto a 100 mm Petri plate containing CZP agar and placed at room temperature for 30 days. A flame-sterilized stainless steel surgical No. 10 blade and scalpel was used to cut a small slit *ca.* 5 mm in diameter and 5 mm in length at a 45 degree angle halfway (*ca.* 3 mm) into the stem between the first and second node at the V5 stage. The scalpel was surface sterilized by dipping it into a container filled with 70% ethanol and flame sterilized between each cut. A 2 cm agar plug from an actively growing culture of each isolate was placed aseptically into the slit. The slit was covered with GLAD Press'n Seal® wrap (GLAD, Oakland, CA) for 7 days to promote fungal colonization and decrease outside contamination.

Syringe inoculation

A spore suspension was prepared from a 2 cm CZP agar plug from an actively growing culture of *A. flavus* strain NRRL 3357 and placed onto seven 60 x 15 mm Petri plates and left at room temperature for 14 days. Plates were flooded with 0.02% Tween 20 and scraped with a glass rod and solution was filtered through 4 layers of cheesecloth into 50 ml Falcon conical tube (Fisher Scientific, Pittsburg, PA). Conidial concentration was determined with a hemacytometer and adjusted with sterile distilled water to $9 \times 10^6$ conidia per ml. Secondly, spore suspensions of the *A. flavus* strain were prepared and a sterile 10 ml Luer-Lok syringe (Fisher Scientific, Pittsburg, PA) equipped with a 26G ½ Precision Guide needle (Fisher Scientific, Pittsburg, PA) was used for stem inoculation at the same sites as above and covered with self-sealing wrap as above.

Toothpick inoculation

Inoculum from *A. flavus* strain NRRL 3357 was prepared with a 2 cm CZP agar plug from an actively growing culture and placed onto a 100 x 15 mm Petri plate containing CZP agar using. Six sterilized round wooden toothpicks were placed aseptically on top of the agar inside the Petri plate containing the fungal isolate and stored at room temperature (22 to 24°C) for 30 days to promote thorough colonization. Sterilized toothpicks were placed in non-inoculated plates of CZP and served as a control. After each of the toothpicks were thoroughly colonized and covered with mycelium, one infested or control toothpick was used to inoculate each maize stem following the procedure by Koehler (1960). The method included; 1) at the vegetative V5, a malleable plastic ruler was used to measure 5 mm from the tip of a stainless steel ice chipper, 2) the distance was marked with painters tape (ScotchBlue, St. Paul, MN) on the ice chipper, 3) the ice chipper was sterilized and was then used to form a 5 mm opening into the maize stalk at a 45 degree angle between the first and second node, 4) one infested (or control) toothpick was chosen at random from the Petri plate with sterile forceps and was inserted into the opening, and 5) the toothpick was cut flush to the stalk with sterilized pruners and covered with self-sealing plastic wrap (GLAD Press'n Seal; GLAD, Oakland, CA) for 7 days to promote fungal colonization and prevent outside contamination.

Inoculation method evaluation

Plants were collected at the V10 stage (2 days consecutive sampling) and each maize stalk was excised with bypass pruning loppers at the soil line just above the brace roots. Each stalk was placed between two 38 mm × 89 mm x 184 mm wooden boards securely clamped with heavy-duty C-clamps. A utility knife was used to make a

longitudinal cut through the entire stem from the brace root to the apical meristem. Visual observation of tissue necrosis was used to select the best method of inoculation.

Experiment 2 - fungal biomass using QPCR evaluation

Greenhouse (GH) trials

Three seeds were chosen and planted (refer to Maize growth parameters discussed above) at random from each of the four genotypes. At the V2 stage, plants were culled to one seedling per pot. Seven GH tests were performed utilizing a randomized complete block design. Sixteen seedlings (1 seedling per pot) were chosen at random from each of the four maize genotypes. There were two treatments; the toothpick inoculation with the *A. flavus* aflatoxigenic strain NRRL 3357 or the control agar. Following the results of the inoculation tests, the toothpick procedure was the preferred method for screening genotypes at the V5 stage. Methodology using the toothpick inoculation procedure for the seven GH trials followed those discussed previously.

Greenhouse parameters including soil media, temperature data collection, and lighting intensity are discussed above. Studies were conducted from March - May 2010 (Trials 1 – 2), August – October 2010 (Trials 3 – 4), and March – June 2011 (Trials 5 – 7). Temperature ranges were as follows; a) trials 1 and 2 were 28°C – 38°C, 2) trials 3 and 4 were between 29°C and 40°C, and 3) trials 3 and 4 were between 32°C - 42°C. The lighting intensity range (PAR measured at leaf / whorl level) at the time of inoculation (V5 stage) was between 600 $\mu mol$ / $m^{-2}s^{-1}$ and 1000 $\mu mol$ / $m^{-2}s^{-1}$, and at the time of sampling (V7 stage) was between 800 $\mu mol$ / $m^{-2}s^{-1}$ and 1300 $\mu mol$ / $m^{-2}s^{-1}$.

Environmental growth chamber (EGC) trials

Three seeds from each of the four genotypes were planted in each plastic square (7.62 cm length x 7.62 cm width x 5.715 cm) in a greenhouse flat (15 flats per tray) (International Greenhouse Company, Danville, IL). Each plastic square was filled with sterilized calcined clay and placed in a Conviron environmental growth chamber (Model CMP 3244; Conviron, Pembina, ND). At the V2 stage, plants were culled to one seedling per square. Four EGC trials were performed utilizing a randomized complete block design. Seven seedlings (1 seedling per square) were chosen at random from each of the four different maize genotypes. There were two different treatments; the toothpick inoculation with the *A. flavus* aflatoxigenic strain NRRL 3357 or the control agar, both inserted into plants at V5.

Environmental growth chamber temperature settings were 37 +/- 2°C for 16 hours with lights on and 28 +/- 2°C for 8 hours with lights off. The lighting intensity or PAR range within the chamber prior to planting was between 900 and 1000 μmol $m^{-2}s^{-1}$. All plastic squares were hand-watered with 7 ml twice per week by removing and placing each one into the top basket of a glassware laboratory cart in order to avoid contamination. Each pot was hand-fertilized once per week with a 0.5 ml mix of nitrogen at a rate of 90.718 kg per 0.405 ha (33% ammonium nitrate) and potassium phosphate at a rate of 9.072 kg per 0.405 ha (28.7% potassium; 22.8% phosphorus) up until sampling at V7. Temperatures were monitored using two WatchDog B-Series Button 3619WD-2K Temperature Loggers placed at opposite sides of the incubator. Data was collected as stated in the above Greenhouse parameters section.

