11-13-2017

Characterization of Aspergillus Flavus Soil and Corn Kernel Populations From Eight Mississippi River States

Jorge A. Reyes Pineda
Louisiana State University and Agricultural and Mechanical College, jorgereyes155@gmail.com

CHARACTERIZATION OF ASPERGILLUS FLAVUS SOIL AND CORN KERNEL POPULATIONS FROM EIGHT MISSISSIPPI RIVER STATES

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Plant Pathology and Crop Physiology

by

Jorge A. Reyes Pineda
B.S., Universidad Nacional de Agricultura-Honduras 2011
December 2017

ACKNOWLEDGEMENTS

I first thank God who gave me the strength and perseverance to complete the requirements for this degree, and second, I thank my family. Without their unconditional support and encouragement, I would have never been able to achieve this endeavor.

I would like to thank my advisory committee, Drs. Kenneth Damann, Christopher Clark, and Vinson Doyle for their advice and support. I would especially like to thank Dr. Damann for his mentoring, encouragement and support throughout these past years and for his energy and passion for science and plant pathology. It has been a huge honor to be one of his trainees and graduate students. My sincerest appreciation to my lab friends, Cathy DeRobertis, Rebecca Sweany and Dr. Subbaiah Chalivendra, for their daily support and advice, and interesting conversations. Special thanks to Ms. Cathy DeRobertis for her altruistic nature and her inspiring energy that generates joy wherever she is, and for always looking out for everyone. I also want to thank Michelle Poole for her support and assistance in my lab procedures and experiments. Thank you all!

I must express my gratitude to Katrina Spillane, Susan Karimiha, Dr. Hector Zapata, Dr. David Picha and Dr. William Richardson for beginning the LSU-UNA internship program that has allowed not only me, but also many other UNA graduates and students to be exposed to science and be able to enroll in internship and graduate programs. I thank my friends, Alejandra Jimenez, Cesar Escalante, Teddy Garcia, and Vondel Reyes, for their friendship and shared experiences during these past years. I thank all my friends for supporting me throughout these two years and for cheering me on through the good and bad times.

I must say thanks to all the people who provided our samples for the isolate collection and the AMCOE support which partially funded my research project. Thanks to the professors from the plant pathology department who shared their passion and knowledge with students, always encouraging us, giving us good advice to always improve and enjoy science.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Aspergillus flavus is a saprophytic ascomycete that can also actively invade the seed of crops and potentially contaminate them with harmful aflatoxins. Management of *A. flavus* currently relies mostly on biocontrol. However, there is still a lot to learn about its biology and ecology so the current approach can be improved. An *A. flavus* population survey was undertaken during the fall of 2014 across eight Mississippi River states to determine population diversity and geographic distribution of VCGs. Isolates from corn and soil were also characterized for VCG, sclerotial morphotype, mating type, cyclopiazonic acid, and aflatoxin production in order to determine trait uniformity within VCGs and those VCGs traits which may be associated with corn infection. From a collection of 339 soil and 204 kernel isolates, 18 multiple-isolate VCGs were determined. Four VCGs accounted for 54% of soil and 48% of total kernel populations and these were the only ones recovered from both niches. Nine VCGs were only recovered from the soil population and 5 from corn kernels. Two VCGs were widely distributed across the 8-state transect and they were the same most frequently isolated from corn kernels in Louisiana in 2007. Results also indicate that the *A. flavus* metapopulation along the Mississippi River was highly genetically diverse as reflected by the diversity of VCGs. Southern populations were more diverse, and the northern region showed higher clonal fractions. Mating type *MAT1-2* and high CPA production were associated with corn infectors, which were mainly of the L-morphotype. The soil niche had both, S and L-sclerotial morphotypes and although ~70% of isolates from both niches produced CPA, kernel isolates produced higher concentrations and support the report that CPA is a pathogenicity factor for *A. flavus.* These results give insight into important traits for corn infection, for both toxigenic and atoxigenic strains, which may be key in the improvement of biological control strategies. Characterizing the diversity of VCGs and their distribution over a 1500 km transect spanning important corn growing areas in the US, has allowed creation of a quantitative baseline of VCGs which can be used for monitoring changes in biodiversity over time.

Chapter 1. INTRODUCTION

Aspergillus flavus Link, Teleomorph: *Petromyces flavus* (Horn *et al.,* 2009) is a filamentous ascomycete placed in the class Eurotiomycetes and order Eurotiales due to the production of ascospores within asci, and asci within a cleistothecial-type ascocarp (Moore *et al.,* 2010). *Aspergillus flavus* was long considered an asexually reproducing fungus but with the discovery of mating type loci and subsequent mating experiments at the lab and field level, its sexual cycle was verified (Ramirez-Prado *et al.,* 2008; Horn *et al.,* 2009; and Horn *et al.,* 2016). *Aspergillus flavus* belongs to section Flavi within the Aspergilli, a section that comprises economically important organisms such as the industrially beneficial *Aspergillus oryzae* but also harmful ones such as *A. flavus* itself, *Aspergillus nomius* and *Aspergillus parasiticus.* These Aspergilli produce carcinogenic aflatoxins, very detrimental secondary metabolites that threaten human and animal health as contaminants of important agricultural commodities (Fountain *et al.,* 2014). Strict regulation and enforcement exists for aflatoxin in developed countries such as the US and European Union (Ehrlich, 2014). However in developing countries, most agricultural products move through local and informal markets, making regulation and enforcement difficult to apply. For example, recent aflatoxin contamination events have occurred in sub-Saharan countries, where aflatoxin contaminated grains are responsible for acute and chronic poisoning, aflatoxicosis and death (Atehnkeng *et al.,* 2015).

Aspergillus flavus represents a good portion of soil mycobiota and is responsible for nutrient cycling by decaying a diversity of organic materials through the release of specialized enzymes that degrade multiple plant components, such as cellulose, lignin, pectin, and lipids (Cotty *et al.,* 1990 and Horn, 2003). The *A. flavus* reservoir in the soil consists of sclerotia, conidia, and mycelial networks, all of which can serve as primary inoculum for continuing propagation. *Aspergillus flavus* is found in virtually any soil that has been sampled around the world and is considered to have a ubiquitous and cosmopolitan distribution (Horn, 2003). The fungus has been found to be more abundant in subtropical and tropical warm climates, conditions that favor its growth and development. It has even been naturally and abundantly found infecting the seeds of wild leguminous trees in the Sonoran desert (Cotty *et al.,* 1994).

In addition to its important saprophytic role in nature, *A. flavus* can also actively invade the seed of crops through its facultative parasitic capabilities (Horn, 2003) and become a problem rather than a beneficial agent in the ecosystem. The infection of corn occurs when conidia from the soil reservoir become airborne or insect vectored onto the silks of developing corn ears. Conidia germinate and the fungal mycelium grows down into the kernels through the naturally dying silks during or just after pollination (Wicklow, 1991). The presence of *A. flavus* in the crop seeds does not represent a problem since the amount of infection causes no significant impact on yield. Its presence becomes an issue with the contamination of grain with harmful aflatoxins (Fountain *et al.*, 2014), secondary metabolites whose biological or physiological significance for the fungus is not completely clear. However, they represent a serious issue for global food safety, human and animal health, and agricultural trade.

It has been suggested that crop composition and crop history influences the relative abundance and persistence of this fungus in the soil of agricultural settings (Garber and Cotty, 2014). Many strains of *A. flavus* increase their populations by producing huge amounts of inoculum when they find rich carbon and nitrogen sources. This regularly replenishes soil populations, especially after harvest seasons with hot and dry environmental conditions (Horn, 2003). This type of stress normally predisposes crop plants (they can even cause seed cracks) and compromises them for opportunistic pathogen invasion due to the lowering of their defense mechanisms (Orum *et al.*, 1997), but it also creates just the right conducive conditions for the fungus to grow and thrive.

Aspergillus flavus is associated with contaminating oil-rich seed crops such as corn, cottonseed, peanuts, and tree nuts, but also rice and peppers (CAST, 2003). It was first recognized as a major issue during post-harvest and storage, but it also affects crops during pre-harvest scenarios when conditions are conducive for its development. Mycotoxin contamination of crops in the United States alone has been estimated to account for a loss of nearly $932 million annually, to which $466 million are added for regulatory enforcement, testing, and quality control measures (CAST, 2003). In addition to direct economic loss, it threatens our global food supplies and health, so management actions and strategies must be developed to counteract *A. flavus*.

In terms of management, growing aflatoxin-resistant hybrids is the most desirable potential approach. Unfortunately, it has not been possible because resistance to *A. flavus* is a quantitative trait controlled by multiple loci of small effect with significant environment-genotype interactions (Fountain *et al.,* 2014), and it has not been incorporated into commercial hybrids. Certain genetic lines with resistance to insect damage and abiotic stresses seem to be more tolerant of aflatoxin contamination. Cultural practices such as proper fertilization and irrigation have a limited positive effect. The use of conventional fungicides is impractical and ineffective. An alternate biological control strategy is the use of atoxigenic strains based on the intraspecific competition concept among different *A. flavus* individuals (Abbas *et al.,* 2009). Non-toxigenic strains outcompete toxigenic ones in the field without causing an increase in the overall *A. flavus* populations, but shifts in the population structure by increasing abundance of the non-toxigenic strain. The population structure shift translates to reduction of total aflatoxin contamination and risk (Bayman and Cotty, 1993). Although the competition or displacement concept is more easily seen in the soil, it has also been demonstrated that cryptic or more complex mechanisms regulate the biocontrol effect of non-toxigenic strains against toxigenic ones during co-infection of crop materials (Huang *et al.,* 2011; and Mehl and Cotty, 2013).

The use of non-toxigenic strains has yielded positive results in several parts of the US as well as in Africa (Atehnkeng *et al.,* 2015). Cotton and corn growers in Arizona and Texas have used a US EPA approved commercial formulation of atoxigenic *A. flavus* (strain AF36) for reducing aflatoxin contamination since the 1990s (Cotty and Bhatnagar, 1994). Growers have accepted the technology and over 50,000 ha of cotton in the southwestern US have been treated with AF36 and successfully reduced aflatoxin levels in cotton seed (Das *et al.,* 2008). Peanut and corn growers in the southern US have two special formulations, one first created for peanut fields and sold as the Syngenta product Aflaguard (NRRL 21882), and a second one, K49, created specifically for corn (Abbas *et al.,* 2011). The United States Department of Agriculture - Agricultural Research Service (USDA-ARS) and the International Institute for Tropical agriculture (IITA) in Africa have also fostered initiatives (and continue doing so) that help growers fight this problematic situation that has not only created production issues and threats, but has also taken lives in Kenya and Nigeria due to severe aflatoxicosis, acute poisoning, and continuing effects on children's growth and development (Lewis *et al.,* 2005). Nigerian corn growers have an IITA product called Aflasafe

that consists of four locally adapted atoxigenic strains that have shown positive results in reducing aflatoxin contamination during pre-harvest, but also during storage of grain. Efforts to expand the technology to other sub-Saharan countries using locally adapted non-toxigenic strains are underway and are expected to contribute to solving one of the agricultural issues that African countries are currently dealing with (Aflasafe.com/IITA).

Although biocontrol has had beneficial effects in the field in the reduction of aflatoxin contamination, the actual antagonistic mechanisms behind the competition interactions are not completely clear and require elucidation. A better understanding of the ecology of *A. flavus*, its biology, and the structure of its populations at the local, regional, and global scales is also needed so biocontrol strategies can be improved (Damann 2015; and Atehnkeng *et al.*, 2015).

1.2 Population biology of *Aspergillus flavus*

In addition to its wide distribution, *Aspergillus flavus* has a high diversity within its populations as determined by phenotypic and genotypic studies (Horn *et al.*, 1996; Giorni *et al.*, 2007; and Pildain *et al.*, 2004), including: mycotoxin profiles (Giorni *et al.*, 2007; and Horn *et al.*, 1996) vegetative compatibility grouping (Bayman and Cotty, 1991; Horn and Greene, 1995; Pildain *et al.*, 2004; and Sweany *et al.*, 2011), and population genetic studies using other molecular markers such as RFLP and SSR fingerprints (McAlpin *et al.*, 2002; and Sweany *et al.*, 2011).

As a species, *A. flavus* is considered a very complex system and its populations have been subdivided in several ways. One of the most common ways to categorize its populations is based on morphology and the type and amounts of toxins they produce. Strains are either assigned to the typical L-strain (large sclerotia) morphotype that produce sclerotia >400 μm in diameter or as the S-strain (small sclerotia) that produce sclerotia <400 μm. Some strains appear incapable of sclerotia formation, but more resemble L-strains (Horn, 2003). Beyond sclerotial size, these two morphotypes also differ in colony morphology and general aflatoxin-producing potential. L-morphotype strains produce relatively few or no sclerotia but copious amounts of conidia, while S-strains produce abundant small sclerotia but few or no conidia (Bayman *et al.*, 1994; and Sweany *et al.*, 2011). L-morphotype strains range in their aflatoxin production from non-producers to

highly toxigenic ones, while S-strains are predominantly high toxin producers. Non-toxigenic S-type strains are rarely found in nature and consequently S-strain individuals are thought to be the major source of aflatoxin contamination in corn and cotton in agricultural fields (Zhang and Cotty, 2007). In addition, L-strains may produce only B-type aflatoxins plus cyclopiazonic acid (CPA), whereas S-strains may produce both B and G-type aflatoxins in addition to CPA (Horn, 2003), something that has caused a lot of controversy in the taxonomy of this fungus. A phylogenetic analysis in 1998, based on the sequences of 5 genes determined that *A. flavus* is non-monophyletic and isolates are segregated into two cryptic groups that are morphologically distinct. Group I consists of L and S-type strains that produce only B-type aflatoxins whereas S-type Group II produces both B and G aflatoxins (Geiser *et al.*, 1998). Some scientists refer to S-trains that produce B-type aflatoxin only as *A. flavus var. parvisclerotigenus* and as *A. minisclerotigenes* (Pildain *et al.,* 2008) if they produce B and G aflatoxins (Moore *et al.,* 2010). A closely related fungus in section Flavi, *Aspergillus parasiticus* resembles *A. minisclerotigenes*, as it produces both types of aflatoxins but it does not produce CPA (Horn, 2003). *Aspergillus flavus* fails to produce G-type aflatoxins due to a partial or total deletion of the *cypA* gene that encodes a P-450 cytochrome monooxygenase, important for the production of G-type aflatoxins (Ehrlich *et al.,* 2004). Generally, non-toxigenic individuals lack the ability to produce mycotoxins due to non-synonymous mutations or deletions in part or the whole aflatoxin gene cluster (Adhikari *et al.,* 2016). To make matters more interesting, some authors suggest that B and G producers of the S-strain morphotype could be hybrids between *A. flavus* and *A. parasiticus*, a strategy that would provide evolutionary advantage through the hybrid vigor and increased capabilities for adaptation (Moore *et al.,* 2010).

The high variability in aflatoxin production coupled with different combinations of other secondary metabolites produced by *A. flavus* (chemotype diversity) has also been useful in the characterization of its populations. Several studies have attempted to characterize populations based solely on chemotype diversity (Giorni *et al.,* 2007), and although it has been informative, the non-monophyletic distribution of toxin producers and the high profile variability has not provided much understanding of genotype diversity and population structure of this problematic fungus. The same is actually the case for morphotype determination. The broad encompassing of

isolates within few categories does not provide information to identify closely related individuals or identify genotype diversity within the whole population.

