Factors Affecting Pre-Harvest Aflatoxin Contamination of Peanut (*Arachis Hypogaea* L.)

by

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Abstract

Aflatoxin contamination of peanuts is one of the greatest concerns in peanut production throughout the world. Aflatoxin in peanut can be severe when plants are subjected to late season drought stress. Available carbon source in the media is proved to be another factor affecting aflatoxin production under *in vitro* studies. Immature and drought-stressed peanuts are reported to have greater carbohydrate levels than mature, non-stressed seed. Most of the research to date to understand the influence of sugars in aflatoxin production has been done to evaluate carbon sources that optimize or support aflatoxin production. However, it is important to understand how sugars in peanut seed influence the synthesis of aflatoxin. Supplemental soil calcium has also been reported to reduce aflatoxin accumulation in peanuts. This project was initiated with the following objectives: 1) to evaluate peanut sugar concentrations for the growth of and aflatoxin production by *Aspergillus flavus* and 2) to determine the effects of drought and soil calcium on aflatoxin contamination and soluble sugar (i.e., sucrose, glucose, and fructose) concentrations of peanut seed in the greenhouse and field.

For objective 1, *A. flavus* was grown in media containing different concentrations of sugars in the range that naturally occurs in peanut. Aflatoxins and mycelial dry weights were determined after defined periods of incubation. Mycelial weight and total aflatoxin produced consistently increased as sucrose concentration within the range in

peanut seed rose. No significant differences in aflatoxin content and mycelial weight were noted due to total reducing sugars.

For objective 2, peanuts were grown in soils at various levels of soil calcium and drought periods in a greenhouse experiment and two field experiments located at Wiregrass Research and Extension Center, Headland, AL and E.V. Smith Research Center, Plant Breeding Unit, Shorter, AL. The greenhouse experiment consisted of 5 soil calcium levels and 3 drought treatments, the Wiregrass experiment consisted of 2 soil calcium levels and 2 drought treatments, and the E.V. Smith experiment consisted of 2 drought treatments. Peanut seeds were analyzed for total aflatoxins (i.e., B_1, B_2, G_1, and G_2) and soluble sugars (i.e., sucrose, glucose, and fructose). In all experiments, total aflatoxin levels were significantly higher in drought-stressed compared to non-drought-stressed peanuts. Total aflatoxins significantly declined with increases in soil calcium in the greenhouse but not in the Wiregrass experiment. In the greenhouse experiment, negative correlations were also observed between aflatoxins and calcium contents of leaves, shells and kernels. In the greenhouse and E.V. Smith experiments, total soluble sugars and sucrose levels were significantly higher in drought-stressed-peanuts compared to non-drought-stressed peanuts. Further, positive correlations were observed with peanut total soluble sugars, sucrose and aflatoxins. However, no differences in the concentrations of total reducing sugars were found due to drought. In the Wiregrass experiment, no differences in peanut sugars were noted due to drought. In all experiments, soil calcium content did not influence sugar levels in peanut.

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Table of Contents

Abstract ii

Acknowledgments iv

List of Tables viii

List of Figures ix

Chapter I. Introduction and Literature Review 1

History of aflatoxins 2

Types of aflatoxins 2

Ecology and biology of aflatoxigenic fungi 3

Effects of aflatoxins 5

Regulations and economic losses 7

Methods of detection 7

Molecular basis of aflatoxin production 10

Factors affecting aflatoxin contamination 11

Peanut 17

Peanut growth process 17

Role of calcium in peanut growth/development 18

Constraints in peanut production 20

Aflatoxin contamination of peanuts 21

Chapter 2. Evaluation of sugar concentrations for growth of and aflatoxin production by *Aspergillus flavus* 39

Chapter 3. Greenhouse and field evaluation of the effects of drought and soil calcium on pre-harvest aflatoxin contamination and sugar allocation of peanut 59

Chapter 4. Summary 91

Cumulative Bibliography.....96

List of Tables

II. Evaluation of sugar concentrations for growth of and aflatoxin production by *Aspergillus flavus*

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1. Values for intercepts and slopes from individual regression lines and differences due to incubation intervals for mycelial dry weight as a function of media sucrose content: Experiment 2 57