Field (FD) trials

Trial one was conducted from April to June 2010 and trial two from May to June 2011. Maize plants were cultivated in soil consisting of Leeper silty clay loam in the R. R. Foil Plant Science Research Center at MSU (Windham and Williams 2007). Each of the four genotypes were planted in individual plots (4 replicate plots) in single rows, 5.1 m in length spaced 0.96 m apart and thinned to 20 plants per row per plot (Windham and Williams 2007). The fertilizers and herbicides were applied according to the standard cultural practices for maize in northern Mississippi. Both FD trials were performed utilizing a randomized complete block design in 2010 and 2011. Four seedlings were chosen at random from each of the four different maize genotypes and each was subjected to two different treatments; toothpick inoculation with the *A. flavus* aflatoxigenic strain NRRL 3357 or the control agar at V5.

Visual inspections from inoculated plants

All inoculated plants were evaluated for visual discoloration or necrosis of tissue indicative of any type of fungal infestation at the inoculation site (=0 cm), 3 cm, 6 cm and / or beyond the 6 cm sampling sight and symptoms were noted. Symptoms included discoloration and darkening of stem tissue and / or sporulation of fungal hyphae. Plants in the GH and EGC were observed daily and the FD inoculated plants were observed every 3 to 5 days. Wilting, stunting, chlorosis, and insect infestation and / or damage were monitored daily.

Tissue sampling from inoculated plant material

Toothpick inoculated plants were harvested at the vegetative V7 stage from all GH, EGC, and FD trials conducted. The sampling procedure for each toothpick-inoculated stem was conducted as follows: 1) the maize stalk was excised with pruners at the soil line just above the brace roots, 2) a 10 cm section was measured from the excised portion from the soil line with pruners, 3) the 10 cm section was placed into a 50-ml Falcon centrifuge tube (Fisher Scientific, Pittsburg, PA) and the remaining stalk material was discarded, 4) tubes containing 10 cm sections were transported to the laboratory, 5) the toothpick was removed from each stem with sterile forceps and placed on a 100 x 15 Petri plate of CZP for each stem, 6) using a sterile stainless steel surgical No. 10 blade and scalpel, a radial cut was made through the entire 10 cm stem section of each stem, inoculated and control, and the total lesion or tissue necrotic area , if present, starting at the inoculation point was measured and recorded, 7) the 10 cm section was taped back together with painters tape (at the top and bottom ends) in order to cut cross sections from three exact points along the 10 cm stem section for further analysis. Two subsamples or cross sections (*ca.* 5 mm thick) were excised at each of the three areas of the 10 cm stem section including 0, 3, and 6 cm. One of the cross sections was used for DNA extraction and subsequent QPCR (refer to Quantitative PCR below) and the other was directly plated onto CZP containing Petri plate to confirm *A. flavus* fungal colonization.

Traditional isolation

To confirm fungal colonization, 100 mm Petri dishes containing Czapeks Dox agar (CZP) (Fisher Scientific, Pittsburg, PA) was employed. On the back of each plate, a permanent marker was used to divide the plate into three sections. For each stem, the 0, 3

and 6 cm tissue subsample was directly and aseptically placed with sterilized tweezers in the appropriate section. Plates were stored at room temperature and examined daily for 14 days for growth.

DNA extraction from inoculated plant material

The CTAB (hexadecyltrimethylammonium bromide EDTA ethylenediaminetetraacetic acid) DNA extraction method was used (Cubero et al 1988). All DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). Samples were evaluated only if the quality of DNA was above 1.7 (Absorbance $_{260}$/Absorbance $_{280}$ ratio).

Quantitative PCR

Oligonucleotide primers and probe are as follows; *OG1*: forward primer: 5' – GCC TCA AAG ATG TTG CGA GT – 3' (melting temperature or Tm) 55.7°C) and, *OG2*: reverse primer: 5' – GGT GAA GGC ACA TTC CAA CT – 3' (Tm 55.6°C) (Geiser et al 1998) and the newly designed *OMG3* TaqMan probe: 5' - FAM – TGT CCT CAC CAG GGA GAC CG – 3'BHQ-3 (Tm 61°C) (*OMG3* in Tables 1-3). The forward and reverse primer and the *OMG3* fluorescent Taqman probe were optimized using IDT (Integrated DNA Technology, Centennial, CO) Primer Quest Analysis and Design Tool (https://www.idtdna.com/pages/scitools 2013; accessed 2009).

The oligonucleotide primers and the dual labeled florescent TaqMan probe by published by (Leinberger et al 2005) were based on the 5.8 rRNA, 28S rRNA, and ITS2 target sequences. A 249 bp amplicon was amplified utilizing both end-point PCR and

QPCR using the primer set and Black Hole Quencher (BHQ2) probe as follows; *Asp1S* forward primer: 5' - ATG CCT GTC CGA GCG T – 3'(Tm 57.1°C), *AflR2* reverse primer: 5' - TTA AGT TCA GCG GGT ATR CC – 3' (Tm 55.1°C) and, the *ASP2* TaqMan probe: 5' – TAM – CGC TTG CCG AAC GCA AAT CAA TCT T – 3'BHQ-2 (Tm 60.8°C) (*ASP2* in Tables 1-3).

Quantitative PCR parameters were optimized based on previous experience and published research (Niessen 2007; Cruz and Buttner 2008; Mideros et al 2009). Quantitative PCR thermocycling considerations were adapted and optimized using the Cephied SmartCycler system (Sunnyvale, CA). Quantitative PCR reactions of 100 µl contained 50 µl of 2X Applied Biosystems (Grand Island, NY) Universal PCR Master Mix, 18 µl of 5 µM forward primer (Integrated DNA Technology, Centennial, CO), 18 µl of 5 µM reverse primer (Integrated DNA Technology, Centennial, CO), 13 µl of 2 µM TaqMan probe (Integrated DNA Technology, Centennial, CO), and 5 µl of DNA sample. Critical and optimized PCR cycling parameters were as follows: initial denaturation for 12 minutes at 96°C; 40 cycles of 95°C for 30 seconds, 59.5°C for 1 minute, and 72°C for 30 seconds, with a final extension of 10 minutes. Quantitative PCR products were randomly selected during each run and were separated on 1% agarose ethidium bromide gels in 1X TBE buffer confirm amplification. The 1000 bp DNA ladder (New England Biolabs, Ipswich, MA) was used as the molecular size marker.

