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The Role of Auxin in Defense Response to Aspergillus Flavus in Zea Mays L

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The role of auxin in defense response to *Aspergillus flavus* in *Zea mays* L.

By

Seval Ozkan

A Dissertation
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The role of auxin in defense response to *Aspergillus flavus* in *Zea mays* L.

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Understanding the role of phytohormone auxin in defense responses is one of the vital tools for plant breeders to develop maize germplasm lines that exhibit high resistance to *Aspergillus flavus* and subsequent aflatoxin accumulation. Besides its critical role in different developmental processes throughout the life cycle of plants, auxin is also involved in the network of plant-pathogen interaction as demonstrated in previous studies. However, the actual mechanism for the auxin signaling pathway leading to resistance is unknown. Therefore, the critical gap in the knowledge base is a lack of understanding of the role of auxin signaling in pathogen resistance in maize. Continuation of this gap is an important problem because fungal resistance is a highly quantitative trait and breeding for resistance is a challenge. A complete understanding of the auxin mechanism in resistance could lead the production of corn hybrids with resistance to *A. flavus* and aflatoxin accumulation.

The focus of this research was to determine the effect of exogenous auxin on *A. flavus* growth and production of aflatoxin in growth media. In addition, auxin levels, the amount of aflatoxin, and fungal growth in three resistant (Mp313E, Mp715, and Mp719)

and one susceptible (B73) germplasm line were determined. As a result, auxin significantly increased mycelium growth and significantly decreased aflatoxin at a high concentration in potato dextrose broth under the lab conditions. Under the field conditions, auxin levels were low in resistant lines but did not change in response to *A. flavus* infection. Susceptible line had high auxin levels and auxin levels significantly decreased in response to *A. flavus* infection.

DEDICATION

I would like to dedicate this dissertation to my parents Hulya and Muhabbet

Ozkan, my husband Alper Coban, my sons Furkan and Kayra Coban. Thank you for all

of your support and always believing in me.

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CHAPTER I

INTRODUCTION

Aspergillus flavus is a weak pathogenic fungus of oil seed crops such as maize, peanut, cottonseed and tree nut. *A. flavus* infection causes an ear rot in maize, aflaroot in peanut and tobacco, and a boll rot in cotton (Klich, 2007; Taubenhaus, 1920). However, besides the infection of crops by *A.flavus*, the presence of aflatoxin produced by the fungus as a secondary metabolite lowers the value of crops. Aflatoxin is evaluated as a Group I hepatotoxic, carcinogenic and allergenic agent, meaning that sufficient evidence exists to link aflatoxin exposure to cancer in humans (IARC, 2002). Both domestically and globally, aflatoxin can place significant burdens on maize trade. It is regulated in international trade and can result in serious economic losses to maize producers. The total cost of aflatoxin damage in the USA has been calculated to be nearly $500 million annually, due to losses to maize, peanut, and other crop growers as well as animal health effects (Wu, 2015). Also, it has been estimated that in the US, the total annual economic loss due to aflatoxin in maize is about $225 million (Wu, 2006). Moreover, aflatoxin management costs an additional $20-50 million per year (Wu, 2015). Hence, a successful management plan of *A. flavus* infection and aflatoxin accumulation can prevent around $250 million losses to maize industry every year in the United States alone (Mejía-Teniente et al.).

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The search for resistance to *A. flavus* and aflatoxin accumulation has been important for breeders because of the deployment of resistant maize cultivars, may be the best way to contain the infection of *A. flavus* and subsequent accumulation of aflatoxin and this reduce the economic loss for sustainable maize production. Breeding for resistance to *A. flavus* and aflatoxin accumulation at USDA-ARS Corn Host Plant Resistance Research Unit (CHPRRU) initiated during the early 1980s (Scott and Zummo, 1988) has resulted in development of resistant inbred germplasm lines such as: Mp313E, Mp715, Mp718, Mp719 (Scott and Zummo, 1990; Williams and Windham, 2001; Williams and Windham, 2012). However, resistance is a highly quantitative trait, meaning that it is controlled by multiple genes that are affected by the environment in which maize is grown. Identification of individual genes in quantitative trait loci (QTL) associated with *A. flavus* resistance is vital to development of resistant lines via Marker Assisted Selection (MAS) using gene-based markers (Warburton and Williams, 2014).

Elucidation of the role of the phytohormones in defense responses is vital for plant breeders in developing germplasm lines that exhibit high resistance to pathogens. Resistance to potential pathogens depends on the interaction between different plant defense signaling pathways such as those regulated by the phytohormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), and auxin (Kazan and Lyons, 2014). Infection of plants with diverse pathogens results in changes in the level of these phytohormones (Adie et al., 2007; Robert-Seilaniantz et al., 2007). During the last decades, our understanding of the role of the plant hormones in plant-pathogen interaction has greatly improved. The role of different plant hormones in the plant defense response to a biotic stress was summarized (Bari and Jones, 2009; Ljung, 2013).

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Among the plant hormones, SA, JA and ET have been described well for their functions in defense response in various plants (Rahman et al., 2013; Smith et al., 2009; Tang et al., 2015). However, only a limited number of studies about the function of other plant hormones such as auxin which is the main hormone in almost every step of plant development and defense response have been conducted.

Auxin was the first fundamental plant hormone discovered in 1881 by Charles Darwin and named in 1926 by the Dutch botanist Frits Warmolt Went. It plays a critical role in different developmental processes throughout the life cycle of plants such as apical dominance, tropic responses, vascular development, organ patterning, flower development and fruit development (Ghanashyam and Jain, 2009). Additionally, the involvement of auxin in the network of plant-pathogen interaction has been also demonstrated (Bari and Jones, 2009; Ludwig-Muller, 2015). Auxin pathways are regulated to balance between developmental processes and pathogen interactions in plants (Ludwig-Muller, 2015). Auxin regulation is thought to be one of the components participating in the regulation of plant defense responses. Direct and indirect effects of auxin and/or its signaling modulation of plant disease resistance have been demonstrated (Kazan and Manners, 2009). Auxin can indirectly influence the resistance to pathogens by causing cell wall growth and subsequent loosening which result in facilitating pathogen entry (Ding et al., 2008a). The direct effect of auxin can be caused by the crosstalk of auxin with other plant hormones which can result in positive or negative regulation of defense responses (Navarro et al., 2006; Wang et al., 2007b). However, the actual mechanism by which the auxin signaling pathway leads to resistance is not clearly understood.

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To decipher the dynamics of plant-pathogen interactions and understand the role of auxin in maize defense would be valuable. Elucidation of the molecular mechanisms underlying maize - *A. flavus* interactions and the effect of auxin on these interactions may allow the design of strategies to improve resistance. The main goal of this research was to determine whether auxin levels and resistance to *A. flavus*/aflatoxin accumulation are associated. The idea was to determine the auxin levels of resistant lines, Mp313E, Mp715, and Mp719 and susceptible line B73 before and after *A. flavus* infection and correlate them with *A. flavus* biomass and aflatoxin accumulation. As an outcome of this research, we intend to ascertain the function of auxin, which will further lead us to identify auxin related genes which play a role in resistance to *A. flavus*. These genes may be used for single nucleotide polymorphisms (SNP) screening to monitor populations and gene flow, genetic alteration in maize via transformation, and comparative genomics with other grasses. Therefore, if we show the direct effect of auxin in resistance mechanism, it may be possible to use information on auxin levels in different maize genotypes to help in identifying new sources of resistance. Such knowledge will be critical for maize breeders in selecting and utilizing genes for *A. flavus* resistance. The main objectives of this research study were:

1. To determine the direct effect of exogenous auxin on *A. flavus* growth and aflatoxin production.

2. To quantify auxin levels in resistant lines Mp313E, Mp715, and Mp719 and susceptible line B73, before and after *A. flavus* infection.

3. To determine the correlation between auxin levels, *A. flavus* biomass and amount of aflatoxin.

CHAPTER II

LITERATURE REVIEW

Aspergillus flavus

Aspergillus flavus is a pathogenic fungus, first described by Link in early 1800's (Link, 1809). This fungus belongs to an anamorphic genus *Aspergillus* which consists of 250 known species. The genus is composed of several sections. Section *Flavi*, also referred as *Aspergillus flavus* group, includes toxigenic species *A. flavus*, *A. parasiticus*, and *A. nomius* as well as nontoxigenic species *A. oryzae*, *A. sojae*, and *A. tamari* (Varga et al., 2009). Currently, the *A. flavus* group includes 23 species or varieties (Hedayati et al., 2007).

Species of *Aspergillus* have been distinguished by using morphological and physiological characters such as growth rate and pigment production. Metabolite production and/or molecular data are also used to describe characters and taxonomy. Phylogenetic analysis of *Aspergillus* species has been done using DNA sequences from four loci (Peterson, 2008). Restriction fragment length polymorphism (RFLP), single stranded conformation polymorphism of internal transcribed spacer (ITS) regions and partial sequences of the mitochondrial cytochrome b gene have also been used as genetic approaches to distinguish species in *Aspergillus flavus* group (Hedayati et al., 2007; Rodrigues et al., 2007).

Aspergillus flavus is the most common saprophyte and the major economically important aflatotoxin producing opportunistic pathogen on a wide range of agricultural commodities and food matrices, including maize, throughout the world (Leslie and Logrieco, 2014). In general, *A. flavus* is characterized by a velvety, yellow to green or brown mould with a goldish to red-brown reverse on Czapek's agar (Figure 2.1) (Hedayati et al., 2007).
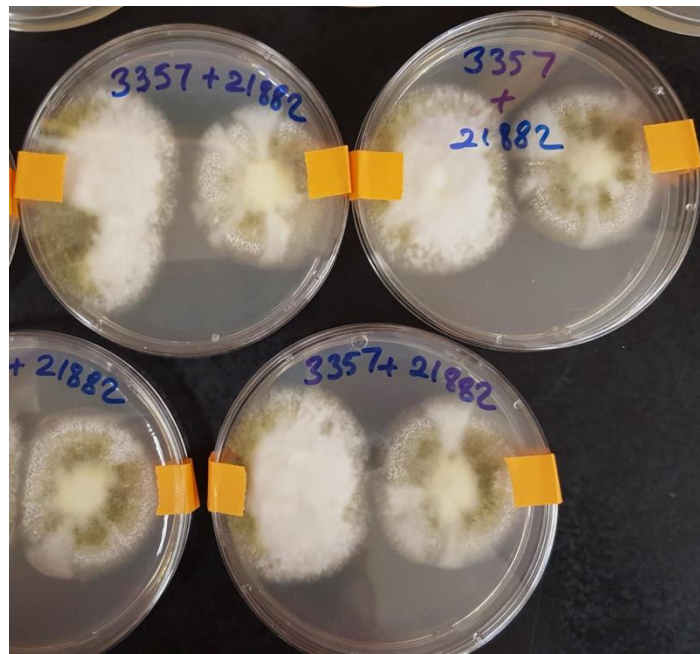
Figure 2.1   Picture of *A. flavus* grown on Czapek's agar
Toxigenic (NRRL 3357) and non-toxigenic (NRRL 21882) strains of *A. flavus*.

A. flavus reproduce asexually forming conidiospores or conidia on specialized structures called conidiophores (Figure 2.2) (Leslie and Logrieco, 2014). A Sexual reproduction is also described from crosses between sexually compatible strains belonging to different vegetative compatibility groups. Recombination during sexual reproduction in *A. flavus* causes the variation in aflatoxin production observed in

populations (Horn et al., 2009). *A. flavus* has two phenotypic isolates (S and L), named based on the size of their sclerotia which are specialized structures that can survive long periods of time, harsh conditions and subsequently produce conidia, as well as hyphae for further colonization. S isolate produces small sclerotia (less than 400 μm in diameter) while L isolate produces large sclerotia (over 400 μm in diameter) (Cotty, 1989). S and L isolates also differ in aflatoxin production. S isolate produces both B and G series aflatoxins whereas L isolate produces only B series aflatoxins (Geiser et al., 2000).
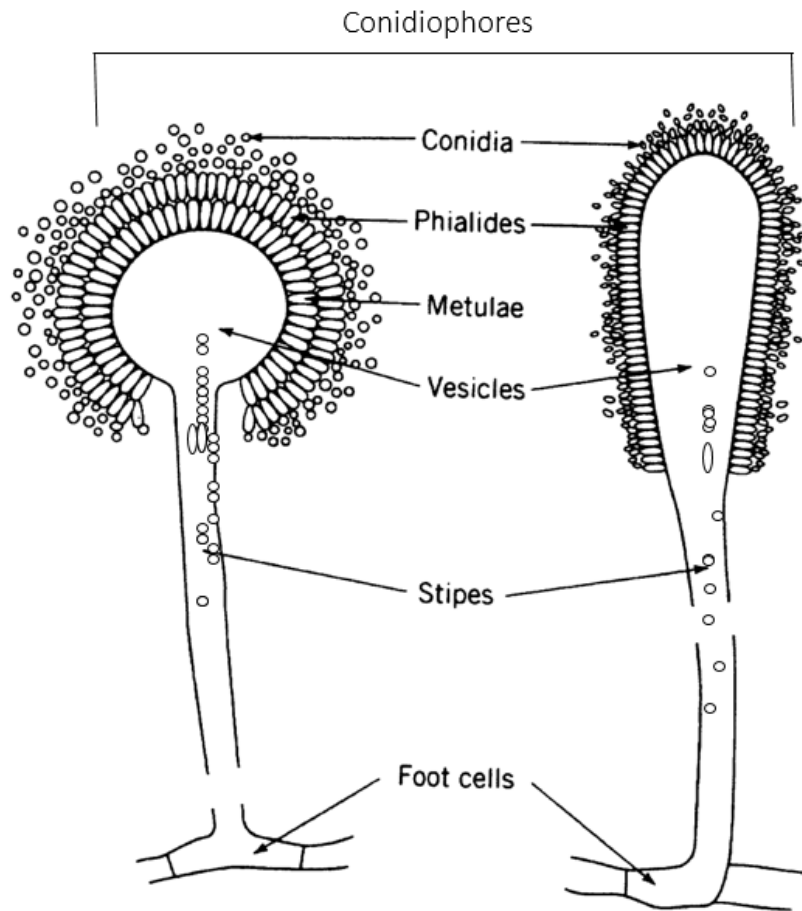
Figure 2.2   Basic morphological structure of conidiophores of *Aspergillus*. The figure was adapted from Klich, (2007).

A. *flavus* has a worldwide distribution because of the formation of numerous airborne conidia, which can be easily dispersed by air and insects. Even though the optimum growth temperature for A. *flavus* is 37°C, it can also grow at temperatures ranging from 12 to 48 $^{\circ}$C. Humidity greatly contributes the growth with water activity (aw) between 0.86 and 0.96 (Vujanovic et al., 2001). A. *flavus* has been found mostly in soil and organic debris associated with plant residues which is the main source for completing its life cycle as well as outdoor and indoor air and water (Hedayati et al., 2007; Klich, 2007). A. *flavus* is capable of surviving and overwintering in plant residues as mycelium (hypha) or sclerotia which can serve as a primary inoculum for infestation of plants parts (Figure 2.3) (Abbas et al., 2009).
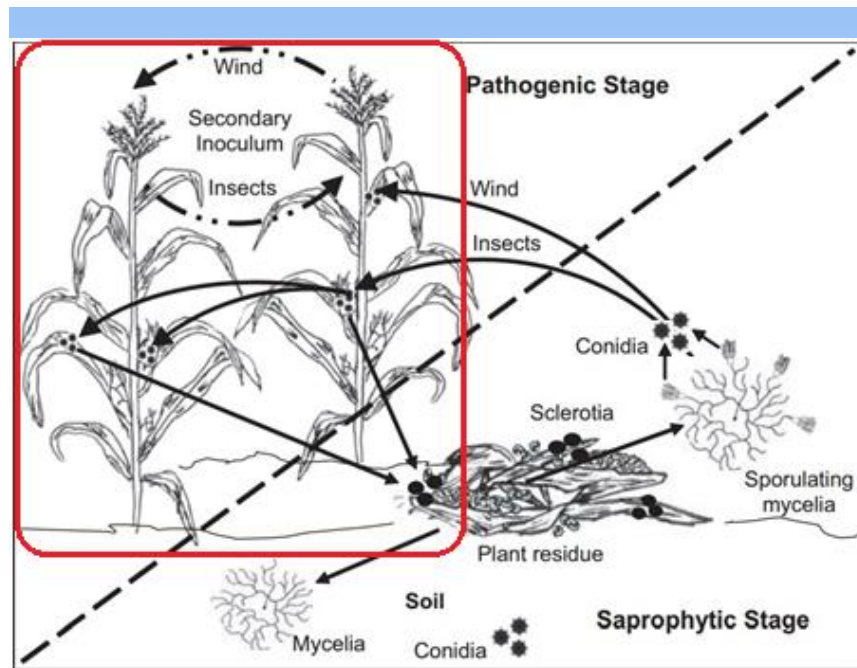
Figure 2.3   Life cycle of A. *flavus* in maize cropping system saprophytic and pathogenic stages of fungal ecology.

Pathogenic stage focused in this study was boxed in red. The figure was adapted from Abbas et al. (2009).

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Aflatoxins

Aflatoxins are highly toxic secondary metabolites produced primarily by *A. flavus* and *A. parasiticus*. The other aflatoxin producing species of *Aspergillus* genus includes *A. arachidicola*, *A. bombycis*, *A. minisclerotigenes*, *A. nominus*, *A. parvisclerotigenus*, and *A. pseudotamarii* (Leslie and Logrieco, 2014).

Aflatoxins were first discovered in 1960 after a disease outbreak caused high mortality among young turkeys, which was termed **"turkey X disease"**. It was soon found that the compound present in the feed was toxic to poultry as well as ducklings and produced by a fungus. This was followed by the identification of the toxin-producing fungus *Aspergillus flavus* (1961) and the toxin was given the name "Aflatoxin" by virtue of its origin (*Aflavus*) (Blount, 1961; Nesbitt et al., 1962).

There are major four aflatoxins named B_1, B_2, G_1, and G_2 based on the color of their fluorescence under the ultraviolet light (B = blue, G = Green) and the numbers indicating their relative migration distance on a thin-layer chromatographic plate (Figure 2.4) (Edite Bezerra da Rocha et al., 2014). Two other aflatoxins, M1 and M2, are metabolites of aflatoxin B1 and B2, respectively, that are present in milk and milk products from animals consuming feed contaminated with aflatoxins (IARC, 2012). Aflatoxin production is greatly affected by several factors such as temperature, pH, water activity, carbon and nitrogen sources, bioreactive agents, oxidative stress and plant metabolites (Yu, 2012).
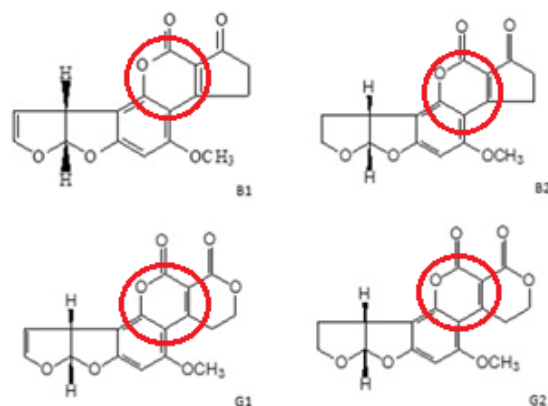
Figure 2.4   Chemical structures of aflatoxins B$_1$, B$_2$, G$_1$ and G$_2$.

Reactive lactame rings were circled in red. The figure was adapted from Edite Bezerra da Rocha et al. (2014).

As many as 30 genes are potentially involved in aflatoxin biosynthesis and clustered within a 75-kb region of the fungal genome on chromosome III roughly 80 kb away from telomere (Figure 2.5). Aflatoxin biosynthesis is controlled transcriptionally and post transcriptionally. Mainly two genes, aflR and aflS modulate transcription of aflatoxin. These positive-acting regulatory genes are located in the middle of the gene cluster adjacent to each other. Additional genes, which are not physically related, laeA and veA, have been shown to exhibit a regulatory role on aflatoxin biosynthesis. External control of the aflatoxin cluster and global regulation of secondary metabolism includes G-protein signaling, cAMP signaling, Ras family GTPase signaling, and Chromosomal remodeling and silencing (Baidya et al., 2014; Conradt et al., 2015; Georgianna and Payne, 2009; Yu, 2012).
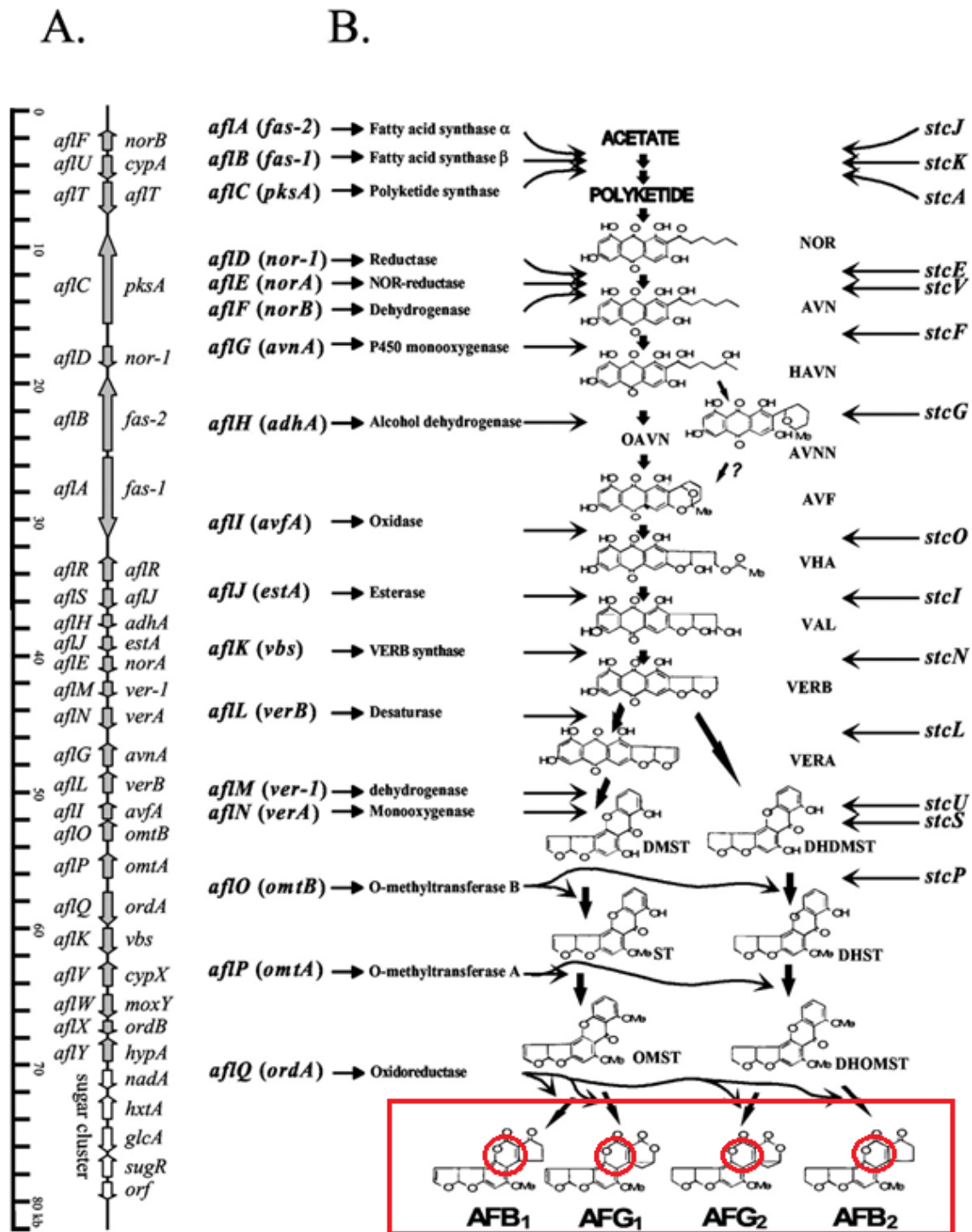
Figure 2.5   Clustered genes (A) and the aflatoxin biosynthetic pathway (B)

Lactone rings of aflatoxins were circled in red. The figure was adapted from Yu et al. (2004).