2. Values for intercepts and slopes from individual regression lines and differences due to media adjustments for total aflatoxin content and mycelial dry weight as a function of media sucrose content: Experiment 3.....58

III. Greenhouse and field evaluation of the effects of drought and soil calcium on pre-harvest aflatoxin contamination and sugar allocation of peanut

1. Total aflatoxins, soluble sugars, and weather parameters from the period of initial drought-stress to peanut harvest: Greenhouse, E.V.Smith and Wiregrass experiments 90

List of Figures

II. Evaluation of sugar concentrations for growth of and aflatoxin production by *Aspergillus flavus*

1. Effect of sucrose (0, 2, and 4% w/v) and on total aflatoxins (A) and mycelia dry weights (B) of *Aspergillus flavus* NRRL 3357 in peptone basal medium after various periods (7, 10, 12 days) of incubation at 28˚C: Experiment 1 54

2. Effect of sucrose (0, 2.5, 3, 5, 10 and 20% w/v) on total aflatoxin (A) and mycelial dry weights (B) of *Aspergillus flavus* NRRL 3357 in peptone basal medium after various periods of (7, 12, 17 days) of incubation at 28˚C: Experiment 2 55

3. Effect of sucrose (0, 2.5, 5, 7.5 and 10% w/v) and media adjustments (CON, DIL, ADD) on total aflatoxin (A) and mycelial dry weights (B) of *Aspergillus flavus* NRRL 3357 in peptone basal medium after 14 days of incubation at 28˚C: Experiment 3 66

III. Greenhouse and field evaluation of the effects of drought and soil calcium on pre-harvest aflatoxin contamination and sugar allocation of peanut

1. Growth season (from sowing to harvest) precipitation data for Wiregrass and E.V. Smith Research and Extension Centers collected from AWIS.....83

2. Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut aflatoxin content during drought or normal greenhouse conditions 84

3. Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut leaf calcium content under greenhouse conditions 85

4. Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut shell calcium content under drought (35 and 60 DBH) or normal greenhouse conditions 86

5. Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut kernel calcium content under drought or normal greenhouse conditions 87

6. Relationship between total aflatoxin and leaf (A), shell (B) and kernel (C) contents of Georgia Green peanut under green house conditions.....88

7. Aflatoxin content of peanut cv. Tifguard grown without and with drought stress (0 and 55 DBH) at E.V. Smith and Wiregrass Research Centers, 2010. 89

I. Introduction and Literature Review

Mycotoxins are toxic metabolites produced by filamentous fungi in association with foods, animal feeds and forages that can cause health problems to humans or farm animals when ingested (Moss, 1996). Thousands of mycotoxins have been identified, but only a few hundred are associated with food, and only a handful present significant food safety issues (Murphy *et al*., 2006). Among different mycotoxigenic fungi, *Aspergillus*, *Fusarium*, and *Penicillium* are predominant (Ciegler, 1978). Mycotoxins affect almost one quarter of global food and feed output every year (Dohlman, 2004). Natural contamination with mycotoxins has been reported for many major agricultural commodities in the world including corn, wheat, rice, barley, oats, sorghum, peanuts, beans, some fruits, nuts, and various forages. Conditions that affect mycotoxin production include fungal starvation, genetic susceptibility of the host plant or commodity, moisture content, commodity composition, temperature, aeration, microbial population, and stress factors (Ciegler, 1978). When consumed, mycotoxins may affect several target organs and systems in animals, notably the liver and kidney as well as nervous, endocrine, and immune systems (Abdulkadar *et al*., 2004). Depending on the concentration and duration of exposure, and age and nutritional status of the animal, mycotoxins can cause acute, chronic and sub-chronic toxic effects in humans and animals (Wood, 1992). Mycotoxins of great concern are aflatoxins (produced by *Aspergillus*

spp.), deoxynivalenol, zearalenone, T-2 toxin, fumonisins (produced by *Fusarium* spp.), ochratoxin, and PR toxin (produced by *Penicillium* spp.) (Whitlow and Hagler, 2005).