Determination of fungal biomass in inoculated maize

The maize stem tissue subsamples; cross sections (*ca.* 5 mm thick) excised at each of the 3 areas of the 10-cm stem section; 0, 3, and at 6 cm from the seven GH, four EGC, and two FD trials were evaluated using the sequence-specific primers (Geiser et al 1998;

Leinberger et al 2005) and Taqman probes (Leinberger et al 2005 and listed above). Quantitative PCR runs were evaluated in duplicate. Between QPCR runs, QPCR products amplified by either of the TaqMan probes (*OMG3*; 199 bp fragment or *ASP2*; 249 bp fragment) were selected at random and were separated on 1% agarose ethidium bromide gels in 1X TBE buffer to confirm amplification. The 1000 bp DNA ladder (New England Biolabs, Ipswich, MA) was used as the molecular size marker.

### Data Analysis

All statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary, NC). Data were subjected to analysis of variance according to the Proc GLM procedure. Means for lesion lengths (cm) and fungal biomass (ng / µl) were separated using a least significant difference of $P < 0.05$. Standard curves were generated and all Ct values and amplification statistics are reported by Cehpied Smartcycler software Version 2.01 as the mean Ct value of two replicates in all experiments conducted in this study.

## Results

### Experiment 1 – stem inoculation determination method evaluation

Three inoculation methods were tested in the GH and evaluated based on visual tissue necrosis comparing the syringe, slit, and toothpick methods. Based on the two trials evaluating inoculation methods genotypes, S (GA209) and R (Mp313E), none of the plants inoculated with the syringe or the slit method exhibited superficial or internal signs of tissue necrosis and / or signs of any type of fungal infestation such as aerial hyphae or sporulation when compared to the non-inoculated controls. The toothpick method was the most suitable as dark brown lesions were clearly visible in the radial section in 83% of

the *A. flavus*-inoculated stems (30 of 36 plants). Interestingly, of the 30 plants (83%), 17 seedlings (58%) were from the susceptible genotype, GA209. The remaining six plants had no visible lesion, five plants were GA209 and one plant was Mp313E.

**Experiment 2 - fungal biomass using QPCR evaluations**

Within all settings (GH, EGC and FD), stem tissue necrosis was observed in the radial sections in *A. flavus* toothpick-inoculated stems. In most cases, the S and R plants examined exhibited lesions in the radial section starting at the inoculation site (0 cm) growing lengthwise up to 3 cm or slightly greater, with an overall average of 2.48 cm. The GH and EGC plant stem tissues usually exhibited lesions that were a darker brown to black and the field plant lesions were light tan, slightly darker within the phloem and pith of the stalk. Microscopic evaluations revealed that necrotic lesions were primarily limited to the phloem in GH and FD inoculations. Microscopic observations within the EGC trials varied, however some susceptible plants showed a clear demarcation of necrosis crossing over from the phloem, into the cambial tissue, then into the pith (Figure 1).

Necrotic lesions observed in susceptible genotypes were significantly larger across 4 of the 7 GH trials (Table 4.1). There were no significant differences in lesion lengths ($P < 0.05$) when comparing between GH, EGC and FD (Tables 4.1 – 4.3). Across trials, necrotic lesion data for EGC trials, when significant, varied between the four genotypes (Table 4.2).

The artificially toothpick-inoculated maize stems, including the control, from the GH, EGC and the FD were also evaluated using traditional isolations (Table 4.4). Fungal identification was confirmed using morphological characteristics and comparing the isolated *A. flavus* cultures to strain NRRL 3357 used for inoculation. Traditional

isolation data from each sampling point (0, 3 and 6 cm), and from *A. flavus* - inoculated plants was analyzed separately. All 7 GH trials were combined and 93% of all tissues sampled from the inoculation point (0 cm) were positive for *A. flavus.* Similar data from EGC and FD at 0 cm showed 88% and 98%, respectively; at the 3 cm sampling site at 83%, 79% and 96% for the GH, EGC and FD, respectively; and at 6 cm, slightly lower percentage of *A. flavus* isolates were recovered. Although, lesion length does not support cultural and QPCR data, DNA fungal biomass was detected within control tissues with no observable necrosis.

Concerning tissue necrosis, there were no significant trends with respect to pooled lesion sampling sites and pooled fungal biomass data across treatment, trial or setting. The overall number of culture isolations confirmed that 91% of all *A. flavus*-inoculated stem tissues sampled were positive using the QPCR *OMG3* probe were also positive using traditional isolation (881 / 968 plants). In comparison, 21% (75 / 357 plants) of the control plants (toothpick non-inoculated) from *EXPERIMENT 2* were positive for *A. flavus* using traditional isolation and identified based on morphological structures compared to the isolate used for inoculation (Table 4.4). Quantitative PCR using both *OMG3* and *ASP2* TaqMan probes verified these findings (Tables 4.1- 4.3). In addition, both *OMG3* and *ASP2* probes positively amplified *Aspergillus* spp. from 2% of all control plant tissues pooled from 0, 3 and 6 cm along the stems.

## Discussion

Optimizing the environmental parameters for fungal growth, infection and toxin production is imperative. Overall, the temperature data collected in the GH was consistent with growing conditions for infection process by *A. flavus* in ear and for stem

inoculations in maize (Kelley 1984; Windham and Williams 2007). Several preliminary experiments comparing different parameters such as lighting intensity, fertilization rates, pot size, and potting media and volume revealed that although plant growth in EGC was not comparable to GH and FD (differences in height, internode length, and stem diameter), the GH provided the best controlled conditions for screening seedlings using the toothpick stem inoculation method. During the initial studies it was also determined that the toothpick method can cause significant damage to the plant tissue during growth if the toothpick is inserted before the V5 stage. The apical meristem does not emerge from the soil line until V4, therefore the toothpick would travel through the whorl if inserted before V5.