A classification system that is definitely more informative for subdividing *A. flavus* populations is vegetative compatibility typing. Vegetatively compatible individuals together form sub-clades or assemblages within a population called vegetative compatibility groups (VCGs) (Leslie, 1993). The vast majority of phenotypic diversity in *A. flavus* populations can be attributed to differences between VCGs (Horn, 2003). Vegetative compatibility is one of the self/non-self-recognition systems that filamentous fungi have and presumably acts as a mechanism to restrict the exchange of genetic material (gene flow) between vegetatively incompatible individuals. It may also restrict the transmission of deleterious viruses, plasmids and parasitic nuclei (Grubisha and Cotty, 2010). More importantly, vegetative compatibility or incompatibility is controlled by a series of unrelated and unlinked loci collectively termed *het* loci, for heterokaryon formation. Heterokaryon formation in vegetatively compatible individuals results in interchange of their cytoplasmic and genetic materials (Leslie, 1993). It has been suggested that in *A. flavus* at least 12 *het* loci govern vegetative (in) compatibility (Ehrlich, 2014) and because 100% identity in those loci must exist for successful fusion, vegetative compatibility tests within a population is an important tool for grouping of closely related individuals. This multi-locus base of VCG subgroupings allows determining identities with a simple phenotypic test (Leslie, 1993). Vegetative compatibility allows determination of genetic diversity, genotype frequency, and distribution in the whole *A. flavus* population.

As discussed earlier, morphological and physiological traits such as morphotypes and chemotypes on their own are not very informative, but when collated with VCGs they provide greater resolution. Genetic relatedness between isolates is most consistent within VCGs, meaning that morphotypes and chemotypes (type, and level of toxins produced) are more uniform within VCGs rather than among them, even non-toxigenic and toxigenic individuals tend to segregate into different VCGs (Horn, 2003; and Pildain *et al.*, 2004). VCG relatedness is also supported by DNA-RFLP fingerprint analysis, where individuals belonging to the same VCG show the same or almost identical band pattern, whereas individuals in different VCGs show their own unique RFLP fingerprint pattern (McAlpin and Wicklow, 2002). VCGs are considered a synonym of "strain" in

this system. The predominantly asexual reproduction or clonality of *A. flavus* may explain the strong correlation of VCGs with morphology, physiology, and other molecular features. Individuals in the same VCG have been shown to possess the same mating type idiomorph (Sweany *et al.*, 2011). However, substantial genetic diversity and variation exists within the whole *A. flavus* population as shown by the presence of multiple VCGs and occasionally slight differences among individuals within the same VCG. Sexual recombination along with random genetic mutations at *het* loci may explain the within VCG genetic diversity, although the rates at which they occur are still unknown. Before discovery of mating type loci in *A. flavus,* this genetic variability was attributed mainly to parasexuality (Batt, 2014).

The VCG classification system, though laborious, has been used to characterize diversity and assess population structure for *A. flavus*. VCG diversity has been widely studied during the last two decades in different agricultural settings, regions, and countries because it represents a way of classifying *A. flavus* populations (Bayman and Cotty, 1991; 1993; Horn and Greene, 1995; and Sweany *et al.*, 2011). VCG diversity is commonly observed by relative abundance in the field, meaning that few VCGs comprise the majority of sampled isolates with many additional VCGs of small representation within the population (Papa, 1986; and Horn *et al.*, 1996). Diversity indices (DI) have been calculated for *Aspergillus* species to compare among different populations. DIs are calculated by dividing the number of VCGs by the total number of isolates identified in a certain region (Horn and Greene, 1995). More informative diversity indices are often used to determine diversity in fungi whose genotypic/phenotypic units are VCGs or similar markers. The Shannon-Wiener and Simpson diversity indices are often used to measure the genotypic richness (number of observed VCGs) and their relative frequencies (evenness) within a population. Although these indices are a composite of richness and evenness, these latter two can also be calculated separately for comparison among populations. *Aspergillus flavus* population studies have been mainly done by using VCGs as genotype identifiers, which are useful to determine population structure. Diversity indices allow a comparison of all these population characteristics with other sympatric or allopatric populations (Milgroom, 2015).

VCG diversity and composition have been associated with different cropping systems, including association and prevalence of certain VCGs from a particular *Aspergillus* species with different

crops. *Aspergillus* species have been found to be non-randomly distributed. *Aspergillus parasiticus* and *A. tamarii* for example have been frequently associated with peanut in the peanut growing areas, whereas *A. flavus* VCGs have been consistently found in corn and cotton growing fields (Horn and Dorner, 1999). However, no VCG had been described or reported to have a particular association infecting specific crop plants. The lack of VCG association with a particular host led to the belief that virtually any strain has the same capabilities to infect a host if the environmental conditions are conducive (Sweany *et al.*, 2011). Strong plant-pathogenic organisms such as *Fusarium oxysporum* have shown correlation between VCG and pathogenicity (specificity) (Bosland and Williams, 1987).

This lack of specificity for *A. flavus* is being questioned. In a three-year Arizona cotton field study, only 5 VCGs out of 11 from the whole collection were sampled from both soil and cotton bolls, giving evidence of specialization (Bayman and Cotty, 1991). A second and more detailed work, from a soil and corn kernels collection in 2007 from Louisiana fields revealed specialization of certain VCGs within the *A. flavus* population for infecting corn. In this comparison 16 VCGs were identified in the soil from which only 6 were determined to infect corn. Interestingly corn infecting individuals were predominantly of the L-morphotype and/non-sclerotia producers and highly skewed for the mating-type *MAT1-2* idiomorph. These characteristics were significantly different from the soil population individuals and tightly correlated and dependent of VCG. VCGs and individuals confined to the soil niche were predominantly of the S-morphotype and their mating-type ratio approximated 1:1. Aflatoxin production was variable among VCGs, but soil populations had a greater proportion of toxigenic representatives than the corn-kernel population overall (Sweany *et al.*, 2011).

A profile for individuals with better infecting abilities within this “opportunistic” pathogen population has not been determined. Although the mechanisms underlying traits that seem to make certain VCGs or strains more fit to infect crops are not clear, it is likely that these traits may confer certain advantages to the VCGs that possess them over their counterparts. In biological and ecological terms, the ability to infect is important for both the toxigenic and atoxigenic strains. Having insight into how the infection process works or what features confer its ability to infect

would play a critical and important role for the implementation or improvement of current biocontrol strategies against *A. flavus* (Damann, 2015).

Sweany *et al.*'s results in 2011 encouraged a series of follow up studies to look at differences between individuals from both soil and plant subpopulations (niches) to better understand what the infecting strains have that their counterparts lack. Besides morphotype and mating-type idiomorph, corn-infecting strains have been shown to have higher growth rates *in vivo* (corn kernels) as determined by biomass marker expression (Chalivendra *et al.*, 2016). Corn infectors have greater conidial fecundity that gives them increased dissemination potential (Sweany *et al.*, 2016), and they also produce more cyclopiazonic acid (CPA) (Chalivendra *et al.*, 2017). CPA is an indole-tetramic acid toxin produced by *A. flavus* and other related fungi that interferes with calcium pumps (Ca^{2+} ATPase), enzymes involved in calcium transport and signal transduction in plants (Kabala and Klobus, 2005) and proper muscle contractions in vertebrates (Ehrlich, 2014). CPA as plant-cell killing toxin has been determined to work as a pathogenicity factor in *A. flavus*¸ so its production would give the fungus an advantage at the moment of colonization of plant tissue (Chalivendra *et al.*, 2017). VCGs with higher levels of CPA production would be expected to more easily infect a crop, so selection would favor them and their frequency should be higher than low or non-CPA producers.

Based on the information gained in previous studies and in search of a better understanding of the biology of *A. flavus*, a population study from 17 fields across eight states along the Mississippi river was undertaken in the fall of 2014. A profile for every VCG/strain identified was created and a comparison made between the soil and corn populations to determine if a correlation holds between VCG specificity and niche. The idea was to determine the characteristics associated with each VCG and the corn-kernel infecting abilities of such VCGs. This should allow identification of traits that the best corn-infecting strains have and that the soil inhabitants lack. Furthermore and no less important, objectives of the work involved determining the population genetic composition and structure utilizing VCGs (genotypes) as a measure of richness and diversity along with other population parameters such as diversity indices, observed and expected genotypic richness, and clonal fractions. Knowing and understanding the ambient diversity and distribution of VCGs at the local, state, and regional levels provides information to implement the best-fit

aflatoxin biocontrol strategies (Cotty 1994; and Pildain *et al.*, 2004). It is important to know the distribution of toxigenic and non-toxigenic individuals to understand the risks associated with them if environmental conditions become conducive (Atehnkeng *et al.*, 2015). More importantly, knowing the population structure and VCG richness is adequate for the appropriate selection of biocontrol strains (Abbas *et al.*, 2009). So, in these regards, a major goal in this study was also to construct a quantitative baseline of the more frequent ambient strains from the soil and corn kernels along the nearly 1500 km transect from Minnesota to Louisiana, and document the diversity of toxigenic and atoxigenic VCGs spanning some of the more important corn growing areas in the US.

Chapter 2. MATERIALS AND METHODS

2.1 Sample collection

Ten corn ears and 5 scoops of soil were randomly collected from each of 17 different fields from eight Mississippi-river states and provided by collaborators during the fall of 2014 (See list in Appendix 1). Two fields from MN, WI, IA, MO and IL, 3 fields from LA and MS and 1 field from AR were sampled. Soil samples were air dried in a fume hood before shipment, although some required drying for a longer time in an oven at 40 °C. Soil samples were stored in paper bags at room temperature and mature corn ear samples were individually shelled and stored in zip-lock bags at room temperature as well.

2.2 Fungal culture isolation and identification

Soil (50g) was suspended in 100 ml of sterile distilled water in a 250 ml Erlenmeyer flask (original dilution) and shaken for 10 minutes in an Eberbach reciprocal shaker (Ann Arbor, MI). A 1-ml aliquot of the suspension was spread onto 5-7 petri dishes containing *Aspergillus flavus/parasiticus* amended medium (AFPA) (Bothast and Fennell, 1974); amended with 50 μg/ml hygromycin, 1.5 μg/ml chlortetracycline, 30 μg/ml streptomycin and 0.04 μg/ml Avermectin (Sweany *et al.*, 2011; and Bayman and Cotty, 1991b). The use of this selective medium allowed rapid identification of *A. flavus* colonies by the bright-orange reverse colony color, indicating the production and release of aspergillic acid, a characteristic of *Aspergillus flavus* (Bothast and Fennell, 1974; Pitt *et al.*, 1983; and Sweany *et al.*, 2011). Another good feature of this medium is that each inoculum unit present in the sample germinates and shows an independent colony due to the scarce or null sporulation of the fungus, which facilitates picking multiple isolates from a plate. Isolations were also attempted with 1:10 and 1:100 serial dilutions from the original suspension but yielded too few isolates per plate in many of the samples, so only 1-ml aliquots from the original dilution were used in further isolation attempts. Plates were incubated at 30 °C for 3-5 days until colonies were visible and well developed. Plugs from the active growing border of each colony were transferred onto V8 medium plates (5% V8 juice, 2% agar, pH adjusted to 5.2 and amended with 0.04 μg/ml Avermectin) and grown for 6-7 days, also at 30 °C. From these cultures, single spore colonies were made by streaking conidia onto PDA plates and leaving them overnight at 30 °C. A single

germinating conidium was transferred onto a new V8 medium-containing plate and grown for 7 more days at 30 °C. Spores from each single spore culture were harvested in 50:50 (v/v) glycerol/water in 2-ml Eppendorf tubes and stored at 8 °C for short term usage and -20 °C for long term storage (Sweany *et al.,* 2011).

One hundred corn kernels from each ear were surface sterilized with 6% bleach solution (plus 400 µg/L of Tween 20 surfactant) in 50 ml plastic centrifuge tubes for 1.5 min and rinsed with sterile distilled water three times for 15 sec. Twenty-five intact corn kernels per 100x15 mm petri dish were plated on AFPA amended medium. Plates were incubated at 30 °C for 3-5 days in the light and examined for the orange-bottom colonies that characterize *A. flavus* in this medium. Plugs from the border of every colony were transferred onto V8-avermectin amended medium and grown for 6-7 days. Single spore colonies were also made in the same way as for soil isolates, and conidial stocks were kept in 50/50 (v/v) glycerol/water at -20C. Isolation attempts were performed 4X for corn samples from the northern states IA, IL, WI, and MN, due to a low frequency of recovery.

2.3 VCGs determination

VCG determination was performed through the creation of nitrate non-utilizing (*nit*) mutants and pairings set up between complementary mutant types within and between locations. The use of nitrate non-utilizing (*nit*) mutants for complementation tests between several strains is a very useful and well-known technique that has been used for population grouping and characterization of *A. flavus* by several investigators (Bayman and Cotty, 1991; Horn and Greene, 1995; Papa, 1986; Pildain *et al.,* 2004; and Sweany *et al.,* 2011).

During this study, *nit* mutants for every isolate were induced by placing 2 µl of conidial suspension onto the center of chlorate amended (*nit* induction) agar plates (Czapek Dox with 25 g of potassium chlorate per liter) and exposed to short wave (254 nm) UV light for 40 seconds at 20 cm of distance from a UV lamp (Spectroline Model XX15G, Westbury, NY). Plates were incubated at 30 °C in the dark and frequently checked, until mutations had occurred. Mutants were identified by the appearance of sparse hyaline hyphae branching out from the main central colony. True mutant sectors normally take around 3-4 weeks to develop (Sweany *et al.,* 2011), but with the exposure

of inoculated plates to short wave ultraviolet radiation, induction of *nit* mutants appeared from day three onward (Papaioannou and Typas, 2015).

Once a mutant sector was identified with the aid of a light microscope, medium plugs containing chlorate-resistant hyphae were transferred onto Czapek Dox (CD) medium (nitrate medium) to confirm that they had actually mutated (true mutants continue sparse growth on nitrate medium and false or poor mutants usually revert to the wild type growth habit). True mutant plugs from CD plates were transferred onto V8 medium plates to produce spores, which after 7 days in the dark at 30 °C were harvested and stored in 50:50 (v/v) glycerol/water solution for further use and kept as conidial stocks.

Nit mutants (2-3 per isolate) were plated onto Czapek Dox medium modified with alternate nitrogen sources to sodium nitrate ($NaNO_3$) in order to identify and characterize the type of mutant created. Alternate nitrogen sources to sodium nitrate were sodium nitrite ($NaNO_2$), hypoxanthine ($C_5H_4N_4O$), and ammonium tartrate ($(NH_4)_2C_4H_4O_6$). Mutants were classified as *niaD* if they were nitrate non-utilizing only (showing robust growth in the three alternate nitrogen sources), *nirA* if they were nitrate and nitrite non-utilizing (sporulating on ammonium tartrate and hypoxanthine, but not on nitrite medium), and *cnx* mutants if they were nitrate and hypoxanthine non-utilizing mutants (Not sporulating on hypoxanthine medium) (Cove, 1976; Pildain *et al.*, 2004; and Sweany *et al.*, 2011). *Cnx* mutants were considered the best candidates for testers because they performed better in complementation pairings (Horn and Greene, 1995; and Sweany *et al.*, 2011), but *niaD* and *nirA* mutants were also selected as testers, because they are also needed to test vegetative compatibility. *Cnx's* needed to be paired against complementary *niaD* or *nirA* mutants since some mutant types are self-incompatible and it was not possible to produce all three types of mutants from every single isolate.

Once mutants from every isolate were identified and characterized, vegetative compatibility tests were performed. Pairings of potential complementary mutants were made on starch modified Czapek Dox medium in order to look at the interaction or border zone between two mutants and confirm whether they complemented each other or not. Mutants from isolates complementing each other are said to be vegetatively compatible because they were able to form a heterokaryon and

were assigned to the same vegetative compatibility group or VCG (Sweany *et al.,* 2011; Leslie, 1993; and Pildain *et al.*, 2004).

Complementation tests between unknown VCG isolates from this collection and the different *nit* mutant testers from the 16 VCGs identified in Louisiana soil and kernel populations in 2007 were also carried out in an effort to try to identify if any of these VCGs was still present in the general population, not only in LA fields but all along the sampled Mississippi river transect. Positive matches for any of these 16 VCG testers were also intended to reduce the number of crosses or complementation pairings between all isolates from the two sampled subpopulations.