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Aflatoxins are extremely toxic and carcinogenic to humans, non-ruminant, and ruminant animals. Aflatoxin B1 (AFB1) is the most potent hepatocarcinogenic substance and extensively linked to human primary liver cancer in which it acts synergistically with Hepatitis B virus (HBV) (IARC, 2012). Also, AFB1 has been found to be contributing agents for Reye's syndrome, which is characterized by encephalopathy and visceral deterioration, and results in liver and kidney enlargement and cerebral edema, and Kwashiorkor syndrome, a disease usually considered a form of protein energy malnutrition (Blunden et al., 1991), with reduced levels of secretory immunoglobulin A in children (Turner et al., 2003). Negative effects of AFB1 on non-ruminants such as chickens, pigs, horses, cats, dogs, mice and rat have been intensively studied. Ruminants such as cattle, sheep, goats, and deer have, generally, been more resistant to the adverse effects of AFB1 because of the rumen microbiota, which degrade aflatoxins. However, in dairy cattle, another problem arises from the transformation of AFB1 and AFB2 into hydroxylated metabolites, aflatoxin M1 and M2 (Zain, 2011).

Worldwide, the presence of the codon 249 mutation in hepatocellular carcinoma (HCC) correlated with high risk of exposure to AFB1 and HBV (Bressac et al., 1991). AFB1 induces the transversion of G-->T in codon 249 of the p53 tumor suppressor gene, resulting in the insertion of serine at position 249 in human hepatocytes (Aguilar et al., 1993; Qi et al., 2015). Mode of action is that: AFB1 is biotransformed into the highly reactive AFB1-8,9-*exo*-epoxide, which binds preferentially to the N7 position of guanine residues in DNA, forming the AFB1-N7-Gua adduct, which can hydrolyze spontaneously to a formamidopyrimidine adduct. Both of these AFB1-adducts can cause mutations that may lead to liver or lung cancer (Mulder et al., 2015).

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Because of the serious effects of aflatoxins on humans and animals, many countries have established a maximum tolerable level (MTL) on aflatoxins in food and feedstuff. Worldwide, MTL for AFB1 in food is 1-20 µg/kg (ppb) where as MTL for the total of the aflatoxins is 0-35 µg/kg (ppb) (Van Egmond and Jonker, 2004). The US Food and Drug Administration (FDA) has established limits of total aflatoxins at 20 µg/kg (20 ppb) in interstate commerce and imports of food and feed, and 0.5 µg/kg (0.5 ppb) of aflatoxin M1 in the sale of milk (Table 2.1) (Brown and Bhatnagar, 2015). However, these regulations put a large burden on food economy. It has been calculated that the total costs of aflatoxin management in the USA to be nearly $500 million annually, due to losses to maize, peanut, and other crop growers as well as through adverse animal health effects (Wu, 2015).

There are several conventional analytical methods such as thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) to quantify aflatoxins in grain and oil seeds. These methods yield results in hours or days (Zheng et al., 2006). Immunological methods including enzyme linked immuno-sorbent assay (ELISA), flow-through assay, lateral flow test or strip test, fluorometric assay, fluorescence polarization immunoassay (FP) are rapid and reliable for aflatoxin determination in the field (Lee et al., 2015; Zheng et al., 2006). Also, a variety of emerging methods have been developed based on novel technologies such as near-infrared reflectance (NIR), Fourier transform near-infrared reflectance (FT-NIR), Fourier FDA has established the following action levels for aflatoxins present in human food, animal feed and animal feed ingredients transform infrared spectroscopy (FTIR), and Raman spectroscopy (Lee et al., 2015; Pascale, 2009).

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Table 2.1   FDA Action Levels for Aflatoxin in Human Food, Animal Feed and
Animal Feed Ingredients

Intended Use	Grain, Grain By-Product, Feed or other Products	Aflatoxin Level [parts per billion (ppb)]
Human consumption	Milk	0.5 (aflatoxin M1)
Human consumption	Foods, peanuts and peanut products, brazil and pistachio nuts	20
Immature animals	Corn, peanut products, and other animal feeds and ingredients, excluding cottonseed meal	20
Dairy animals, animals not listed above, or unknown use	Corn, peanut products, cottonseed, and other animal feeds and ingredients	20
Breeding cattle, breeding swine and mature poultry	Corn and peanut products	100
Finishing swine 100 pounds or greater in weight	Corn and peanut products	200
Finishing (i.e., feedlot) beef cattle	Corn and peanut products	300
Beef, cattle, swine or poultry, regardless of age or breeding status	Cottonseed meal	300

Adapted from FDARegulatoryGuidanceforMycotoxins8-2011.pdf

Maize (*Zea mays* L)

Maize or corn (*Zea mays* L) is an annual plant belonging to the family of grasses (*Poaceae*). The number of chromosomes in *Zea mays* is 2 n = 20 with the genome size of approximately 2,500 Mbp (Martienssen et al., 2004). The maize genome is the product of a segmental allotetraploid event which, occurred approximately between 16.5 million years ago (mya) and 11.4 mya. Its two diploid progenitors diverged 20.5 mya. One of the two ancestral diploids shares a more recent common ancestor with sorghum than it does with the other ancestral diploid (Gaut and Doebley, 1997). After the allopolyploid event, the maize genome rearranged and diploidized rapidly before 11.4 mya (Gaut et al., 2000). The maize genome has doubled in size in the last 5–6 million years with the proliferation of repetitive DNA (transposable elements). It contains from 60% to 80% transposable elements (SanMiguel et al., 1998).

Maize is a diclinous monoecious monocot. It produces large, narrow, opposite leaves, borne alternatively along the length of stem. Ears develop above a few of the leaves in the midsection of the plant, between the stem and leaf sheath. There are two main stages, vegetative and reproductive, that last about 125 days in maize life cycle. Vegetative stages include seedling/sprouting stage, grand growth stage, and tasseling/flower initiation stage. Reproductive stages include silking stage, soft-dough/milky stage and hard- dough/maturity stage. Maize is essentially cross-pollinated crop species. Pollen of a given plant rarely fertilizes the silks of the same plant. However, self-pollination can be forced (Bennetzen and Hake, 2009; Strable and Scanlon, 2009).

Genetic and archeological studies about the origin of maize have demonstrated that maize (Zea mays ssp. mays) has been domesticated from a wild indigenous grass

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called teosinte (Zea mays ssp. parviglumis) about 9000 years ago in the tropical Balsas river valley in Mexico (Hake and Ross-Ibarra, 2015). Domestication of maize from teosinte improved its usefulness as food source from an inedible grass into edible crop while kept the wind-born pollen (Doebley, 2004). This process involved selective cultivation of plants in favor of larger ears with more kernel rows. Remarkable changes from teosinte to corn can be seen in Figure 2.6 (Hake and Ross-Ibarra, 2015). Visible differences are in female inflorescence on the left and tassels on the right (A-teosinte and B-maize), in kernel size (C-left-teosinte and right-maize), and ear size (D-left-teosinte, middle-F1, right-maize). Today, in modern maize cultivars, other targets of selection include kernel size, kernel composition, palatability, rapid germination, stalk strength, leaf size, high seed yield, better nutrient content and more. After the domestication, maize has been cultivated in a broadest range from the south of Chile 40°S to Canada 50°N. It spread to Europe and other parts of the world around the 15th century by the travelers (Tenaillon and Charcosset, 2011).

The high adaptability of maize to very different environments is the result of its extraordinary diversity (Tenaillon and Charcosset, 2011). This diversity has a great potential for interdisciplinary fields such as genetic, cytogenetic, genomic research and agriculture and has placed the maize in the center as a model organism since early 1900's. Many scientific discoveries have first been shown in maize, such as transposable elements (McClintock, 1950), the correlation between cytological and genetic crossing over (Creighton and McClintock, 1931), and the discovery of maize heterosis (Crow, 1998; Shull, 1908). Because of its exceptional characteristics maize serves as both a commodity and a leading model for basic research (Lawrence et al., 2008).

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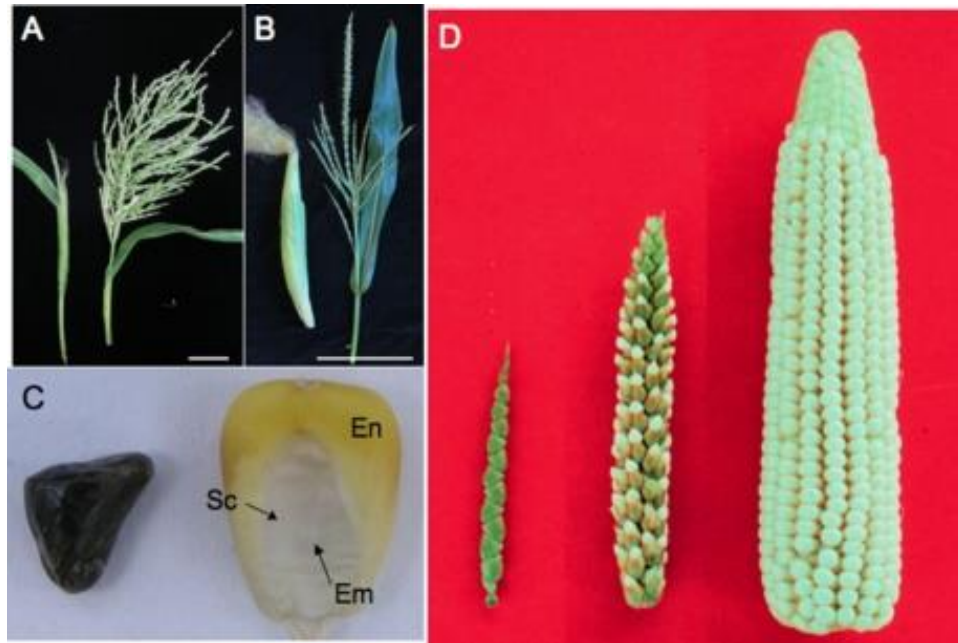
Figure 2.6   Comparison of teosinte to maize.

Endosperm (En), embryo (Em), and scutellum (Sc). Images: (D) was from John Doebley, Department of Genetics, University of Wisconsin– Madison; all other images were from, Sarah Hake. The figure was adapted from Hake and Ross-Ibarra, (2015).

The United States is, by far, the largest producer of maize in the world, producing 40% of the world's maize crop. Other top producing countries include China, Brazil, Mexico, Indonesia, India, France and Argentina (NCGA, 2015). In the United States, maize is the number one crop in terms of harvested acres (83.1 million acres in 2014) and selected crop value ($51.89 billion in 2014) (NCGA, 2015). In the US, 43% of maize production has been used as livestock feed. It is followed by ethanol production (30%), export (15%), starch production (8%), and human consumption (3%) (USDA, 2015).

Aspergillus flavus and Maize Infections

Since maize has a great economic value in agriculture, several ubiquitous fungal pathogens can cause significant losses worldwide during the maize growing season.

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Pathogens account for at least 31 % of the world's maize losses (Lange et al., 2014; Oerke, 2006). Maize can be infected by the pathogen *A. flavus* which produces the toxin, aflatoxin. While Infection of maize by *A. flavus* causes ear and kernel rots which lower the grain yield and quality, aflatoxin contamination affects the feed value and marketability of the grain (Leslie and Logrieco, 2014). Ear rot is more problematic in the area with high temperatures, humidity, and droughty environments (Marin et al., 1998). However, aflatoxin is a chronic problem (Marsh and Payne, 1984; Payne, 1998).

A. flavus can colonize on maize at the early stage of silking. Colonization starts with the landing of spores on silk or kernel by wind and/or insects. Over wintered fungal spores (conidia), mycelium (hypha) or sclerotia in the air, soil and plant debris such as old cobs, kernels, or other plant tissues can serve as a primary source for infestation (Figure 2.3) (Abbas et al., 2009; Olanya et al., 1997; Wilson and Payne, 1994). However, infection rate is highly affected by the physical characters such as age of the silk and plant, the morphological characters such as tight, adherent husk, less open apical parts of the ear, and thicker pericarp as well as environmental conditions such as temperature, relative humidity, and moisture (Leslie and Logrieco, 2014). After colonization, *A. flavus* grow down into the kernel region rapidly if the conditions are favorable (Marsh and Payne, 1984). The mode of entry into kernel has been clearly demonstrated that fungus enters the unwounded kernels from the rachis and also from the bracts. It grows through the aerenchmya in the rachillae to the floral axis and into the pericarp. It cannot penetrate to the endocarp from the exterior of the pericarp (Smart and Caldwell, 1990). However, wounded pericarp can provide an easy entrance for the fungus (Payne, 1998; Wilson and Payne, 1994). Physical wounding of ears and kernels may be caused by insects. This can

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provide additional entry sites for the fungus and play an important role in aflatoxin contamination (Williams et al., 2002a; Williams et al., 2005; Windham et al., 1999). Internal infection of *A. flavus* may lead to aflatoxin production, alternatively, infection can remain silent or extensive growth of fungus may be observed without aflatoxin production depending on environmental conditions (Wilson and Payne, 1994).

Aflatoxins have been held responsible in poisoning (aflatoxicosis) outbreaks that killed hundreds of people in developing countries especially in Asia and Africa, and it has been thought that many aflatoxin-related losses go unreported (Schmidt, 2013). One of the largest and most severe aflatoxicosis outbreaks occurred in Kenya in 2004, resulting in 317 cases and 125 deaths. The source of outbreak was aflatoxin contaminated homegrown maize with the higher level of aflatoxin more than the regulatory limit of 20 ppb (Lewis et al., 2005). In 1981, 12 out of 20 people hospitalized died from eating maize which contained as much as 12,000 ppb of aflatoxin B_1 (Ngindu et al., 1982). Also, in 2010, another outbreak occurred in Kenya which resulted in destroying 2.3 million bags (estimated at \$69 million) of contaminated maize (Okoth et al., 2012). In Europe, in 2003 and periodically in the following years, a hot and drying season caused severe *A. flavus* infection of maize in Northern Italy (Perrone et al., 2014). In 2013, aflatoxin contamination of milk for human consumption has been reported in several European countries, including Romania, Serbia, and Croatia. However, in the United States and other developed countries, the risk of toxicity from aflatoxins is very low because of close monitoring and tight regulations of aflatoxins (Schmidt, 2013). Contamination of maize with aflatoxins is a sporadic problem in Midwest states such as Iowa, Illinois, and Indiana while it is a chronic problem in Southeast states such as Mississippi, Louisiana,

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and Texas. Severe losses from aflatoxins occurred in 1983 and 1988 in Midwest and in 1998 in the southern states (Robens and Cardwell, 2003; Windham and Williams, 2002). Resulting cost of contamination in the southern states ranged from $85 million to $100 million (Windham and Williams, 2002). In a very conservative calculation, the total cost of aflatoxins in maize industry in the US has been estimated between $500 million and $1.5 billion a year (Robens and Cardwell, 2003).

Crop Management Practices to Minimize *A. flavus* Infection and Aflatoxin Contamination

Managing *A. flavus* infection and aflatoxin accumulation requires a wide-ranging approach that begins before planting and continues through the final utilization of the maize or maize products (Leslie and Logrieco, 2014). Management practices to prevent aflatoxin contamination can be divided into 3 stages: preharvest, harvest and postharvest.

Preharvest strategies including field selection, hybrid selection, crop rotation, tillage, planting date and density, fertilization, irrigation, insect and weed management, and biological control greatly influence aflatoxin accumulation in maize (Jones and Duncan, 1981; Jones et al., 1981; Payne et al., 1986). The most important preharvest practice is insect management for reducing aflatoxin risk in maize (Leslie and Logrieco, 2014). Insecs act as vectors for fungal spores and they cause kernel damage which can create infection sites for fungi (Williams et al., 2002b; Williams et al., 2005). Even though insecticides can greatly reduce the risk of aflatoxin contamination associated with insects, a more effective way is to use transgenic maize hybrids containing insect resistance genes, known as Bt genes (Williams et al., 2005).

Biological control strategy is one of the most promising preharvest practices that utilize the active ingredients such as bacteria, yeast and fungus. Large quantitaties of atoxigenic strain of *A. flavus* is applied to maize to compete with toxigenic strain to limit preharvest aflatoxin contamination (Abbas et al., 2011). Several atoxigenic strains of *A. flavus* have been proven to be most effective to prevent contamination in different agro-environmental conditions (Accinelli et al., 2014). NRRL 21882 from Afla-Guard, AF36, and Aflasafe^{TM} are commercially available biopesticides. Also, recently, two more atoxigenic strains, CT3 and K49, have been evaluated and patented as biocontrol agents (Abbas et al., 2011).

Harvest date and handling during harvest can have a major impact on the aflatoxin level in maize. Earlier planting and harvest before maize reaches the industry level of 15.5% moisture, and/or drying to 15.5% or less immediately or within 24 hours can reduce aflatoxin contamination significantly (Larson, 1997; Leslie and Logrieco, 2014).

Postharvest practices which mostly improve storage condition include sanitation, loading, aeration and monitoring (Maier et al., 1997). Preventing kernel damage during harvesting, drying, loading, and transporting can also prevent *A. flavus* infection by minimizing the easy entry of the fungus (Maier, 2004).

If aflatoxin contamination is not avoidable, several physical and chemical methods can be used for decontamination in maize. Physical methods for removal of contaminated material are sorting, floating and density segregation, dehulling, and irradiation (Afolabi et al., 2006; Bennett and Anderson, 1978; Herzallah et al., 2008; Huff, 1980; Siwela et al., 2005). Sorting has reduced aflatoxin levels by 81% from an

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initial average of 53 ppb in Kansas maize (Pearson et al., 2004). Floating and density segregation has removed contaminated maize kernels resulting in 87% aflotoxin reduction (Huff, 1980). Similarly, physical dehulling and irradiation reduced aflatoxin contamination 92% and 40-100%, respectively (Herzallah et al., 2008; Siwela et al., 2005). Chemical methods, which effectively detoxify aflatoxin in maize, include ammoniation, ozonation, nixtamalization, by using sodium bisulfate and citric acid (Doyle et al., 1982; Hoogenboom et al., 2001; McKenzie et al., 1998; McKenzie et al., 1997; Méndez-Albores et al., 2005; Price and Jorgensen, 1985). These chemical methods can remove or degrade aflatoxin from 90% to 100%. However, decontaminated or detoxified crops usually are considered low quality and sell for less than uncontaminated, untreated material and they also add extra costs for maize production (Leslie and Logrieco, 2014).

Breeding Maize for Resistance to *A. flavus* and Aflatoxin Contamination

Even though management strategies may reduce aflatoxin contamination of maize, the best strategy for elimination of aflatoxin is to develop preharvest host resistance to aflatoxin accumulation (Brown et al., 1999). Breeding for resistance to *A. flavus* and aflatoxin accumulation at USDA-ARS Corn Host Plant Resistance Research Unit (CHPRRU) was initiated during the early 1980s (Scott and Zummo, 1988). Breeding programs mainly focused on several areas: identify, develop, and release maize germplasm with resistance to *A. flavus* infection and aflatoxin accumulation (Williams, 2006). Identification of resistant sources requires reliable and efficient screening techniques. Silk inoculation technique, kernel screening assay (KSA), infesting maize ears with insect larvae infected with *A. flavus* conidia, pin-bar and side-needle inoculation

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techniques have been developed and are regularly used in breeding programs (Brown et al., 1999; Wilson and Payne, 1994; Windham et al., 2003; Zummon and Scott, 1989). Also, development of new technologies in high-through aflatoxin analysis such as high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS), enzyme-linked immunoabsorbent assay (ELISA), fluorometric assays with immunoaffinity column clean-up, biosensors, near-infrared reflectance (NIR)/fourier transform near-infrared reflectance (FT-NIR), fourier transform infrared spectroscopy (FTIR), and Raman spectroscopy has allowed fast screening for resistant genotypes.

The first resistant maize germplasm lines released by CHPRRU were Mp313E and Mp420 (Scott and Zummo, 1990). Other sources of resistant germplasm are Mp715, Mp717, Tex 6, Mo18W, LB31, CI2, and MI82, GT-MAS:gk population, TZAR 101–106, GT-603, Mp718 and Mp719 (Guo et al., 2011; Guo et al., 2001; Maupin et al., 2003; Menkir et al., 2008; Williams and Windham, 2006; Williams and Windham, 2001; Williams and Windham, 2012). However, these resistant germplasms are either unsuitable or are unacceptable agronomically and cannot be used immediately in commercial hybrids. A desirable trait of the resistant germplasm is the ability to combine aflatoxin resistance with superior agronomic quality (Williams, 2006). On the other hand, aflatoxin resistance is a quantitative trait which is encoded by two or more genes and has extremely variable heritability which is highly affected by genotypes, location, and years (Leslie and Logrieco, 2014). Even though conventional breeding based on phenotype can be used to develop recombinant inbred lines with aflatoxin resistance, no commercial lines so far have been marketed (Brown et al., 2013b; Williams, 2006).

Progresses in genomics, proteomics, metabolomics, marker development, genetic engineering technology, and RNAi technology can greatly facilitate developing host-resistance against *A. flavus* infection and aflatoxin contamination (Bhatnagar-Mathur et al., 2015) . Also, identifying quantitative trait loci (QTL) associated with aflatoxin resistance and practicing marker-assisted selection (MAS) provide an alternate approach to the phenotype based selection (Williams, 2006).