History of aflatoxins

Aflatoxins were discovered in 1960 when more than 100,000 young turkeys died in England over the course of a few months from an apparently new disease that was termed "Turkey-X disease" (Asao *et al*., 1965). It was soon found that the mortality was not limited to turkeys. Ducklings and young pheasants were also affected. After a careful survey of the outbreaks, the disease was associated with Brazilian peanut meal. Intensive study of that peanut meal revealed its toxic nature as it produced typical symptoms of Turkey-X disease when consumed by poultry and ducklings. Study on the nature of the toxin suggested its origin from *A. flavus*. Thus, the toxin was named "aflatoxin" by virtue of its origin from *A. flavus* (Guo *et al*., 2008). This was the event which stimulated scientific interest and gave rise to modern mycotoxicology. Research on aflatoxins led to a 'golden age' of mycotoxin research during which several new mycotoxins were discovered (Bennett, 2010). Other important mycotoxins produced by *Aspergillus* include ochratoxin, patulin and fumigillin (Bennett and Klich, 2003; Cole and Cox, 1981). Among all mycotoxins and polyketide compounds synthesized by fungal species, aflatoxins continue to receive major attention and are most intensely studied. They are the most potent hepatotoxic, carcinogenic metabolites (Diener *et al*., 1987).

Types of aflatoxins

There are four major types of aflatoxins (B_1, B_2, G_1 and G_2). Aflatoxins designated B_1 and B_2 show strong blue fluorescence under UV light, whereas the G_1 and G_2 forms show greenish yellow fluorescence (Swick, 1984). Among these four, aflatoxin B_1 is best known because of its hepatocarcinogenic nature. Aflatoxin M_1 and M_2 are other significant members of the aflatoxin family and are oxidative forms of aflatoxin B_1, which are modified in the digestive tract of some animals and humans and can be isolated from milk (Squire, 1989).

Ecology and biology of aflatoxigenic fungi

Aflatoxins are produced by four species of *Aspergillus*: *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarii* (Payne, 1992). Among these four, *A. flavus* and *A. parasiticus* are the most extensively studied aflatoxigenic fungi. Dominant aflatoxins produced by *A. flavus* are B_1 and B_2, whereas *A. parasiticus* produces two additional aflatoxins G_1 and G_2 (Payne, 1998). *A. flavus* of the section Flavi is the most common species involved in pre-harvest aflatoxin contamination of crops. These fungi produce aflatoxins on various commodities, but they are a concern on corn, peanuts, cottonseed, and nuts (Cotty *et al*., 1994).

Aflatoxigenic fungi are soil-borne imperfect filamentous fungi, which are saprophytic during most of their life cycle, and grow on wide variety of substrates including decaying plant and animal debris. Two major factors that influence soil populations of these fungi are soil moisture and soil temperature. These fungi can grow at temperatures of 12 to 48°C, with optimum of 25 to 42°C, and at water potentials as low as -35 MPa (Klich *et al*., 1992). Under high soil temperatures and low moisture, which are

associated with drought stress, these fungi become highly competitive and dominant, produce abundant inoculum, and outcompete other microflora on corn, peanut, cotton, and nuts (Payne, 1998). Neither *A. flavus* nor *A. parasiticus* has a known sexual stage; they reproduce only by asexual means but undergo genetic recombination through a parasexual cycle (Payne and Brown, 1998). Morphology of the conidiophore, which bears asexual spores, is the most important taxonomic character in the identification of *Aspergillus*. Other important morphological structures used in identification are cleistothecia, hulle cells, and sclerotia (Bennett, 2010). These fungi overwinter either as mycelium or as resistant structures known as sclerotia (Hedayati *et al*., 2007). *A. flavus* type fungi are genetically and phenotypically diverse. There are of two types, L isolates producing abundant conidiophores, large sclerotia, and variable amounts of aflatoxin, while the S isolates produce abundant, small sclerotia, fewer conidiophores, and high levels of aflatoxins (Cotty, 1989).