2.4 Aflatoxin production determination and quantification

Screening for aflatoxin and measurement was done for aflatoxin B1 since this is the most abundantly produced by *Aspergillus flavus*. Aflatoxin B1 is the most potent carcinogen known to be produced by natural means. It is second just after the synthetic derived polychlorinated biphenyls (Batt, 2014).

Aflatoxin production for all *A. flavus* isolates was determined and quantified by growing every isolate on rice as a substrate, a carbohydrate rich medium that allows *A. flavus* isolates to express their maximum aflatoxin producing potential. Five ml (g) of rice was soaked overnight in 5 ml of water in a 20-ml scintillation vial, autoclaved for 20 minutes at 20 psi-121°C and allowed to cool. Cooled rice was stirred with a sterile spatula to aerate it and break up the compacted mass left after autoclaving. Vials were inoculated with 10 µl of conidial suspension from every isolate, vial caps were left loosely fitted for oxygen and gas exchange and incubated at 30 °C for 7 days in the dark. Vials were filled with chloroform and left to soak overnight in a fume hood. The extract was filtered through Whatman no. 1 (100 mm) filter paper in a funnel and into a 100-ml glass beaker. Chloroform was allowed to evaporate overnight in the fume hood and aflatoxins contained in the solid residue were re-suspended in 1.2 ml of 80:20 (v/v) methanol/water. Methanol-resuspended extract was filtered through a cleanup column packed with 200 mg of basic aluminum oxide and collected into a 2-ml glass autosampler vial (Sweany *et al.,* 2011). Approximately one ml of methanol containing aflatoxins was recovered after filtering the samples and represented a

sufficient volume for the HPLC system to pick up a 10-µl aliquot for proper measurement. The extracted samples in glass vials were screened for blue fluorescence in a UV light box in order to make dilutions (10 or 100 fold) when necessary before quantification in the HPLC system.

Aflatoxin was quantified using reversed-phase high performance liquid chromatography with a Waters Alliance HPLC system (Waters Corporation, Massachusetts USA) equipped with a 2695 separation module and a 2475 dual FLR Detector; a fluorescence detector with 365 nm of excitation wavelength and 440 nm of emission wavelength. The mobile phase for sample separation was 62.5% HPLC-grade and degasified water and 37.5% HPLC-grade degasified methanol, set to run in mixture at flow rate of 0.8 ml/min. Aflatoxin B1 peak appeared at ~12 minute in a 16-min run per sample and data was collected using the software Empowered 3 (Waters Corporation, Massachusetts USA).

2.5 Cyclopiazonic acid production determination

Isolates were grown in YES + soytone liquid medium (2% yeast extract, 15% sucrose and 1% soytone) with pH adjusted to 6.0 with HCl before autoclaving. Ten µl of conidial suspension from each isolate was inoculated into 5 ml liquid medium in a 20-ml glass vial. Stationary cultures were grown at 30 °C in the dark for 7 days for maximal CPA production. Two milliliters of chloroform were added to each vial to extract metabolites, fast frozen (incubated at -80 °C for three hours) to break down fungal cells and recover as much CPA as possible from the medium (99% of CPA is recovered with this procedure). A 1 ml aliquot was collected into a new Eppendorf microcentrifuge tube, vortexed for a few seconds and spun at 10,000 rpm for 5 min to separate the aqueous and organic phases. Six hundred µl of the organic layer were transferred to a clean microcentrifuge tube and left to air dry in a fume hood until average volumes reached 50-60 µl. Fifteen µl of chloroform was used to resuspend the CPA per sample which was then spotted onto thin layer chromatography (TLC) silica gel 60 plates (Merck, Darmstadt, Germany) for detection. TLC plates were dip-treated in 2% oxalic acid in methanol before spotting of samples. Plates were run in a glass tank containing ethyl acetate/methanol/ammonium hydroxide (85:15:10) for 40-45 min, left to dry, and sprayed with Ehrlich's reagent (1 g of 4-dimethylaminobenzaldehyde dissolved in 75 ml methanol and 25 ml concentrated HCl). CPA appeared as blue-purple spots as confirmed by

proper CPA standards (Chang *et al.*, 2009). CPA was qualitatively recorded in a scale of Negative, Low, Medium and High production for every isolate based on the intensity of the spots in comparison to standards of known quantity (5, 10 and 20 µl of CPA at [336.38 g/mol]).

2.6 Sclerotia production, sclerotia size measurement, and sclerotial density

All *A. flavus* isolates from the collection were grown on 15mm x 100mm petri dishes containing modified Wickerham medium, a special medium for induction of sclerotia production (Chang *et al.*, 2012), at 30 °C in the dark and for 30 days (Sweany *et al.*, 2011). Ability to produce sclerotia was first observed and annotated, separating sclerotia producers from non-producers. Sclerotia size and diameters were measured with the aid of an ocular micrometer under a conventional light microscope. Sclerotia were classified as large if the diameter surpassed 400 µm in diameter and small if less than 400 µm (Horn, 2003). *A. flavus* colony morphotypes were designated for every isolate based on the production and type of sclerotia; which were designated either as L-morphotype or S-morphotype. L-morphotype comprise non-producers and large sclerotia producers, and S-morphotype the small sclerotia producers.

Density of sclerotial production was determined by counting sclerotia per square centimeter from five randomly selected squares per plate. Sclerotia within them were counted and the sum divided by 5 to estimate the number of sclerotia produced per square centimeter per isolate.

2.7 Mating type determination

2.7.1 DNA extraction

Isolates were grown on potato dextrose agar (PDA) in petri dishes at 30 °C for 7 days in order to get an abundant production of conidia. Conidia were then scraped into a 1.5 ml Eppendorf micro-centrifuge tube containing 600 µl of Promega lysis solution, ground with a micropestle, vortexed, and incubated at 65 °C for 20-60 minutes. This solution was centrifuged for 5 min at 14,000 rpm and the aqueous layer was transferred into a new 1.5-ml micro-centrifuge tube. Proteins were precipitated using 200 µl of Promega protein precipitation solution plus another centrifugation step for 5 minutes at 14,000 rpm. The aqueous layer was collected into a new micro-centrifuge tube

and DNA was precipitated by the addition of 600 µl of room temperature isopropanol (Promega, Wisconsin). DNA was washed in a final step with 70% ethanol and allowed to dry overnight. DNA was rehydrated using 50 µl of pH 8 TE buffer and quantified using a Nanodrop spectrophotometer for subsequent dilutions and standardization to 10-ng/µl for PCR reactions (Sweany *et al.,* 2011).

2.7.2 DNA Amplification and mating type determination

DNA was amplified using a multiplex polymerase chain reaction (PCR) in order to determine mating type loci from the whole isolate collection.

Twenty-five µl of total solution for each reaction were used, and composed of:
12.5 µl of Hot Start PCR-to-gel TAQ Master Mix 2X
5.5 µl of sterile Milli-Q water
1.25 µl of each of 4 primers:
2, 10-µM *Mat 1-1 Aspergillus oryzae* (Forward and Reverse) primers (Wada *et al.*, 2012)
2, 10-µM *Mat 1-2 Aspergillus flavus* (Forward and Reverse) primers (Ramirez -Prado, 2008)
2 µl (10 ng/µl) DNA solution for a total of 20 ng of DNA needed for reaction.

Reaction mixtures were kept at 4 °C. PCR amplification was performed in a Perkin Elmer Cetus DNA thermal cycler, with an initial denaturation step at 95°C for 5 minutes, and 25 cycles that include 30 s at 94°C denaturation step, a 30 s at 53°C annealing step and a 60 s at 72°C extension step. PCR products were separated in an agarose gel electrophoresis system. A mixture of 10 µl of DNA and 2 µl of a tracking dye were loaded into each well in a 2% agarose gel. Ten µl of a 100 bp ladder (Omega bio-tek, Norcross, GA) with 2 µl of tracking dye (EZ Vision Three, Amresco, Solon, OH) were also loaded into the gel to be able to size the PCR products separated in the gel system. Gels were run at 100 volts for 5 minutes and then at 70 volts for 1 hour. As dyed DNA bands fluoresce when exposed to UV light, images of the gel were captured with a Gel Doc Imaging System equipped with a fluorescence camera from UVP Company (Upland CA) and edited with Vision Works LS analysis software. Presence of *Mat1-1* mating type was indicated by an amplicon of ~780-800 bp (Wada *et al.*, 2012) and *Mat1-2* by an amplicon of 273 bp (Ramirez Prado *et al.,* 2008).

2.8 Statistics and indices of genotypic diversity

Ecological based approaches and the "vegan" R package were used to calculate diversity indices and performed using the R statistical software (www.r-project.org; version 3.3.3). Diversity indices of VCGs for the whole population were estimated in three different scenarios for comparison; first, diversity indices for individual state populations; second, diversity indices for southern and northern state populations, where states were sub-grouped according to the region they belong to (South ~29 – 37^{o} N latitude, LA, MS and AR) and North (~38 - 49^{o} N latitude, MO, IA, IL, MN and WI); and third, diversity indices for niche comparisons, soil versus corn kernel populations. Shannon's diversity index was estimated by the formula: $H' = [-\Sigma (ni/N) \times \ln (ni/N)]$, where ni is the number of isolates in the ith VCG, and N is population size (total number of isolates) (Shannon and Weaver, 1949). VCG richness was expressed as both observed richness (G obs), the number of observed VCGs per population, and the expected richness (E(Gn), which was the expected number of VCGs in a random sample equivalent to the smallest population size, in this case AR (N=29) and calculated by rarefaction analysis. Evenness (*E1*), the measure of relative abundance of genotypes (frequency of each VCG per population) was calculated by dividing *H'* by the natural logarithm of corresponding maximum genotypic richness in that population $E1 = (H' / \mathrm{Ln}(gMax))$ (Grünwald *et al.,* 2003). Simpson's diversity indices were estimated using the formula $G' = 1/[\Sigma(ni/N)^2]$ where again, ni/N is the frequency of the ith VCG in N, the total population size (Grünwald *et al.,* 2003). A simple diversity index used in previous studies was also calculated for comparison, where the VCG diversity index (DI) was estimated as the VCG observed richness divided by their correspondent population size (Mauro *et al.,* 2013). DIs have values ranging between 0 and 1, where a value of 1 indicates a maximum diversity, whereby each isolate represents a distinct VCG. The clonal fraction per population was also calculated by the formula (1-g/N) (Milgroom, 2015). Aflatoxin and cyclopiazonic acid, sclerotia production and sclerotial density produced by VCGs were subjected to statistical analysis using the MIXED procedure of SAS (version 9.2; Cary, NC, USA). For clustering analysis and identification of variables conferring the most variation among the morphological and physiological variables, a Pearson (n) type principal component analysis was performed using XLSTAT 2016.07.38968.

Chapter 3. RESULTS

3.1. Isolate / Culture Collection

The complete collection consisted of 543 *A. flavus* isolates, 339 recovered from 17 soil samples across the 8 Mississippi River states and 204 isolates from corn kernels from 170 corn ears (10 from each of the 17 sampled locations). The frequency of *A. flavus* recovery from all soil samples was uniform across the 17 locations and averaged 42 (±12 SD) isolates per state comprising the eight subpopulations. The mean number of isolates recovered from corn kernel samples was 26 (±11 SD) isolates per state. Isolates from different fields from the same state were grouped together to form eight state populations and were also separated by niche, soil and corn kernels (Figure 1).

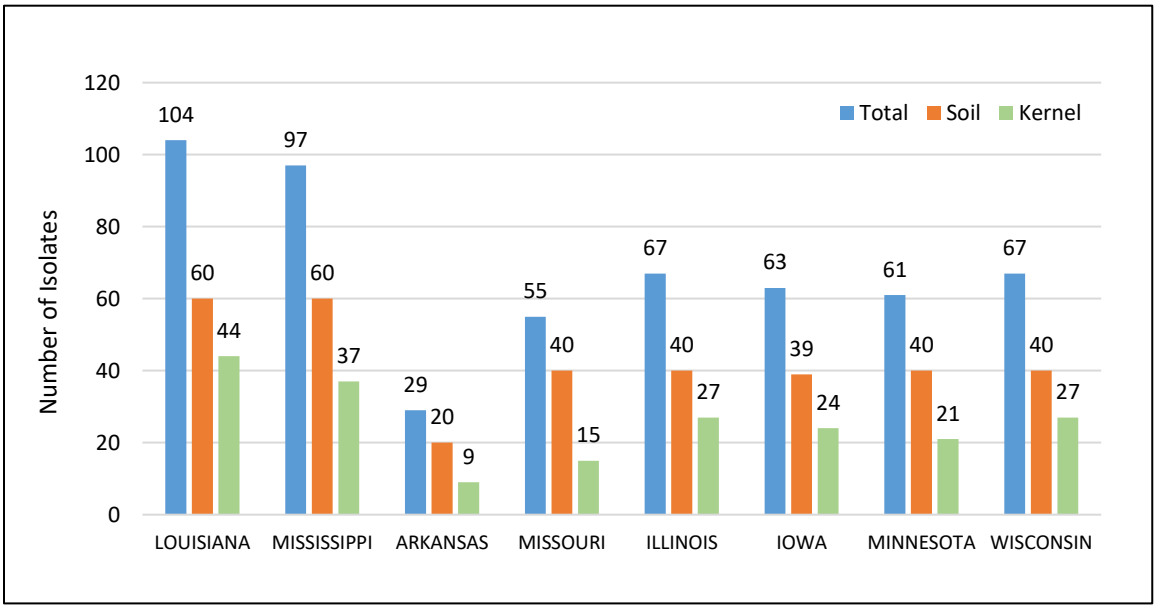

Figure 1. Number of isolates of *Aspergillus flavus* by state populations, for soil and corn kernels

3.2 Sclerotial morphotype and sclerotial density

In total, 36% of isolates from the 8 states did not produce sclerotia, 54% produced large and 10% produced small sclerotia, so ~90% of the whole population is the L-morphotype (Figure 2). More importantly and looking at different niche populations, isolates from the corn kernels were 100% L-morphotype (including 25% of non-sclerotia producers).The soil population is comprised of

16% small sclerotia producers, 42% large and 42% non-producers of sclerotia. Differences of least square means (LSM) between subpopulations measured at the 0.05 level of significance for sclerotia production varied significantly across the three different levels of comparison (niche, state, and region). Differences among states were mainly attributed to differences between southern and northern regions, where Iowa and Illinois populations were the most significantly different from Louisiana and Mississippi in regards to sclerotial type production. Northern state populations were more similar to one another (predominantly L-type strains) and not statistically different but significantly different from the whole southern states population, which had a greater representation of S-types (Table 1).

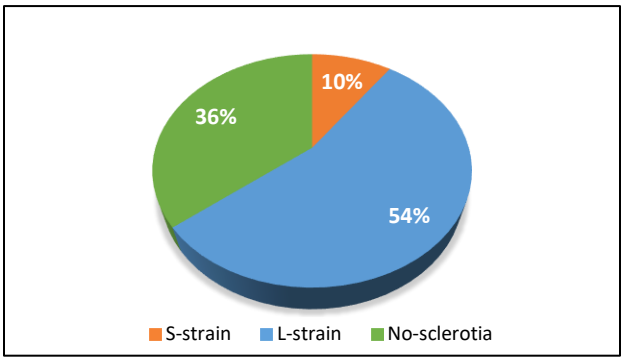

Figure 2. Total proportions of sclerotial morphotypes of *Aspergillus flavus* among the eight MS River states

The mean number for sclerotial density for soil isolates across the whole population was 44.2 sclerotia/cm^2 and 35.7 sclerotia/cm^2 for kernel isolates. Kernel isolates are consistent with the type and amount of sclerotia produced across the different states, however MS and AR have the representatives with higher density values of sclerotia/cm^2 than any other state. The density means for the soil isolate population are affected by density of sclerotial production per state. For example, the mean sclerotial density produced for Louisiana soil isolates is 78 sclerotia/cm^2 and 136 sclerotia/cm^2 for Mississippi (Table 1). These two states have representatives of the small sclerotia producing isolates (15% of total soil population in LA and MS) whose average density is 457.2 small sclerotia/cm^2. If these unusual small sclerotia producers from the soil of LA and MS are excluded, the mean sclerotial density for the soil population across the eight states is 46.9 sclerotia/cm^2, actually similar to the total population mean.