Aflatoxin resistance candidate gene identification

Since resistance to aflatoxin accumulation in maize is a complex, highly quantitative trait with the interaction of multiple genes, knowledge of these genes, their function and expression under various environmental conditions can enhance the breeding strategies (Williams et al., 2015). Candidate genes that may play an important role in resistance to *A. flavus* infection and aflatoxin contamination from kernel, rachis, and silk tissues are selected based on biochemical, molecular and genetics studies (Zhu and Zhao, 2007). They can be used to determine QTLs or as molecular markers for implementing MAS on a broad scale for resistance to aflatoxin accumulation in maize breeding programs (Chen et al., 2014; Zhu and Zhao, 2007).

Candidate gene identification at transcript levels

Several research studies have been performed to identify candidate genes through analysis of gene expression at transcript levels. A comparison study of resistant (Mp313E) and susceptible (Va35) maize inbred lines after *A. flavus* infection has identified 236 genes as significant, and 67 of these could be directly mapped to the maize chromosomes in known QTL associated with resistance (Kelley et al., 2009). A

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comparative analysis of the growth performance of *Aspergillus flavus* revealed differences in fungus growth, aflatoxin biosynthetic pathway, and host gene expression on Mp313E and Va35 during infection and also several potential candidate genes such as IAA13 auxin-responsive gene, indole-3-glycerol phosphate lyase, and a predicted transposon were identified (Ankala et al., 2011).

Similarly, a combination of microarray analysis, qRT-PCR analysis, and QTL mapping methods has identified maize genes associated with resistance or susceptibility to *A. flavus* infection. In this study, a gene encoding glycine-rich RNA binding protein which involved in post-transcriptional gene expression processes was found to be associated with the host hypersensitivity and susceptibility in Va35. Other up regulated genes in susceptible line encodes a stress-induced small heat shock protein, a ribosomal protein, a cinnamoyl-CoA reductase (CNCR2) that catalyses the lignin pathway, a phytochrome A, and a nuclease-phosphatase domain superfamily protein. Sixteen genes were found highly expressed in kernels of resistant maize inbred line Mp313E, including a NUP85-like gene that functions for transport of RNA and other macromolecules from nucleus to cytoplasm, a heat shock protein HSP101 that functions in protein folding, a metallothionein like protein that is involved in the binding and detoxification of heavy metal ions, a lecithin cholesterol acyltransferase (LCAT)-like gene, an ethylene responsive protein, a HSP26, and a prenylated rab acceptor (PRA1) family protein (Kelley et al., 2012). Likewise, seven chitinase genes were characterized that had alleles associated with increased resistance to aflatoxin accumulation and *A. flavus* infection in field grown maize using sequence diversity and expression patterns (Hawkins et al., 2015; Mylroie, 2011).

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Histological methods were also used to follow transcriptional changes of two maize defence-related genes. RNA *in situ* hybridization showed that transcripts of the maize pathogenesis-related protein, maize seed (PRms) gene, and the maize sucrose synthase-encoding gene, shrunken-1(Sh1), were observed in the embryo of non-infected kernels, but were induced on infection by *A. flavus* in the aleurone and scutellum (Shu et al., 2015).

The potential roles of six WRKY transcription factor genes in response to *A. flavus* inoculation in B73 (susceptible) and TZAR101 (resistant) were examined. It was demonstrated that several WRKY transcription factors exhibited differential expressions between resistant and susceptible maize lines in response to *A. flavus* inoculation (Luo et al., 2011). In a similar study, it was shown that WRKY transcription factors (*Zm*WRKY19, -53 (two isoforms), and -67) may play a role in defense responses to *A. flavus* infection and suppress aflatoxin production through reducing oxidative stress in the resistant line with the coordination of salicylic acid and ethylene (Fountain et al., 2015b).

In another custom designed DNA multi-species (*A. flavus*-maize) microarray study for simultaneous detection of disease-associated transcript in the plant-pathogen interaction, more than 4000 maize genes were found differentially expressed after *A. flavus* infection. They observed notable changes in the transcription of genes associated with carbohydrate utilization and defense signaling pathways involving salicylic acid (SA), jasmonic acid (JA), and ethylene. Defense related genes, signaling pathways, and genes encoding maize hydrolytic enzymes, which involved in the degradation of host reserves, were up regulated whereas starch biosynthetic genes were down regulated

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during infection. Their findings suggested that *A. flavus* infection induced defense response as well as a disruption in kernel development (Dolezal et al., 2014).

Asters et al. (2014) developed an analysis method to demonstrate the significance and relations among maize candidate genes based on the empirical gene expression data obtained by RT-qPCR technique from maize inbred lines using diverse programs to provide statistical analysis and data visualization. They also applied network-based methods to describe empirical gene expression data to determine genes that are potentially important in the regulation of maize-*A. flavus* defense responses. This method can be used on prioritizing candidate genes. RT-qPCR gene expression analysis indicated that genes in RNA transport pathways, especially in nuclear pore complexes (NPC) and survival motor neuron (SMN) complexes, were highly upregulated and involved in maize resistance.

Identifying aflatoxin resistance associated proteins

Identification and characterization of biochemical activities of resistance associated proteins (RAPs) has been performed in various crops in early studies (Brown et al., 2013a). In one of the earliest studies, SDS-PAGE and western blot analysis reveal that germinating kernels contained zeamatin which increase the permeability of fungus cell wall and ribosome-inactivating proteins (RIP) which inactivate foreign ribosome. Purified RIP and zeamatin exhibited antifungal activity against *A. flavus* hyphal growth (Guo et al., 1997).

Similarly, a 14-kDa trypsin inhibitor (TI) protein which showed high expression levels in seven resistant maize kernels was found to be associated with resistance to *A. flavus* (Chen et al., 1998). Investigation of the involvement of TI in aflatoxin resistance

through RNAi gene silencing also demonstrate that TI reduced transgenic kernels were more susceptible to *A. flavus* colonization and aflatoxin production (Chen et al., 2015).

A 36-kDa α-amylase inhibitor protein from Lablab purpurea (AILP) was identified after screening protein extracts from 200 different plant species. Fungus α-amylase converts kernel starch into simple sugars, which is crucially important to both infection of maize host tissue and the production of aflatoxin. AILPs are lectins that have chitin binding ability and inhibit the growth of *A. flavus* independently of their α-amylase inhibiting activity (Fakhoury and Woloshuk, 2001).

Constitutive and inducible proteins have been identified and appeared to be necessary for kernel resistance in resistant maize genotypes. Among these, as a first layer of defense, constitutively expressed 58- and 46-kDa proteins were identified as globulin-1 (GLB-1) and globulin-2 (GLB-2), respectively. The 22-kDa zeamatin was found to be an induced and specific antifungal protein (Chen et al., 2001).

In a different study, catalase activity was determined in immature and mature embryos, pericarp, and rachis tissues of maize lines that are resistant and susceptible to *A. flavus* infection. Catalase plays a key role in maintaining H_2O_2 homeostasis in cells and has been implicated in ROS signaling in response to pathogen attack. Catalase activity was found significantly higher in the embryos of resistant lines compared to susceptible ones. Sequence analysis of catalase 3 from resistant (Mp313E) and susceptible (SC212m) lines reveal a 20-aa deletion in the resistant line that might affect enzymatic activity (Magbanua et al., 2007).

The maize lipoxygenase (LOX) gene ZmLOX3 involved in catalysis of the oxidation of polyunsaturated fatty acids has been inactivated to study its function in *A.*

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flavus resistance. Kernels with the mutated gene exhibited high susceptibility to *A. flavus* infection, implicating that LOX actively functions in resistance to *A. flavus* (Gao et al., 2009; Gao et al., 2007).

A proteomics approach has been used, for the first time, to identify variations in maize kernel embryo protein expression between aflatoxin resistant and aflatoxin susceptible genotypes (Chen et al., 2002). Several potential markers were identified in resistant maize lines including storage proteins such as GLB-1, GLB-2, late embryogenesis abundant proteins (LEA3), stress-responsive proteins such as a water stress-inducible protein (WSI18), aldose reductase (ALD), and heat shock proteins (HSP). Same proteins were also identified as response to water or drought stress, suggesting that aflatoxin resistant maize lines may also include a high level of expression of stress related proteins (Chen et al., 2002). One of the protein spots #1175 from resistant genotype from the previous proteomics study was sequenced and identified as glyoxalase I (GLX-I) which functions in the conversion of cytotoxic methylglyoxal (MG) (induces aflatoxin production) into D-lactate. Higher GLX-I activities observed in resistant lines with or without *A. flavus* infection suggested an important role for GLX-I in resistance through the control of MG levels and, therefore, aflatoxin induction in *A. flavus* infected kernels (Chen et al., 2004). Another protein (#717) from the same study, which was expressed fivefold higher in three resistant lines compared with three susceptible ones, was identified as a pathogenesis-related protein (PR-10). Ribonucleolytic and antifungal activities of PR-10 suggested that PR-10 plays a role in kernel resistance by inhibiting fungal growth of *A. flavus* (Chen et al., 2006). RNA interference (RNAi) gene silencing technology was employed to examine effect of PR-10

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on *A. flavus* colonization and aflatoxin production. In RNAi-silenced transgenic kernels, fungal colonization and aflatoxin accumulation were significantly increased, and these kernels showed a significant reduction in PR10 protein level, suggesting a direct role for PR10 in maize aflatoxin resistance (Chen et al., 2010).

A novel antifungal β-1,3-glucanase, fungal cell wall degradative enzyme, was identified in five resistant lines developed from crosses between five African maize inbreds and five temperate aflatoxin-resistant lines by using a proteomics approach (Chen et al., 2012). An increased β-l,3-glucanase activity in maize kernels was also correlated with lower *A. flavus* infection observed in the resistant genotype (Tex 6) compared with a susceptible one (B73). These studies indicate that β-l,3-glucanase activity may have a role in the inhibition of the growth of *A. flavus* (Lozovaya et al., 1998).

In another proteomics study, maize endosperm proteins including GLB-2, LEA3 and LEA14, a stress-related peroxiredoxin antioxidant (PER1), heat-shock proteins (HSP17.2), a cold-regulated protein (COR), and an antifungal trypsin-inhibitor protein (TI) were found to be up regulated twofold or higher in resistant lines compared with susceptible ones. Significant induction of PER1 upon *A. flavus* infection and its peroxidase activity suggested that it may play an important role in enhancing kernel stress tolerance and aflatoxin resistance (Chen et al., 2007b).

Proteins that could be correlated with the resistance to *A. flavus* infection and aflatoxin accumulation have also been investigated in maize rachis and silk tissues (Pechanova et al., 2011; Peethambaran et al., 2010). Protein profile differences between resistant and susceptible maize lines in rachis tissues with or without *A. flavus* infection indicated that resistant rachis contains higher levels of abiotic stress-related proteins such

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as superoxide dismutase, peroxidases, and chaperonins and proteins from phenylpropanoid metabolism, whereas susceptible rachis contains biotic stress inducible pathogenesis-related proteins. This study also demonstrated that resistant rachis relies on constitutive defenses, while susceptible rachis is more dependent on inducible defense (Pechanova et al., 2011). A Comparative proteomics study identified several proteins that were differentially expressed in the silk tissue of resistant and susceptible maize lines including three chitinases. Chitinase activity in resistant silk has been found to be significantly higher than that from susceptible inbreds. Therefore, it has suggested that chitinases may inhibit *A. flavus* growth in the silk and contribute to the resistant phenotype (Peethambaran et al., 2010).

Most of the candidate genes and proteins examined in above studies are commonly expressed in both aflatoxin and abiotic stress resistance. Therefore, selecting candidate genes targeting both aflatoxin resistance and abiotic stress may greatly enhance the breeding efforts for resistance (Fountain et al., 2015a).

Molecular markers and QTL mapping

As previously stated, highly expressed genes and proteins could be potentially utilized as molecular markers for use in Marker Assisted Selection (MAS) in breeding applications. Even though conventional breeding strategies based on phenotypic selection can efficiently increase aflatoxin resistance, a high level of genetic resistance is currently not available in maize (Williams and Windham, 2015). MAS based on genotype rather than phenotype can provide some advantages such as analyzing plants at the seedling stage, screening multiple characters, minimizing linkage drag, and rapidly recovering a recurrent parent's genotype (Ortega and Lopez-Vizcon, 2012). MAS can be used as an

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alternative approach to enhance aflatoxin resistance. The quantitative nature of the aflatoxin resistance and environmental factors that strongly influence the trait limits the success of transferring resistance to commercial hybrids (Warburton et al., 2011; Williams and Windham, 2015). Also, assessing aflatoxin accumulation and test sites with consistently high disease pressure requires expensive methods (Leslie and Logrieco, 2014).

Using molecular markers for resistance to *A. flavus* and aflatoxin accumulation to transfer specific quantitative trait loci (QTL) from resistant lines to commercial lines via MAS could greatly speed resistance breeding efforts, allowing for easier introgression of this resistance into commercial lines while maintaining the agronomic integrity of the commercial lines (Mylroie et al., 2013; Warburton et al., 2011).

The first gene-based maker has been developed by Mylroie et al. (2013) for tracking resistance to aflatoxin accumulation in maize. The gene, encoding a chloroplast precursor, was found to contain multiple polymorphisms that were used to design a marker designated Mississippi Marker 1 (MpM1). MpM1 can successfully distinguish the resistant individuals from the more susceptible individuals in a fast and economical manner. A trypsin Inhibitor and three chitinase genes, exhibiting strong association with aflatoxin accumulation resistance, have been validated in an association mapping panel which can make excellent markers for marker assisted improvement of aflatoxin accumulation resistance (Warburton and Williams, 2014).

QTL mapping methods is a powerful tool to understand the generic relationships between correlated traits. QTL are regions of the genome associated with a particular quantitative trait. By selecting for individuals containing markers tightly linked to the

genetic regions associated with resistance, the trait can be transferred from resistant inbreds into select commercial lines. Many QTL reported on resistance to *A. flavus* infection and aflatoxin contamination in maize account for less than 5% of the phenotypic variation observed in the population and the environment in which it was measured (Bhatnagar-Mathur et al., 2015; Warburton and Williams, 2014). They were identified on chromosomes 2, 3, and 7 (bins 2.01 to 2.03, 2.08, 3.08, and 7.06). However, two QTL account for up to 20% of the phenotypic variation in multiple environments were identified on chromosome 4 (Busboom and White, 2004). A meta-QTL analysis can be done if data for more than three or four QTL studies are available. In this analysis, mapping data and QTL effect data from all individual studies are combined into a single projected map. Sixty two meta-QTL created by the combination of QTL from multiple studies or ear rot traits were found on all chromosomes except 9 and 10. The 12 QTL from the meta-QTL analysis fell into bins 4.07 to 4.09 which indicates that the 4.07/8 region of the maize genome contains a cluster of genes influencing the response of maize to multiple pathogens (Warburton and Williams, 2014).

The Corn Fungal Resistance Associated Sequences Database (CFRAS_DB; http://www.agbase.msstate.edu/cgi-bin/maizecandidates/index.cgi) was created to identify important genes for *A. flavus* and aflatoxin resistance. The database contains many types of data such as microarrays, proteomics, QTLs, and SNPs (Kelley et al., 2010).

Auxin

Auxin was the first plant hormone discovered by Charles Darwin in canary grass (Phalaris canariensis) coleoptiles in 1880 while investigating plant tropisms, especially

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phototropism. Darwin demonstrated that when the tip of the coleoptile was covered, the characteristic bending of the coleoptile toward light did not occur. It was concluded that a signal produced in the tip, traveled down, and caused bending of the tip which was caused by unequal growth (Darwin and Darwin, 1880) . An unknown substance (auxin)-containing agar blocks stimulated the growth of the excised coleoptile tips of *Avena sativa* (oat) *in vitro* on a dosage-dependant manner (Jensen, 1911). Based on these experiments, the role of auxin in plant growth were first described by the Dutch scientist Frits Warmolt Went in 1926, and he named this hormone as "auxin" which means "to grow/increase" in Greek (Hohm et al., 2013). Chemical structure of the principal auxin in higher plants was determined as indole-3-acetic acid (IAA) by K. V. Thimann (Abel and Theologis, 2010).

Chemically, auxins are characterized by a ring system as a nucleus which has at least one double band and a side chain containing carboxyl group with at least one atom removed from the ring. A particular space is required between carboxyl group and the ring for the auxin activity (Magnus and Kojic-Prodic, 1999). Beside IAA as principle auxin, plants synthesize other auxins, namely, 4-chloroindole-3-acetic acid (4-Cl-IAA), indole-3-butyric acid (IBA) and phenylacetic acid (PAA) (Figure 2.7). Synthetic auxins such as 2,4-dichlorophenoxy acetic acid (2,4-D) and naphthalene-1-acetic acid (NAA) have been frequently used as tools in plant research (Simon and Petrášek, 2011).

Identification of the first auxin receptor, Auxin binding protein 1 (ABP1), on maize coleoptile membranes provided the first molecular basis for the auxin activity (Ray et al., 1977). A model proposed for the auxin binding site for maize ABP1 describes three regions of the protein required for binding: a planar aromatic ring binding platform, a

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carboxylic acid binding site which is somewhat flexible and a hydrophobic transition

region which separates the two binding sites (Edgerton et al., 1994).
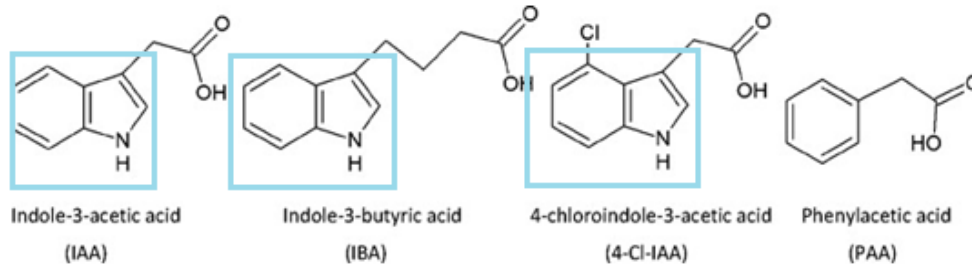
Figure 2.7   Chemical structure of four endogenous auxins IAA, IBA, 4-Cl-IAA, and
             PAA.

Indole groups were boxed with blue. The figure was adapted from Simon and Petrášek,
(2011).

Even though other auxins are found in plants, IAA has probably been the most

abundant and intensely studied hormone in physiological research. It functions virtually

in every aspect of plant growth and development such as cell enlargement, cell division,

vascular tissue differentiation, root initiation, tropistic response, apical dominance, leaf

senescence, leaf and fruit abscission, fruit setting and growth, fruit ripening, flowering,

and growth of flower parts (Davies, 2004).

Many genetic and biochemical studies demonstrated that IAA is mainly

synthesized from L-tryptophan (Trp) via indole-3-pyruvate (IPA) by two-step reactions.

In the first step, conversion of Trp to IPA is catalyzed by tryptophan aminotransferase

(TAA). A flavin-containing monooxygenase (YUC) catalyzing the conversion of IPA to

IAA in the second step (Brumos et al., 2014; Mashiguchi et al., 2011). IAA is also

synthesized from Trp, via indole-3-acetaldoxime (IAOx) (Sugawara et al., 2009), Indole-

3-acetamide (IAM) (Pollmann et al., 2003), indole-3-acetaldehyde (IAAld), and

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tryptamine (TAM) (Quittenden et al., 2009). Besides the Trp-dependent IPA, IAOx, IAM, and TAM pathways, a Trp-independent pathway was proposed as a major route of IAA biosynthesis (Normanly et al., 1993; Wright et al., 1991). A summary of the IAA biosynthesis in plants is shown in Figure 2.8 (Kasahara, 2015).
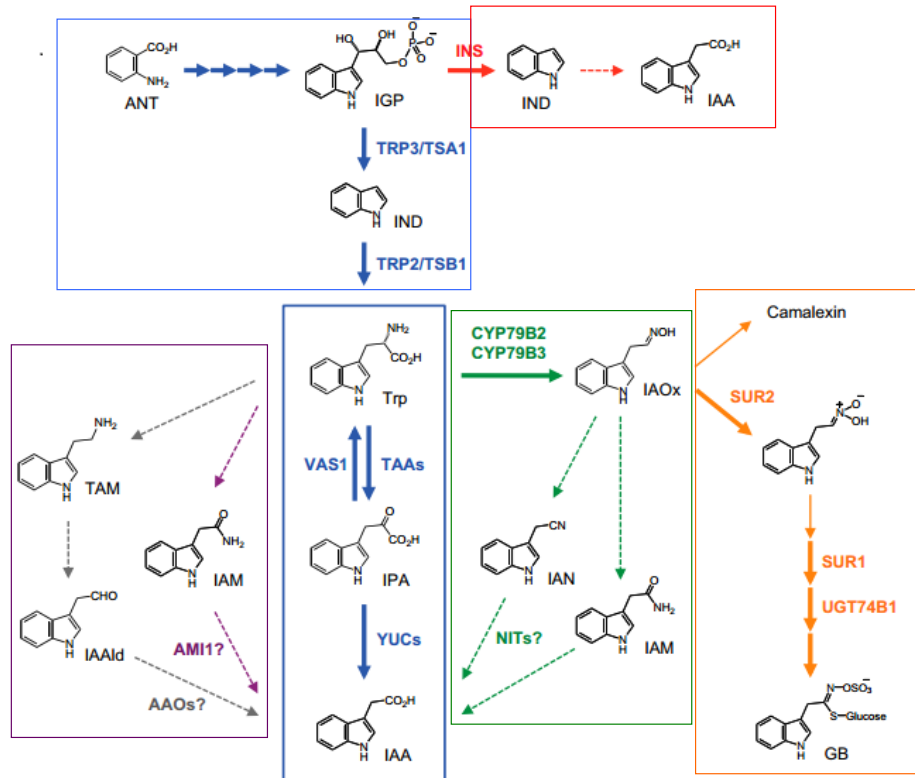
Figure 2.8   IAA biosynthetic pathways and secondary metabolic pathways
The figure was adapted from Kasahara, (2015).