Although the GH, EGC, and FD did not have significant differences between lesion lengths and fungal biomass data four of the seven GH trials did display significantly greater lesion lengths in the susceptible genotypes across the seven trials. These results indicate there is potential to further refine the method for GH screening in the future. Especially the limited tissue sampling sites at 0, 3 and 6 cm need to be refined to reflect the results from this investigation. Previous studies have shown that stem inoculations can be successful in other field grown crops (Drepper and Renfro 1990; Pratt 1991; Windham and Williams 2007; Twizeyimana et al 2012). Windham and Williams (2007) used the toothpick method to examine the systemic infection of *A. parasiticus* in stalks and ears inoculated between the $5^{th}$ and $6^{th}$ node. Results from their study showed that aflatoxin resistant or susceptible genotypes were not clearly separated. Based on the previous studies and current research, future studies could be improved by; 1) inoculating between nodes, 2) creating a larger size wound before inserting toothpick(s), 3) moving

the inoculation site to a higher node, such as the 3rd or 4th node, 4) allowing the plant to grow past the V5 stage, possibly inoculate at V6 or V7, and 5) sampling the entire stem instead of choosing sampling sites.

Overall average lesion length was 2.48 cm and a range across all tests and genotypes from 1.47 to 3.52 cm (Tables 4.1 – 4.3; Figures 4.1 and 4.2). It is important to note that tissue necrosis was not observed at the sampling site of 6 cm, however *A. flavus* fungal biomass was still detected in all four genotypes when sampled at 6 cm (Tables 4.1 – 4.3). Studies conducted by G. L. Windham (personal communication; 2013), regularly isolated *A. flavus* from stalk tissue that exhibited no symptoms from *A. flavus* or from *A. parasiticus* infestation using cultural isolation.

Lesion and fungal biomass data collected from the GH, EGC and FD trials are shown in Tables 4.1, 4.2 and 4.3. Mean lesion length for the four genotypes combined for each study location was 1.78, 2.56 and 2.61cm, respectively. Based on this data, another improvement to this research would be to sample the stem tissue at the following points; 0, 0.5, 1, 1.5, 2, 2.5 and 3 cm. For resistance studies, adding additional sampling sites and only sampling up to 3 cm should be sufficient to determine susceptibility or resistance to the fungus.

There are many reasons plants can be resistant to fungal disease and those resistant factors may be phenotypic or genotypic mechanisms. A study by Barros-Rios et al (2011) reports that a thicker and stiffer cell wall is found within the pith of the R and S maize genotypes than the S genotypes used in his studies (Barros-Rios et al 2011). Resistant genotypes exhibited an increased level of xylose, arabinose, lignin, and total cell wall material (Barros-Rios et al 2011). It was also observed that xylose is present in

the R genotypes he used and it is 19% greater than in S genotypes. It is possible that simple sugars within the cell wall may have resulted in the movement of the fungus responsible for tissue necrosis and possible pathogen colonization of the entire x-section of the S stalk at that specific point of sampling (Figure 4.1). Susceptible genotypes may have more vulnerable pith tissues because a higher level of glucose is found within the cell walls (Barros-Rios et al 2011). The study also found that susceptible genotype pith tissue cell walls also released specific phenolic compounds in response to wounding compared to resistant genotypes (Barros-Rios et al 2011), thus possibly inhibiting fungal colonization to occur or establish within the stalk. In the current study, QPCR results varied and were not consistent over the tissue sites sampled. The phenolic compounds found within the pith tissue cell walls may have inhibited QPCR results, therefore skewing fungal biomass data, which would explain the high variability. Although there was no clear separation between R and S genotypes using QPCR, the study did demonstrate that fungal biomass could be detected and quantified within the maize stem tissue regardless of lesion size, genotype, stem sampling site, or setting. The results further demonstrate that QPCR is more sensitive than traditional isolation, especially when using the *OMG3* and *ASP2* probes in maize tissue. Quantitative PCR confirmed isolations from tissues using probes and amplified additional *Aspergillus* spp. in maize tissue that were not isolated on CZP media.

## Conclusion

Although there was no significant difference between lesion length and relative resistance, four of the seven GH trials determined that the S genotype, GA209, had significantly larger lesions than the other three evaluated. Based on the results of the

study, lesion length may be a good indicator to determine fungal colonization once more is understood about the physiological nature of maize stalks and the toothpick inoculation method is refined *in vivo.* This work focused on inoculating at the first node at the earliest possible stage (V5) to essentially speed up the screening process. No other nodes or stages were investigated. Only the three tissue-sampling sites per stem, the inoculation point (0 cm), 3 cm and 6 cm, were sampled. Data from this study shows that lesion lengths from the inoculation points, regardless of genotype, were generally always less than 3 cm in length and the overall means were 1.78, 2.56 and 2.61cm for the GH, EGC and FD, respectively. Based on this data, further research needs focus on refining tissue-sampling methods; sampling in 0.5 increments in length up to 3 cm or even increasing length of time for tissue sampling following inoculation. Some slight changes in sampling procedures may provide more useful data when evaluating maize genotypes.

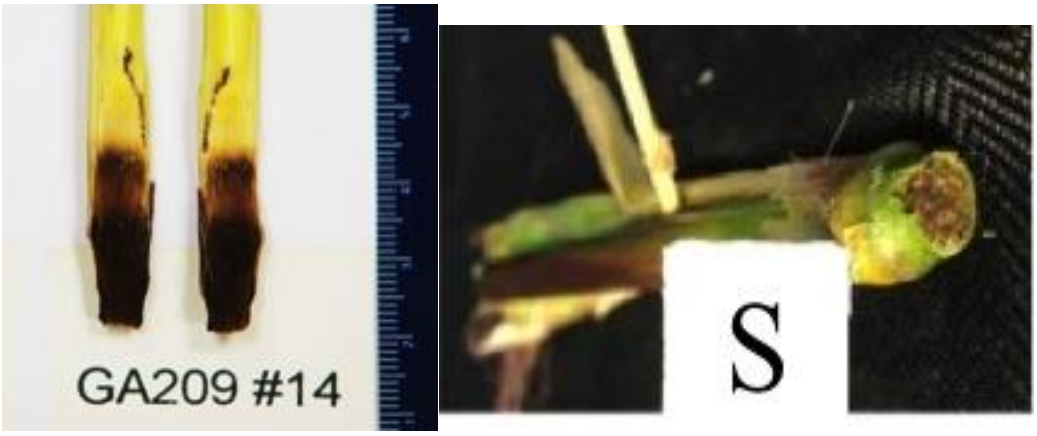


Figure 4.1 Macroscopic observations.