Aflatoxigenic fungi are ubiquitous in nature and have important roles in natural ecosystems and human economy. *Aspergillus* spp. are capable of recycling starches, hemicelluloses, celluloses, pectins and other sugar polymers. Some species of *Aspergillus* degrade more refractory compounds, such as fats, oils, chitin and keratin. Maximum decomposition occurs in the presence of sufficient nitrogen, phosphorus and other essential inorganic nutrients. Foods utilized by humans and domestic animals are also good nutritional sources for *Aspergillus* spp. (Bennett, 2010).

Aflatoxins, like any other mycotoxins, are a subclass of substances which originated as a result of secondary metabolism of fungi. Unlike primary metabolites, these secondary metabolites are not essential for the growth of the fungi (Luchese and

Harrigan, 1993) but have survival functions in nature (Demain, 1986). Genes required for aflatoxin production have persisted in fungi for more than 100 million years (Cary and Ehrlich, 2006). According to Demain (1986), expression of secondary metabolite biosynthesis genes does not occur at high growth rates, which indicates that the synthesis of these metabolites occurs during growth repression.

Effects of aflatoxins

Exposure of humans to aflatoxins is difficult to avoid as the fungi grow aggressively in many foods at all stages of the food chain from field production to food storage in the home (Bennett, 2010). Aflatoxins impair child development, suppress the immune system, cause cancer, and result in death under severe acute exposure. Aflatoxins are the most potent hepatocarcinogens known. In a study by Peers *et al*. (1986) in Swaziland, a strong association between liver cancer incidence and aflatoxin intake was found. More recently, a severe outbreak of aflatoxin poisoning was reported in Kenya in 2002 (CDC, 2004) where half of the maize food samples tested had B_1 levels >20 µg/kg with 3 to 12% of samples, depending on the district, containing more than 1000 µg/kg, and some samples containing as much as 8000 µg/kg B_1. This outbreak led to a 39% incidence of death.

Alpert *et al*. (1969) surveyed the incidence of primary hepatocellular carcinoma in Uganda and determined aflatoxin levels in food samples stored for consumption between harvests. They observed a positive association between the frequency of aflatoxin contamination of food samples and liver cancer incidence. Bioassays in various species

of fish, birds, rodents, and subhuman primates revealed B_1 as a liver carcinogen (Wogan, 1999).

In 1974, unseasonal rains resulted in extensive damage to corn crops and extremely high concentrations of aflatoxins (6.3 to 15.6 mg/kg) in western India. This led to an acute toxicity to liver in humans, which was characterized by jaundice, rapidly developing ascites, portal hypertension and a high mortality rate. Dietary calculations and analysis of contaminated samples showed a correlation between aflatoxin consumption (2 to 6 mg daily over a period of one month) and illness (Krishnamachari *et al*., 1975). In a ten year follow up study in 145 men with chronic hepatitis B virus (HBV), aflatoxin increased the risk of hepatocellular carcinoma (HCC) in patients with HBV by 3.3 fold, 5.6 fold if there was a family history of HCC and 5.8 fold if concomitant exposure to hepatitis C virus (HCV) occurred (Sun *et al*., 1999).

Teratogenic effects of aflatoxin B_1 were evaluated by Wangikar *et al*. (2005) by administering aflatoxin B_1 (0.025, 0.05 and 0.1 mg/kg body weight) orally to New Zealand white rabbits during 6-18 days of gestation. Neither maternal mortality nor decrease in percent of live fetuses was observed. However, a significant reduction in mean fetal weight and anomalies such as wrist drop, enlarged eye sockets, incomplete ossification of skull bones, and bent metacarpels were noted with 0.1 mg/kg body weight oral administration. Turner *et al*. (2007) reported a strong effect of *in utero* aflatoxin exposure during second and third trimesters of pregnancy on the growth of Gambian infants in the first year of life. They reported that a reduction of maternal aflatoxin-albumin adduct level from 110 to 10 µg/kg would lead to a 0.8 kg increase in weight and 2 cm increase in height of infants in the first year of life.