In plants, meristematic regions and actively growing organs have the highest IAA concentration. Tissue type and its growth state are important factors for determining IAA concentrations found at various locations within a plant during development. Different cellular responses are produced based on the auxin level in the cell. Auxin levels are regulated by biosynthesis, conjugation/deconjugation of IAA to amino acids, peptides, or

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carbohydrates and several degradation pathways (Figure 2.9) (Ljung, 2013; Normanly, 1997). The genes encoding biosynthesis enzymes have been shown to alter IAA levels in transgenic plants (Klee et al., 1994). IAA conjugates are biologically inactive and function to serve as IAA storage forms in seeds and hormonal homeostasis (Ludwig-Müller, 2011; Szerszen et al., 1994). Major IAA catabolism occurs via oxidation to oxindole-3-acetic acid and subsequent glycosylation through an added 7-hydroxyl group (Normanly, 1997).
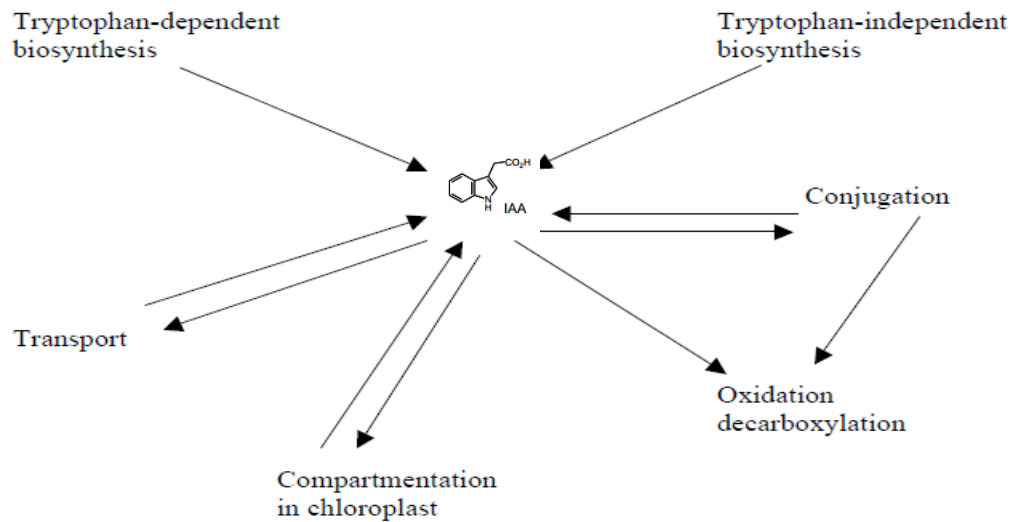
Figure 2.9   Homeostatic control of the active IAA
The figure was adapted from Poli, (2005).

The endosperm of the maize kernel has been used extensively for the biochemical analysis of IAA metabolism, since developing kernels are the most active IAA biosynthetic tissue in maize and relatively large quantities of IAA esters are stored in the endosperm (Kriechbaumer et al., 2006). For example, the endosperm of a germinating maize seed was measured to contain 308 pmoles of IAA while its shoot contained 27

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pmoles of IAA (Epstein et al., 1980). A Trp-dependent IAA biosynthesis has been identified as the main pathway in the maize endosperm (Glawischnig et al., 2000). In this pathway, IAA is synthesized from Trp via IAM by a nitrilase (ZmNIT2) (Kriechbaumer et al., 2006).

Auxin regulates diverse plant developmental processes through auxin signaling. Auxin signaling controls auxin responsive gene expression via DNA-binding Auxin Responsive Factors (ARFs), transcriptional repressor proteins (AUX/IAA), and two F-box proteins Transport Inhibitor Resistant 1 (TIR1) and Auxin Signaling F-Box (AFB). TIR1 and AFB are substrate recognition components of the E3 ubiquitin ligase SKP–Cullin–F-box (SCF) complex. At the low auxin concentration, AUX/IAA proteins heterodimerize with the ARF transcription factors, thereby inhibiting the transcription of specific auxin response genes. Auxin binding to the TIR1/AFB receptors promotes the recruitment of AUX/IAA proteins to the SCF complex. Subsequent degradation of AUX/IAA protein via ubiquitin/26S proteasome pathway results in derepressing responsive gene expression (Figure 2.10) (Naseem et al., 2015a; Quint and Gray, 2006). Auxin can induce the expression of three groups of genes: Small Auxin-Up RNAs encoding small proteins (SAURs) as effectors of hormonal signals in plant growth (Ren and Gray, 2015), GH3-related transcripts encoding IAA–amino acid conjugating enzymes to diminish the auxin signal by inactivating IAA via conjugation, and Aux/IAA family (Woodward and Bartel, 2005).
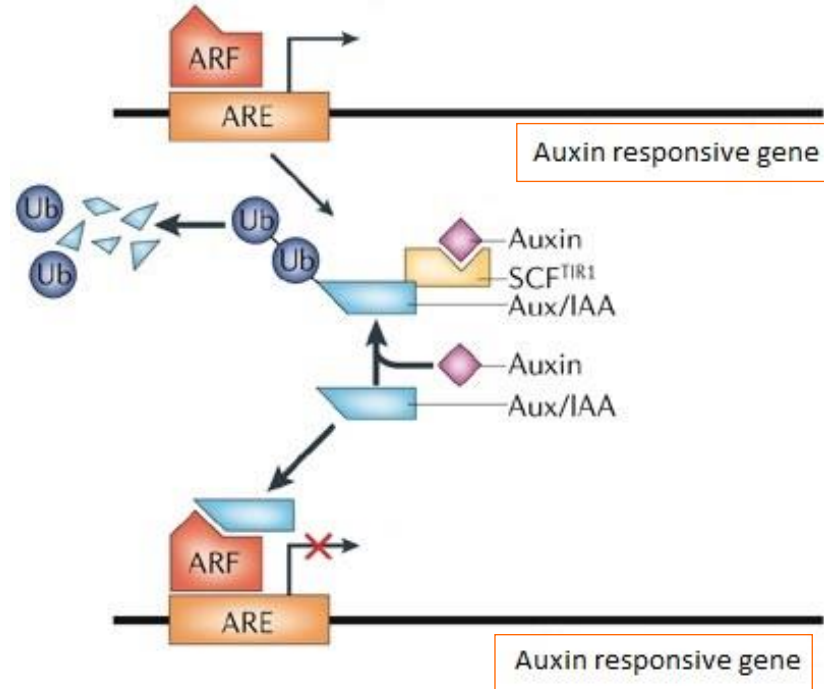
Figure 2.10 Auxin regulation of gene expression

The figure was adapted from Teale et al. (2006).

Auxin can be synthesized by many bacteria and fungi during their interactions with plants (Fu and Wang, 2011). Several models have been suggested to elucidate the function of pathogen produced auxin in plant-pathogen interaction. Auxin may act as microbial signaling molecule that can have a direct effect on bacterial physiology for their colonization strategy and circumvention of basal plant defense mechanisms and/or effector molecule to regulate auxin metabolism in host plants (Naseem et al., 2015b; Spaepen and Vanderleyden, 2011; Spaepen et al., 2007). It has been demonstrated that *A. flavus* produces auxin when grown on synthetic medium (Tsavkelova et al., 2006; Youssef and Mankarios, 1975). However, the function of *A. flavus* produced auxin has not been elucidated.

Auxin quantification techniques have been highly improved during the last 15 years in terms of sensitivity and accuracy (Porfírio et al., 2015). However, sample preparation is still the major limiting step in accurate auxin quantification. Sample preparation consists of mainly two steps: extraction and purification, and it may take 80% of the total time of analytical process (Chen et al., 2008). Auxin is extracted from plants into a liquid phase by using organic solvents such as methanol, ethanol, acetone, diethyl ether and dimethyl sulfoxide or aqueous alkaline solutions such as basic buffers (George et al., 2008). Two of the most common techniques used in auxin purification are liquid-liquid extraction (LLE) and solid-phase extraction (SPE). Less common purification strategies include immunoaffinity column purification, vapor phase extraction (VPE), dual-cloud point extraction (dCPE), HPLC fractionation, and QuEChERS (quick, easy, cheap, effective, rugged and safe) methodology. Many options are also available for sample analysis including GC, GC/MS, GC/MS/MS, LC, LC/MS, LC/MS/MS, CE, CE/MS, immunoassays, Immunosensors, fluorimetric assays, and colorimetric methods. Figure 2.10 summarizes auxin quantification techniques (Porfírio et al., 2015).
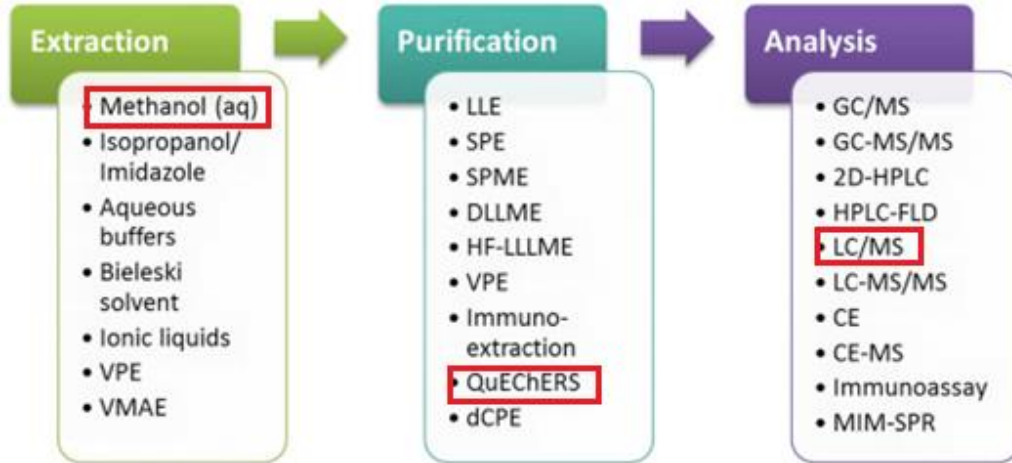
Figure 2.11 Methods used in auxin analysis

Methods used in this study were boxed with red. The figure was adapted from Porfírio et al. (2015).

Role of auxin in plant defense response to pathogens

Beside its critical role in plant growth and development, auxin is known to be involved in plant defense response to biotic stress. Exogenous application of IAA and an auxin anolog, 2,4-dichlorophenoxyacetic acid (2,4-D) to plants, promoted disease symptoms and induced susceptibility of maize, tobacco and Arabidopsis to Helminthosporium leaf spot, tobacco mosaic virus (TMV), and Pst DC3000, respectively (Chen et al., 2007a; Hoffmann and Zscheile, 1973; Navarro et al., 2008; Simons et al., 1972). Auxin treatment increased the transcription of GH3 genes which encode for IAA conjugating enzymes to reduce free auxin levels in rice (Jain et al., 2006). Similar to exogenous IAA, auxin produced by pathogens such as *Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Pseudomonas savastanoi, Corynebacterium fascians, Erwinia* milletiae, and *Pst* DC3000 induced tumor production and involved in the inhibition of the hypersensitive response (HR) in their host plants (Robinette and Matthysse, 1990;

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Yamada et al., 1991). These findings suggest that auxin reduces plant defense response. If auxin response is blocked by another plant hormone such as SA, disease resistance can be increased (Wang et al., 2007b). On the contrary, exogenously supplemented IAA and IBA on healthy and phytoplasma (plant pathogenic bacteria)-infected periwinkles induce recovery of phytoplasma-infected periwinkle shoots. It was speculated that auxins affect already disturbed endogenous levels of auxins which would trigger differential regulation of gene expression resulting in a metabolic change unfavorable for phytoplasma (Ćurković Perica, 2008).

Endogenous auxin levels and auxin signaling in plants responding to pathogens have been studied. Auxin levels increased in Arabidopsis after infection by *Xanthomonas campestris* pv. campestris (*Xcc*) and *Pseudomonas syringae* type III effector AvrRpt2 (Chen et al., 2007a; O'Donnell et al., 2003). Mutated Arabidopsis plants constitutively expressing type III effector AvrRpt2 exhibited phenotypes with longer primary roots, increased number of lateral roots, and increased sensitivity to exogenous auxin. Free auxin levels were also high in these plants and increased more after pathogen inoculation. Pathogen infection induced auxin biosynthetic genes where as repressing Aux/IAA and auxin transporter genes, suggesting that AvrRpt2 can be the virulence factor that can modulate auxin physiology to promote disease (Chen et al., 2007a). Another effector (Penetration-Specific Effector 1 (PSE1)) from the oomycete *Phytophthora parasitica* enhanced the susceptibility of Arabidopsis by disrupting auxin accumulation (Evangelisti et al., 2013). Similarly, a root-infecting fungal pathogen *Fusarium oxysporum* induced the transcription of auxin biosynthesis genes in infected Arabidopsis whereas auxin signaling mutants exhibited more resistance to infection (Kidd et al., 2011). A bacterial

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pathogen *Xanthomonas oryzae* pv *oryzae* and a fungal pathogen *Magnaporthe grisea* induced IAA biosynthesis which induces the production of expansins, the cell wall-loosening proteins, and makes rice vulnerable to pathogens in rice (Ding et al., 2008a; Fu et al., 2011).

Even though exact mechanism of how auxin promotes susceptibility in plants is not known it has been suggested that auxin signaling is part of SA- mediated host defense and suppresses it antagonistically (Navarro et al., 2006; Wang et al., 2007b). In contrast to these findings described previously, auxin signaling might positively regulates disease resistance. It has been demonstrated that repression of auxin signaling pathway enhances susceptibility of Arabidopsis to the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea* (Llorente et al., 2008). This suggested that synergetic crosstalk between auxin and JA occurs during defense to necrotrophic pathogens instead of antagonistic interaction between auxin and SA (Kazan and Manners, 2009). Synergetic interaction between auxin and JA has been also shown in Arabidopsis during defense responses to the necrotrophic pathogen *Alternaria brassicicola* (Qi et al., 2012).

Aspergillus flavus is an auxin producing pathogen but the role of produced auxin and/or if it is produced during maize-*A. flavus* interaction is not known. However, there are some evidences demonstrated that maize auxins play a role in defense responses to *A. flavus*. Comparisons of the biological profile responses of resistant (Mp313E) and susceptible (Va35) inbred maize lines 48 hours post-*A. flavus* infection revealed that Mp313E increased ABA and auxin signaling while Va35 reduced auxin signaling (Kelley et al., 2009). Our recent high-throughput analysis in maize demonstrated that gene onthology (GO) term "response to auxin stimuli" was overrepresented in aflatoxin and *A.*

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flavus resistant maize genotype compared to susceptible maize genotype. In addition, a genome-wide association study (GWAS) to identify metabolic pathways involved in maize aflatoxin accumulation resistance revealed that the most significant metabolic pathway identified was jasmonic acid (JA) biosynthesis which might be working synergistically with auxin (Tang et al., 2015).

To understand the molecular mechanisms by which auxin regulates maize defense responses to *A. flavus*, integration of findings from auxin biosynthesis, conjugation, transport and signaling will be necessary. Recent progress in auxin biosynthesis makes it practical to alter auxin levels with temporal and spatial precision, providing exciting tools to tackle complex questions regarding the mechanisms of how auxin regulates plant defense responses to pathogens.

CHAPTER III

EFFECT OF EXOGENOUS AUXIN ON *ASPERGILLUS FLAVUS* GROWTH AND AFLATOXIN PRODUCTION

Abstract

The objectives of this study were to evaluate the capacity of the *A. flavus* isolate NRRL 3357 to grow and produce aflatoxin under different culture conditions and optimize experimental conditions for aflatoxin (AFB1) production. AFB1 production was determined in two different media and culture conditions (temperature) which were varied to achieve maximal toxin production. Also, two different plant hormones (auxins), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were evaluated for their effects on fungal growth and AFB1 production. Stability of IAA was determined under the experiment conditions. AFB1 and IAA were extracted directly from the cultures by QuEChERS (quick, easy, cheap, effective, rugged and safe). High performance liquid chromatography (HPLC) was used to identify AFB1 and IAA. It was observed that *A. flavus* isolate NRRL 3357 did not produce AFB1 when grown in Czapek dox broth (CD). IBA did not affect fungal growth under any culture conditions. Potato dextrose broth (PD) and IAA were determined to be suitable medium and auxin for further experiment in this study. IAA was stable at low and middle concentrations for 7 days. Nonbiological destruction of IAA in culture medium was observed at high concentration during the incubation period. However, *A. flavus* isolate inoculation increased IAA degradation

significantly at all concentrations. IAA significantly increased mycelium growth and significantly decreased AFB1 at a high concentration (10000 ppb) in PDB when compared to control.

Introduction

Aspergillus flavus is a ubiquitous fungus that can produce toxic, carcinogenic, teratogenic, and mutagenic secondary metabolites, aflatoxins. Among aflatoxins (B1, B2, G1, G2 and M2) aflatoxin B1 (AFB1) is the most potent carcinogen (Payne and Brown, 1998). Since these toxins were first isolated from Brazilian peanut meal in 1961 they have been found in many other commodities, including maize and other grains, Brazil nuts, pistachio nuts, and cottonseed (Campbell et al., 2003) Aflatoxin contamination is a serious problem both in developing and postharvest grains particularly in the southeastern United States (Zummon and Scott, 1989).

Due to the significant health and economic impacts of AFB1 contamination, numerous studies have been focused on *in vitro* production of aflatoxins. Quantitative data on aflatoxins production have been produced in various media (Codner et al., 1963; Davis et al., 1966; Mateles and Adye, 1965). Factors influencing production of aflatoxin such as temperature, humidity, pH, nutrients, carbohydrate source, nitrogen source, and specific aminoacids have been reported (Mateles and Adye, 1965; Park and Bullerman, 1983; Payne and Hagler, 1983b; Schindler et al., 1967). However, results have been reported with different isolates of *A. flavus* and not directly comparable. Optimum nutritional and physiological conditions vary for different isolate of *A. flavus*. Therefore, optimization of conditions for toxigenic *A. flavus* isolate NRRL 3357 is necessary to

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evaluate the growth capacity and toxin production potential under further experimental conditions in this study.

Plant hormones are involved in different stages of plant growth and development. Earlier reports on the effect of plant hormones on fungal growth and production of secondary fungal metabolites are inconsistent (Chatterjee et al., 2008a). Stimulatory as well as inhibitory effects of auxins on microbial growth have been reported. Indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) increase the fresh weight of *Claviceps purpurea* mycelia in static culture (ReRabek, 1970). Similarly, IAA enhanced biomass production of *Agaricus campestris*, *Kluyveromyces fragilis* and *Pleurotus sajor-caju* grown under submerged condition and in the whey medium, respectively (Guha and Banerjee, 1974; Mukhopadhyay et al., 2005; Paul et al., 1994). Prusty et al. (2004) demonstrated that yeast can be inhibited or stimulated by IAA depending on its concentration. At high concentrations, IAA inhibited growth, whereas at lower concentrations, it induced growth and adhesion of *Saccharomyces cerevisiae* as well as *Ustilago maydis* and *Fusarium oxysporum* (Prusty et al., 2004). Stimulatory and inhibitory effects of IAA on growth were also observed in a dose dependent manner in Lichen-forming fungi (LFF), *Nephromopsis ornate* (Wang et al., 2010). Also, it has been clearly demonstrated that production of secondary fungal metabolites could be affected by plant hormones (Lisowska et al., 2006). IAA and IBA influenced secondary metabolites production of aposymbiotically grown LFF (Wang et al., 2010). Therefore, it has been the subject of great interest to study whether auxins can affect the growth of *A. flavus* isolates NRRL 3357 and the production of AFB1 in various conditions.

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In this work, nutritional and physiological conditions for *A. flavus* isolate NRRL 3357 were optimized. The influence of auxins on *A. flavus* growth and production of AFB1 in various conditions was investigated. The knowledge gained from this study may help in utilizing aflatoxin to produce commercially important aflatoxin free maize.

Material and Methods

Fungus material and auxin/IAA/IBA Preparation

The *A. flavus* isolate NRRL 3357, which is known to produce aflatoxin in maize grain, was used as inoculum. Inoculum was increased on sterile maize cob grits in 500-milliliter flasks, each containing 50 g of grits and 100 ml of water, and then incubated at 28°C. Conidia were washed from the grits using sterile distilled water containing 20 drops of Tween 20 per liter and filtered through four layers of sterile cheesecloth. The concentration of conidia was determined with a hemacytometer and adjusted with sterile distilled water to 10 million per milliliter (10^7spores/ml). Stock solutions of 1mg/ml IAA (Sigma Chemical Co., USA) and IBA (Sigma Chemical Co., USA) were prepared by dissolving 50 mg of IAA/IBA in 50 ml of 10 % ethanol and then filter sterilized.

Culture media

The following culture media were used in the present work: Czapek dox broth (CD): 3 g sucrose, 0.3 g sodium nitrate, 0.1 g dipotassium phosphate, 0.05 g magnesium sulphate, 0.05 g potassium chloride, and 100 ml distilled water. Potato dextrose broth (PDB): 0.4 g potato starch, 2 g dextrose, and 100 ml distilled water. The media were autoclaved at 121°C for 20 min.

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A. flavus growth and culture conditions

To optimize the experiment conditions, czapek dox broth (CD; Difco) and potato dextrose broth (PDB; Difco) were examined for their effects on *A. flavus* growth and aflatoxin (AFB1) production in the presence of different concentration of either IAA or IBA. The flasks containing 65 ml CD or PDB were amended with IAA or IBA to give final concentration of 0, 100, 1000, 10000 ppb. Amount of alcohol in all media including control was kept constant (10%). A spore suspension (2 μl) of *A. flavus* containing 10^7 spores/ml was inoculated to flasks. The flasks were incubated at 28° on a rotary shaker at 200 rpm for 7 days. The cultures were harvested after 7 days. Three replicates of each treatment were performed. To determine whether higher temperatures affect *A. flavus* growth and aflatoxin production, the experiments were repeated at 30 and 32°C. Since CD was not found to be suitable for the toxin production and IBA did not affect the fungal growth, further experiments were carried out using PDB as growth medium and IAA as the main auxin.

The growth of *A. flavus* and AFB1 production was determined in PDB supplemented with IAA at various concentrations. The flasks containing 65 ml PDB and IAA (0, 100, 1000, 10000 ppb) were inoculated with a spore suspension (2 μl) of *A. flavus* containing 10^7 spores/ml and incubated at 28°C for 7 days. AFB1 and IAA were extracted and measured from 1, 3 and 7 day cultures by removing 10 ml liquid-extract from each flask with a micropipette into 50 ml sterilized polypropylene tubes. After the incubation period, mycelium was harvested from the medium using Whatmann no.11 paper (DX102, Xinhua Paper Co., Ltd., Hangzhou, China) and the dry weight was

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determined by placing it in a hot air oven at 60°C until a constant dry weight was attained.

Stability of IAA in PDB under the experiment conditions was determined simultaneously by incubating another set of the flask containing PDB and various concentration (0, 100, 1000, and 10000 ppb) of IAA in the absence of *A. flavus*. IAA was extracted and measured at 1, 3 and 7 days.

In vitro plate assay

As a parallel growth experiment, *in vitro* plate assay was performed. Potato dextrose agar (PDA; Difco) was amended with IAA (Sigma, UK). IAA was added at a final concentration of 0, 100, 1000, 10000 ppb to PDA. Plates were inoculated with 2 μl of 10^7 spores/ml *A. flavus* strain NRRL 3357. Plates were incubated at 28°C. After 7 days, the diameter of the colony (in cm) was measured. The experiment was carried out twice, each containing three technical replicates per treatment.