Table 1. Percentage of sclerotial morphotypes of *Aspergillus flavus* isolated by state and niche (soil or kernel) and density of sclerotial production.

Population		Percentage of Sclerotial Morphotypes per State			Density
		Small	Large	No-sclerotia	No. / cm2
Kernel	**N= 204**	**0.0**	**74.5**	**25.5**	**35.7**
Louisiana	44	0.0	72.7	27.3	29
Mississippi	37	0.0	78.4	21.6	68
Arkansas	9	0.0	66.7	33.3	62
Missouri	15	0.0	80.0	20.0	18
Iowa	24	0.0	83.3	16.7	29
Illinois	27	0.0	92.6	7.4	29
Minnesota	21	0.0	71.4	28.6	34
Wisconsin	27	0.0	48.1	51.9	18
Soil	**N= 339**	**15.6**	**42.5**	**41.9**	**44.2**
Louisiana	60	56.7	25.0	18.3	78
Mississippi	60	23.3	73.3	3.3	138
Arkansas	20	10.0	20.0	70.0	15
Missouri	40	0.0	77.5	22.5	14
Iowa	39	0.0	17.9	82.1	2
Illinois	40	2.5	62.5	35.0	14
Minnesota	40	0.0	12.5	87.5	2
Wisconsin	40	5.0	32.5	62.5	11
Total	**100.0**	**9.8**	**54.5**	**35.7**	**41.0**

3.3 Aflatoxin production and distribution of aflatoxigenicity

Aflatoxin B1 was extracted with chloroform from isolates after seven days growth on rice and quantified using reversed-phase high performance liquid chromatography (HPLC). Out of the 339 soil and 204 corn kernel isolates from the eight states, 41.2% of the soil and 36.5% of the kernel isolates were non-aflatoxigenic (Table 2). An additional 24.2% of the soil isolates produced less than 20 ppb of aflatoxin while only 3.3% of corn isolates produced less than 20 ppb. The other 34.5% of soil and 60.2% of corn kernel isolates were producers of aflatoxin at different levels (low, medium or high) with production ranging from slightly more than 20 ppb to close to 80,000 ppb per gram of rice. The soil population has a good representation of highly toxigenic individuals, ~20% of the total population produce more than 10,000 ppb, but the corn kernel population has a total proportion of 41.8 % of highly toxigenic individuals. By looking at differences between southern and northern populations (Table 2), a higher proportion of toxigenic individuals occurred in the northern states both from soil and corn kernels (41% and 70% respectively), than in the southern states. The total toxigenic proportion of individuals from the south consisted of 24% and 43% for soil and corn kernel isolates, respectively. Absolute non-aflatoxigenicity was in higher

proportions for the soil and kernel populations in the southern region (55% and 51%), than in the northern region (33% and 28%).

Table 2. Proportion of aflatoxin producing isolates of *Aspergillus flavus* from soil and kernel populations, per state and region. Aflatoxin classes: Zero; U20 under 20 ppb; Low 20-300 ppb; Medium 300-10K ppb; High 10K-80K ppb.

State	Percentage of aflatoxin producing isolates by Class / State / Niche / Region									
	Soil Population					Kernel Population				
	Zero	U2O	Low	Medium	High	Zero	U2O	Low	Medium	High
Louisiana (s)	16.7	61.7	10.0	5.0	6.7	15.9	6.8	11.4	15.9	50.0
Mississippi (s)	68.3	1.7	5.0	3.3	21.7	73.0	8.1	2.7	2.7	13.5
Arkansas (s)	80.0	0.0	0.0	10.0	10.0	66.7	0.0	11.1	0.0	22.2
Missouri (n)	15.0	10.0	2.5	27.5	45.0	0.0	0.0	0.0	20.0	80.0
Illinois (n)	30.0	0.0	0.0	40.0	30.0	25.9	0.0	0.0	22.2	51.9
Iowa (n)	84.6	2.6	2.6	2.6	7.7	20.8	4.2	0.0	20.8	54.2
Minnesota (n)	27.5	55.0	7.5	0.0	10.0	38.1	0.0	4.8	23.8	33.3
Wisconsin (n)	7.5	62.5	2.5	2.5	25.0	51.9	7.4	0.0	11.1	29.6
Total	**41.2**	**24.2**	**3.8**	**11.4**	**19.5**	**36.5**	**3.3**	**3.7**	**14.6**	**41.8**
South = (s)	55.0	21.1	5.0	6.1	12.8	51.8	5.0	8.4	6.2	28.6
North = (n)	32.9	26.0	3.0	14.5	23.5	27.3	2.3	1.0	19.6	49.8

3.4 VCG composition and distribution

In total, 1371 mutants were induced from both soil and kernel isolates, 836 from soil and 535 from corn kernels that represents an average of 2-3 mutants per isolate. Out of the 543 isolates only 33 isolates did not yield any *nit* mutant. The proportion of mutants produced from the whole population was 27 % *cnx*, 11% *nirA* and 62% *niaD*. VCG was determined by pairing tests between complementary mutant types. In total, eighteen (18) multiple-isolate VCGs were identified. From the whole isolate collection ~70% of soil isolates were assigned to VCGs as well as ~57% of the kernel isolates. Morphological, physiological and molecular trait comparisons between VCGs were confined to the 18 multiple-isolate VCGs, however these data were generated for all 543 isolates. Measures of genotypic diversity included the remaining 188 singleton VCGs not assigned to any multiple-isolate VCG group.

VCG naming in this study was a continuation of the 16 VCGs identified by Sweany *et al.* throughout LA fields in 2007 (2011). Fifteen (15) new VCGs were determined from the eight-state study, (VCG-17 through VCG-31). Three VCGs previously identified in Louisiana (VCG-1, VCG-

4 and VCG-10) were also found across the 8-state population by doing complementation tests with known testers. These 3 VCGs alone accounted for 44% of the total population, VCG-1 and VCG-4 being the more abundant and found in all eight states (Table 3). These 3 VCGs accounted for 46% of the total soil subpopulation and 42% of the corn kernel subpopulation. The fifteen newly identified VCGs accounted collectively for 21.4% of the total population. These VCGs were not widely distributed across the whole geographic area but rather they were only sampled at the local or state level, in a very specific fashion. The 35% of isolates of undetermined VCG (188) were identified at different proportions across the whole area under study but were more frequent in the southern region.

Table 3. The number of isolates of *Aspergillus flavus* from each VCG by state

VCG	1	4	10	22	17	18	19	20	21	23	24	25	26	27	28	29	30	31	ND	T
LA	5	20	1		2	11	6	2	12					3					42	104
MS	2	7		40															48	97
AR	13	1	1																14	29
MO	1	17	3							5	5								24	55
IA	31	13														2	3		14	63
IL	11	23	3									10			5				15	67
MN	34	12		2									2				3	3	5	61
WI	25	16																	26	67
Total	122	109	8	42	2	11	6	2	12	5	5	10	2	3	5	2	6	3	188	543
%	**22.5**	**20**	**1.5**	**8**	**0.4**	**2**	**1.1**	**0.4**	**2.2**	**0.9**	**0.9**	**1.8**	**0.4**	**0.6**	**1**	**0.4**	**1.1**	**0.6**	**35**	**100**

The most widely distributed VCGs, VCG-1 and VCG-4, were found in all 8 states in both, soil and corn kernel subpopulations. VCG-1 had the higher representation in the soil population at 34% whereas it was only found in 3.5% in the kernel population. VCG-4 on the other hand was mostly associated with the corn kernel population at 35% and only at 11% from the soil. VCG-10 was scarcely recovered from both subpopulations and represented 1% and 3% for the soil and kernel populations respectively. The rest of VCGs of small representation were recovered from their respective niche (soil or kernel) in a very low proportion, with the exception of VCG-22 that was recovered predominantly from Mississippi in 9% and 6% from the soil and corn kernel respectively (Figure 3).

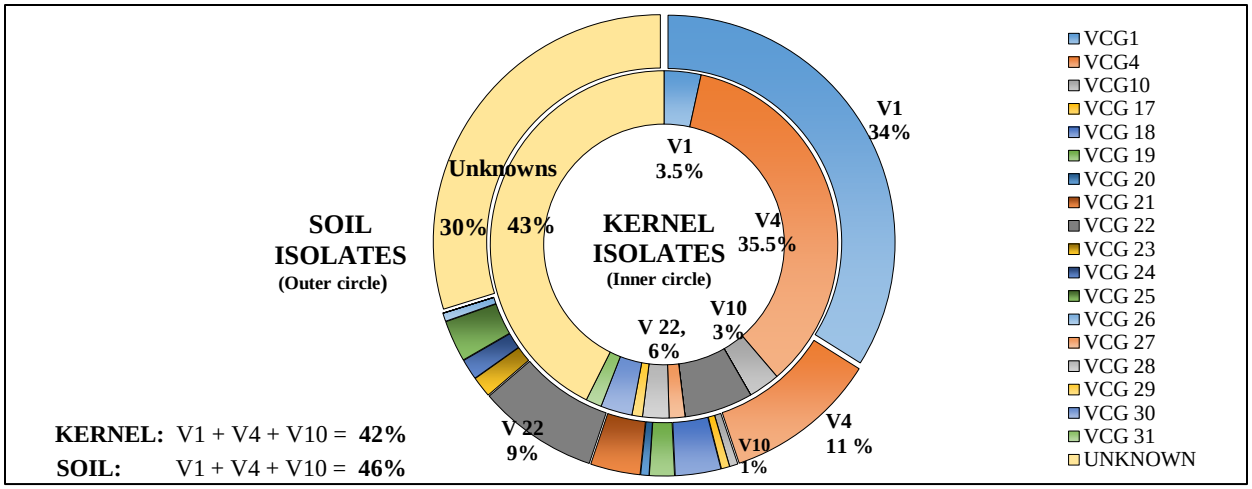

Figure 3. Proportion of different VCGs among isolates of *Aspergillus flavus* obtained from soil (outer circle) or corn kernels (inner circle) across the eight states sampled in 2014.

VCG-1 and VCG-4 were highly represented in northern (MO, IA, IL, MN and WI) (~38 - 49^{o} N latitude) and southern (LA, MS, AR) (~29 - 37^{o} N latitude) state populations. VCG-1 occurred in the soil population at 68% in the northern states, but 33% in the overall northern population and it was only recovered at 9% in the southern population. VCG-4 was mostly recovered from corn kernels at 26% in the northern and 12% in the southern populations (Figure 4).

3.6 VCG profiles and VCG-associated traits

A profile for every multiple-isolate VCG identified in this study was created and a comparison made between the soil and corn populations to determine if a correlation holds between VCG specificity and niche. The idea was to determine the characteristics associated with each VCG and the relation of these characteristics to the frequency of recovery of those VCGs from *A. flavus*-infected corn kernels. Sclerotial morphotype, mating type, aflatoxin, and cyclopiazonic acid production were characterized and compared among different VCGs and niches.

Close to 70% of soil (238) and 57% (117) of corn-kernel isolates were assigned to one of the 18 multiple-isolate VCGs. Four VCGs (VCG-1, VCG-4, VCG-10 and VCG-22) accounted for 54% of the total soil population and 48% of the total corn kernel population. Interestingly, these four VCGs were the only ones isolated from both the soil and corn kernel samples (Figure 4).

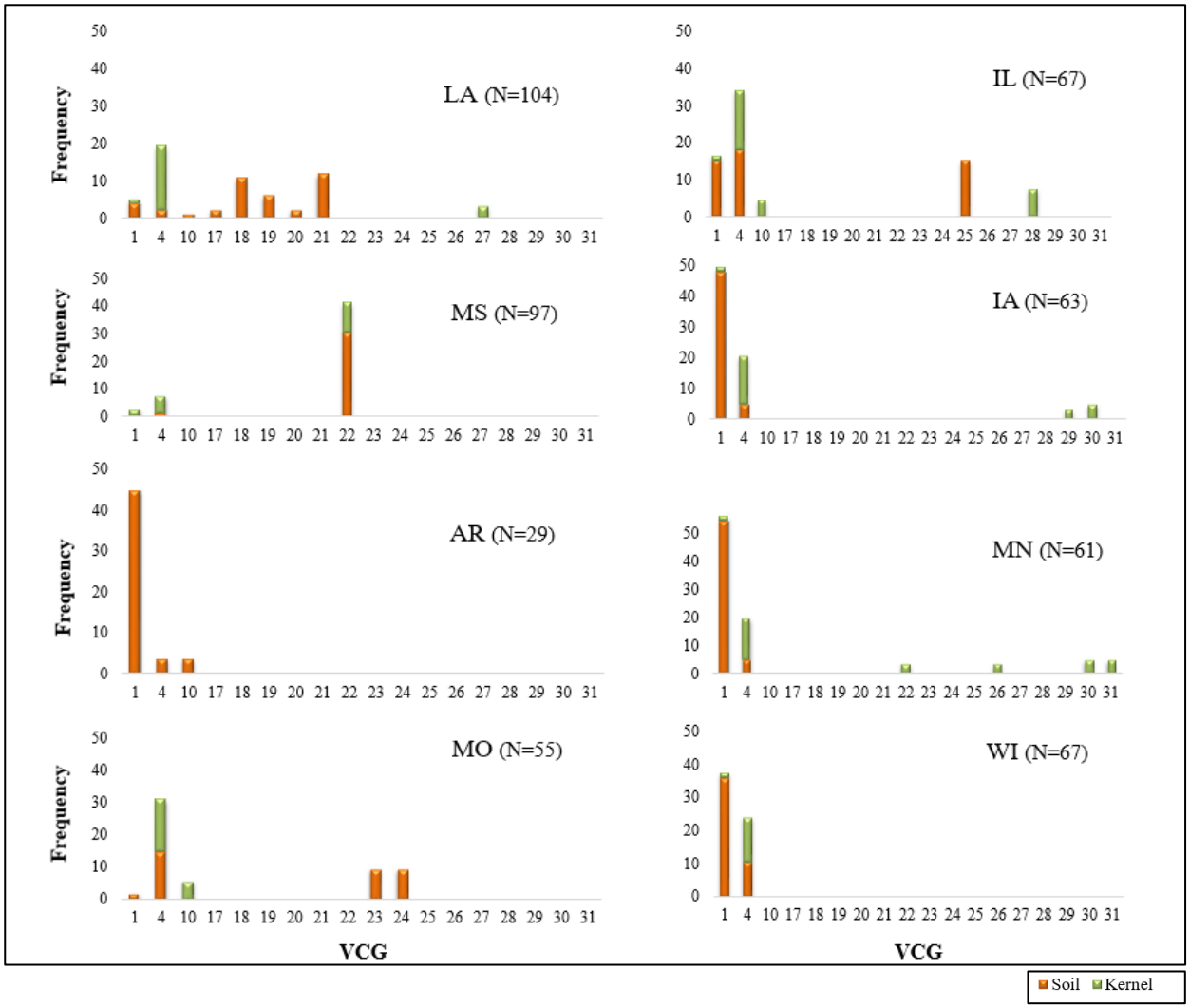

Figure 4. Frequency of different vegetative compatibility groups (VCGs) of Aspergillus flavus by state and niche.