Longitudinal section of the inoculated susceptible genotype GA209 #14 within the EGC trials shows a clear demarcation of necrosis crossing over from the phloem, into the cambial tissue, then into the pith. In the S genotype GA209, the lesion is moving through the cambium to the pith to exhibit complete tissue necrosis. The entire cross section of the S stem at that specific point of sampling (3 cm sample site)

Table 4.1 Greenhouse trials 1 through 7.

| GREENHOUSE | INOCULUM[v] | | | GENOTYPE | | | | |
|---|---|---|---|---|---|---|---|---|
| **TRIAL 1** | Control[x] | 3357[y] | LSD | MP717(R) | MP313E(R) | SC212M(S) | GA209(S) | LSD |
| **ASP2** | **Fungal Biomass (ng/ul) [z]** | | | | | | | |
| *0 cm sample site* | 11.73 | 10.11 | 2.68 | - | 11.59 | - | 10.28 | 2.69 |
| *3 cm sample site* | 13.09a | 17.43b | 3.56 | - | 13.10a | - | 17.65b | 3.46 |
| *6 cm sample site* | 13.91a | 10.91b | 2.69 | - | 11.71 | - | 12.67 | 2.72 |
| *Pooled sample sites*[w] | 33.04 | 34.05 | 4.79 | - | 31.36 | - | 27.93 | 4.60 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 11.65 | 11.26 | 2.76 | - | 12.02 | - | 10.32 | 2.81 |
| *3 cm sample site* | 12.02 | 11.75 | 2.87 | - | 9.56a | - | 12.54b | 2.87 |
| *6 cm sample site* | 11.22 | 11.15 | 2.75 | - | 11.22 | - | 11.15 | 2.75 |
| *Pooled sample sites* | 31.36 | 27.93 | 4.65 | - | 30.81a | - | 36.80b | 4.65 |
| **TRIAL 2** | | | | | | | | |
| **Lesion Length (cm) N = 242** | 1.98 | 1.56 | 0.41 | 1.73 | 1.55 | 2.02 | 1.82 | 0.77 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 11.76 | 11.25 | 2.10 | 12.37 | 10.68 | 11.89 | 11.14 | 3.92 |
| *3 cm sample site* | 10.26 | 13.08 | 1.98 | 14.47a | 10.36b | 10.74b | 11.56ab | 3.69 |
| *6 cm sample site* | 10.55 | 12.40 | 1.98 | 10.17 | 11.77 | 11.37 | 12.74 | 3.70 |

Table 4.1 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Pooled sample sites* | 28.66a | 32.53b | 3.46 | 31.48 | 29.64 | 31.10 | 30.25 | 6.51 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 10.39 | 10.79 | 1.80 | 9.29b | 13.36a | 9.86a | 9.67b | 3.26 |
| *3 cm sample site* | 11.46 | 12.00 | 2.79 | 11.63 | 11.13 | 12.30 | 11.93 | 3.14 |
| *6 cm sample site* | 11.53 | 11.40 | 1.89 | 10.41 | 11.03 | 10.47 | 13.82 | 3.47 |
| *Pooled sample sites* | 31.03 | 31.85 | 3.12 | 29.61 | 34.41 | 28.83 | 32.83 | 5.73 |
| **TRIAL 3** | | | | | | | | |
| **Lesion Length (cm) N = 237** | 1.46a | 2.18b | 0.45 | 2.82a | 1.45a | 1.52a | 1.57b | 0.81 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 10.77a | 13.79b | 1.92 | 14.00 | 11.23 | 11.25 | 12.80 | 3.61 |
| *3 cm sample site* | 11.31 | 12.13 | 1.83 | 11.40 | 11.11 | 12.30 | 12.08 | 3.41 |
| *6 cm sample site* | 12.27 | 11.19 | 1.91 | 12.91 | 11.20 | 12.03 | 10.84 | 3.55 |
| *Pooled sample sites* | 32.17a | 35.29b | 2.79 | 34.38 | 31.80 | 33.92 | 34.83 | 3.66 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 11.45 | 11.87 | 1.86 | 13.49 | 10.07 | 12.42 | 10.73 | 3.42 |
| *3 cm sample site* | 12.85a | 10.94b | 1.78 | 11.45ab | 14.10a | 10.61b | 11.41ab | 3.29 |
| *6 cm sample site* | 11.98a | 9.94b | 1.77 | 10.14 | 10.27 | 10.83 | 12.65 | 3.30 |
| *Pooled sample sites* | 34.04a | 30.86b | 2.99 | 33.67 | 32.67 | 30.86 | 32.59 | 3.66 |

Table 4.1 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **TRIAL 4** | | | | | | | | |
| **Lesion Length (cm) N = 244** | 1.49 | 1.47 | 0.25 | 1.58 | 1.42 | 1.45 | 1.48 | 0.47 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 10.78 | 10.38 | 1.78 | 13.68a | 10.14b | 9.77b | 9.15b | 3.22 |
| *3 cm sample site* | 13.12 | 13.21 | 1.91 | 13.07ab | 11.08b | 14.59a | 14.20ab | 3.51 |
| *6 cm sample site* | 9.42a | 12.26b | 1.87 | 11.39ab | 9.86ab | 12.98a | 9.22b | 3.48 |
| *Pooled sample sites* | 30.49 | 33.63 | 3.30 | 34.64a | 29.67ab | 35.55a | 28.39b | 6.02 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 10.58 | 10.32 | 1.74 | 9.97ab | 12.70a | 9.00b | 10.11ab | 3.19 |
| *3 cm sample site* | 12.26 | 12.08 | 1.93 | 11.00b | 15.03a | 11.17b | 11.26b | 3.50 |
| *6 cm sample site* | 11.71 | 11.39 | 1.97 | 10.68 | 13.92 | 10.47 | 11.28 | 3.63 |
| *Pooled sample sites* | 32.27 | 31.48 | 2.79 | 30.30b | 38.86a | 27.77b | 30.56b | 3.66 |
| **TRIAL 5** | | | | | | | | |
| **Lesion Length (cm) N = 254** | 1.53 | 1.74 | 0.43 | 1.71 | 1.60 | 1.76 | 1.48 | 0.80 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 13.39 | 11.79 | 1.91 | 12.63 | 12.98 | 12.32 | 12.42 | 3.59 |
| *3 cm sample site* | 12.34 | 12.14 | 2.05 | 10.88 | 11.37 | 12.90 | 13.77 | 3.79 |
| *6 cm sample site* | 13.07 | 10.46 | 1.92 | 11.52ab | 14.02a | 11.54ab | 10.38b | 3.58 |