Extraction of AFB1 and IAA

AFB1 and IAA were extracted from the PDB culture supernatant using QuEChERS (quick, easy, cheap, effective, rugged and safe) method. After incubation, 10 ml liquid-extract were pooled out from each flask with the help of a micropipette into the 50 ml sterilized polypropylene tubes and 15 ml of acetonitrile (Fisher Scientific, Pittsburgh, PA) was added followed by vortexing for 1 min. A QuEChERS pre-packed extraction pack (containing 6 g of magnesium sulfate and 1.5 g of sodium acetate) (Agilent Technologies, Santa Clara, CA) was added, and the solution was vortexed immediately for another 1 min. Tubes were centrifuged for 5 min at 5000 rpm. The

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supernatant was collected into a clean 15 ml plastic centrifuge tube. A total of 1 ml of the clarified supernatant was filtered through a PTFE syringe filter (0.45 μm pore size, 13 mm diameter) (Fisherbrand®, Fisher Scientific, Pittsburgh, PA, USA) prior to HPLC−LS/MS analysis.

High performance liquid chromatography (HPLC)

The samples were placed in auto-sampler vials and analyzed using an Agilent 6460 LC/MS Triple Quadruple, which uses electrospray ionization. The HPLC used a Zorbax Eclipse Plus-C18 Narrow Bore 2.1 x 50mm, 5μm column with a temperature of 40°C. The injection volume was 10 μl. The mobile phase consisted of 5mM ammonium acetate with 0.1% formic acid in HPLC-grade water and 5mM ammonium acetate with 0.1% formic acid in methanol. The flow rate was 0.6 ml/min. The mobile phase was on a gradient where it transitioned from 95% water to 100% methanol then back to 95% water over the course of the six minute run. The total run time of the method was nine minutes which includes a six minute run time and an additional three minutes for the system to get back to equilibrium.

The parameters for the mass spectrometer were the following. The gas temperature was 325°C while the gas flow was set to ten liters per minute. The nebulizer gas pressure was set to 50 psi and the capillary voltage was 4000V. The Sheath Gas Flow had an output of 11 liters per minute and the sheath gas temperature reached temperatures of 350°C. While in MRM mode, the mass spectrometer was set to look for certain daughter ions after the AFB1 ion entered the collision cell. These transitions for AFB1 included 313.1 > 285.1 with a collision energy of 20kEV, 313.1 > 269.1 with a collision energy of 25kEV, and 313.1 > 241.1 with a collision energy of 35kEV. The transitions

for IAA are 176.1>130.1, 176.1>103.1, and 176.1>77.0. The fragmentor (130) and the cell accelerator (7) values were the same for all the AFB1 transitions. The retention time of the AFB1 was 3.4 minutes and IAA was 2.6 minutes. There was an eight point standard curve used for IAA including the points 10ppb, 50ppb, 100ppb, 200ppb, 400ppb, 600ppb, 800ppb, and 1ppm. A standard graph was prepared with AFB1 (Supelco, Bellefonte, USA) in the range of 1 to 100 ppb.

Statistical Analysis

The experiment was designed as a completely randomized design. An analysis of variance (ANOVA) was conducted to determine whether there was a significant difference in fungal growth and/or aflatoxin production among IAA concentrations of 0, 100, 1000, 10000 ppb. The means of the four IAA concentrations were compared using Fisher's Protected least significance difference (LSD). The LSD analysis and ANOVA (performed using Proc GLM) were performed with the SAS® (SAS Institute Inc.; Cary, NC) software using a significance level of $\alpha = 0.05$.

Results

In this study, Czapek Dox (CD) media and Potato Dextrose Broth (PDB) were used to determine the influence of variable IAA and IBA concentrations on mycelium growth and the production of AFB1. The effect of different type of auxin (IAA and IBA) on *A. flavus* growth and AFB1 production in two different culture media supplemented with 0, 100, 1000, and 10000 ppb of IAA and IBA was studied by inoculating *A. flavus* strain NRRL 3357 into 65 ml of CD media and PDB and culturing at 28 °C. Temperature

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effect was also studied on *A. flavus* growth and AFB1 production by growing cultures at 30 °C and 32 °C. Results are represented in Table 3.1 and Table 3.2.

Table 3.1   Effect of IAA on *A. flavus* growth in CD at different temperatures

IAA (ppb)	Dry weight of mycelium (g)		
	28 °C	30 °C	32 °C
0	0.592[ab]	0.141[a]	0.243[a]
100	0.554[b]	0.169[a]	0.158[a]
1000	0.765[a]	0.218[ab]	0.178[a]
10000	0.239[c]	0.445[b]	0.275[a]

Different letters following means in a column represent means that were statistically different at the 0.05 level.

The effect of auxins on *A. flavus* growth at different temperatures in the CD medium was shown in (Table 3.1). At 28 °C, the lowest (100 ppb = 0.57µM) and middle concentration (1000 ppb = 5.7µM) of IAA did not change mycelium growth significantly. The highest concentration (10000 ppb = 57µM) of IAA significantly decreased mycelium growth by 60 % compared to control concentration (0 ppb). At 30 °C, the lowest and middle concentration of IAA did not significantly change mycelium growth. The highest concentration of IAA significantly increased mycelium growth. However, there was no significant change in mycelium growth in any concentration of IAA at 32 °C. IBA treatments at different concentrations did not significantly change mycelium growth in CD media when compared to the control without IBA at any growth temperatures (Table 3.2).

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Table 3.2   Effect of IBA on *A. flavus* growth in CD at different temperatures

IBA (ppb)	Dry weight of mycelium (g)		
	28 °C	30 °C	32 °C
0	0.167[a]	0.121[a]	0.124[a]
100	0.187[a]	0.090[a]	0.110[a]
1000	0.166[a]	0.129[a]	0.139[a]
10000	0.202[a]	0.106[a]	0.112[a]

Different letters following means in a column represent means that were statistically different at the 0.05 level.

After the filtration and separation of mycelium from the supernatant AFB1 production was determined in CD media. No detectable quantities of AFB1 were formed at the above conditions. This shows that toxigenic potential of *A. flavus* NRRL 3357 cannot be evaluated in CD. Because IBA did not affect mycelium growth significantly at any concentrations and temperatures and AFB1 was not produced in CD media, experiments were carried out using IAA as the main auxin and PDB as growth media.

To elucidate fully the potential of IAA in fungal culture, we developed a better understanding of how to regulate IAA presence in artificial growing systems. This was accomplished by separating the loss of auxin that occurs in sterile medium alone from the loss associated with fungal metabolism. Stability of IAA was determined in PDB at 28 °C for 7 days in the absence and presence of *A. flavus*. In the absence of *A. flavus* (Figure 3.1), the analyses of IAA in PDB indicated that no detectable losses occurred over 7 days at the lowest IAA concentration (100 ppb). However, IAA was much less stable at middle (1000 ppb) and high (10000 ppb) concentrations, its concentration declined to approximately 64 and 33 % of the original, respectively, within 7 days. The results indicate that physical or nonbiological component of PDB media contributed to the degradation of IAA. In the presence of *A. flavus* (Figure 3.2), it was observed that IAA

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degraded to non-detectable levels at the initial IAA concentrations of 100 and 1000 ppb compared to un-inoculated. At high (10000 ppb) initial concentration of IAA, IAA degraded by 64 % after *A. flavus* inoculation where as it degraded by 50 % without *A. flavus* inoculation. The results suggest that *A. flavus* inoculation increased the degradation of IAA *in vitro*.

Natural IAA degradation
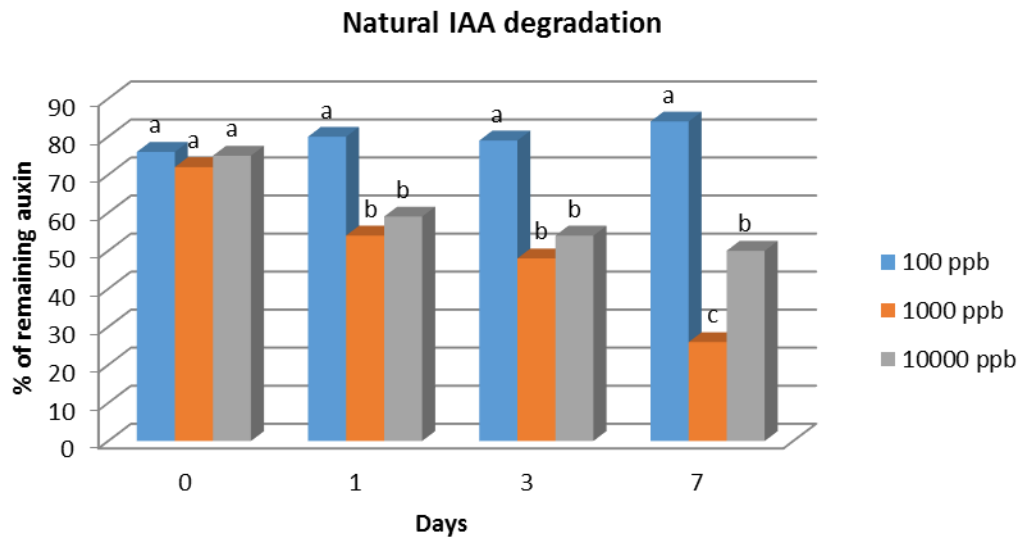
Figure 3.1   Stability of IAA in PDB for 7 days

The amount of hormone remaining was expressed as the percentage of the initial concentration. Different letters represent means that were statistically different at the 0.05 level.
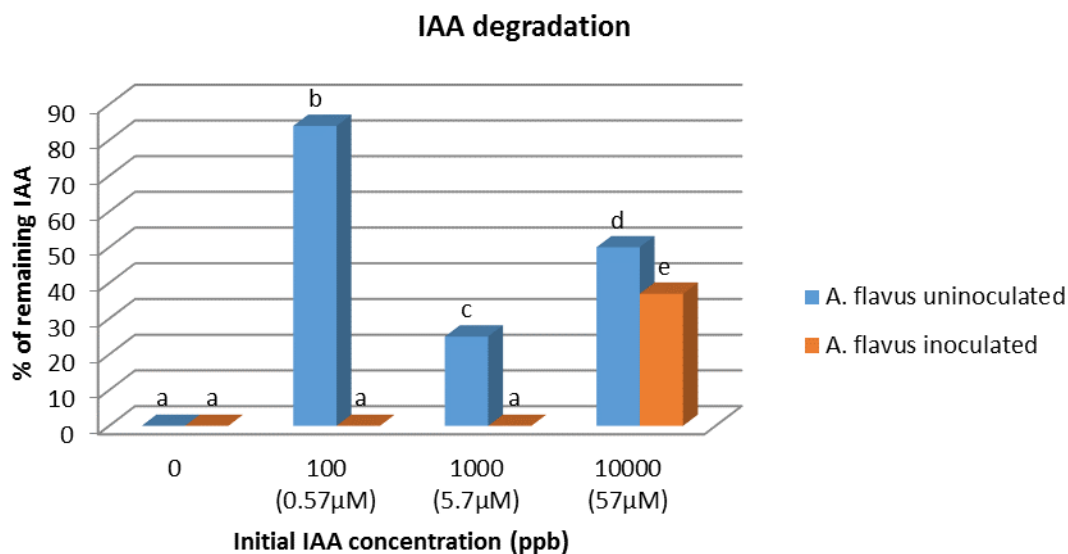
IAA degradation

Figure 3.2   Determination of IAA degradation in presence of *A. flavus* at 7 days

The amount of hormone remaining was expressed as the percentage of the initial concentration. Different letters represent means that were statistically different at the 0.05 level.

Results shown in Table 3.3 present the mycelium growth, AFB1 production and IAA degradation by *A. flavus* in PDB 7 days after inoculation. The treatments of 100 and 1000 ppb of IAA in PDB did not significantly affect the mycelium growth. IAA treatment significantly increased the mycelium growth at high concentration (10000 ppb) (Figure 3.3). *A. flavus* produced detectable level of AFB1 in PDB (Figure 3.4). It produced 643 ppb of AFB1 in the absence of IAA. Similar to the mycelium growth, the treatments of 100 and 1000 ppb of IAA did not significantly affect the AFB1 production. However, in contrast to mycelium growth, AFB1 production was significantly decreased at high IAA concentration. Determination of remaining IAA concentrations at 7 days (Figure 3.5) reveals that *A. flavus* either utilizes or exogenously degrades IAA to undetectable levels at where the initial IAA concentration 100 and 1000 ppb. Remaining IAA concentration was determined as 3694 ppb (approximately 37 % of initial

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concentration) at high IAA. The results suggest that high IAA treatment may induce

fungal growth whereas decrease aflatoxin in PDB *in vitro*.

Table 3.3   Dry weight estimation, aflatoxin production and auxin degradation by *Aspergillus flavus* NRRL 3357 in different IAA concentration at 7 days.

	IAA (ppb)	Dry weight of mycelium (g)	AFB1 (ppb)	Remaining IAA (ppb)
A.flavus	0	0.206^a	643^a	0^a
	100	0.216^a	720^a	0^a
	1000	0.223^{ab}	679^a	0^a
	10000	0.239^b	446^b	3694^b

Different letters following means in a column represent means that were statistically different at the 0.05 level.
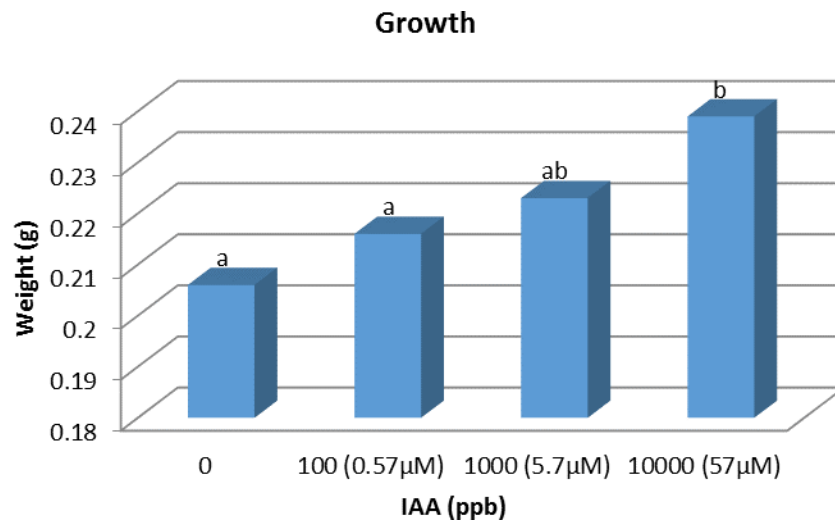
Figure 3.3   Determination of fungal dry weight based on IAA levels

Different letters represent means that were statistically different at the 0.05 level.

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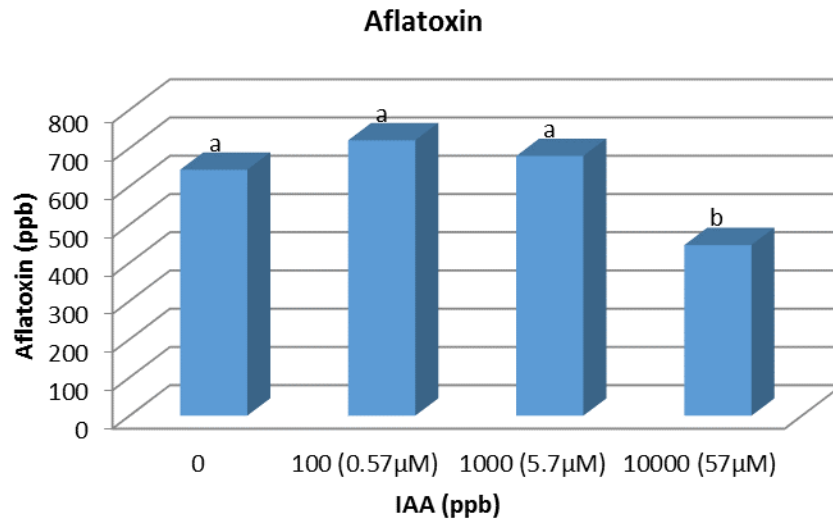
Aflatoxin

Figure 3.4   Determination of aflatoxin based on IAA levels

Different letters represent means that were statistically different at the 0.05 level.
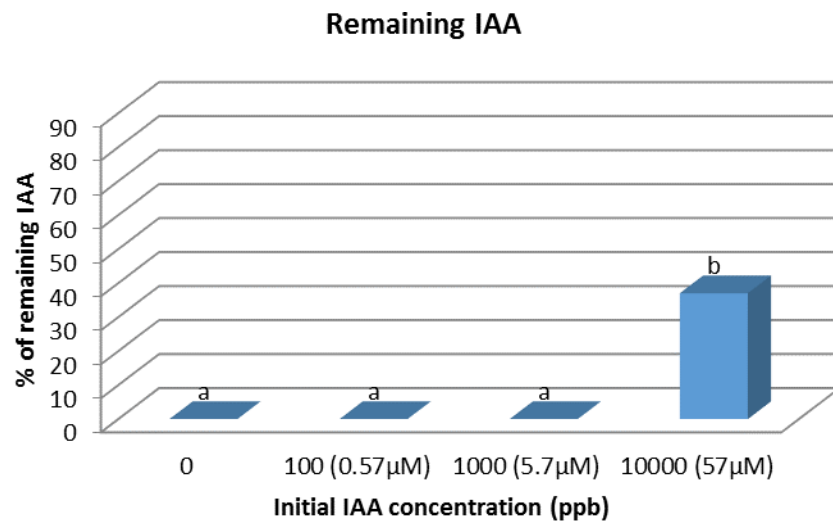
Remaining IAA

Figure 3.5   Determination of remaining IAA levels in the presence of *A. flavus*

Different letters represent means that were statistically different at the 0.05 level.

No evidence that IAA directly affect fungal growth *in vitro* in PDA

An *in vitro* plate assay was conducted in order to assess the effect of hormone

supplementation on PDA on the growth of *A. flavus* strain NRRL 3357 (at 7 days post

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inoculation). Irrespective of the hormone concentration used, IAA did not significantly affect the growth of the fungus, relative to control plates.

Discussion

The manipulation of environment and nutrition has been shown to have significant impacts on the quantity of biomass and secondary metabolite production by fungus and optimization of this initial step is necessary to determine an ideal culture medium to evaluate the toxin production (Bode et al., 2002; Mohanty and Prakash, 2009). Therefore, small changes can have a big effect on growth and highly flexible production of aflatoxins. Similarly, media that facilitate increased fungal growth may not produce aflatoxin (Bode et al., 2002; Mohanty and Prakash, 2009).

In this study, two different culture media, CD and PDB supplemented with auxins (IAA and IBA) were used to evaluate the fungal growth of *A. flavus* strain NRRL 3357 and AFB1 production (Table 3.1). CD supplemented with low and middle concentration of IAA tended to produce more mycelium growth (even though it was not significant) whereas high concentration of IAA significantly decreased mycelium growth compared to control at 28 °C. However, at 30 °C, high IAA treatment significantly induced mycelium growth whereas no significant effect of IAA was observed at 32 °C. These results suggest that whether mycelium growth is inhibited or stimulated by IAA in CD depends on its concentration and temperature. Stimulatory and inhibitory effects of IAA on mycelium growth of *Saccharomyces cerevisiae* have been reported. At high concentrations, IAA inhibits growth, whereas at lower concentrations, it induces filamentation and adhesion (Prusty et al., 2004).

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IBA treatments in CD have no influence on mycelium growth at various temperatures. In contrast to our finding, IBA showed a growth promoting effect on several fungi such as *Nephromopsis ornata* and *Rhizopus oryzae* (Chatterjee et al., 2008b; Wang et al., 2010) Even though *A. flavus* strain NRRL 3357 produced mycelium and various IAA effects were observed on mycelium growth, it did not produce AFB1 in CD. Similarly, Mehta (2013) reported that two *A. flavus* isolates from sugar cane (SC), SC-3 and SC-5, grew but did not produce aflatoxin in CD. CD is a semisynthetic media used for the general cultivation of fungi. A number of studies reported that semisynthetic and synthetic media are unfavorable for aflatoxin production because they mainly contain precipitated salts (Adye and Mateles, 1964; Davis et al., 1966). PDA was found to be suitable media for our experiments. Toxigenic potential of *A. flavus* strain NRRL 3357 grown in PDB resulted in production of maximum mycelium as well as AFB1 (Table 3.3). Among the various liquid media, PDB has been reported to be favorable for maximum production of AFB1 (Davis et al., 1966; Mehta, 2013). Therefore, PDB was used to evaluate growth and AFB1 production in the presence of IAA. *A. flavus* strain NRRL 3357 was found to produce AFB1 at a concentration of 643 ppb in PDB witout IAA after 7 days of incubation.

Tissue culture procedures can significantly affect the stability and availability of IAA. Factors affecting IAA stability include temperature, pH, light vs. dark during incubation, and salts in the medium (Dunlap and Robacker, 1988; Nissen and Sutter, 1990). The contribution of the physical or nonbiological component of culture media such as salt concentration to the degradation pattern of several auxins has been reported (Dunlap et al., 1986). It is clearly demonstrated that in as little as 10 days of storage, the

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majority of auxin could be degraded. Thus, the time of medium preparation in relation to incubation of tissue samples was a potential source of variation (Nissen and Sutter, 1990). The actual level of a particular exogenous auxin has rarely been measured continuously over the entire length of the culturing period. Knowing the actual exogenous auxin levels over time is therefore essential to evaluate the effects on mycelium growth and AFB1 production. Consistent with the previous studies, our findings revealed that IAA was stable at lower concentration (100 ppb) in PDB for 7 days in the absence of *A. flavus* (Figure 3.1). There was significant loss in IAA at higher concentrations. The most nonbiological degradation occurred at the initial concentration of 1000 ppb. The presence of *A. flavus* in the culture (Figure 3.2) increases the degradation of IAA. The results from this study suggest the involvement of two possible mechanisms. First*, A. flavus* was able to degrade IAA *in vitro* or second, it was able to utilize IAA as a source of carbon and energy. Similarly, exogenous degradation of IAA by bacteria and fungi such as *Pseudomonas putida* and *Trichoderma atroviride* has been reported (Gravel et al., 2007; Leveau and Lindow, 2005). However, to the best of our knowledge, this is the first report of IAA degradation by *A.flavus*. Such degradation could have reduced the concentration of IAA to prevent the detrimental or inhibitory affects of IAA. However, the brief exposure to IAA prior to degradation was apparently sufficient to initiate physiological changes required for mycelium growth and AFB1 production.

Various effects of IAA on fungi have been reported. IAA was reported to affect yeast sporulation and cell elongation, but the effects of IAA were not uniform and varied according to growth conditions, such as vitamin content in the culture medium (Wang et al., 2010). As in this study, both stimulatory and inhibitory effects of IAA on mycelium

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growth of *A. flavus* were observed in CD (Table 3.1) whereas only stimulatory effect was observed in PDB (Figure 3.3). High IAA concentration greatly reduced AFB1 concentration in PDB (Figure 3.4) after 7 days. Factors influencing aflatoxin production *in vitro* have been reported. The influence of carbohydrate source, nitrogen source, and various trace elements has been studied (Davis et al., 1966). Effects of specific amino acids as sole nitrogen source on growth and aflatoxin production have also been studied. Toxin production was stimulated by proline and asparagines whereas it is inhibited by methionine and tryptophan (Payne and Hagler, 1983a). Tryptophan also serves as a precursor for IAA biosynthesis. It is highly possible that *A. flavus* may degrade IAA to its precursor that results in increased concentration of tryptophan in PDB. External application of IAA has been shown to have various effects on different fungal species, but it has been difficult to determine whether the observed phenotypes represent the physiological effects of endogenous fungal IAA (Degani et al., 2015).