Regulations and economic losses

Due to the toxic and carcinogenic nature of aflatoxins, humans and animals need to be protected from aflatoxins by limiting or regulating consumption. Since their discovery in the 1960s, regulations have been enacted in many countries to protect consumers from harmful effects of mycotoxins. The U.S Food and Drug Administration (FDA) has enforced regulatory limits on the concentrations of aflatoxins in foods and feeds that are involved in interstate commerce since 1965. According to the FDA, the maximum permissible level of aflatoxin is 20 µg/kg in all products (except milk) that are destined for human consumption (Wood, 1992). As of 2003, there are roughly 99 countries with specific regulations for mycotoxins (FAO, 2004).

Aflatoxin contamination not only affects health, but also impacts the agricultural economy through the loss of produce and time and expense associated with monitoring and decontamination (Shane, 1994). Furthermore, commodities contaminated with aflatoxin have a lower market value and cannot be exported. The Council for Agricultural Science and Technology (CAST, 2003) has estimated crop losses (corn, peanuts and wheat) in United States due to mycotoxin contamination at $932 million annually. Regulatory enforcement, testing, and other control measures add an additional $466 million annually to the losses due to aflatoxin.

Methods of detection

Aflatoxins pose food safety problems in both developed and developing countries. In developed countries aflatoxin contamination is usually monitored by expensive, highly-specific, sensitive, quantitative, and reliable methods such as enzyme-

linked immunosorbent assay (ELISA) or high-performance liquid chromatography (HPLC) (Abbas *et al*., 2004). Immunoassays combine high sensitivity and specificity. They require minimal sample preparation and contribute to high rates of sample analysis (Sekhon *et al*., 1996). Chu and Ueno (1977) developed ELISA for the first time for the detection of B_1 with a sensitivity of 0.4 to 4 µg/L sample. Sekhon *et al*. (1996) were able to improve the sensitivity of dot-ELISA from 500 to 1 pg by including an additional pre-incubation step. Commercially available ELISA kits provide relatively easy quantification of total aflatoxin concentration but do not identify individual aflatoxins in the sample. Most recent technologies for mycotoxin detection include evanescent wave technologies, lateral flow and dipstick devices, fluorescence polarization, microbead assays, electrophoretic immunoassays, and flow injection liquid bilayer assays (Maragos, 2004).

Some inexpensive cultural methods such as detection of blue fluorescence of aflatoxin B_1, yellow pigment production, and color change on exposure to ammonium hydroxide serve as alternatives for determining the presence of aflatoxigenic fungi in developing countries (Abbas *et al*., 2004). The natural fluorescence of aflatoxins arises from their oxygenated pentaheterocyclic structure (Fente *et al*., 2001). Gupta and Gopal (2002) used seven different types of solid media for growing *A. flavus* isolates and observed the reverse side of the plate for fluorescence under long-wave (366 nm) UV (ultraviolet) light in order to differentiate plates with aflatoxin from others. They described this method as a simple, rapid method needing minimum equipment. Among the different media tested, they found commercial coconut milk powder agar medium (CCMPA) as the best medium for this method of aflatoxin detection as the media highly

absorbs UV light and provides an effective background for fluorescence detection. Enhanced detection of aflatoxin production by *A. flavus* and *A. parasiticus* was observed by Fente *et al*. (2001) with the addition of β-CD (a methylated cyclodextrin derivative) to a suitable agar medium and detection under long-wavelength (365 nm) UV light. They observed the presence of a bright blue or blue-green fluorescence coincident with the production of aflatoxins. Presence of aflatoxin was confirmed by extraction of the medium with chloroform and examination of extracts by HPLC with fluorescence detection.