Table 4.1 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Pooled sample sites* | 36.87 | 31.44 | 3.57 | 33.31 | 33.67 | 34.38 | 35.22 | 6.77 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 10.18 | 11.14 | 1.65 | 10.15 | 10.91 | 11.55 | 10.11 | 3.07 |
| *3 cm sample site* | 11.28 | 11.55 | 1.93 | 11.00 | 9.16 | 12.10 | 12.66 | 3.54 |
| *6 cm sample site* | 10.73 | 12.49 | 1.81 | 11.00ab | 9.77b | 11.75ab | 14.00a | 3.33 |
| *Pooled sample sites* | 29.72 | 32.81 | 3.20 | 29.80ab | 27.48b | 33.73a | 34.11a | 5.88 |
| **TRIAL 6** | | | | | | | | |
| **Lesion Length (cm) N= 255** | 1.38a | 2.19b | 0.54 | 1.40 | 1.36 | 2.13 | 2.27 | 1.00 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 12.62 | 11.03 | 1.90 | 11.17b | 9.97b | 14.88a | 11.51ab | 3.45 |
| *3 cm sample site* | 11.38 | 10.74 | 1.83 | 9.20 | 11.27 | 11.92 | 12.00 | 3.37 |
| *6 cm sample site* | 10.13a | 12.23b | 1.85 | 13.27 | 10.89 | 10.73 | 9.85 | 3.43 |
| *Pooled sample sites* | 31.30 | 31.98 | 3.12 | 32.53 | 30.20 | 33.32 | 30.55 | 5.79 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 10.27 | 11.69 | 1.83 | 9.72 | 11.68 | 10.24 | 12.39 | 3.40 |
| *3 cm sample site* | 13.51 | 11.36 | 2.79 | 12.24 | 13.27 | 11.02 | 13.00 | 4.10 |
| *6 cm sample site* | 11.88 | 10.81 | 2.79 | 12.03 | 11.57 | 10.10 | 11.69 | 3.31 |
| *Pooled sample sites* | 33.15 | 30.94 | 3.37 | 31.14 | 34.67 | 28.78 | 33.52 | 6.21 |

Table 4.1 (continued)

**TRIAL 7**

| **Lesion Length (cm) N=256** | 1.30 | 2.70 | 2.79 | 1.36b | 1.30b | 1.98b | 3.37a | 3.66 |
|---|---|---|---|---|---|---|---|---|
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 10.42 | 9.45 | 1.73 | 8.33b | 9.49ab | 11.88a | 9.87ab | 3.18 |
| *3 cm sample site* | 11.15 | 13.83 | 3.91 | 11.13 | 11.68 | 13.57 | 13.58 | 3.54 |
| *6 cm sample site* | 11.66a | 10.63b | 2.00 | 10.26bc | 14.67a | 11.96ab | 7.87c | 3.55 |
| *Pooled sample sites* | 30.31 | 31.92 | 3.17 | 29.08b | 31.48ab | 35.06a | 28.83b | 3.66 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 12.43 | 13.39 | 2.00 | 12.84 | 14.36 | 11.11 | 13.47 | 3.70 |
| *3 cm sample site* | 10.79a | 13.98b | 1.88 | 13.23 | 12.13 | 12.97 | 10.93 | 3.57 |
| *6 cm sample site* | 10.67 | 10.10 | 1.74 | 9.27 | 10.93 | 10.33 | 11.08 | 3.24 |
| *Pooled sample sites* | 32.91 | 34.91 | 2.79 | 34.11 | 35.23 | 34.41 | 31.88 | 6.02 |

Mean lesion lengths and fungal biomass following toothpick inoculation in maize at the V5 growth stage.[v]*Aspergillus flavus* inoculum used in this study was prepared with the aflatoxigenic strain NRRL 3357. [w]All plants and lesion data were pooled Each plant was subjected to toothpick inoculation with NRRL 3357. [x]All plants and lesion data were pooled. Each plant was non-inoculated with a toothpick inserted into the stem and served as the control (represented in the first column). In environmental growth chamber (EGC) trial 1, only one R and one S genotype was evaluated. [y]Fungal biomass (DNA) in ng / ul detected by Quantitative PCR either the *ASP2* or *OMG3* TaqMan probe at each site sampled within the lesion. [z]Pooled sampled sites is the total fungal biomass combined for each trial. For example; 0 cm + 3 cm + 6 cm = *Pooled sample site*. Means for lesion lengths (cm) and fungal biomass (ng / µl) were separated using a least significant difference of $P < 0.05$.

Table 4.2 Growth chamber trials 1 through 4.

| **GROWTH CHAMBER** | | | **INOCULUM**[v] | | | **GENOTYPE** | | |
|---|---|---|---|---|---|---|---|---|
| **TRIAL 1** | Control[x] | 3357[y] | LSD | Mp717(R) | Mp313E(R) | SC212M(S) | GA209(S) | LSD |
| **Lesion Length (cm) N = 56** | 2.65a | 5.25b | 2.55 | - | 2.56b | - | 5.34a | 2.53 |
| **ASP2** | | | | **Fungal Biomass (ng/ul)**[z] | | | | |
| *0 cm sample site* | 9.14 | 11.00 | 3.29 | - | 10.14 | - | 10.00 | 3.33 |
| *3 cm sample site* | 11.57a | 16.82b | 4.19 | - | 14.14 | - | 14.25 | 4.43 |
| *6 cm sample site* | 13.14 | 14.39 | 3.60 | - | 14.75 | - | 12.79 | 3.58 |
| *Pooled sample sites*[w] | 33.86a | 42.21b | 7.04 | - | 37.04 | - | 37.04 | 7.38 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 10.32 | 10.04 | 2.93 | - | 10.00 | - | 10.36 | |
| *3 cm sample site* | 13.00 | 13.32 | 3.09 | - | 11.43a | - | 14.89b | 2.95 |
| *6 cm sample site* | 11.86 | 13.04 | 3.91 | - | 12.18 | - | 12.71 | 3.92 |
| *Pooled sample sites* | 35.18 | 36.39 | 6.24 | - | 33.61 | - | 37.96 | 6.14 |
| **TRIAL 2** | | | | | | | | |
| **Lesion Length (cm) N = 55** | 1.35a | 5.46b | 1.90 | 4.04 | 3.22 | 3.31 | 3.05 | 4.19 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 11.04 | 11.61 | 3.44 | 12.50 | 9.86 | 10.79 | 12.14 | 6.47 |
| *3 cm sample site* | 16.29 | 12.79 | 4.34 | 18.29a | 13.00ab | 17.43ab | 9.43b | 7.61 |