The scavenging activity of different indoles has been reported. Indole-3-carbinol (I3C) and IAA are both capable of acting as a scavenger of free radicals in an *in vitro* system (Arnao et al., 1996). Dietary treatment of I3C elevated hepatic microsomal metabolism of AFB1 in male rats by increasing initial rates of AFM1 (a detoxication product of AFB1) (Stresser et al., 1994). Because of the structural similarities of I3C and IAA, it is possible that IAA may have a similar function on AFB1 degradation in *A. flavus in vitro.*

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CHAPTER IV

THE POTENTIAL ROLE OF PLANT HORMONE, AUXIN, ON *ASPERGILLUS*
FLAVUS GROWTH AND AFLATOXIN PRODUCTION

Abstract

The plant hormone auxin (indole-3-acetic acid-IAA) is central to regulation of
plant growth and defense to biotic stresses such as pathogens. Quantification of the
hormone levels can reveal different plant strategies to cope with the stress, e.g.,
suppression of growth or mobilization of plant metabolism. *Aspergillus flavus* infection
and the subsequent accumulation of aflatoxin in maize continue to be a chronic problem.
Since *A. flavus* resistance is a quantitative trait loci (QTL), gaining knowledge about the
contribution of each individual gene in the QTL is crucial. Understanding the role of IAA
in resistance to *A. flavus* infection may be critical to identify the individual genes
underlying each QTL. Determining the precise role of IAA and its regulation in the
maize-*A. flavus* interaction requires quantification of its concentration in maize lines
before and after infection. We determined IAA levels of resistant lines, Mp313E, Mp715,
and Mp719 and a susceptible line B73 before and after *A. flavus* infection and correlated
them with *A. flavus* biomass and aflatoxin accumulation. B73 had higher IAA than that of
resistant lines. *A. flavus* infection significantly lowered IAA levels, but increased
aflatoxin accumulation in B73. However, very low *A. flavus* biomass was observed in
B73, suggesting that IAA may play a role in susceptibility of B73 to aflatoxin

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accumulation, but not to *A. flavus* growth. Resistant lines exhibited lower levels of IAA. *A. flavus* infection did not have an effect on IAA levels and aflatoxin accumulation in resistant lines. Two resistant lines (Mp313 and Mp719) had the highest *A. flavus* growth.

Introduction

Aspergillus flavus infection and the subsequent accumulation of aflatoxin place a significant economic burden on food and feed industries because contaminated products are prevented from reaching the food and feed supply. This is one of the biggest challenges in food and feed industry in the Southern United States. Moreover, it has been estimated that economic loss to the maize industry due to aflatoxin damage accounts for over $190 million annually. Developing maize germplasm lines that exhibit resistance to *A. flavus* is a key step to prevent these losses. However, resistance is a highly quantitative trait, meaning that it is controlled by multiple genes that are affected by the environment in which the corn is grown. Identification of individual genes in quantitative trait loci (QTL) associated with *A. flavus* resistance is vital to develop resistant lines via Marker Assisted Selection (MAS) using gene-based markers. The search for resistance has been slow, and the development of new tools is necessary for successful identification.

Understanding the role of the phytohormone, auxin, in defense responses is vital for plant breeders to develop germplasm lines that exhibit high resistance to pathogens. The pathogen, *A. flavus,* attacks oil seed crops such as maize, *Zea mays mays,* and produces the toxin aflatoxin. Besides its critical role in different developmental processes throughout the life cycle of plants (Ghanashyam and Jain, 2009), the involvement of auxin in the network of plant-pathogen interaction has been demonstrated (Bari and Jones, 2009). Endogenous auxin levels and auxin signaling in plants responding to

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pathogens have been studied. Auxin levels increased in Arabidopsis after infection by *Xanthomonas campestris* pv. campestris (*Xcc*) and *Pseudomonas syringae* type III effector AvrRpt2 (Chen et al., 2007a; O'Donnell et al., 2003). Mutated Arabidopsis plants constitutively expressing type III effector AvrRpt2 exhibited phenotypic traits with longer primary roots, increased number of lateral roots, and increased sensitivity to exogenous auxin. Free auxin levels were also high in these plants and increased after pathogen inoculation. Pathogen infection induced auxin biosynthetic genes and repressed Aux/IAA and auxin transporter genes, suggesting that AvrRpt2 can be a virulence factor that can modulate auxin physiology to promote disease (Chen et al., 2007a). Another effector, Penetration-Specific Effector 1 (PSE1), from the oomycete *Phytophthora parasitica* enhanced susceptibility of Arabidopsis by disrupting auxin accumulation (Evangelisti et al., 2013). Similarly, a root-infecting fungal pathogen *Fusarium oxysporum* induced the transcription of auxin biosynthesis genes in Arabidopsis, and auxin signaling mutants exhibited more resistance to infection (Kidd et al., 2011). A bacterial pathogen *Xanthomonas oryzae* pv *oryzae* and a fungal pathogen *Magnaporthe grisea* induced IAA biosynthesis, which induced the production of expansins, the cell wall-loosening proteins, and made rice vulnerable to pathogens in rice (Ding et al., 2008a; Fu et al., 2011).

Even though the exact mechanism of how auxin promotes susceptibility in plants is not known, it has been suggested that auxin signaling is part of salicylic acid (SA) - mediated host defense and SA suppresses auxin signaling antagonistically (Navarro et al., 2006; Wang et al., 2007b). In contrast to these findings described previously, auxin signaling might positively regulate disease resistance. It has been demonstrated that

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repression of auxin signaling pathway enhances susceptibility of Arabidopsis to the necrotrophic fungi *Plectosphaerella cucumerina* and *Botrytis cinerea* (Llorente et al., 2008). This suggested that synergistic crosstalk between auxin and jasmonic acid (JA) occurs during defense to necrotrophic pathogens instead of antagonistic interaction between auxin and SA (Kazan and Manners, 2009). Synergetic interactions between auxin and JA have also been shown in Arabidopsis during defense responses to the necrotrophic pathogen *Alternaria brassicicola* (Qi et al., 2012).

Aspergillus flavus is an auxin producing pathogen but the role of pathogen-produced auxin or auxin produced during maize-*A. flavus* interaction is not well characterized. However, there is some evidence demonstrating that auxins in maize play a role in defense responses to *A. flavus*. Comparisons of the biological profile responses of resistant (Mp313E) and susceptible (Va35) inbred maize lines 48 hours post-*A. flavus* infection revealed that Mp313E increased ABA and auxin signaling while Va35 reduced auxin signaling (Kelley et al., 2009). Our recent high-throughput microarray analysis demonstrated that gene onthology (GO) term "response to auxin stimuli" was over-represented in the aflatoxin and *A. flavus* resistant maize genotype compared to the susceptible maize genotype. In addition, a genome-wide association study (GWAS) to identify metabolic pathways involved in maize aflatoxin accumulation resistance revealed that the most significant metabolic pathway identified was JA biosynthesis which might be working synergistically with auxin (Tang et al., 2015).

In this study, we quantified auxin in resistant and susceptible lines. We also correlated auxin levels with *A. flavus* biomass and aflatoxin accumulation. Our approach elucidated the role of auxin in *A. flavus* resistance in maize and provided important

insights into plant-pathogen interactions. This knowledge can be used for developing

maize lines and hybrids with resistance to both *A. flavus* growth and aflatoxin

accumulation.

Material and Methods

Plant Materials, inoculums, and treatments

Three resistant (Mp313E, Mp715, and Mp719) and one susceptible (B73)

germplasm lines were planted in single row plots that were 4m long and spaced 0.97 m

apart in randomized complete block design with three replications and a split plot

treatment arrangement. Plots were thinned to 20 plants after seedlings emerged. Standard

production practices were followed. Supplemental irrigation was applied as needed.

A. flavus isolate NRRL 3357 was grown on sterile corn cob grits (size 2040, Grit-

O-Cobs, Maumee, Ohio) in 500 ml flasks, each containing 50 g of grits and 100mL

sterile distilled water, and incubated at 28°C for 21 days. Conidia in each flask were

washed from the grits with 500 ml sterile distilled water containing 0.1% Tween 20 per

liter and filtered through four layers of sterile cheese cloth. Concentration of conidia was

determined with a hemacytometer and adjusted to 9×10^7 conidia per mL with sterile

distilled water. Inoculum not used immediately was stored at 4°C.

There were 3 treatments including a control. Resistant and susceptible genotypes

were inoculated with water and *A. flavus* isolate NRRL 3357, which is known to produce

aflatoxin, using a tree-marking gun fitted with a 14-gauge needle. Control plants were not

inoculated. Three plants were used for each treatment and control. Because the time of

silk emergence varied between two genotypes, all plots were not inoculated on the same

day. Seven days after silks had emerged from 50% of the plants in a plot, the primary ear

of each plant was inoculated with 3.4 ml of the conidial suspension injected underneath the husk into the side of the ear using an Indico tree-marking gun fitted with a 14-guage hypodermic needle. We harvested ears 3, 7, and 14 d after inoculation and stored at -80 °C.

IAA and aflatoxin extraction

IAA and AFB1 were extracted from the same aliquot of plant homogenate. Two grams of maize tissue from the inoculation site was extracted and ground in dry ice. Ground samples were placed into a 50 ml centrifuge tubes. Ceramic homogenizing beads and 5 ml of water were added into the tubes. Samples were homogenized in a GenoGrinder 2010 (SPEX Sample Prep, LLC, Metuchen, NJ, USA) for 1 min. Ten milliliter of 1% acetic acid in acetonitrile were added and homogenized for 1 min. A QuEChERS (Anastassiades et al., 2003) pre-packed extraction pack (containing 6 g of magnesium sulfate and 1.5 g of sodium acetate) (Agilent Technologies, Santa Clara, CA) was added into each tubes, and the solutions were homogenized immediately for another 1 min. Sample tubes were centrifuged for at least 10 min at 4,000 rpm to induce phase separation between acetonitrile and water layer. The supernatants were collected into a clean 15 ml plastic centrifuge tubes. A total of 1 ml of the clarified supernatant was filtered through a PTFE syringe filter (0.45 μm pore size, 13 mm diameter) (Fisherbrand®, Fisher Scientific, Pittsburgh, PA, USA) prior to high performance liquid chromatography / mass spectrometry (HPLC/MS) analysis.

Quantification of IAA and aflatoxin

The samples were placed in auto-sampler vials and analyzed using an Agilent 6430 LC/MS Triple Quadruple which uses electrospray ionization. A Zorbax Eclipse Plus-C18 Narrow Bore 2.1 x 50 mm, 1.8 μm column was used at a temperature of 40°C. The injection volume was 5 μl. The mobile phase consisted of 5 mM ammonium formate with 0.1% formic acid in HPLC-grade water and 5 mM ammonium formate with 0.1% formic acid in methanol. The flow rate was 0.4 ml/min. The mobile phase was on a gradient where it transitioned from 90% water to 90% methanol and then back to 90% water over the course of the 6 minute run. The total run time of the method was 9 min which included a 6 min run time and an additional 3 min post time for the system to get back to equilibrium.

The parameters for the mass spectrometer were the following: The gas temperature was 300°C while the gas flow was set to 10 L/min. The nebulizer gas pressure was set to 20 psi and the capillary voltage was 4,000V. While in MRM mode, the mass spectrometer was set to look for certain daughter ions after the AFB1 ion entered the collision cell. These transitions for AFB1 included 313.1, 285.1 with a collision energy of 21kEV, and 313.1, 241.1 with a collision energy of 41kEV. The transitions for IAA are 176.1, 130.1 and 176.1, 77.1. The fragmentor (162) and the cell accelerator (4) values were the same for all the AFB1 transitions. The retention time of the AFB1 was 3.4 minutes and IAA was 2.6 minutes. There was an eight point standard curve used for IAA including the points 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200ppb. A standard graph was prepared with AFB1 (Supelco, Bellefonte, USA) in the range of 1 to 100 ppb.

Determination of fungal biomass

Aspergillus flavus biomass was determined from the ratio of *A. flavus* DNA (ng) to maize genomic DNA (ng) in each sample using quantitative real-time polymerase chain reaction (qRT-PCR) assays. Genomic DNA was extracted from *A. flavus* culture and *A. flavus* inoculated, water inoculated and uninoculated maize tissue. Species specific primers Af2 (forward primer: 5´ATCATTACCGAGTGTAGGGTTCCT-3´; reverse primer: 5´GCCGAAGCAACTAAGGTACAGTAAA-3´; amplicon 73 bp) designed in the internal transcribed spacer 1 (ITS1) of ribosomal DNA sequence and Zmt3 (forward primer: 5´-TCCTGCTCGACAATGAGGC-3´; reverse primer: 5´-TTGGGCGCTCAATGTCAA-3´; amplicon 63 bp) amplifying maize α-tubulin, were used to quantify *A. flavus* and maize DNA, respectively. These primers were designed and reported by Mideros et al. (2009).

The Light Cycler SYBR Green I Master (Roche Applied Science, Germany) was used at 1× concentration with 2µl of sample template (≈10 ng/µl) in 10µl reaction volumes. The PCR conditions were 95°C for 10 min for initial denaturation, followed by 45 cycles of 95°C for 5 s, 59°C for 10 s, and 72°C for 5 s and a final extension at 72°C for 5 min. Also, for the purpose of biomass quantification, two standards containing both *A. flavus* DNA and maize DNA in the ratio 1:1 and 20:1 were included in each run. Both *A. flavus* and maize DNA were quantified in each biological sample and in the standards using the species specific primers Af2 and Zmt3 in separate wells in the same run. Two technical replicates were run for each biological sample and included in the same run on the same 96-well plate.

For the determination of primer efficiencies and for DNA quantification, separate standard curves for each set of primers were generated. For Zmt3, several standards with serial dilutions of maize DNA, 20, 10, 5, 2.5 and 1.25 ng/µl, and for Af2 primers, a series of mixed DNA standards containing varying concentrations of *A. flavus* DNA, 20, 10, 1, 0.1, 0.01, 0.001, 0.001 ng/µl, in a constant maize DNA concentration of 1 ng/µl were used to construct the standard curve.

Statistical Analyses

The experiment was designed as a split-plot design. Data were analyzed using the SAS General Linear Models procedure and the variance was partitioned as appropriate for a split plot arrangement of genotypes as the main plot and treatments as the subplot. Means for ear IAA levels, aflatoxin concentration, and fungal biomass were compared using Fisher's Protected Least Significant Difference (LSD) at $P = 0.05$ (Steel and Torrie, 1960).

<div align="center">

Results

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To gain insight into the function of IAA in defense response to *A. flavus* infection and subsequent aflatoxin accumulation, IAA levels, aflatoxin concentrations, and *A. flavus* biomass of resistant (Mp313E, Mp715, and Mp719) and susceptible (B73) genotypes were examined and compared after *A. flavus* inoculation.

The HPLC / MS was used to measure free IAA levels in uninoculated (control) ears to determine the basal level of auxin at 3 (17 days after pollination=DAP), 7 (21 DAP), and 14 (28DAP) days after inoculation (DAI). Indole-3-acetic acid was

successfully quantified in ears and the overall recovery of internal standard was 93% for IAA.

In the analysis of variance for IAA levels of control ears, genotypes and days were highly significant sources of variation. Interactions of genotypes with days were also significant. Mean levels of IAA for genotypes and days were highly correlated ($r^2=$ 0.98, $P < 0.0001$).

Overall IAA levels of control ears in B73, Mp719, Mp313E, and Mp715 were measured as 26.3, 21.5, 1.47, and 0.82 ppm, respectively. Susceptible genotype B73 had significantly higher IAA levels than that of the resistant genotypes Mp719, Mp313E, and Mp715. Among the resistant genotypes, the average IAA level of Mp719 was significantly higher ($\approx$ 20-fold) than that of Mp313E and Mp715. There was no significant difference observed between Mp313E and Mp715 (Figure 4.1). This suggested the potential role of IAA in resistance response. Indole-acetic-acid levels were not significantly changed from 3 DAI (16.42 ppm) to 7 DAI (14.54 ppm). However, there was significant decrease in IAA levels at 14 DAI (6.5 ppm) (Figure 4.2) which suggested the developmental regulation of IAA in corn ears.

Figure 4.1   Distribution of IAA in different maize genotypes

Different letters represent means that were statistically different at the 0.05 level.

Figure 4.2   Distribution of IAA between days

Different letters represent means that were statistically different at the 0.05 level.

Examination of IAA levels in each genotype over the time course revealed that susceptible genotype B73 had high IAA levels as 29.6, 26.67, and 22.7 ppm at 3, 7, and 14 DAI, respectively. Indole -3-acetic acid level significantly decreased at 14 DAI. Similar to B73, Mp719 had high levels of IAA at 3 and 7 DAI being 32.4 and 28.3 ppm,

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respectively. However, there was significant decrease (≈20-fold) in IAA at 14DAI.
Indole-acetic-acid levels of Mp313E were measured as 2.79, 1.05, and 0.41 ppm at 3, 7,
and 14 DAI, respectively. There was no significant change observed between the days.
Among the genotypes, Mp715 had the lowest levels of IAA with 0.9, 0.56, and 0.99 ppm
at 3, 7, and 14 DAI, respectively. Similar to Mp313E, there was no significant difference
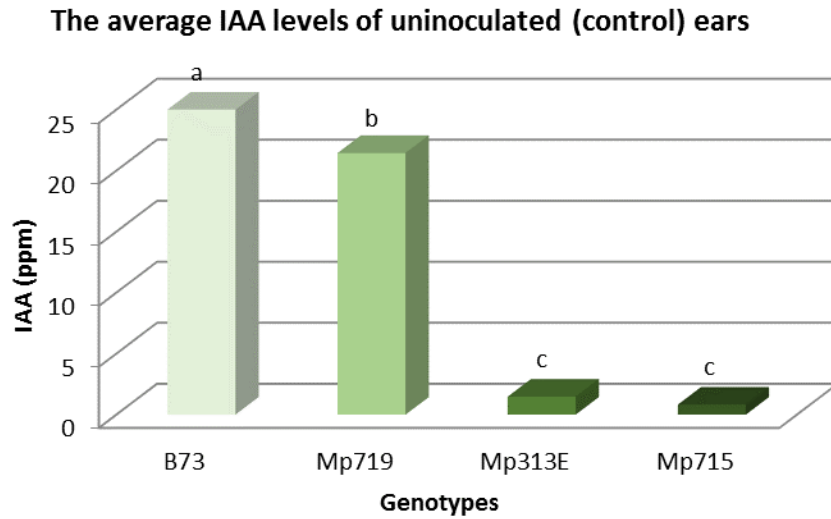between the days (Figure 4.3).
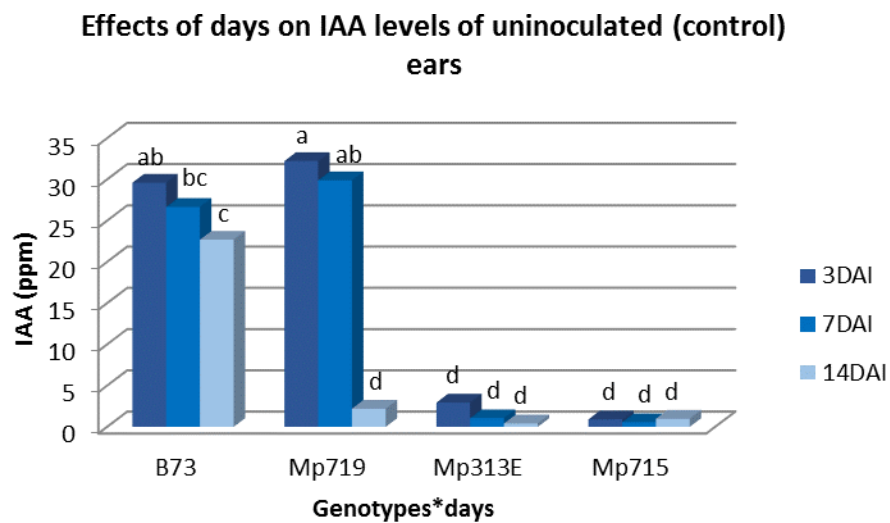
Figure 4.3   Distribution of IAA in maize genotypes at different days
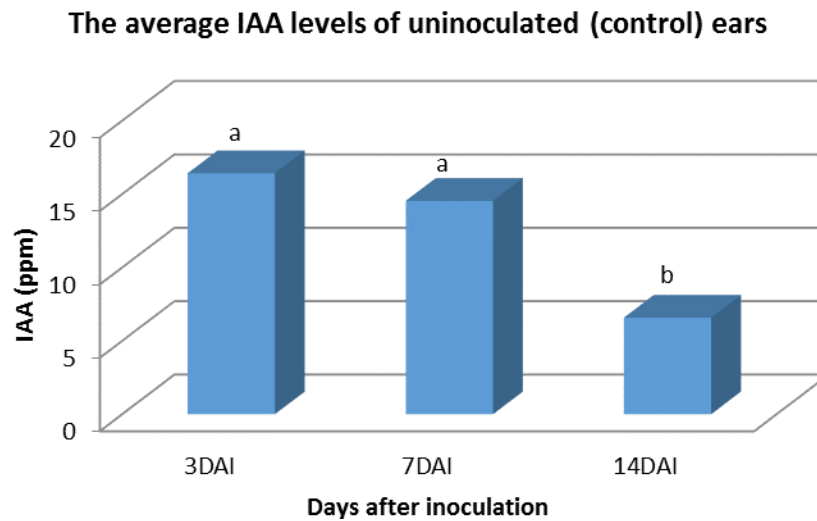Different letters represent means that were statistically different at the 0.05 level.

The alterations in host auxin physiology during *A.flavus* infection were also
investigated. To determine whether *A.flavus* was associated with a change in IAA levels
during pathogenesis, free IAA levels were measured in water and *A.flavus* inoculated ears
and compared to uninoculated control ears. Since inoculations were performed using
needles during the experiment, water inoculation was included to determine whether
wounding by the needle had an effect on IAA levels.

In the combined analysis of variance for IAA levels, genotypes, treatments, and days were highly significant sources of variation ($P < 0.0001$). Interactions of genotypes with treatments ($P = 0.0033$) and with days were also significant ($P < 0.0001$). Interactions of treatment × days and genotype × treatment × days were not significant sources of variation for IAA levels. Mean levels of IAA for genotypes inoculated with water and/or *A. flavus* and control (uninoculated) were highly correlated ($r^2 = 0.97$, $P < 0.0001$) (Table 4.1).