Lin and Dianese (1976) noted a consistent association of orange-yellow pigmentation on CCMPA medium with the production of fluorescence and the aflatoxin production by *A. flavus* and suggested the production of orange-yellow pigmentation as an alternative to the use of UV illumination for aflatoxin detection. Davis *et al*. (1987) found the CCMPA described by Lin and Dianese (1976) unsuitable as medium preparation was laborious, ingredients were not readily available, the medium did not support vigorous growth of *A. flavus* and *A. parasiticus*, and assay results were inconsistent. They modified the medium in order to make it simple, rapid and useful for large scale screening. Nine isolates of *A. flavus* and *A. parasiticus* were grown on modified coconut agar medium (containing Baker's and Tropical brand shredded coconut or unimproved Baker's Gem coconut) and observed under long-wave UV light (365nm) after 2-5 days of growth. Six toxigenic strains showed blue fluorescence indicating the presence of aflatoxin. According to Saito and Machida (1999), ammonia vapor can detect aflatoxin production by changing the color of toxigenic colonies from yellow to pink upon exposure. They introduced this rapid, sensitive method for differentiation of

aflatoxin producing and nonproducing strains of *A. flavus* and *A. parasiticus*. For this method, a single colony of the fungus was grown in the center of a Petri dish containing media suitable for aflatoxin production. The dish was inverted and one drop (0.2 ml) of concentrated 25% ammonium hydroxide solution was placed on the inside of the lid. They observed pink color from aflatoxin producing colonies and no change in the color from non-aflatoxin-producing colonies.

High performance liquid chromatography (HPLC) is a rapid method with more specificity and sensitivity for the detection of aflatoxins than cultural methods. Several reports have been published on identification and quantification of aflatoxins and other mycotoxins by HPLC (Engstrom *et al*., 1977; Pons, 1976; Stack *et al*., 1976). Aflatoxin detection by HPLC is based on their photophysical properties, such as by light absorption and fluorescence emission spectra. The B toxins are named for their blue fluorescence (425 nm), and the G toxins for their green-blue fluorescence (540 nm) under UV irradiation (Do *et al*., 2007).

Molecular basis of aflatoxin production

Enzymes and regulatory proteins for aflatoxin synthesis in *A. flavus* and *A. parasiticus* are encoded by more than two dozen clustered genes in a 66 kb region (Ehrlich *et al*., 2005; Yu *et al*., 2004). According to Cary and Ehrlich (2006), aflatoxin biosynthesis requires at least 25 enzymes and two regulatory proteins encoded by contiguous genes in a 70 kb cluster. The aflatoxin gene clusters of S and L types of *A. flavus* are > 99% identical. The *A. flavus* gene cluster is 96% identical to that of *A. parasiticus* and 91% identical to that of *A. nominus*. Aflatoxins are produced during

idiophase, after primary growth has slowed. Induction of aflatoxin biosynthesis involves the interplay of transcriptional regulatory elements and physiological factors that affect fungal metabolism. Experiments with *A. flavus*, *A. parasiticus* and *A. nidulans*, showed that *aflR* is the transcriptional regulator for the biosynthesis of aflatoxin (Payne and Brown, 1998). The altered and elevated transcription of *afl*R leads to changes in the expression of the pathway genes and high levels of aflatoxin production (Flaherty and Payne, 1997).

Factors affecting aflatoxin contamination

Substrate

Several papers have been published on the influence of identity (Abdollahi and Buchanan, 1981; Davis *et al*., 1967; Davis and Diener, 1968; Mateles and Adye, 1965) and concentration (Davis *et al*., 1966; Shih and Marth, 1974) of carbon source on the growth of and aflatoxin production by aflatoxigenic fungi under *in vitro* conditions. Mateles and Adye (1965) tested 17 carbon sources for aflatoxin production by growing *A. flavus* in a basal medium with glucose, ammonia salts and each carbon source at concentration of 5%. They found that 14 of 17 tested sources supported aflatoxin production and that sucrose, glucose, and fructose supported higher aflatoxin production than other carbon sources tested. Davis *et al.* (1967) also studied the effects of various carbon sources (each at 20% w/v concentration) on the production of aflatoxin B_1 and G_1 by *A. flavus* in a chemically defined medium. They reported mycelial growth and aflatoxin production with all carbon sources (glucose, sucrose, fructose, raffinose, mannitol, galactose) tested except lactose. High aflatoxin production was observed with