Table 4.2 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *6 cm sample site* | 11.68 | 13.79 | 3.90 | 13.14 | 11.93 | 11.29 | 14.57 | 7.40 |
| *Pooled sample sites* | 39.00 | 38.18 | 6.21 | 43.93 | 34.79 | 34.79 | 36.14 | 11.28 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 8.89 | 8.11 | 2.91 | 10.93 | 8.43 | 6.21 | 8.43 | 5.28 |
| *3 cm sample site* | 10.25 | 10.18 | 3.59 | 11.07 | 7.71 | 14.07 | 8.00 | 6.29 |
| *6 cm sample site* | 8.79 | 11.00 | 3.78 | 8.93 | 6.79 | 10.57 | 13.29 | 6.86 |
| *Pooled sample sites* | 27.93 | 29.29 | 5.78 | 30.93 | 22.93 | 30.86 | 29.71 | 10.50 |
| **TRIAL 3** | | | | | | | | |
| **Lesion Length (cm) N = 56** | 1.42a | 5.29b | 1.68 | 2.13 | 2.52 | 2.80 | 3.96 | 3.61 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 11.29 | 15.43 | 5.02 | 8.36b | 12.00ab | 15.21ab | 17.86a | 9.10 |
| *3 cm sample site* | 15.29 | 15.43 | 4.05 | 13.21 | 14.00 | 15.93 | 18.29 | 7.45 |
| *6 cm sample site* | 13.61 | 12.00 | 3.70 | 10.71 | 12.71 | 16.21 | 11.57 | 6.77 |
| *Pooled sample sites* | 40.18 | 42.86 | 7.61 | 32.29b | 38.71ab | 47.36a | 47.71a | 12.96 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 3.72a | 12.18b | 3.59 | 8.71b | 9.36ab | 15.14a | 12.43ab | 6.35 |
| *3 cm sample site* | 13.04 | 14.21 | 3.72 | 10.79 | 17.36 | 12.57 | 13.79 | 6.66 |
| *6 cm sample site* | 12.50 | 10.82 | 3.51 | 12.50 | 14.50 | 9.14 | 10.50 | 6.41 |

Table 4.2 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Pooled sample sites* | 36.18 | 37.21 | 6.63 | 32.00 | 41.21 | 36.86 | 36.71 | 12.19 |
| **TRIAL 4** | | | | | | | | |
| **Lesion Length (cm) = 56** | **N** 2.16a | 3.73b | 1.56 | 1.40 | 2.43 | 1.78 | 1.23 | 3.48 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 13.89 | 18.46 | 5.11 | 1.50b | 1.41b | 5.49a | 3.46ab | 2.54 |
| *3 cm sample site* | 13.44 | 13.82 | 4.16 | 14.29 | 13.86 | 13.86 | 12.46 | 7.91 |
| *6 cm sample site* | 10.70 | 12.68 | 3.94 | 10.07 | 14.07 | 9.93 | 12.85 | 7.36 |
| *Pooled sample sites* | 38.04 | 44.96 | 7.80 | 38.36 | 48.36 | 41.64 | 37.62 | 14.66 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 9.00 | 12.93 | 3.42 | 9.57 | 12.21 | 12.14 | 10.00 | 6.72 |
| *3 cm sample site* | 12.74 | 11.54 | 3.86 | 11.21 | 15.14 | 13.29 | 8.62 | 6.96 |
| *6 cm sample site* | 6.89 | 9.11 | 3.07 | 7.43 | 10.29 | 7.86 | 6.39 | 5.79 |
| *Pooled sample sites* | 28.63 | 33.57 | 6.21 | 28.21ab | 37.64a | 33.29ab | 25.00b | 11.04 |

Lesion lengths and fungal colonization following toothpick inoculation stage in maize V5 growth stage.[v]*Aspergillus flavus* inoculum used in this study was prepared with the aflatoxigenic strain NRRL 3357. [w]All plants and lesion data were pooled. Each plant was non-inoculated with a toothpick inserted into the stem and served as the control (represented in the first column). [x]All plants and lesion data were pooled  Each plant was subjected to toothpick inoculation with NRRL 3357. In environmental growth chamber (EGC) trial 1, only one R and one S genotype was evaluated. [y]Fungal biomass (DNA) in ng / ul detected by Quantitative PCR either the *ASP2* or *OMG3* TaqMan probe at each site sampled within the lesion. [z]Pooled sampled sites is the total fungal biomass combined for each trial. For example; 0 cm + 3 cm + 6 cm = *Pooled sample site*. Means for lesion lengths (cm) and fungal biomass (ng / µl) were separated using a least significant difference of $P < 0.05$.

Table 4.3 Field trials 1 and 2.

| FIELD | | | INOCULUM[v] | | | GENOTYPE | | |
|---|---|---|---|---|---|---|---|---|
| **TRIAL 1** | Control[x] | 3357[y] | LSD | Mp717(R) | Mp313E(R) | SC212M(S) | GA209(S) | LSD |
| **Lesion Length (cm) N = 22** | 1.52 | 1.98 | 1.21 | 1.89 | 0.90 | 1.68 | 2.75 | 2.23 |
| **ASP2** | | | | **Fungal Biomass (ng/ul)[Z]** | | | | |
| *0 cm sample site* | 12.00 | 11.10 | 5.90 | 11.71 | 16.00 | 6.80 | 10.75 | 10.41 |
| *3 cm sample site* | 9.75 | 14.50 | 6.53 | 16.14 | 6.50 | 11.80 | 12.75 | 12.23 |
| *6 cm sample site* | 10.50 | 11.80 | 5.40 | 11.57 | 10.17 | 11.80 | 10.75 | 11.09 |
| *Pooled sample sites*[w] | 32.25 | 37.40 | 10.83 | 39.43 | 32.67 | 30.40 | 34.25 | 21.76 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 8.83 | 7.70 | 4.74 | 11.00 | 7.67 | 5.60 | 11.00 | 9.00 |
| *3 cm sample site* | 9.17 | 10.40 | 6.25 | 10.86 | 11.17 | 9.20 | 6.25 | 12.43 |
| *6 cm sample site* | 8.92 | 6.20 | 2.84 | 8.00 | 8.33 | 7.80 | 6.00 | 6.20 |
| *Pooled sample sites* | 26.92 | 24.30 | 9.51 | 29.86 | 27.17 | 22.60 | 20.25 | 18.38 |
| **TRIAL 2** | | | | | | | | |
| **Lesion Length (cm) N = 20** | 2.49 | 4.28 | 2.97 | 5.18 | 1.85 | 5.10 | 2.17 | 7.27 |
| **ASP2** | | | | | | | | |
| *0 cm sample site* | 15.91 | 8.17 | 9.98 | 10.50 | 14.67 | 14.75 | 11.67 | 21.92 |
| *3 cm sample site* | 14.27 | 14.67 | 7.34 | 17.00 | 14.17 | 12.50 | 14.00 | 14.67 |