Overall IAA levels were measured in B73, Mp719, Mp313E, and Mp715 as 22.97, 20.81, 1.59, and 0.76 ppm, respectively. Indole-3-acetic acid levels of B73 and Mp719 were not significantly different from each other. Likewise, IAA levels in Mp313E and Mp715 were not significantly different. However, B73 and Mp719 had significantly higher (≈ 20-fold) IAA levels than that of Mp313E, and Mp715 (Figure 4.4). Overall IAA levels significantly decreased from 3 DAI to 14 DAI. Most significant decline (≈2-fold) in IAA level occurred from 7 DAI to 14 DAI (Figure 4.5).

Table 4.1   Analysis of variance for IAA and *A.flavus* biomass in maize ears

Source	Degree of freedom	Mean squares	
		IAA	*A. flavus* biomass
Reps	2	8.63	0.000056
Genotypes	3	3887.05****	0.0096****
Reps*Genotypes	6	14.4	0.00064
Treatments	2	66.15***	0.0296****
Days	7	798.02****	0.0045**
Genotypes*Treatments	6	31.17*	0.0095****
Reps*Genotypes*Treatments	16	13.92	0.0004
Genotypes*Days	6	585.6****	0.0056**
Treatments*Days	4	5.24	0.0044****
Genotypes*Treatments*Days	12	14.24	0.0055****
Error	48	8.12	0.0005
R2		0.97	0.91

*Significant at P = 0.0033 ** Significant at P = 0.0007
*** Significant at P = 0.0009 **** Significant at P < 0.0001

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The average IAA levels of fungus /water inoculated and uninoculated (control) ears

Figure 4.4   Distribution of IAA in different maize genotypes

Different letters represent means that were statistically different at the 0.05 level.

The average IAA levels of fungus/water inoculated and uninoculated (control) ears
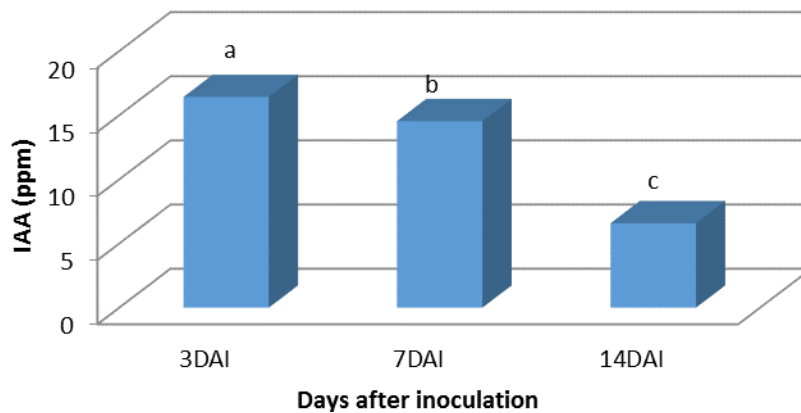
Figure 4.5   Distribution of IAA between days

Different letters represent means that were statistically different at the 0.05 level.

Examination of IAA levels after the treatments revealed that *A. flavus* inoculation significantly decreased IAA in ears compared to uninoculated (control) and water inoculated ears. There was no significant difference between control and water inoculated

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ears. These results suggested that wounding of ears by needle did not have any effects on IAA levels where as *A. flavus* inoculation had significant effect on decreasing IAA levels (Figure 4.6).
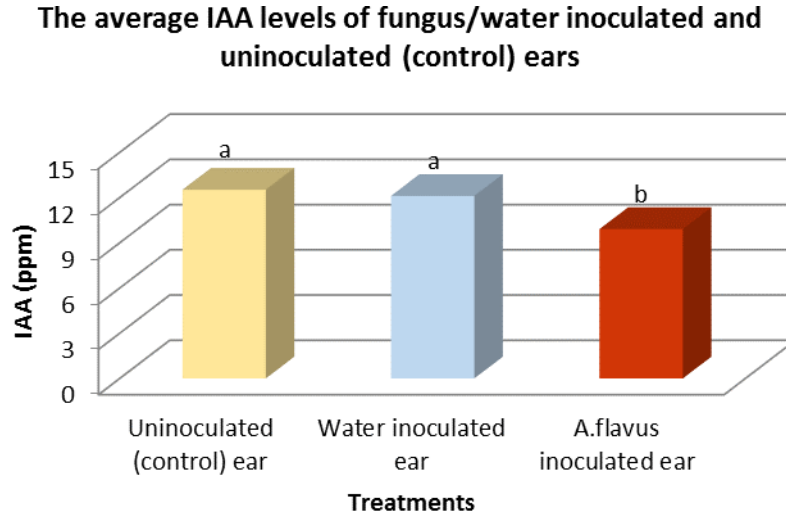
Figure 4.6   Distribution of IAA between treatments
Different letters represent means that were statistically different at the 0.05 level.

The interaction of genotype × treatment (Figure 4.7) was significant source of variation ($P < 0.0001$). Means of treatments were averaged over days in each genotype (Table 4.2). Water inoculation and *A.flavus* inoculation did not significantly affect IAA levels in Mp719, Mp313E, and Mp715. In B73, *A.flavus* inoculation significantly decreased IAA levels whereas water inoculation did not affect significantly. These results suggested that IAA may play a role in the susceptibility of B73 to *A.flavus* infection and aflatoxin accumulation. The interaction of genotype × days was also significant source of variation ($P < 0.0001$) (Figure 4.8). Means of days were averaged over the treatments in each genotype (Table 4.3). Indole-3- acetic acid levels did not significantly change at 3,

7, and 14 DAI in B73. There was significant decrease at 14 DAI in Mp719, at 7 and 14 DAI in Mp313E, and at 14 DAI in Mp715. Effect of fungus and water inoculation on IAA levels in each genotype over the time course was summarized in Figure 4.9.
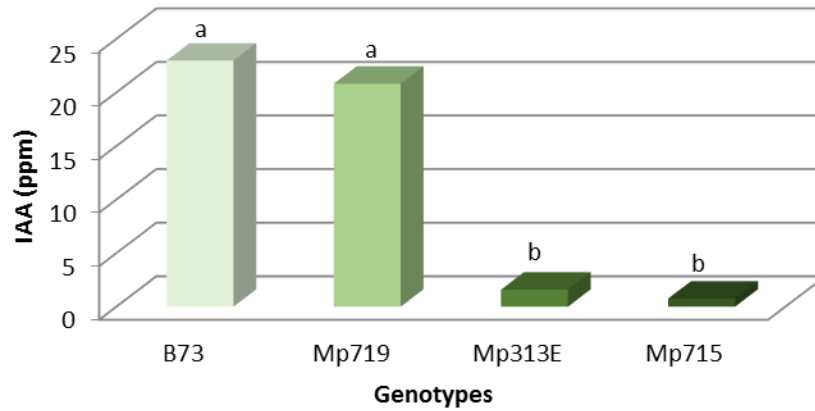
IAA distribution

Figure 4.7   Effect of treatments on IAA levels in different maize genotypes

Different letters represent means that were statistically different at the 0.05 level.

Table 4.2   Mean IAA levels (ppm) of treatments averaged over days in each genotype

Treatments	Genotypes			
	B73	Mp719	Mp313E	Mp715
Uninoculation (control)	26.32[a]	21.44[a]	1.47[a]	0.82[a]
Water inoculation	23.67[ab]	22.32[a]	1.74[a]	0.66[a]
A.flavus inoculation	18.91[b]	18.67[a]	1.54[a]	0.81[a]

Different letters following means in a column represent means that were statistically different at the 0.05 level.

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IAA distribution
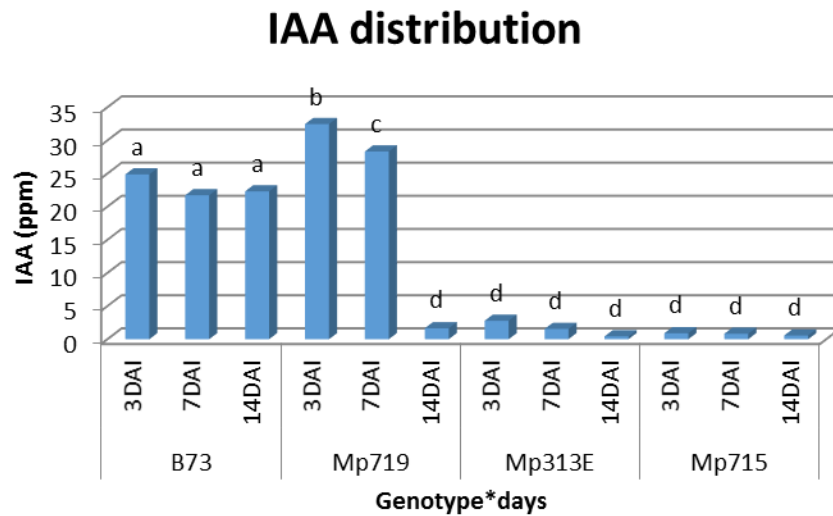
Figure 4.8   Effect of days on IAA levels in different genotypes

Different letters represent means that were statistically different at the 0.05 level

Table 4.3   Mean IAA levels (ppm) of days averaged over the treatments in each
genotype

Days After Inoculation (DAI)	Genotypes			
	B73	Mp719	Mp313E	Mp715
3	24.86^a	32.45^a	2.79^a	0.91^a
7	21.72^a	28.34^a	1.54^b	0.8^a
14	22.32^a	1.65^b	0.4^c	0.56^b

Different letters following means in a column represent means that were statistically
different at the 0.05 level.

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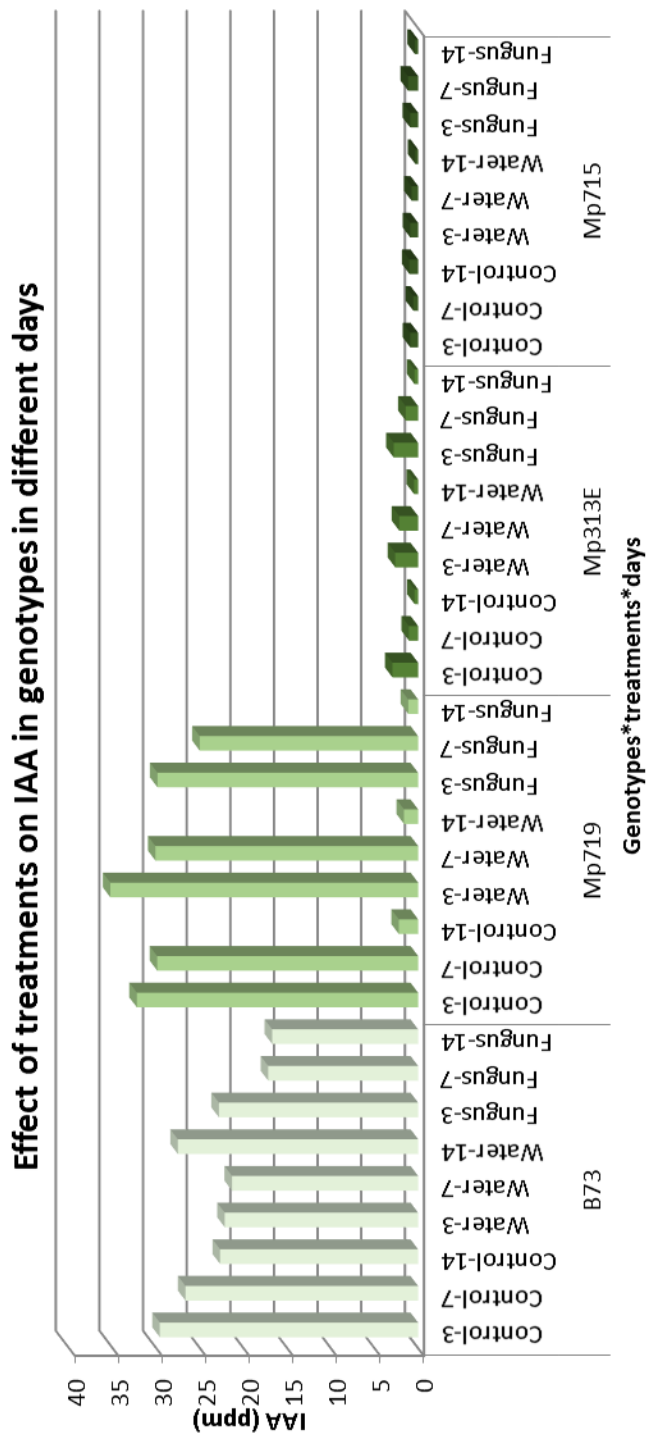
Figure 4.9   Effect of fungus and water inoculation on IAA levels in each genotype over the time course

A. flavus biomass was determined by quantitative real-time polymerase chain reaction (qRT-PCR) assays. In the combined analysis of variance for *A. flavus*/maize ratio (an indicator of *A. flavus* biomass), genotypes, treatments ($P < 0.0001$) and days were significant source of variation ($P = 0.0007$). Interactions of genotypes with treatments and with days ($P < 0.0001$), treatment × days and genotype × treatment × days ($P < 0.0001$) were also significant sources of variation for *A. flavus*/maize ratio. Mean levels of *A. flavus*/maize ratio for genotypes inoculated with water and/or *A. flavus* and control (uninoculated) were highly correlated ($r^2 = 0.91$, $P < 0.0001$).

The average *A. flavus*/maize ratios were calculated in Mp313E, Mp719, Mp715, and B73 as 437.5×10^{-4}, 144.7×10^{-4}, 71.2×10^{-4}, and 12.5×10^{-4}, respectively. *A. flavus*/maize ratio of Mp313E was significantly higher than that of other genotypes. The average *A. flavus*/maize ratios of Mp719, Mp715, and B73 were not significantly different (Figure 4.10). The average *A. flavus*/maize ratios significantly increased from 3 DAI to 7 DAI. There was no significant change from 7 DAI to 14 DAI (Figure 4.11).

Examination of *A.flavus*/maize ratios after the treatments revealed that *A. flavus* inoculation significantly increased *A.flavus* growth (from 0.6×10^{-4} to 498.2×10^{-4}) in ears compared to uninoculated (control) ears. There was no significant difference between control and water inoculated ears (Figure 4.12).
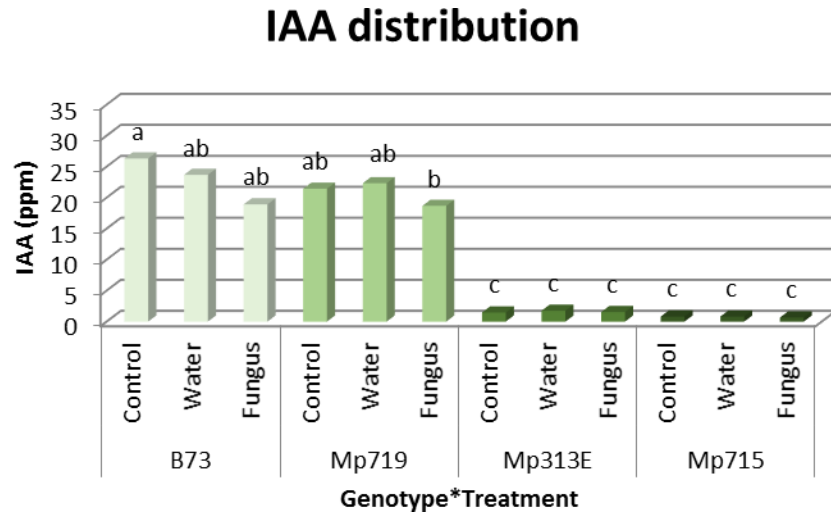
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A.flavus biomass
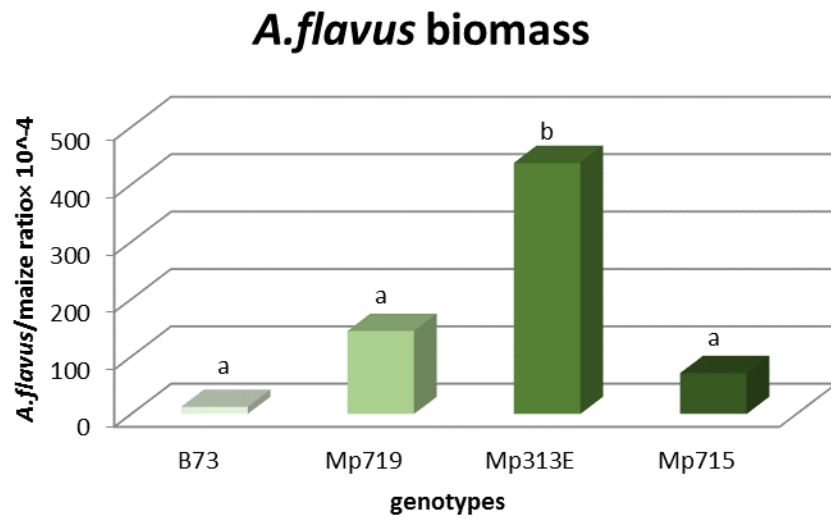
Figure 4.10 Distribution of *A.flavus*/maize ratios in different maize genotypes

Different letters represent means that were statistically different at the 0.05 level.

A.flavus biomass
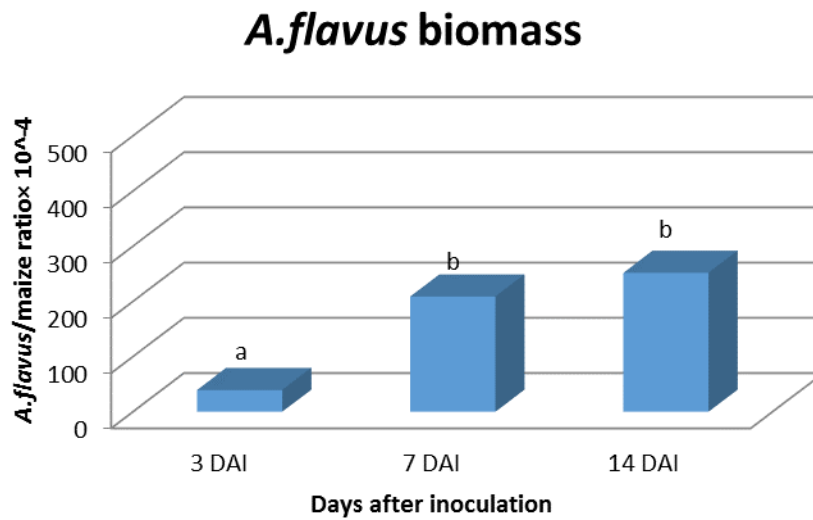
Figure 4.11 Distribution of *A.flavus*/maize ratios between days

Different letters following means in a column represent means that were statistically different at the 0.05 level.

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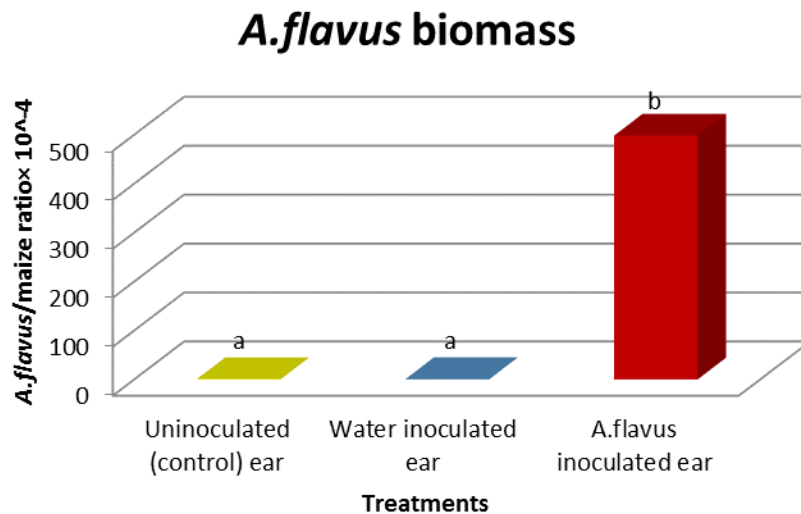
Figure 4.12 Distribution of *A.flavus*/maize ratios between treatments
Different letters represent means that were statistically different at the 0.05 level.

The interaction of genotype × treatment was significant source of variation. Mean *A.flavus*/maize ratios of treatments were averaged over days in each genotype (Table 4.4). *A. flavus* inoculation significantly increased *A.flavus*/maize ratios in all genotypes. There was no significant difference between water inoculation and control. Also, means of genotypes were averaged over *A.flavus* inoculation exhibited significant differences with days (Table 4.5). There was no significant difference between genotypes at 3DAI. At 7 DAI, Mp313E had highest level of *A.flavus*/maize ratio. Mp719 had the highest *A.flavus*/maize ratio at 14 DAI.

Table 4.4   Mean *A.flavus*/maize ratios ($\times 10^{-4}$) of treatments averaged over days in each genotype

Treatments	Genotypes			
	B73	Mp719	Mp313E	Mp715
Uninoculation (control)	0.25[a]	0.26[a]	2.0[a]	0.15[a]
Water inoculation	0.09[a]	0.63[a]	2.1[a]	0.21[a]
A.flavus inoculation	37.32[b]	433.44[b]	1308[b]	213.38[b]

Different letters following means in a column represent means that were statistically different at the 0.05 level.

Table 4.5   Mean *A.flavus*/maize ratios of genotypes inoculated with *A.flavus* at 3, 7, and 14 DAI

	A.flavus/maize ratios ($\times 10^{-4}$)		
Genotypes	3 DAI	7 DAI	14 DAI
B73	20.4[a]	418[a]	40[a]
Mp719	9.5[a]	18.9[a]	1200[b]
Mp313E	205.6[a]	2680[b]	1030[ab]
Mp715	235.4[a]	260[a]	140[a]
Mean	117.7	844.2	602.5

Different letters following means in a column represent means that were statistically different at the 0.05 level.

The analysis of variance for aflatoxin accumulation indicated that there were no significant differences between genotypes, treatments and days. Overall high aflatoxin accumulation (5 ppm) was observed only in B73 followed by Mp313E (0.03 ppm), Mp715 (0.01 ppm), and Mp719 (0.002 ppm). Aflatoxin accumulation was high (3.81 ppm) in *A.flavus* inoculated ears. There was no aflatoxin detected in water inoculated and control ears (0 ppm). Aflatoxin accumulation increased over the time course resulted in

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high concentration (3.75 ppm) at 14 DAI. Even though results were not statistically significant (Table 4.6) they were consistent with the previous findings that susceptible genotypes accumulated more aflatoxin than that of resistant genotypes.