glucose, sucrose and fructose which was in agreement with Mateles and Adye (1965). In addition, Abdollahi and Buchanan (1981) tested sucrose, glucose, and fructose at concentrations of 6%, along with other compounds, for their ability to support aflatoxin biosynthesis by *A. parasiticus*. The fungus was initially plated on peptone basal medium without any test compound for 48 hours and then transferred to a replacement medium having test compounds. Aflatoxins were not detected in the media without the test compounds. Sucrose, glucose, fructose, galactose, ribose, sorbose, xylose, maltose, raffinose, and glycerol supported aflatoxin production, while lactose, α-methyl D-glucose, pyruvate, citrate, acetate, and oleic acid did not. They suggested that the synthesis of one or more of the enzymes responsible for aflatoxin formation is regulated by the availability of specific readily metabolized carbon sources. All these studies indicate the importance of carbon source, and sucrose, fructose and glucose in particular for aflatoxin production.

Davis *et al*. (1966) evaluated increasing concentrations of sucrose (0, 1, 5, 10, 15, 20, 30, and 50%) by growing *A. flavus* in 2% yeast extract medium for 6 to 8 days at 25ºC in stationary cultures; they found optimal aflatoxin accumulation at 20% sucrose content. Davis *et al*. (1966) also noted marked increases in mycelial dry weights when sucrose concentrations rose from 0 to 10% but found few differences in fungal mass with greater amounts of sucrose. Shih and Marth (1974) evaluated glucose concentrations up to 50% by growing *A. parasiticus* in a synthetic medium at 28ºC for 6 days and observed maximum aflatoxin production at 30% glucose with maximum fungal growth at 10% glucose. These studies suggest that simple sugars such as glucose, sucrose and fructose, which are also primary peanut soluble sugars, are excellent carbon sources for both

growth of the aflatoxigenic fungi and aflatoxin biosynthesis. Davis *et al*. (1966) showed that fungal growth was greatest in media containing 20% sucrose with > 3% yeast extract, while maximum aflatoxin production was observed in media containing only 2% yeast extract, which suggests that the trigger for toxin production may be a nutritional stress imposed on the fungus.

Under natural conditions, occurrence of aflatoxin is restricted to certain agricultural commodities such as corn, peanut, cottonseed and tree nuts (Cotty *et al*., 1994; Guo *et al*., 2008). Mellon *et al*. (2005) studied the utilization of substrate by *A. flavus* in inoculated whole corn kernels and found that concentrations of sucrose and raffinose in inoculated seed decreased to nearly zero after 6 days. Total seed carbohydrates in peanuts have also been shown to decrease rapidly after inoculation with aflatoxigenic fungi (Basha and Pancholy, 1986). Furthermore, atoxigenic isolates utilize seed carbohydrates more slowly than toxigenic isolates (Basha and Pacholy, 1986), which suggests that aflatoxin production requires additional carbohydrate.

From these studies, it is evident that carbohydrates play an important role in aflatoxin production. Pickett (1949) studied carbohydrate composition of Virginia bunch peanut seeds for two seasons as they developed. In the first season, the age of the peanuts were judged by appearance and it was observed that total sugars decreased from 3.6% (2.4% sucrose and 1.2% reducing sugars) in immature peanut seeds to 1.9% (1.5% sucrose and 0.4% reducing sugars) in mature peanut seeds on a dry weight basis. In the second season, composition of Virginia bunch peanuts were studied for a longer period and peanuts of different age were separated based on the date of entry of gynophores into the soil. It was observed that total sugars decreased from 9.6% (8.2% sucrose and 1.4%

reducing sugars) in immature peanuts to 4.4% (0.1% reducing sugars and 4.3% sucrose) in mature peanut seeds. Manda *et al*. (2004) reported that aflatoxins are greater in juvenile peanut seed, which have a higher sugar content compared to mature peanuts. Significant positive correlations of sucrose, glucose, and fructose with aflatoxin contents of peanuts at the time of harvest were also noticed (Manda *et al*., 2004). However, they did not look at changes in peanut sugar composition due to drought and their effect on aflatoxin production.