VCGs of small representation were isolated from either soil or corn kernels but not from both niches. Including the 4 previously mentioned VCGs, 13 out of 18 VCGs were isolated from the soil whereas only 9 were identified from corn kernels. Nine VCGs were strictly recovered only from the soil population and 5 only from the kernel population. Out of the 4 most represented VCGs identified from both niches, 2 VCGs were widely distributed across the 8 states (VCG-1 and VCG-4).

The soil population included three sclerotial morphotypes; 3 VCGs did not produce any sclerotia at all (VCG-1, VCG-17 and VCG-26), 7 were of the L-morphotype (VCG-4, 10, 19, 22, 23, 24,

and VCG-25), and 3 of the S-morphotype (VCG-18, VCG-20, and VCG-21); however, VCG-20 sclerotia were extremely small and copious. Kernel isolate VCGs were strictly non-sclerotia producers or of the L-morphotype. Isolates that weren't assigned to any of the 18 VCGs also showed the same trend for sclerotial morphotype characteristics; soil isolates had a combination of all the previously described morphotypes whereas kernel isolates were strictly of the non-sclerotial or of L-sclerotial morphotype.

The mating type composition was skewed toward the mating type idiomorph *MAT1-2* for both, total soil and corn kernel populations. The soil population consisted of 90 % *MAT1-2* individuals and the kernel population of 85 % of the same type (Figure 5). From the 18 identified VCGs, and from the 13 strictly soil and 9 kernel VCGs, only 3 from each niche were found of the mating type *MAT1-1*. These six VCGs represented, however, just a small proportion of the population comprised of multiple isolate VCGs (~5%), so accounting only for the population comprised of multiple-isolate VCGs, ~95 % of both populations consisted of *MAT1-2* individuals across the eight states.

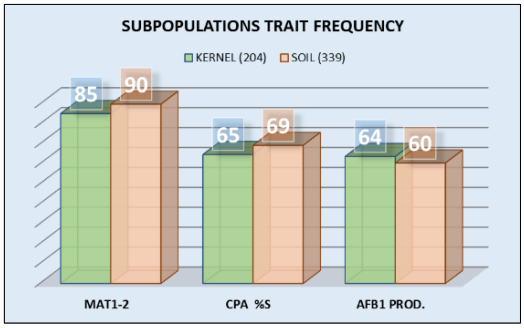

Figure 5. Frequency of the *MAT1-2* mating type, cyclopiazonic acid production (CPA %s) and aflatoxin B1 production (AFB1 Prod.) for soil and corn kernel populations of *Aspergillus flavus.*

Aflatoxin production was VCG dependent and variable across niches, states and regions. VCGs from both subpopulations, soil and corn kernel ranged from 0 ppb of aflatoxin production up to close to 80,000 ppb. Aflatoxin-producing VCGs from soil exhibited more diversity in aflatoxin levels which reflects the greater soil VCG diversity. It is interesting to point out that the isolate portion not assigned to VCGs from the soil population had higher mean aflatoxin values than the corn kernel population. Aflatoxin was also variable between VCGs of the different mating types,

MAT1-2 VCGs are very variable in aflatoxin production ranging again from non-producers up to highly toxigenic individuals; however VCGs of the *MAT1-1* were generally high aflatoxin producers in this study (Table 4). Mean aflatoxin values for isolates of unknown VCG can be seen as well for mating types (Table 4). Isolates of the *MAT1-1* mating type have higher mean aflatoxin values than their counterparts of the *MAT1-2* idiomorph. No clear inferences were possible in regards to aflatoxin and morphotype in this study, since most of the population was of the L-morphotype and aflatoxin production very variable.

Cyclopiazonic acid (CPA) production was rated on a scale ranging from non-producers, through low, medium and high CPA producers. In total, 69% of the soil population and 65% of the corn kernel population produced CPA at different levels and with no evident correlation with aflatoxin production and mating type. Different aflatoxin levels, from no-aflatoxin to high aflatoxin production was observed for each class or level of CPA (Figure 6). Some VCG examples in these regards are: VCG-1, a low aflatoxin producer, produced high amounts of CPA whereas VCG-4, a high aflatoxin producer, produced medium levels of CPA. The kernel-only VCGS with mating type *MAT1-1* (VCG-27, VCG-30 and VCG-31) and mating type *MAT1-2* (VCG-28 and VCG-30), all produced high levels of CPA. Interestingly, despite similar proportions of isolates recovered from both niches being positive for CPA production, the kernel isolates and VCGs in general tended to produce higher amounts of CPA than soil-isolate VCGs (Table 4). Out of the four more abundant VCGs and the total 9 kernel-isolate VCGs, only VCG-22 was found not to produce any detectable CPA. This VCG is also a non-aflatoxigenic VCG recovered mainly from 2 Mississippi fields in soil (29 isolates) and corn(11 isolates) and found scarcely (2 isolates) in MN corn.

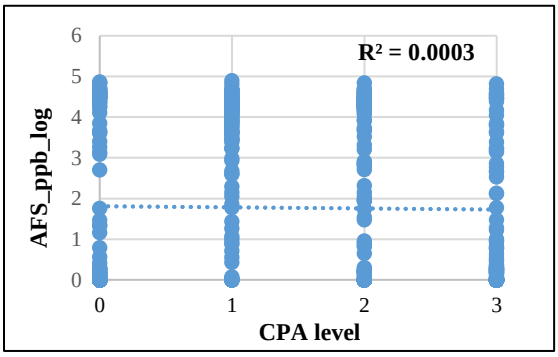

Figure 6. Correlation plot of aflatoxin production (AFS_ppb_log) versus levels of CPA production (cero: no CPA; 1: low; 2: medium; 3: high levels of CPA).

Aflatoxin and CPA production was variable among VCGs and statistical differences were found at the 0.001 level of significance for populations across states and regions. These differences were attributed to the different VCGs recovered from every state, with the exception of VCG-1 and VCG-4 that were present in all states. No clear correlation was observed between CPA and aflatoxin production and although close to 67% of the whole population produced CPA and ~62% produced aflatoxin B1, different chemotypes or combinations of aflatoxin and CPA levels were observed. Aflatoxin production varied a lot across niches because aflatoxigenic VCGs were recovered at different frequencies from both, soil and corn kernels. However, despite that CPA was also produced with variation across niches and VCGs as well, corn-kernel isolates from multiple-isolate VCGs exhibited higher amounts of CPA (Table 4).

Each of the 18 identified VCGs are shown in Table 4 with their respective phenotypic trait profile, aflatoxin and CPA production, sclerotial morphotypes, mating types, and their distribution.

Table 4. VCG profiles for *Aspergillus flavus* isolates based on phenotypic characteristics (morphological and physiological) and their distribution across states. Mating type (1 or 2) for *MAT1-1* and *Mat1-2* respectively; Sclerotial type: (-): No sclerotia, L: large, S: small; Mean aflatoxin in ppb; Isolate numbers (counts); CPA: cyclopiazonic acid (No, low, medium or high).

VCG	Mat. Type	Scler. type	Mean AF (ppb)		Soil Population					Kernel Population			
					Isol. #	State distribution and No.		CPA		Isol. #	State distribution and No.		CPA
VCG 1	2	-	3.1		115	**7**	LA AR, MO, IA, IL, MN, WI	Med / High		7	**6**	LA, MS, IA, IL, MN, WI	Med / High
VCG 4	2	L	30814.4		37	**8**	LA, MS, AR, MO, IA, IL, MN, WI	Low / Med		72	**7**	LA, AR, MO, IA, IL, MN, WI	Med
VCG 10	2	L	6113.4		2	**2**	LA, AR,	Low		6	**2**	MO, IL	Med
VCG 17	2	-	0.04		2	**1**	LA	No					
VCG 18	2	S	0.1		11	**1**	LA	No					
VCG 19	2	L	8.1		6	**1**	LA	Low					
VCG 20	1	S	10738.8		2	**1**	LA	Low / Med					
VCG 21	2	S	2.6		12	**1**	LA	No					
VCG 22	2	L	0		29	**1**	MS	No		13	**2**	MS, MN	No
VCG 23	1	L	19533.3		5	**1**	MO	Med					
VCG 24	1	L	1552.9		5	**1**	MO	Low / Med					
VCG 25	2	L	5542.8		10	**1**	IL	Low					
VCG 26	2	-	0.3		2	**1**	MN	Med					
VCG 27	1	-	46041.8							3	**1**	LA	High
VCG 28	2	L	0							5	**1**	IL	High
VCG 29	1	-	33715.7							2	**1**	IA	Med / High
VCG 30	2	L	0							6	**2**	IA MN	Med / High
VCG 31	1	L	2031.6							3	**1**	MN	Med
UNKN.	1	MS/S/L	15325.5		22	**5**		Low / Med					
UNKN.	2	S/L	7170.1		79	**8**		No-L-M-H					
UNKN.	1	N/L	4353.5							22	**4**		Med / High
UNKN.	2	L	4344.5							65	**8**		Med / High

Most of the diversity and variation of morphological, physiological and molecular traits in this study are related to specific VCGs. Morphotype characteristics, aflatoxin, CPA production and mating type, varies significantly among isolates from different VCGs and consequently differences are observed between soil and corn kernel populations, between different states, and across regions (Table 4).

Principal component analysis (PCA) for VCGs based on these multiple variables (Figure 7) revealed the homogeneity of isolates within a VCG. This PCA analysis revealed that all individuals belonging to the same VCG were clustered together toward the same direction and quadrant and set apart from individuals from different VCGs. Seven factors or components of variation were created from which only two were necessary to explain the higher variation among VCGs rather than within. Factors 1 and 2 accounted for 71.24 % of the total variation and were subsequently plotted for illustration (Figure 7). Aflatoxin production and sclerotial types accounted for most of the variation in factor 1 (Axis X in the plot) and sclerotial density and CPA in factor 2 (Axis Y). Aflatoxin, sclerotial type, and sclerotial density have a positive load value whereas CPA has a negative load value (Table 5). The lower an isolate is plotted (more negative values) on the F2 or Y axis, the more CPA it produces, the more to the right on the F1 or X axis, the more aflatoxin it produces. In the same fashion, the higher the value (positive values) on the F2 axis, the more sclerotia those isolates produced, and if they are to the left on the X axis (on the negative quadrant), they produce less or no aflatoxin. For example, VCG-1, a low aflatoxin producing, high CPA and no-sclerotia producer has all its isolates clustered on the lower-left quadrant in the plot (negative values), reflecting exactly those characteristics (blue dots). VCG-4 on the other hand, a high aflatoxin, high CPA, L-morphotype with low sclerotial density, is located on the right lower quadrant of the plot (numerous dark green dots). Similarly, VCG-29 represented by the grey dots just below VCG-4 isolates, is a high CPA, high aflatoxin producer and it does not produce any sclerotia. Another interesting case is the one of VCG-22 (Red dots) in the slight left of zero on F1 but high positive values on F2. This VCG does not produce aflatoxin nor CPA, but its isolates produce reasonable amounts of large sclerotia. All active variables are also shown in the plot. It is important to mention that because aflatoxin and sclerotial density values vary a lot, their log transformed data was also used to plot this PCA analysis. However, because distribution

of data was observed to be the same with or without the transformed data, the original non-transformed variables were used in the plot.

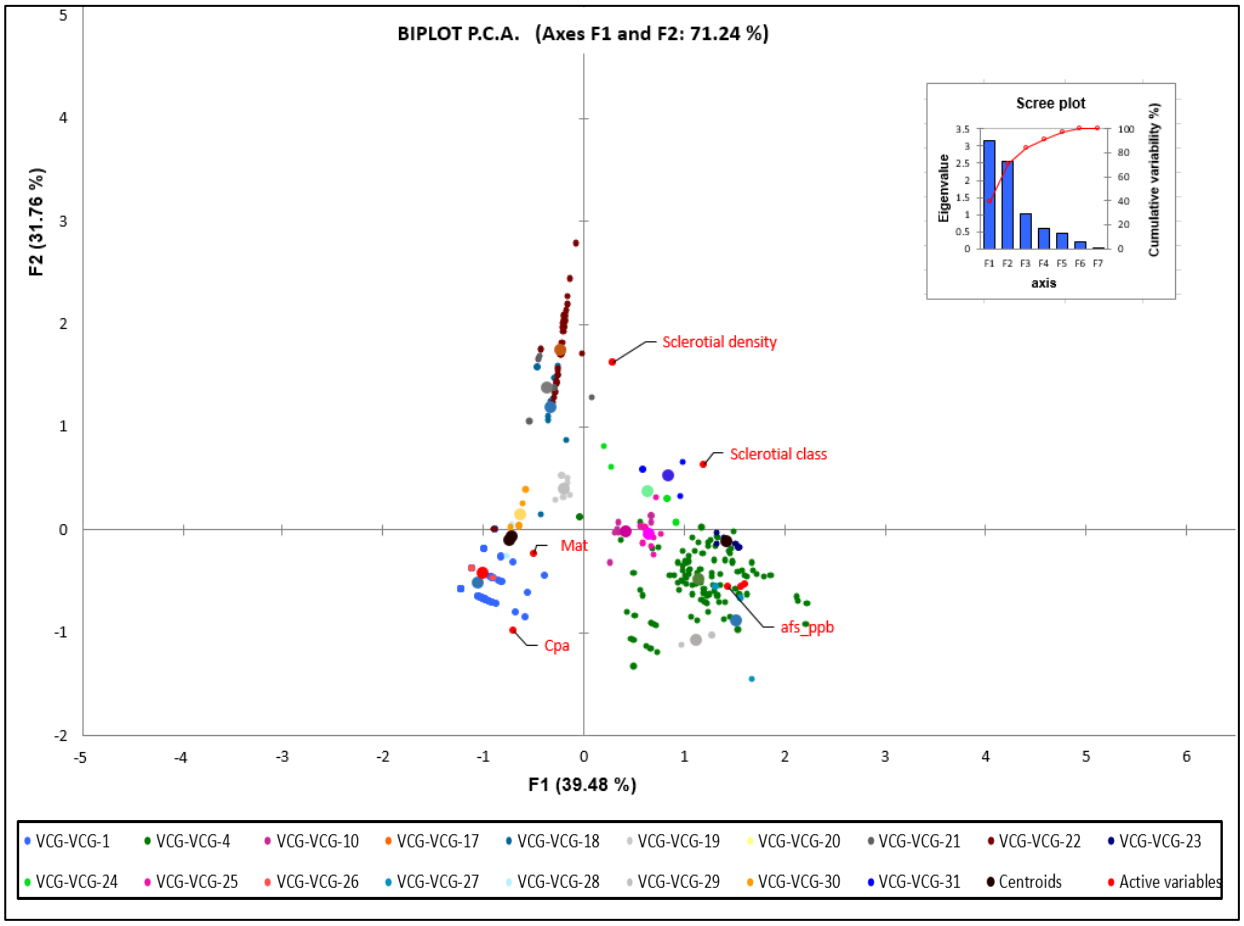

Figure 7. Principal component analysis (PCA) plot for isolates of *Aspergillus flavus* from different vegetative compatibility groups (VCGs) showing clustering of isolates with other isolates of the same VCG.

Table 5. Factor of variation loadings determined by principal components analysis from the different variables determined for isolates of *Aspergillus flavus* (* Plotted factors).

	F1*	**F2***	F3	F4	F5	F6	F7
Mating type	**-0.287**	**-0.138**	0.879	0.349	-0.038	-0.050	0.001
Sclerotia	**0.691**	**0.366**	0.279	-0.137	0.529	0.110	-0.006
Scle. density	**0.165**	**0.940**	-0.119	0.266	-0.061	-0.009	0.000
CPA	**-0.408**	**-0.569**	-0.391	0.510	0.302	0.068	-0.002
Afs_ppb	**0.833**	**-0.319**	0.048	0.131	-0.249	0.352	-0.009
Afs_class	**0.907**	**-0.319**	-0.060	0.113	-0.022	-0.227	-0.081
Log_afsppb	**0.927**	**-0.303**	-0.050	0.112	-0.006	-0.158	0.091
Log_scle. density	**0.165**	**0.940**	-0.119	0.266	-0.061	-0.009	0.000

3.7 Indices of genotypic diversity

Ecological-based approaches and the "vegan" R package were used in this study to calculate diversity indices. Shannon-Wiener and Simpson diversity indices were calculated, as well as observed and expected genotypic richness, evenness and Jaccard distance coefficients for the different subpopulations under study.