Table 4.6   Aflatoxin accumulation (ppm) after treatments in genotypes at 3, 7, and 14 DAI

| Treatments | Genotypes | | | |
	B73	Mp719	Mp313E	Mp715
Uninoculation (control)	0.0^a	0.0^a	0.0^a	0.033^a
Water inoculation	0.0^a	0.0003^a	0.0002^a	0.0^a
A.flavus inoculation	15^a	0.006^a	0.097^a	0.0^b
Days				
3 DAI	0.01^a	0.002^a	0.0002^a	0.0008^a
7 DAI	0.14^a	0.003^a	0.037^a	0.029^a
14 DAI	14.96^a	0.0007^a	0.059^a	0.033^a

Different letters following means in a column represent means statistically different at the 0.05 level.
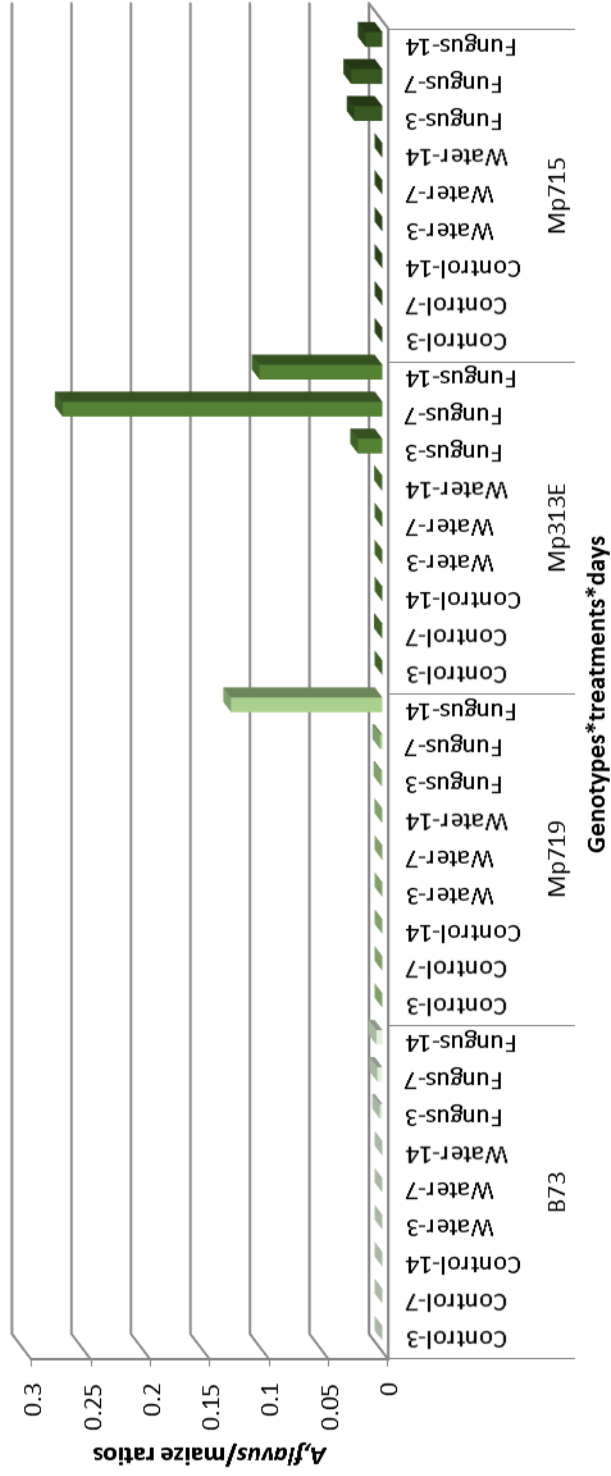
Figure 4.13 Effect of fungus and water inoculation on *A. flavus* biomass in each genotype over the time course

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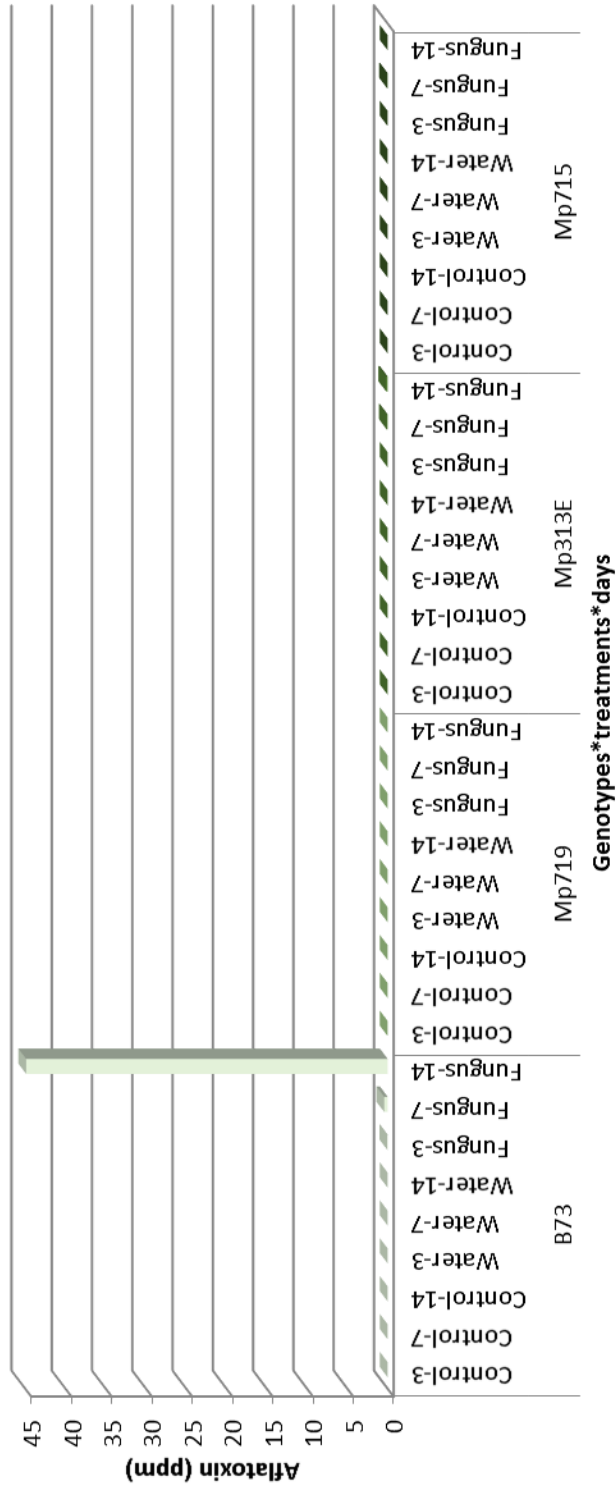
Figure 4.14   Effect of fungus and water inoculation on aflatoxin accumulation in each genotype over the time course

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Discussions

Maize kernels have been shown to be good sources of IAA (Carnes and Wright, 1988), and the temporal patterns of IAA accumulation during development of maize kernels or endosperms have been reported (Lur and Setter, 1993). Extremely rapid rates of auxin accumulation were observed between 12 and 15 days after pollination coinciding with the embryo development and starch grain initiation (Carnes and Wright, 1988). The timing of developmental events and regulatory roles of auxin were examined in maize endosperms, and an abrupt increase in auxin induced endoredupliction and expression of particular zein storage proteins (Lur and Setter, 1993). Besides having a regulatory role in developmental events, auxin has also been shown to function in defense response to various pathogens in various plants (Bari and Jones, 2009). However, very little is known about IAA involvement in the interactions between maize and *A. flavus*. To the best of our knowledge, this is the first study report of IAA involved in maize resistance to *A. flavus* infection and aflatoxin accumulation.

In this study, examination of free IAA levels of susceptible and resistant lines to determine the basal level of IAA before the infection revealed that susceptible line B73 had higher levels of IAA over the time course. Resistant lines Mp313E and Mp715 had 20-fold lower IAA levels than that of B73 (Figure 4.1). Interestingly, resistant line Mp719 had higher IAA levels at 3 and 7 DAI. However, at 14 DAI, IAA levels were abruptly decreased to the levels as low as other two resistant lines (Figure 4.3). The differences in IAA levels of susceptible and resistance lines suggested that low IAA levels might contribute to maize resistance to *A. flavus* infection and aflatoxin

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accumulation. Our results supported the idea that reductions of auxin levels enhanced plant defense responses (Chen et al., 2007a; Kidd et al., 2011; O'Donnell et al., 2003).

To understand whether IAA modulation conferred susceptibility or resistance in maize to *A. flavus* infection and aflatoxin accumulation, the effect of *A. flavus* infection on IAA levels, *A. flavus* biomass and aflatoxin accumulation was studied in B73, Mp719, Mp313E, and Mp715. Analysis of free IAA levels of maize lines after *A. flavus* infection indicated that lowered levels of endogenous plant IAA was observed during *A. flavus* inoculation in B73. Free IAA level of B73 significantly decreased from 3 DAI to 7 DAI and did not change afterwards. This may suggest that a rapid response was generated by B73 to lower the free IAA level to deal with pathogen infection. However, the decrease of IAA was insufficient and far from the IAA levels of resistant lines. Therefore, it may be concluded that high IAA is involved in the susceptibility of B73. Our results were consistent with a previous study that compared the biological profile responses of resistant (Mp313E) and susceptible (Va35) inbred maize lines 48 hours post-*A. flavus* infection. The study revealed that Mp313E increased ABA and auxin signaling while Va35 reduced auxin signaling (Kelley et al., 2009). High levels of free IAA in B73 could potentially change plant physiology to promote *A. flavus* infection. It has been shown that increased auxin levels caused cell wall loosening, increased membrane permeability, and stomatal opening (Perrot-Rechenmann, 2010), resulting in easy pathogen entry and growth. Inoculation with *A. flavus* did not cause significant local changes in the IAA levels in resistant maize lines Mp719, Mp313E and Mp715 for the duration of the experiment. This suggested that the resistant plants have already re-established auxin

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homeostasis by this stage and unusually low levels of IAA may function as a constitutive defense against *A. flavus*.

In previous studies, many pathogens have been reported to alter host auxin metabolism during infection (Chen et al., 2007a; Navarro et al., 2008; Navarro et al., 2006; Wang et al., 2007a). For example, *Pseudomonas syringae* increased free IAA levels during infection as a strategy to promote disease susceptibility in *Arabidopsis thaliana* (Chen et al., 2007a). Also, repressing auxin signaling in *Arabidopsis* conferred increased antibacterial resistant (Navarro et al., 2006). Taken together, our results suggested that the role of IAA in maize resistance *A. flavus* may differ from that observed in *Arabidopsis* and rice to various pathogens (Ding et al., 2008b; Navarro et al., 2006). First, the basal levels of IAA in resistance maize were 20-fold lower than that of susceptible line. Second, no changes were detected in the IAA levels in resistance maize lines after interaction with *A. flavus*. The relationship between IAA levels and *A. flavus* resistance support our hypothesis that in resistant lines, IAA and/or signaling might be suppressed. However, a complete genetic analysis of maize populations segregating for *A. flavus* resistance and IAA levels is required for more definite conclusions. Breeding for *A. flavus* resistance and aflatoxin accumulation over the years produced maize lines with constitutively low IAA levels. Because of the correlation of lowered IAA with *A. flavus* resistance in maize, it may be possible to use IAA levels as a biochemical marker for *A. flavus* resistance in maize breeding programs.

Plant pathogens can modulate host defense signaling and resistance mechanisms by regulating hormonal balance of the plant cells. Susceptibility or resistance to pathogens can be established by the crosstalk between hormones (Robert-Seilaniantz et

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al., 2011). It has been shown that IAA and SA work antagonistically to confer resistance in *Arabidopsis* to pathogen growth (Wang et al., 2007a). Our attempt to quantify SA in maize lines to investigate whether lower IAA levels coincided with higher SA levels failed because of the undetectable levels of SA in maize ears. Constitutively high SA levels were found in rice seedlings resistant to bacterial and fungal pathogens (Silverman et al., 1995).

   A. flavus biomass and aflatoxin accumulation were also quantified in maize lines. In this study *A. flavus*/maize ratio was used as an indicator of fungal biomass. Surprisingly, *A. flavus/*maize ratio of B73 was significantly lower than Mp313E. Mp313E had significantly higher *A. flavus*/maize ratio than Mp719 and Mp715. There was no significant difference between B73, Mp719 and Mp715. Even though it is not significantly different, the *A. flavus*/maize ratio was lower for B73 than the resistant lines (Figure 4.10). This result was not consistent with previous studies that susceptible lines accumulated more *A. flavus* growth (Mideros et al., 2009; Williams et al., 2013; Williams et al., 2011). There are two possible explanations. First, maturity may play a role in this aspect. Number of days required from planting to silking are 54, 66, 71, and, 82 for B73, Mp719, Mp313E, and Mp715, respectively. Mp313E is one of the late maturing maize lines that can have larger exposure to potentially unfavorable conditions such as higher temperatures, high humidity, greater *A. flavus* inoculums and higher insect activity. Second, differences in time course of experiments may cause this conflict. In previous experiments, quantification of *A. flavus* biomass was performed at the end of the harvesting season, approximately 63 days after mid-silk. However, in this experiment, *A. flavus* biomass was quantified at 3, 7, and 14 DAI which were relatively early compared

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to the whole season. As expected, only *A. flavus* inoculation significantly increased levels *A. flavus*/maize ratios in all maize lines (Table 4.4). A rapid significant increase was observed in the levels of *A. flavus*/maize ratios from 3 to 7 DAI. No significant change was observed from 7 to 14 DAI.

The ANOVA for aflatoxin accumulation did not reveal any significant differences in aflatoxin accumulation even though numeric values of means were different in genotypes, treatments, and days (Table 4.6). High variability in aflatoxin accumulation may have been caused by quantifying limited number of samples in each genotype. Increasing sample size/numbers may help to solve this problem. We evaluated aflatoxin accumulation based on the mean numeric value differences. Overall high aflatoxin accumulation (5 ppm) was observed only in B73 and followed by Mp313E (0.03 ppm), Mp715 (0.01 ppm), and Mp719 (0.002 ppm). Aflatoxin accumulation was high (3.81 ppm) in *A. flavus* inoculated ears. There was no aflatoxin detected in water inoculated and control ears (0 ppm). Aflatoxin accumulation increased over the time course resulted in high concentration (3.75 ppm) at 14 DAI. Even though results were not statistically significant in this study, they were consistent with the previous findings that susceptible genotypes accumulated more aflatoxin than that of resistant genotypes (Mideros et al., 2009; Williams et al., 2013; Williams et al., 2011). In contrast to low *A. flavus* growth high accumulation of aflatoxin in B73 may suggest that aflatoxin may play a role in the susceptibility. Previous studies demonstrated that mycotoxins produced by pathogens had the ability to act like effectors to alter hormone-regulated host defenses and increased the susceptibility (Desmond et al., 2008; Kazan and Lyons, 2014; Lorang et al., 2012).

CHAPTER V

SUMMARY

Aspergillus flavus is a weak fungal pathogen that affects a wide variety of agricultural crops. This fungus produces a mycotoxin known as aflatoxin that can be fatal or cause serious diseases even at very small quantities in animal and human. In the crop industry, maize is one of the most negatively affected crops by aflatoxin contamination. Economic loss in maize due to aflatoxin was estimated as a $225 million/yr in the U.S. Even though pre and post harvest prevention strategies have been very effective in preventing aflatoxin contamination, the most promising approach is to develop resistant maize lines. For years, interdisciplinary approaches such as genomics, proteomics, and transcriptomics have been used to investigate the process underlying the mechanism of maize resistance to *A. flavus* at the USDA-ARS Corn Host Plant Resistance Research Unit, Mississippi State University.

In this study, we investigated the role of a plant hormone, auxin, in resistance mechanism. Plant hormones function in every aspect of plant growth and development and they have been studied for decades. The role of hormones in plant-pathogen interaction is very intricate and has been receiving great attention lately. Several hormones such as salicylic acid, jasmonic acid and ethylene have been widely studied in plant resistance to pathogens and enlightened the mechanism in various plants. In

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contrast, the potential role of auxin in resistance has received considerably less attention due to its critical role in growth and development.

The main objectives of this research study were:

1. To determine the direct effect of exogenous auxin on *A. flavus* growth and aflatoxin production.

2. To quantify auxin levels in resistant lines Mp313E, Mp715, and Mp719 and susceptible line B73, before and after *A. flavus* infection.

3. To determine the correlation between auxin levels, *A. flavus* biomass and amount of aflatoxin.

To meet the first objective, we evaluated the capacity of the *A. flavus* isolate NRRL 3357 to grow and produce aflatoxin under different culture conditions and optimize experimental conditions for aflatoxin (AFB1) production. The manipulation of environment and nutrition has been shown to have significant impacts on the quantity of biomass and secondary metabolite production by the fungus and optimization of this initial step is necessary to determine an ideal culture medium to evaluate the toxin production (Bode et al., 2002; Mohanty and Prakash, 2009). Therefore, small changes can have a big effect on growth and highly flexible production of aflatoxins. Similarly, media that facilitate increased fungal growth may not produce aflatoxin (Bode et al., 2002; Mohanty and Prakash, 2009).

AFB1 production was determined in two different media and culture conditions (temperature) were varied to achieve maximal toxin production. Also, two different plant hormones (auxins), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) were evaluated for their effects on fungal growth and AFB1 production. Stability of IAA was determined under the experiment conditions. AFB1 and IAA were extracted directly from the cultures by QuEChERS (quick, easy, cheap, effective, rugged and safe). High

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performance liquid chromatography (HPLC) was used to identify AFB1 and IAA. It was observed that *A. flavus* isolate NRRL 3357 did not produce AFB1 when grown in Czapek dox broth (CD). Indole-3-butyric acid did not affect fungal growth under any culture conditions. Potato dextrose broth (PD) and IAA were determined to be the suitable medium and auxin for further experiment in this study. IAA was stable at low and middle concentrations for 7 days. Nonbiological destruction of IAA in culture medium was observed at high concentration during the incubation period. However, *A. flavus* isolate inoculation increased IAA degradation significantly at all concentrations. IAA significantly increased mycelium growth and significantly decreased AFB1 at a high concentration (10000 ppb) in PDB when compared to control. The knowledge gained from this study may help in utilizing aflatoxin to produce commercially important aflatoxin free maize.

To meet the second and third objectives, we determined endogenous IAA levels in resistant lines, Mp313E, Mp715, and Mp719 and susceptible line B73 before and after *A. flavus* infection and correlated them with *A. flavus* biomass and aflatoxin accumulation. Since *Aspergillus flavus* resistance is a quantitative trait loci (QTL), gaining knowledge about the contribution of each individual gene in the QTL is crucial. Understanding the role of IAA in resistance to *A. flavus* infection is critical to identify the individual genes underlying each QTL. Determining the precise role of IAA and its regulation in maize-*A. flavus* interaction requires quantification of its concentration in maize lines before and after infection. B73 had higher IAA than that of resistant lines. *A. flavus* infection significantly lowered IAA levels whereas increased aflatoxin accumulation in B73. However, very low *A. flavus* biomass was observed in B73

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suggesting that IAA may play a role in susceptibility of B73 to aflatoxin accumulation but not to *A. flavus* growth. Resistant lines exhibited lower levels of IAA. *A. flavus* infection did not have an effect on IAA levels and aflatoxin accumulation in resistant lines. Two resistant lines (Mp313 and Mp719) had higher *A. flavus* growth. A model for susceptibility/resistance for both susceptible and resistant maize lines has been proposed and it was shown in Figure 5.1.

Overall this work provides a basis for future investigation of maize hormones, including biochemical and genetic experiments that may provide key insights into maize-*A. flavus* interaction. Next step may be the quantification of IAA, SA, JA and ET in more susceptible and resistant lines to have insights into crosstalk of hormones to confer susceptibility or resistance. Quantification of *A. flavus* biomass and aflatoxin accumulation in these lines is also necessary to understand the correlation between hormones and resistance.
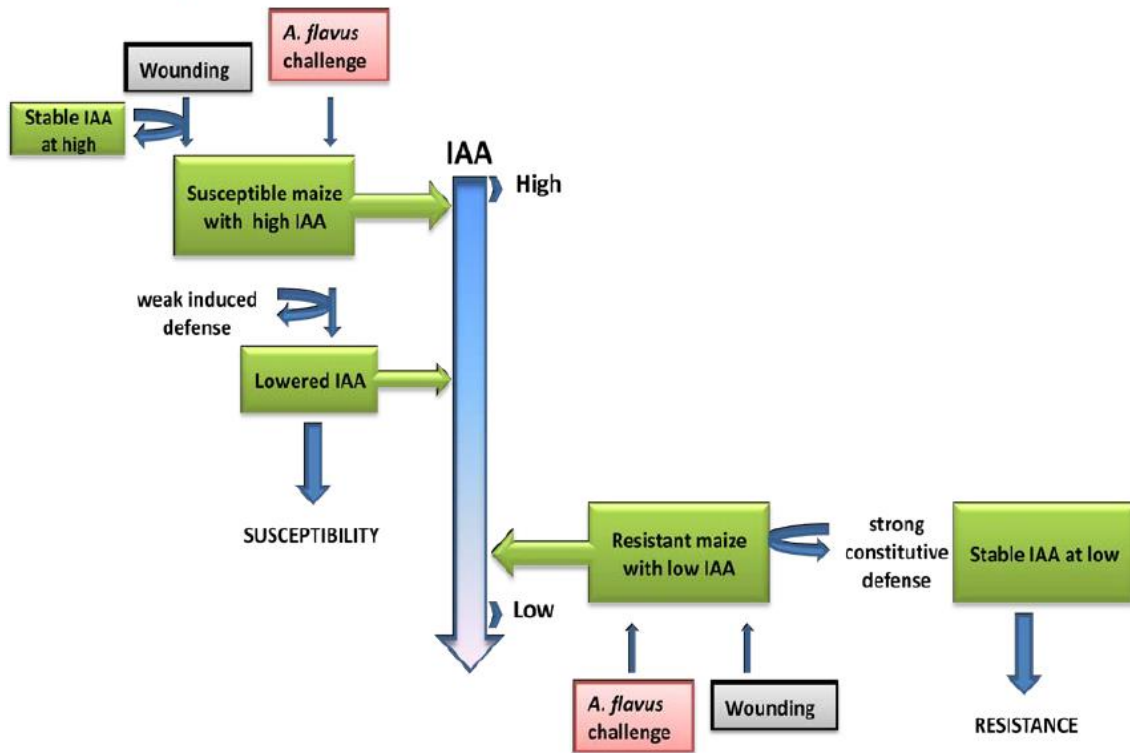
Figure 5.1   Model for susceptibility/resistance for both susceptible and resistant maize lines

A rapid response is generated by the susceptible maize line to lower the free IAA level to deal with *A. flavus* infection. However, the decrease of IAA is insufficient and far from the IAA levels of the resistant line. This may account for plants susceptibility. High levels of free IAA in the susceptible maize could potentially change the plant physiology in several ways including cell wall loosening, increased membrane permeability, and stomatal opening to promote *A. flavus* infection. *A. flavus* infection does not cause significant local changes in the IAA level in resistant maize because resistant plants already have low IAA level which confers efficient resistance. The low levels of IAA function as a strong constitutive defense in response to *A. flavus* infection.

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