Table 4.3 (continued)

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *6 cm sample site* | 10.09 | 9.33 | 6.54 | 9.00 | 12.67 | 12.51 | 8.00 | 12.51 |
| *Pooled sample sites* | 40.27 | 32.17 | 18.68 | 36.50 | 41.50 | 35.00 | 33.67 | 38.86 |
| **OMG3** | | | | | | | | |
| *0 cm sample site* | 14.82a | 3.00b | 8.88 | 7.25 | 9.50 | 15.75 | 10.67 | 21.50 |
| *3 cm sample site* | 13.27 | 11.17 | 9.44 | 14.00 | 10.83 | 9.50 | 18.00 | 18.27 |
| *6 cm sample site* | 10.27 | 9.00 | 8.24 | 5.75 | 11.33 | 12.25 | 9.00 | 4.15 |
| *Pooled sample sites* | 38.36 | 23.17 | 18.33 | 27.00 | 31.67 | 37.50 | 37.67 | 40.31 |

Lesion lengths and fungal colonization following toothpick inoculation stage in maize V5 growth stage. [v]*Aspergillus flavus* inoculum used in this study was prepared with the aflatoxigenic strain NRRL 3357. [w]All plants and lesion data were pooled. Each plant was non-inoculated with a toothpick inserted into the stem and served as the control (represented in the first column). [x]All plants and lesion data were pooled  Each plant was subjected to toothpick inoculation with NRRL 3357. In environmental growth chamber (EGC) trial 1, only one R and one S genotype was evaluated. [y]Fungal biomass (DNA) in ng / ul detected by Quantitative PCR either the *ASP2* or *OMG3* TaqMan probe at each site sampled within the lesion. [z]Pooled sampled sites is the total fungal biomass combined for each trial. For example; 0 cm + 3 cm + 6 cm = *Pooled sample site*. Means for lesion lengths (cm) and fungal biomass (ng / µl) were separated using a least significant difference of $P < 0.05$.

Table 4.4 Overall percent frequencies from all inoculated plant tissues using traditional isolations.

| | INOCULUM[z] | | GENOTYPE | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Across All Trials | **Control** | **3357** | **Mp717E(R)** | | **Mp313E(R)** | | **SC212M(S)** | | **GA209(S)** | |
| | | | Control | 3357 | Control | 3357 | Control | 3357 | Control | 3357 |
| **Greenhouse** | | | | | | | | | | |
| *0 cm sample site* | 22 | 93 | 20 | 93 | 22 | 89 | 24 | 92 | 22 | 97 |
| *3 cm sample site* | 9 | 88 | 11 | 85 | 9 | 89 | 8 | 92 | 8 | 86 |
| *6 cm sample site* | 4 | 72 | 1 | 75 | 3 | 72 | 4 | 71 | 6 | 72 |
| **Environmental Growth Chamber** | | | | | | | | | | |
| *0 cm sample site* | 5 | 88 | 5 | 88 | 4 | 89 | 8 | 85 | 4 | 85 |
| *3 cm sample site* | 3 | 79 | 2 | 79 | 3 | 77 | 4 | 77 | 3 | 79 |
| *6 cm sample site* | 2 | 75 | 2 | 72 | 2 | 74 | 2 | 79 | 2 | 77 |
| **Field** | | | | | | | | | | |
| *0 cm sample site* | 28 | 98 | 27 | 96 | 29 | 98 | 26 | 94 | 29 | 97 |
| *3 cm sample site* | 12 | 96 | 11 | 98 | 13 | 96 | 11 | 96 | 12 | 94 |
| *6 cm sample site* | 4 | 79 | 4 | 69 | 3 | 81 | 4 | 81 | 5 | 85 |

[z]*Aspergillus flavus* inoculum used in this study was prepared with the aflatoxigenic strain NRRL 3357. All plants subjected to toothpick inoculation were inoculated with NRRL 3357. A non-inoculated toothpick inserted into the stem served as the control (represented in the first column). To confirm fungal colonization, 100 mm Petri dishes containing Czapeks Dox agar (CZP) (Fisher Scientific, Pittsburg, PA) was employed. On the back of each plate, a permanent marker was used to divide the plate into three sections. For each stem, the 0, 3 and 6 cm tissue subsample was directly and aseptically placed with sterilized tweezers in the appropriate section. Plates were stored at room temperature and examined daily for 14 days for growth. Each isolate was compared morphologically to the NRRL strain 3357 used to inoculate to confirm colonization. Each section of each plate was counted as a positive or negative for the presence of *A. flavus*. The percent frequency was calculated as the total positive divided by the total the number of sections plated for that subsample multiplied by 100.

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# CHAPTER V

# GENERAL CONCLUSION

A specifically designed oligonucleotide, dual-labeled florescent probe (*OMG3*) to amplify DNA within a specific gene target *aflP* (within the aflatoxin biosynthetic pathway) was used. A standard curve was developed based on known DNA concentrations, and the curve could then be used to evaluate comparing resistance and susceptible genotypes. The QPCR method and the novel *OMG3* probe were tested. Both *in vivo* and *in situ* procedures were developed and refined. The toothpick inoculation method was used to artificially inoculate maize stems in the vegetative stage. This method was 91% consistent for infecting maize plants however inoculation must occur during the vegetative V5 stage of growth to avoid mechanical tissue damage. Only lesion length data in 4 out of the 7 greenhouse trials showed significantly greater measured lengths in resistant lines than in susceptible lines. Based on this data, additional research needs to focus on refining tissue-sampling methods; sampling in 0.5 increments in lengths up to 3 cm or even increasing length of time for tissue sampling following inoculation. Procedural changes in sampling procedures may provide more useful data when evaluating maize genotypes. Solid phase microextraction gas chromatography – mass spectrometry was used to determine the predominant microbial volatile organic compounds (MVOC's) extracted by from both aflatoxigenic and non-aflatoxigenic strains. The major MVOC's from both strains were alcohols, ketones and hydrocarbons.

Dimethylsulfide and 2-heptanol were key MVOC biomarkers and were produced only by the aflatoxigenic strain of *A. flavus* and distinguished the two strains.