The overall observed genotypic richness (number of VCGs) identified across the eight states consisted of 206 unique genotypes. Eighteen multiple-isolate VCGs with different relative abundance and 188 single-isolate VCGs were observed across the sampled geographic area. The highest relative abundance of VCGs was represented by 3 VCGs (VCG1, VCG4 and VCG10) which accounted for ~43% of the whole *A. flavus* population. Based on the number of 206 unique VCGs, the overall Shannon diversity index (*H'*) for the whole population was 3.70 and the maximum value observed among states was of *H'*= 3.29 and *H'*=2.90 for Louisiana (LA) and Mississippi (MS), respectively, representing the states with higher diversity of VCGs. These higher *H'* values for LA and MS are correlated with their highly observed (G Obs) and expected genotypic richness (E(Gn)) (Table 6); LA with a G Observed value of 51 and E(Gn) of 18.2 and MS with a G Obs of 51 and E(Gn) richness of 16.8 when rarefied to the smallest population size (Arkansas N= 29). The states with the lowest diversity were IA and MN with an *H'* value of 1.85 and 1.50 respectively and expected number of VCGS (E(Gn)) of 10.0 and 7.6 when rarefied to the smallest population size. Simpson's diversity indices (*G'*) were consistent with the Shannon *H'* values giving the highest value to LA with 13.76 and the lowest to MN with 2.80, although the second highest diversity value based on *G'* was for MO instead of MS as with *H'* indices. Evenness (*E1*), which is a measure of how is the frequency of VCGs within each population and across states, ranged from 0.63 in MN to 0.84 in LA. The overall evenness value for the population across the eight states was *E1*= 0.70 (Table 6).

Although population size and number of VCGs observed varied among states in this study, the rarefied richness value E(Gn) was relatively similar for LA, MS, AR and Missouri (MO) with a value ranging from 16.4 -18.2. E(Gn) richness values for Iowa, Illinois (IL), Minnesota (MN) and Wisconsin (WI) were more similar between them with an average of 10.5 (range=7.6 -13.3). These later values were significantly different and lower from the E(Gn) values of the 4 southernmost

states (LA, MS, AR and MO), reflecting a marked difference in genotypic diversity among states. When comparing the whole population across regions, southern versus northern collectively, indices of genetic diversity based on VCGs were higher for the southern states (LA, MS and AR) which had an *H'*= 3.81 and G'= 16.48. For the northern states (from MO to MN) their values were *H'*= 2.86 and G'=5.66 specifically. These differences are dependent on their observed richness of 114 for southern and 96 for northern, and their respective evenness values of E1= 0.80 and E1= 0.63 for southern and northern populations, respectively. The simplest diversity indices (DI) also reflect the lower diversity of northern state population in comparison to the south, by having the highest clonal fractions (Table 6). Higher clonal fractions in the north reflect that relatively few VCGs comprise most of the isolates identified in this study.

Table 6. Indices of genotypic diversity by state for populations of *Aspergillus flavus* isolated from soil and corn kernels. (N: Population size; G Obs: number of observed genotypes (VCGs); E(Gn): expected number of VCGs when populations are rarefied to the lowest N (AR, 29); S.E: Standard Error for E(Gn); H'**:** Shannon's diversity index; G': Simpson's diversity index; DI: simplest diversity index (g/N); E1: (H'/ Ln(gMax)) evenness value; (1-g/N): clonal fraction per population.

Indices	Statistic	Indices of Genotypic Diversity per State								
		LA	**MS**	**AR**	**MO**	**IA**	**IL**	**MN**	**WI**	**Total**
	N	104	97	29	55	63	67	61	67	543
Richness	**G Obs**	51	51	17	29	18	20	11	28	206
	E(Gn)	18.2	16.8	17.0	17.0	10.0	11.3	7.6	13.3	
Stand. Er.	**S.E *E(Gn)***	2.18	2.23	0	1.83	1.69	1.71	1.26	1.99	
Evenness	***E1***	0.84	0.74	0.78	0.83	0.64	0.74	0.63	0.70	0.70
Shannon	***H'***	3.29	2.90	2.22	2.78	1.85	2.22	1.50	2.34	3.70
Simpson	***G'***	13.76	5.53	4.55	8.11	3.43	5.62	2.80	4.95	10.05
Simple DI	***DI***	0.49	0.53	0.59	0.53	0.29	0.30	0.18	0.42	0.38
Clonal Fr.	**(1-g/N)**	0.51	0.47	0.41	0.47	0.71	0.70	0.82	0.58	0.62

The Jaccard distance or dissimilarity coefficient (JDC) was also calculated for population comparisons based on VCG diversity and across the three different scenarios; states, regions and niches using the "vegdist" function in the "vegan" R package. The Jaccard coefficient for dissimilarity between populations in most of the cases was very high ranging from 0.74 to 0.97 genetic dissimilarity in state pairwise comparisons. Exceptions were the case of IA::WI and MN::WI, where the JDCs were 0.58 and 0.59 respectively being the lowest dissimilarity values among all the populations and state comparisons. The JDC between southern and northern state *A.*

flavus populations was 0.89 (0.11 similarity) and the JDC between soil and corn kernel populations was 0.87 (0.13 similarity) (Table 7).

Table 7. Jaccard dissimilarity coefficient for state pair's comparison of *Aspergillus flavus* populations (samples) obtained from soil and/or corn kernels.

	AR	IA	IL	LA	MN	MO	MS
IA	0.82						
IL	0.84	0.77					
LA	0.94	0.88	0.82				
MN	0.82	0.41	0.78	0.89			
MO	0.96	0.87	0.79	0.86	0.87		
MS	0.98	0.94	0.94	0.95	0.93	0.94	
WI	0.83	0.59	0.75	0.86	0.59	0.84	0.94

The genetic (dis-)similarity between populations in state pairwise comparisons as determined by JDC seems to be highly correlated to geographical distance between populations. A Mantel correlation test @ 0.005 level of significance was performed between these two variables of distance (Genetic vs geographic) and returned a correlation value of 0.60. This indicates that two variables are not independent of each other and actually the genetic distance between state populations is determined and dependent on the geographical distance (Table 8) between one another. Two geographically closer populations show higher similarity in VCG composition and frequencies of VCGs.

Table 8. Geographic distance in Km, state pairwise comparisons of *Aspergillus flavus* populations obtained from soil and/or corn kernels.

	AR	IA	IL	LA	MN	MO	MS
IA	896.0						
IL	615.0	390.0					
LA	234.0	1108.0	848.0				
MN	1220.0	336.0	719.0	1423.0			
MO	539.0	361.0	269.0	747.0	681.0		
MS	75.0	915.0	608.0	263.0	1244.0	566.0	
WI	1083.0	345.0	472.0	1313.0	455.0	616.0	1080.0

Chapter. 4. DISCUSSION

One overarching goal of research on *Aspergillus flavus* on corn is to determine the characteristics associated with each VCG and the corn-kernel infecting abilities of VCGs in order to identify the traits that the best corn infecting strains have and that the soil-only inhabitants lack. In biological and ecological terms, the ability to infect is important for both the toxigenic and atoxigenic strains. Insight into how the infection process works and what features confer that ability would aid in implementation and improvement of current biocontrol strategies against *A. flavus* (Damann, 2015). As a result of this study, a quantitative baseline of the most common strains of *A. flavus* from the soil and corn kernels along the nearly 1500 km transect from Minnesota to Louisiana now exists and the diversity of toxigenic and atoxigenic VCGs spanning an important corn growing area in the US has been documented. By collating different morphological, physiological and molecular traits between the pool of corn-infective VCGs and those VCGs that only occupy the soil and by comparing them with those previously determined by Sweany, I was able to confirm the niche specialization concept (Sweany *et al.,* 2011) and the clear differentiation of soil and corn kernel-infecting populations in a broader sampled area than Louisiana. The knowledge and understanding of ambient diversity and distribution of VCGs at the local, state and regional levels provides information to implement the best fit aflatoxin biocontrol strategies (Cotty 1994; and Pildain *et al.,* 2004). It is important to know the distribution of toxigenic individuals to understand the risks associated with them if environmental conditions become conducive (Atehnkeng *et al.,* 2015) and more importantly, knowing the population structure and VCG richness for the appropriate selection and deployment of biocontrol strains (Abbas *et al.,* 2009) based on the intrinsic characteristics of the target population.

Results indicate that the *A. flavus* population along the Mississippi river states is highly genetically diverse as reflected by the richness of VCGs and their associated diversity indices. Based on the 206 unique VCGs, a high diversity within the whole *A. flavus* population was determined in this study, but especially higher for the southernmost states. The higher *H'* values for LA and MS were highly dependent on their observed and expected genotypic richness, which were higher than any other state. Relatively lower Shannon diversity values to those observed in this study have been reported for an *A. flavus* population in Nigeria (*H'*=2.60) (Atehnkeng *et al.,* 2015) and northern

Italy (*H'* ranging from 2.4 - 3.2) (Mauro *et al.,* 2013). Lower values are usually explained as consequence of population size, since diversity is known to increase with population size, and in order to compare different populations, rarefaction analysis to interpolate diversity to a standard population size is needed (Grünwald *et al.,* 2003). Diversity indices observed in this study were in general similar to those reported in Arizona, DI=0.54 (Bayman and Cotty, 1991b) but relatively lower than those reported in Georgia, DI=0.69 (Papa, 1986) and Argentina, DI=0.64 (Pildain *et al.,* 2004).

The high VCG diversity in this study is also reflected in the variability of production of mycotoxins, particularly aflatoxin and CPA, and their distribution. A uniform trend and form of directional selection is observed in regards to the morphotype associated with corn fields, for both soil and corn kernel-infecting strains. Overall, ~90% of the whole population is of the L-morphotype and just a small portion of the soil isolated individuals produced small sclerotia. In fact, 100% of the corn infectors were L-type strains. These results are similar to two studies of the genetic diversity and distribution of VCGs in Nigeria, (Atehnkeng *et al.,* 2008; and Atehnkeng *et al.,* 2015). In both studies they reported a >90% representation by L-type strains. Similar reports of dominance of L-morphotype strains for corn-associated strains were done in northern Italy (Giorni *et al.,* 2007; Mauro *et al.,* 2013), in Ghana (Perrone *et al.,* 2014) and in the peanut growing areas of Argentina (Pildain *et al.,* 2004). It is believed that these strains are the most important source of aflatoxin contamination in these areas and that they should be the primary target in the development of management strategies (Atehnkeng *et al.,* 2008). Small sclerotia producers were primarily observed in Louisiana and Mississippi soils in this study; consistent with the observations of Sweany *et al.* in 2011, where ~56% of soil representatives produced small sclerotia and the other 44% large, however, even for fields in these states, only L-type strains were recovered from the corn-kernel population. Even when S-type strains are present in the soil and these strains tend to have higher aflatoxin producing potential than L-type strains, the higher incidence of L-type strains in the crop represent the largest contribution of aflatoxin contamination in such crops. These reports are also in concordance to that reported by Okun *et al.* in 2015 in a study looking at the distribution of toxigenicity by Aspergilli in Kenyan soils, from corn and peanut fields; the S-type strains represent only a small portion of the whole population and thus, L-type strains are the most important source of aflatoxin contamination in these crops (Okun *et*

al., 2015). However, in contrast to Okun *et al.,* during a severe aflatoxicosis outbreak in 2004 in Kenya, the high S-strain incidence from corn samples collected from major agricultural markets and storage facilities were attributed as the cause of aflatoxin poisoning events (Probst *et al.,* 2007). This could also be explained by the improper handling practices and storage conditions of grain after harvest, especially if grain had come in contact with soil at any point during these activities as occurs in African countries (Probst *et al.,* 2011). In the southern US the L-morphotype strains have been documented to be more abundant and widely distributed from eastern New Mexico to Virginia with the exception of Louisiana, Mississippi and eastern Texas, where S-type strains are either in close proportion or slightly higher than L-strains. Such observations are thought to be associated with adaptation of S-strains to areas of cotton cultivation. L-morphotype strains seem to be predominant in areas historically cultivated with peanuts and corn (Horn and Dorner, 1998).

Aspergillus flavus strains have been documented to be quite variable in their aflatoxin-producing ability. The aflatoxin producing potential of ambient strains or VCGs in this study was relatively high and variable, especially for the northern state populations. In this study, 41% of soil and 70% of kernel isolates from the northern states (MO, IA, IL, WI and MN) were highly toxigenic, whereas 24% and 43% were toxigenic in the southern region (LA, MS and AR) for soil and kernels, respectively. The vast majority of non-aflatoxigenic individuals were found in the soil populations of the southern states with 55% of the isolates being non-toxigenic and an additional 21% producing less than 20 ppb. The northern population was composed of ~58% non-aflatoxigenic individuals in the soil, but only 30% of the corn kernel isolates were of this type. Most of the corn-infecting isolates from the northern states (60%) are highly toxigenic. A highly toxigenic population has also been documented for a transect spanning the southern U.S. from New Mexico to Virginia where most individuals (~80%) produced varying levels of aflatoxins (Horn and Dorner, 1999). Most of the highly aflatoxigenic isolates in this study were from the highly aflatoxigenic VCGs, VCG-4 and VCG-10, both first described and identified in Louisiana fields in 2007 (Sweany *et al.,* 2011). VCG-4 produced mean aflatoxin concentrations of 30,800 ppb and VCG-10 ~6,000 ppb. VCG-4 was primarily isolated from corn kernels in the states of MO, IA, IL, MN and WI, with proportions up to 34% of the whole kernel population. In 2007, VCG-4 represented only ~10% of the whole Louisiana population (Sweany *et al.,* 2011), but in this study represented ~20% of the LA kernel population and much higher frequencies for the northern states

(~35%). With its wide distribution from LA to MN, VCG-4 appears to be the single most toxigenic and threatening VCG along the Mississippi River corn-growing areas and its presence and dominance is surprisingly seen 7 years after its first identification in LA in 2007. Others have not been able to recover predominant strains from fields sampled over two consecutive years in a third year of sampling from the same field (Bayman and Cotty, 1991). It is known that high temperatures and dry conditions favor infection and development of *A. flavus* and consequently it contaminates corn with aflatoxins (Guo *et al.,* 2005; Atehnkeng *et al.,* 2008). It is curious that no serious aflatoxin contamination events have been reported in the last decade, in either the southern or northern states. It is possible that corn crops have not been subjected to highly stressful conditions that often predispose contamination with aflatoxins or that indigenous atoxigenic strains, similar to the widely distributed VCG-1 in Louisiana and along the whole MS River transect are acting as a natural biocontrol against toxigenic VCGs. In fact VCG-1 is also the most dominant non-toxigenic VCG found across the eight states, although not as frequently isolated in the corn as seen in LA in 2007 (Sweany *et al.,* 2011) but isolated from the soil samples in high frequencies.

The knowledge of genetic diversity of *A. flavus* based on VCGs, especially that of non-toxigenic strains along with their environmental distribution generates information that may facilitate the selection and deployment of atoxigenic VCGs to mitigate aflatoxin contamination in corn growing areas (Atehnkeng *et al.,* 2015). Of course, additional characterization of non-aflatoxigenicity and lab and field efficacy of selected strains for their potential use in biocontrol needs to be determined (Atehnkeng *et al.,* 2014; Damann, 2015). Several successful cases exist in which non-toxigenic *A. flavus* strains are being used in the U.S. and Africa for the mitigation and control of aflatoxin contamination of economically important commodities (Cotty and Bhatnagar, 1994; Abbas *et al.,* 2011; and Bandyopadhyay and Cotty, 2013) and the identification of non-aflatoxigenic strains from this study has created a rich baseline of individuals that may be screened and tested for the potential use in biocontrol in the region.

Close to 70% of soil and 57% of the kernel isolates were assigned among 18 VCGs, which varied in relative abundance and frequencies among the different state populations. Three of these VCGs (VCG-1, VCG-4 and VCG-10) had been previously identified in Louisiana in 2007 (Sweany *et al.,* 2011) and accounted for ~44% of the total population in this study. The first two were found

widely distributed across the 8-state population and their dominance is still observed 7 years after their first identification and report in LA fields (Sweany *et al.*, 2011). This may be due to the observation that of the 16 LA-observed VCGs, VCG-1, 4, and 10 had the greatest conidial fecundity *in vitro* ($2\text{-}3X10^7/cm^2$) suggesting a greater potential for dissemination (Sweany *et al.*, 2016). VCG-1 accounted for 87% of the identified VCGs in the kernel population and 3% of the soil VCG population in 2007, but it was only 3% of kernel isolates in this study. VCG-1 was found primarily in the soil population at 68% in the northern states of MN, WI, and IA and AR in the south; however its overall distribution included 7 of the 8 sampled states. This observation is interesting because VCG-1 comprised 87% of the corn kernel population in 2007 as determined by Sweany *et al.*, and although highly present in the soil it was only occasionally found in the kernel pool in this study. VCG-1 is also known to be highly infective and fecund VCG in field trials in LA (Damann, 2015). It is possible that VCG-1 had not produced sufficient inoculum due to low germination caused by lack of moisture, or there was not enough inoculum disseminated into those fields from elsewhere. It is known that VCG-1 also happens to be the same VCG as Syngenta's Afla-Guard biocontrol strain NRRL 21882 (VCG-24 from Horn and Dorner, 1998) a widely distributed VCG in the southern US (Damann, personal communication). NRRL 21882 is known to be missing the whole aflatoxin gene cluster and consequently is non-aflatoxigenic, but many members of VCG-1 in the southern US and along the MS River produce aflatoxin in low levels (~3 ppb). Molecular characterization needs to be done to determine the extent of the relationship between these different aflatoxin phenotype individuals belonging to VCG-1 and whether or not they are products of recombination. Vegetative compatibility for isolates within the same VCG would allow non-meiotic recombination to occur between them (Leslie 1993).This means that atoxigenic isolates that belong to VCGs with toxigenic members should not be selected as biocontrol strains (Mehl *et al.*, 2012). VCG-4 on the other hand accounted for 10% of the kernel population in 11 LA fields in 2007 (Sweany *et al.*, 2011) but was found in 27% of the population from kernels in this study. VCG-4 was present in 7 out of the 8 states in kernels and in all 8 in the soil population (8%). This VCG also has a high aflatoxin producing potential in culture but to our knowledge there were no significant aflatoxin contamination events in the fields during 2014, when samples were collected, nor since a severe contamination event in 1998 in Louisiana. Perhaps environmental conditions in the mid-west were not conducive to aflatoxin production in

spite of infection or maybe other VCGs such as VCG-1 are acting as natural biocontrol against toxigenic VCG-4 (Damann, 2015).

The fifteen VCGs (VCG-17 through VCG-31) which were identified in this study accounted collectively for 21.4% of the total population. These VCGs were not widely distributed across the whole geographic area but rather they were only sampled and recovered from their respective niche (soil or corn kernels) at the local or state level, in a very specific fashion. The relative abundance of VCGs in this study, in which few VCGs comprised the vast majority of sampled isolates and multiple VCGs had just a small representation within the population has been observed since Papa in 1986, Bayman and Cotty in 1991, Horn and Greene in 1995 and Pildain *et al.,* 2004 and other research groups in western and eastern Africa during the last decade (Perrone *et al.,* 2014 and Atehnkeng *et al.,* 2015). The high diversity of VCGs identified in a region may be due to the widespread aerial dispersal of *A. flavus*. Just as Horne and Greene reported in 1995 that *A. flavus* is the dominant species within section Flavi of the Aspergilli because of its better ability to infect above-ground portions of crops, the dominance of certain VCGs within the *A. flavus* population may be due to the differential ability of certain VCGs to infect their associated crops as well as their greater potential for dispersal (Horne and Greene, 1995). Bayman and Cotty in 1991 reported shifts in the VCG composition of a single field from year to year in a three year-study. They postulated that these shifts in the population could be due to the influx of airborne conidia from outside the fields. If they had sampled a larger area they could have identified a potential common VCG to a broader area (Horn and Dorner, 1999).

The multi-locus nature of genetic control of vegetative compatibility allows determination of identities (Leslie, 1993) and is useful in the characterization of population genetic structure and diversity (Bayman and Cotty, 1991). In this study the close genetic relatedness between isolates belonging to the same VCG was evidenced by morphotypes, mating type idiomorphs, aflatoxin, and CPA production just as reported by others in previous studies (Bayman and Cotty, 1991; Horne and Greene, 1995; and Pildain *et al.,* 2004) and is clearly illustrated by a principal component analysis (PCA). Toxigenic and non-toxigenic individuals were segregated as well into different VCGs and represent a great feature to identify potential strains for use in biocontrol (Abbas *et al.,* 2009). Non-toxigenic strains outcompete toxigenic ones in the field without causing an increase

in the overall *A. flavus* populations, but shift the population structure by increasing abundance of the non-toxigenic genotype, that translates to reduction of total aflatoxin contamination and risk (Bayman and Cotty, 1993). All individuals within the same VCG in this study had the same mating type idiomorph, and although not significantly different from each other, slight differences were observed in sclerotial densities, aflatoxin, and CPA production for certain isolates. Grubisha and Cotty in 2010 suggested that gene flow does not occur among VCGs in nature and that the differences observed within VCGs are likely to be caused by genetic mutation, especially due to the high clonality of *A. flavus* (Grubisha and Cotty, 2010). These mutations can originate phenotypes with varying morphotype and sclerotial densities, or differences in mycotoxin production within VCGs just as the ones observed and reported here. They may also change VCGs by mutations at the *het* loci (Leslie, 1993).

Aspergillus flavus has been previously determined to be ubiquitous in distribution (Horn, 2003) and it has been suggested that crop composition and crop history influences the relative abundance and persistence of this fungus in agricultural soils (Garber and Cotty, 2014). Many strains of *A. flavus* produce huge amounts of inoculum when they find rich carbon and nitrogen sources, thereby not only increasing their populations which regularly replenishes soil populations but also allowing long range dispersal (Horn, 2003). It has been reported that certain VCGs or strains are more fecund in inoculum production and consequently may be more widely dispersed (Sweany *et al.*, 2016). Both, in this study and Sweany *et al*., 2011, the three more widely distributed VCGs along the MS River states were highly fecund and produced abundant amounts of conidia. However, the success of certain VCGs in infecting crops does not seem to be just about producing inoculum and dispersing it. In fact, as previously mentioned, ~90% of the whole population in this study was the L-morphotype and produced great amounts of inoculum. Still, just a few VCGs seem to be dominant and better adapted to infect corn and to wide dispersal. The success of these few VCGs has to be related to their abilities to infect the crop, establish a population, reproduce, disseminate, survive and continue to reproduce. These VCGs seem to be better in tissue invasion and ramification during infection and consequently display different life strategies (Mehl and Cotty, 2010), which may help explain their differential adaptive abilities (Mauro *et al.*, 2015). The differential adaptation to infect plant host materials by *A. flavus* genotypes has been attributed to their different abilities in production of hydrolytic enzymes such as pectinases (Mehl *et al.*, 2012).

It has also been found that L-morphotype strains produce pectinases in higher frequency and amounts than S-strains, which may help explain why the whole corn-associated population in this study is highly skewed toward the L-morphotype (Mellon *et al.,* 2007). However, even within the L-morphotype population, few genotypes seem to dominate in total frequency and distribution. Besides the predominance of L-morphotype strains in both soil and corn kernel niches , the corn kernel isolates were determined to produce higher amounts of cyclopiazonic acid, an indole-tetramic acid toxin known to interfere with calcium pumps and consequently act as plant cell killing toxin (Kabala and Klobus, 2005; and Ehrlich, 2014). Although ~70% of isolates from both niches were found to produce CPA, the corn kernel infecting VCGs were found to produce higher concentrations of the toxin. In fact, the highest CPA producer in this study, toxigenic VCG-4, is one of the most widely distributed along the transect. Recently, Chalivendra *et al.*, in 2017 reported that CPA is a pathogenicity factor for *A. flavus*, which would also explain why in natural populations higher CPA producers are more fit and associated with infecting corn.

Sweany *et al.* in 2011 also found that the *MAT1-2*-idiomorph isolates were more frequently recovered from the kernel population, accounting for ~95% of the kernel isolates whereas the soil population had close to a 50/50 representation of both mating types. *MAT1-1* and *MAT1-2* are transcriptional activators turning on different sets of genes, which may give relative advantage in infecting plant tissue to those carrying the *MAT1-2* idiomorph at the mating type locus (Damann, 2015). In this study ~90% of the whole population (corn and soil) was of the *MAT1-2* mating type and is consistent with better fitness of *MAT1-2* strains interacting with corn.

Aflatoxin production has never been correlated with pathogenicity or virulence in *A. flavus.* Aflatoxin production varied a lot in this study and among different VCGs without regard to soil or crop-associated individuals. However, although not of importance for corn infectors, high aflatoxin amounts are suggested to benefit individuals residing in the soil (Fountain *et al.,* 2014). Mehl *et al.,* suggest that aflatoxin production for soil inhabitants may give these isolates better competing abilities against the huge array of organisms living in the soil as well as against grazing insects (Mehl *et al.,* 2012). S-morphotype strains are usually high aflatoxin producers which was also true for the small fraction of S-type VCGs isolated in this and previous studies (Horn *et al.,* 1996; Horn and Dorner, 1999; Sweany *et al.,* 2011).

In conclusion, from the total collection of 339 soil and 204 corn kernel isolates, 70% of soil and 57% of kernel isolates were assigned to 18 multiple-isolate VCGs using *nit* mutants. Four VCGs accounted for 54% of the total soil and 48% of total kernel population and these 4 were the only ones recovered from both niches. Out of the 18 multiple-isolate VCGs determined in this study; 15 were newly identified and 3 had been previously identified in a 2007 survey in Louisiana (Sweany *et al.,* 2011). These three main corn infecting VCGs (1, 4 and 10) were found in 2014 among the 17 corn fields in 8 states sampled across the transect and it is noteworthy that 7 years after their identification in Louisiana fields VCG 1 and 4 still occurred at high frequencies in the 2014 population. There were 101 singleton VCGs from soil and 87 singleton VCGs from kernels which accounted for 188 of the 206 vegetative compatibility groups identified in the whole population. Results also indicate that the *A. flavus* population along the Mississippi river states is highly genetically diverse as reflected by the richness of VCGs and their associated diversity indices. Southern state populations are more diverse than northern populations and the northern region shows higher clonal fractions. Morphotype characteristics, aflatoxin and CPA production, and mating type varies significantly among VCGs and consequently differences are observed between the soil and corn kernel populations, across states and across regions. However, all individuals shown consistency in every trait when they belonged to the same VCG. Soil and corn populations were characterized for VCG, sclerotial morphotype, mating type, cyclopiazonic acid (CPA) production, and aflatoxin production, in order to determine trait uniformity within a VCG and those VCGs associated with corn infection. The mating type *MAT1-2* and high CPA production were associated with corn infectors, all of which were of the L-morphotype. The soil niche had both S and L-sclerotial morphotypes but predominately L-type. Although ~70% of the isolates from both niches produced CPA, kernel isolates produced higher amounts and support the idea that CPA is a pathogenicity factor (Chalivendra *et al.,* 2017). These results give insight into important traits for corn infection, for both toxigenic and atoxigenic strains, which may be key in the improvement of biological control strategies for successful biocontrol. The aflatoxin producing potential of ambient strains or VCGs in this study was relatively high and variable and especially higher for the northern state populations. Much of the high aflatoxigenicity in this study is attributed to the highly toxigenic VCG-4 that comprises ~35% of the total population. The identification of ~55% non-aflatoxigenic strains from this study provides a pool of individuals that may be screened and tested for the potential use in biocontrol by region. Characterizing the ambient

diversity of VCGs and their distribution in a nearly 1500 km transect spanning an important corn growing area in the US, from Minnesota to Louisiana, has allowed us to create a quantitative baseline of *A. flavus* VCGs which can be used for improving the current aflatoxin biocontrol strategies and determination of the traits that confer selective advantage for corn infection within the *A. flavus* population. The distribution of toxigenic individuals will contribute to understanding the risks if environmental conditions become conducive. More importantly, the knowledge of the population structure and VCG richness will be useful for the initial selection and tests of potential biocontrol strains that can later be used in the efforts for aflatoxin contamination control.

REFERENCES

"Aflasafe." International Institute for Tropical Agriculture (IITA), 20 Oct. 2017, aflasafe.com/aflasafe/.

Abbas, H.K., Weaver, M.A., Horn, B. W., Carbone, I., Monacell, J.T., and Thomas-Shier, W. 2011. Special double issue—*Aspergillus*, aflatoxins, cyclopiazonic acid and biological control of aflatoxins. Toxin Reviews 30: 59–70.

Abbas, H.K., Wilkinson, J.R., Zablotowicz, R.M., Accinelli, C., Abel, C.A., Bruns, H.A., and Weaver M.A. 2009. Ecology of *Aspergillus flavus*, regulation of aflatoxin production, and management strategies to reduce aflatoxin contamination of corn. Toxin Reviews 28: 142–153.

Adhikari, B.N., Bandyopadhyay, R., and Cotty, P.J. 2016. Degeneration of aflatoxin gene clusters in *Aspergillus flavus* from Africa and North America. AMB Expr 6:62.

Atehnkeng, J., Donner, M., Ojiambo, P.S., Ikotun, B., Augusto, J., Cotty, P.J., and Bandyopadhyay, R. 2015. Environmental distribution and genetic diversity of vegetative compatibility groups determine biocontrol strategies to mitigate aflatoxin contamination of maize by *Aspergillus flavus*. Microbial Biotechnology 9:75–88.

Atehnkeng, J., Ojiambo, P.S., Cotty P.J., Bandyopadhyay R. 2014. Field efficacy of a mixture of atoxigenic *Aspergillus flavus* Link: Fr vegetative compatibility groups in preventing aflatoxin contamination in maize (Zea mays L.). Biological Control 72:62–70.

Atehnkeng, J., Ojiambo, P.S., Donner, M., Ikotun, T., Sikora, R.A., Cotty P.J., and Bandyopadhyay, R. 2008. Distribution and toxigenicity of *Aspergillus* species isolated from maize kernels from three agro-ecological zones in Nigeria. International Journal of Food Microbiology 122: 74–84.

Batt, C.A. 2014. Encyclopedia of Food Microbiology. Academic Press (2): 3248 pages.

Bayman, P., and Cotty, P. J. 1991. Vegetative compatibility and genetic diversity in *Aspergillus flavus* population of a single field. Canadian Journal of Botany 69:1707-1711.

Bayman, P., and Cotty, P.J., 1991b. Improved media for selecting nitrate-non utilizing mutants in *Aspergillus flavus*. Mycologia 83: 311–316.

Bayman, P., and Cotty, P.J., 1993. Genetic diversity in *Aspergillus flavus:* association with aflatoxin production and morphology. Canadian Journal of Botany 71, 23–31.

Bothast, R. J., and Fennell, D. I. 1974. A medium for rapid identification and enumeration of *Aspergillus flavus* and related organisms. Mycologia 66:365-369.

Chalivendra, S., DeRobertis, C., and Damann, K. E., Jr., 2016. Hypoxia tolerance as a factor in *Aspergillus flavus* invasion and toxin contamination of developing maize seeds. APS Abstracts/ 2016 APS Annual Meeting.

Chalivendra, S., DeRobertis, C., and Damann, K. E., Jr., 2017. Cyclopiazonic acid is a pathogenicity factor for *Aspergillus flavus* and a promising target for screening germplasm for ear rot resistance. Molecular Plant-Microbe Interactions 30(5), 361-373.

Chang P.K., Horn, B.W., and Dorner, J.W. 2009. Clustered genes involved in cyclopiazonic acid production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. Fungal Genetics and Biology 46: 176-182.

Chang, P.K., Ehrlich, K.C., and Fujii I. 2009. Cyclopiazonic acid biosynthesis of *Aspergillus flavus* and *Aspergillus oryzae.* Toxins 1: 74-99.

Chang, P.K., Scharfenstein, L.L., Mack, B., and Ehrlich, K.C. 2012. Deletion of the *Aspergillus flavus* orthologue of *A. nidulans* fluG reduces conidiation and promotes production of

sclerotia but does not abolish aflatoxin biosynthesis. Applied Environmental Microbiology 78:7557-7563.

Cotty, P. J. 1994. Influence pf field application of an atoxigenic strain of *Aspergillus flavus* on the populations of *A. flavus* infecting cotton bolls and the aflatoxin content of cottonseed. Phytopathology 84:1270-1277.

Cotty, P.J. 1994. Influence of field application of an atoxigenic strain of *Aspergillus flavus* on the populations *of A. flavus* infecting cotton bolls and on the aflatoxin content of cottonseed. Phytopathology 84, 1270–1277.

Cotty, P.J., and Bhatnagar, D. 1994. Variability among atoxigenic *Aspergillus flavus* strains in ability to prevent aflatoxin contamination and production of aflatoxin biosynthetic pathway enzymes. Applied and Environmental Microbiology 60:2248–2251.

Cotty, P.J., Bayman, P., Egel, D.S., and Elias, K.S. 1994. Agriculture, aflatoxins and *Aspergillus.* In the genus *Aspergillus*: from taxonomy and genetics to industrial application. Plenum Press Eds: 1–27. New York.

Cotty, P.J., Cleveland, T.E., Brown, R.L. and Mellon, J.E. 1990. Variation in polygalacturonase production among *Aspergillus flavus* isolates. Applied and Environmental Microbiology 56: 3885– 3887.

Council for Agriculture Science and Technology. 2003. Mycotoxins: Risks in plant, animal, and human systems. Task Force Report 139. Ames, IA

Cove, D.J. 1976. Chlorate toxicity in *Aspergillus nidulans*: studies of mutants altered in nitrate assimilation. Molecular Genetics146: 147-159.

Damann, K.E. 2015. Atoxigenic *Aspergillus flavus* biological control of aflatoxin contamination: what is the mechanism? World Mycotoxin Journal 8: 235-244.

Das, M.K., Ehrlich, K.C., and Cotty, P.J. 2008. Use of pyrosequencing to quantify incidence of a specific *Aspergillus flavus* strain within complex fungal communities associated with commercial cotton crops. Phytopathology 98: 282-288.

Dyer, P.S., and O'Gorman, C.M. 2012. Sexual development and cryptic sexuality in fungi: insights from *Aspergillus* species. FEMS Microbiology Reviews 36:165–192.

Ehrlich, K.C. 2014. Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: advantages and limitations. Frontiers in Microbiology 5:50.

Ehrlich, K.C. 2014. Non-aflatoxigenic *Aspergillus flavus* to prevent aflatoxin contamination in crops: advantages and limitations. Frontiers in Microbiology 5:50.

Ehrlich, K.C., and Cotty, P.J. 2004. An isolate of *Aspergillus flavus* used to reduce aflatoxin contamination in cottonseed has a defective polyketide synthase. Applied Microbial Biotechnology 65:473-8.

Ehrlich, K.C., Chang, P., Yu, J., and Cotty, P.J. 2004. Aflatoxin biosynthesis cluster gene cypA is required for G aflatoxin formation. Applied and Environmental Microbiology 70, 6518–6524.

Fountain, J.C., Kheraa, P., Yanga, L., Spurthi N. N., Scullye, B.T., Leef, R.D., Cheng, Z-Y., Kemeraita, R.C., Varshneyb, R.K., Guoc, K. 2016. Resistance to *Aspergillus flavus* in maize and peanut: Molecular biology, breeding, environmental stress, and future perspectives. The Crop Journal 3: 229-237.

Fountain, J.C., Scully, B.T., Ni, X., Kemerait, R.C., Lee, R.D., Chen, Z-Y., and Guo, B. 2014. Environmental influences on maize-*Aspergillus flavus* interactions and aflatoxin production. Frontiers in Microbiology 5:40.

Garber, N. P., and Cotty, P. J. 2014. *Aspergillus parasiticus* communities associated with sugarcane in the Rio Grande Valley of Texas: Implications of global transport and host association within *Aspergillus* section Flavi. Phytopathology 104:462-471.

Geiser, D.M., Pitt, J.I., and Taylor, J.W. 1998. Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. Proceedings of the National Academy of Sciences USA 95:388–393.

Giorni, P., Magan, N., Pietri, A., Bertuzzi, T., and Battilani, P. 2007. Studies on *Aspergillus* section Flavi isolated from maize in northern Italy. International Journal of Food Microbiology 113: 330–338.

Grubisha, L.C., and Cotty, P. J. 2010. Genetic isolation among sympatric vegetative compatibility groups of the aflatoxin-producing fungus *Aspergillus flavus.* Molecular Ecology 19: 269-280.

Grünwald, N.J., Goodwin, S.B., Milgroom, M.G., and Fry, W.E. 2003. Analysis of genotypic diversity data for populations of microorganisms. Phytopathology 93, 738–746.

Horn, B. W., and Dorner, J. W. 1998. Soil populations of A*spergillus* species from section Flavi along a transect through peanut-growing regions of the United States. Mycologia 90: 767-776.

Horn, B. W., and Dorner, J. W. 1999. Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Applied Environmental Microbiology 64: 1444–1449.

Horn, B. W., Moore, G. G., Carbone, I. 2009. Sexual reproduction in *Aspergillus flavus.* Mycologia 101:423-429.

Horn, B.W. 2003. Ecology and population biology of aflatoxigenic fungi in soil. Toxin Reviews Journal/ Toxicology 22: 351-379.

Horn, B.W., and Dorner, J.W. 1999. Regional differences in production of aflatoxin B1 and cyclopiazonic acid by soil isolates of *Aspergillus flavus* along a transect within the United States. Applied Environmental Microbiology 65, 1444–1449.

Horn, B.W., and Greene, R.L. 1995. Vegetative compatibility within populations of *Aspergillus flavus, A. parasiticus, and A. tamarii* from a peanut field. Mycologia 87:324-332.

Horn, B.W., Gell, R.M., Singh, R., Sorensen, R.B., Carbone, I. 2016. Sexual reproduction in *Aspergillus flavus* sclerotia: acquisition of novel alleles from soil populations and uniparental mitochondrial inheritance. PLoS ONE 11:1.

Horn, B.W., Greene, R.L., Soboler, V.S., Dorner, J.W., Powell, J.H., Lyton, R.C., 1996. Association of morphology and mycotoxin production with vegetative compatibility groups in *Aspergillus flavus, Aspergillus parasiticus and Aspergillus tamarii.* Mycology 88: 574–587.

Huang, C., Jha, A., Sweany, R., DeRobertis, C., and Damann, K.E. 2011. Intraspecific aflatoxin inhibition is thigmoregulated, independent of vegetative compatibility group and is strain dependent. PLoS ONE 6: e23470.

Leslie, J.F. 1993. Fungal vegetative compatibility. Annual Reviews of Phytopathology 31: 127-150.

Mauro. A., Battilani, P., and Cotty, P.J. 2015. Atoxigenic *Aspergillus flavus* endemic to Italy for biocontrol of aflatoxins in maize. Bio Control 60:125–134.

Mauro. A., Battilani, P., Callicott, K.A., Giorni, P., Pietri, A., and Cotty, P.J. 2013. Structure of an *Aspergillus flavus* population from maize kernels in northern Italy. International Journal of Food Microbiology 162:1–7.

McAlpin, C. E., Wicklow, D. T., and Horn, B. W. 2002. DNA fingerprinting analysis of vegetative compatibility groups in *Aspergillus flavus* from a peanut field in Georgia. Plant Disease 86:254-258.

McAlpin, C. E., Wicklow, D. T., and Platis, C. E. 1998. Genotypic diversity of *Aspergillus parasiticus* in an Illinois corn field. Plant Disease 82:1132-1136.

McAlpin, C.E., and Wicklow, D.T. 2002. DNA fingerprinting analysis of vegetative compatibility groups in *Aspergillus flavus* from a peanut field in Georgia. Plant Disease 86:254-258.

Mehl, H. L., and Cotty, P. J. 2010. Variation in competitive ability among isolates of *Aspergillus flavus* from different vegetative compatibility groups during maize infection. Phytopathology 100:150-159.

Mehl, H. L., and Cotty, P.J. 2013. Nutrient environments influence competition among *Aspergillus flavus* genotypes. Applied Environmental Microbiology 79: 1473-1480.

Mehl, H.L., and Cotty, P.J. 2013. Influence of plant host species on intraspecific competition during infection by *Aspergillus flavus*. Plant Pathology 62:1310–1318.

Mehl, H.L., Jaime, R., Callicott, K.A., Probst, C., Garber, N.P., Ortega-Beltran, A., Grubisha, L.C., and Cotty, P.J. 2012. *Aspergillus flavus* diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health. Annals of the New York Academy of Sciences 1273:7–17.

Mellon, J.E., Cotty, P.J. and. Dowd, M.K. 2007. *Aspergillus flavus* hydrolases: their roles in pathogenesis and substrate utilization. Applied Microbial Biotechnology 77: 497–504.

Milgroom, M.G. 2015. Population biology of plant pathogens: Genetics, ecology and evolution. APS Press: 399 pages.

Moore, G. G. 2010. Global Population structure and aflatoxin chemotype diversity in *Aspergillus* section Flavi. Dissertation NCSU.

Okun, D.O., Khamis, F.M., Muluvi, G.M., Ngeranwa, J.J., Ombura, F.O., Yongo, M.O. and Kenya, E.U. 2015. Distribution of indigenous strains of atoxigenic and toxigenic *Aspergillus flavus* and *Aspergillus parasiticus* in maize and peanuts agro-ecological zones of Kenya. Agriculture & Food Security 4:14.

Orum, T.V., Bigelow, D.M., Nelson, M.R., Howell, D.R., and Cotty, P.J. 1997. Spatial and temporal patterns of *Aspergillus flavus* strain composition and propagule density in Yuma County, Arizona, soils. Plant Disease 81:911-916.

Papa, K.E. 1986. Heterokaryon incompatibility in *Aspergillus flavus.* Mycologia 78:98-101.

Papaioannou I.A., and Typas M.A. 2015. High-throughput assessment and genetic investigation of vegetative compatibility in *Verticillium dahliae*. Phytopathology 163: 475–485.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakarnchanakul, W. and Samson R.A. 2014. Biodiversity of *Aspergillus* species in some important agricultural products. Studies in Mycology 59: 53–66.

Pildain, M. B., Vaamonde, G., and Cabral, D. 2004. Analysis of population structure of *Aspergillus flavus* from peanut based on vegetative compatibility, geographic origin, mycotoxin, and sclerotia production. International Journal of Food Microbiology 93: 31-40.

Pitt, J.I., Hocking, A.D., and Glenn, D.R. 1983. An improved medium for the detection of *Aspergillus flavus* and *A. parasiticus.* Journal of Applied Bacteriology 54:109-114.

Probst, C., Bandyopadhyay, R., Price, L.E., and Cotty, P.J. 2011. Identification of atoxigenic *Aspergillus flavus* isolates to reduce aflatoxin contamination of maize in Kenya. Plant Disease 95, 212-218.

Probst, C., Njapau, H. and Cotty, P.J. 2007. Outbreak of an acute aflatoxicosis in Kenya in 2004: identification of the causal agent. Applied and Environmental Microbiology 73: 2762–2764.

Ramirez-Prado, J., Moore, G.G., Horn, B.W., and Carbone, I. 2008. Characterization and population analysis of the mating-type genes in *Aspergillus flavus* and *Aspergillus parasiticus*. Fungal Genetics and Biology 45: 1292–1299.

Shannon, C.E., and Weaver, W. 1949. The mathematical theory of communication. University of Illinois Press, Urbana, IL.

Sweany, R. R., Damann, K. E., Jr., and Kaller, M.D. 2011. Comparison of soil and corn kernel *Aspergillus flavus* populations: Evidence for niche specialization. Phytopathology 101:952-959.

Sweany, R. R., DeRobertis, C., and Damann, K. E., Jr. 2016. Conidial versus sclerotial production: implications for corn and soil niche specialization in *Aspergillus flavus* Phytopathology 107(12):S4.47.

Wada, R., Maruyama, J., Yamaguchi, H., Yamamoto, N., Wagu, Y., Paoletti, M., Archer, D.B., Dyer, P., and Kitamoto K. 2012. Presence and functionality of mating type genes in the supposedly asexual filamentous fungus *Aspergillus oryzae*. Applied and Environmental Microbiology 78: 2819–2829.

Wicklow, D.T. 1991. Epidemiology of *Aspergillus flavus* in corn. New Perspectives, ed. O. L. Shotwell & C. R. Hurburgh pp.315-328. Iowa Agriculture and Home Economics Experiment Station Research Bulletin 599: Ames, Iowa.

Wicklow, D.T. and Horn, B.W. 2007. Association between vegetative compatibility and aflatoxin production by *Aspergillus* species during intraspecific competition. Mycoscience 48:267-273.

APPENDICES

Appendix A. List of collaborators who provided soil and corn kernel samples during the Fall of 2014.

Sample Provider	Affiliation	Location
Harold Lambert	Lambert Ag. Consulting	Batchelor, LA
Patrick Colyer	Louisiana State University	Bossier City, LA
Ken Damann	Louisiana State University	Baton Rouge, LA
Mark Weaver	USDA-ARS	Stoneville, MS
Gary Windham	USDA-ARS	Starkville, MS
Terry Spurlock	University of Arkansas	Monticello, AR
Bradley Schad	MO. Corn Growers Assoc.	Jefferson City, MO
Robert Bellm	University of Illinois	Brownstown, IL
Virgil Schmitt	Iowa State University	Muscatine, IA
Derrick Mayfield	Iowa State University	Ames, IA
Dean Malvick	University of Minnesota	Saint Paul, MN
Bruce Potter	University of Minnesota	Lamberton, MN
Damon Smith	University of Wisconsin	Madison, WI

VITA

Jorge Alberto Reyes Jr. was born in Lempira, Honduras and since a very young age he was passionate about agriculture. He earned a high school degree with orientation to agricultural sciences at the "Technical and Community Institute, David H. Navarro" in 2007. He received his B.S. degree in Agronomy at the National Agricultural University (UNA) of Honduras, in 2011. During his last year at UNA he visited Purdue University for a three-month Internship where he did research in the Weed Sciences lab at the Horticulture department. After graduating from UNA he worked with Jicatuyo Foundation; a private institution where his role was to assist growers in seeking financial support through proposals and grant applications and also provide consulting services to coffee growers in western Honduras. He became a Graduate Student under the mentoring of Dr. Ken Damann in 2015 and started working on a research project about population biology of the aflatoxin producing fungus *Aspergillus flavus* where he has had excellent training and learned a lot about Plant Pathology. He anticipates graduating with his master's degree in December 2017.