When subjected to drought and temperature stress similar to the conditions under which aflatoxin contamination occurs, carbohydrate content of peanut seed increases. Musingo *et al*. (1989) observed increases in total carbohydrates in drought-stressed peanuts by 3 to 5% (in jumbo market size), 5% (in medium market size), and 6% (in No. 1 market size), compared to irrigated, non-stressed peanuts. Similar results were observed by Timpa *et al.* (1986) who demonstrated 2 to 5% greater levels of carbohydrates in water-stressed cotton plants compared to irrigated samples.

Other substrate-related factors affecting aflatoxin production

Nitrogen source

Nitrogen source in culture medium affects aflatoxin production. Organic nitrogen is reported to stimulate aflatoxin synthesis (Luchese and Harrigan, 1993). Thapar (1988) reported high levels of aflatoxin B_1 (3.8 mg/L) when organic nitrogen sources such as casamino acids were supplied in a synthetic medium. When NH_4Cl and $NaNO_3$ were supplied in the media, aflatoxin levels were significantly lower than organic nitrogen sources.

Versicolorin and averufin are two intermediates in the biosynthesis of aflatoxin. Bennett *et al*. (1979) studied the effect of nitrogen sources on the growth and versicolorin production by a mutant strain *A. parasiticus* in a defined medium. Unlike nitrates, which support only mycelial growth, ammonium salts supported both growth and versicolorin production. Kachholz and Demain (1983) demonstrated that ammonium was a favored nitrogen source for production of averufin in averufin producing mutant ATCC 24551 of *A. parasiticus*, while nitrate repressed enzyme activity. According to Martin and Demain (1980), the effect of nitrogen source on secondary metabolite synthesis is mediated by repression of enzymes involved in assimilation of ammonium ions or metabolism of amino acids.

Concentration of nitrogen also affects the production of aflatoxin. In synthetic media, Shih and Marth (1974) reported maximum growth of *A. parasiticus* NRRL 2999 with 3% $(NH_4)_2SO_4$, while maximum accumulation of aflatoxin occurred at 1% $(NH_4)_2SO_4$. Thus, they concluded that a sub-optimal concentration of nitrogen for maximum growth as the optimum concentration for aflatoxin production.

Metal nutrients

Elements such as Fe, Cu, Mn, Mg, Zn and Mo are essential for the growth of fungi, and most of these elements are involved as prosthetic groups of enzymes (Fe, Cu, Zn) or as enzyme activators (Mn, Zn, Co, Fe, Ca). Among these metals, Mn, Zn, and Fe are the most important in secondary metabolite biosynthesis (Luchese and Harrigan, 1993).

Zinc is needed at a concentration greater than that required for fungal growth in order for high production of aflatoxin (Gupta *et al*., 1976). Mateles and Adye (1965) studied various concentrations of Zn (0.04 to 40 mg/L) in a basal medium containing glucose, ammonia, and salts. They reported that the minimum concentration of Zn required for the production of aflatoxin was 0.4 mg/L. Lee *et al*. (1966) tested various concentrations of Zn from 0.2 to 3 mg/L in basal medium with glucose, ammonia, and inorganic salts for aflatoxin production by *A. flavus* and observed maximum aflatoxin production at 0.8 mg/L of Zn. Jones *et al*. (1984) analyzed Zn, Mn, Fe, Cu, Cd, and aflatoxin contents of five commercial chicken feed samples. They observed a significant positive correlation between aflatoxin and Zn in the feed. Positive correlations between aflatoxin and Zn and Cu were also observed in Virginia corn (Failla *et al*., 1986). Marsh *et al*. (1975) observed a 30 to 1000 fold increase in aflatoxin production with less than a threefold increase in *A. parasiticus* mat weight when Zn concentration in the medium was evaluated from 0 to 10 mg/L.

Bennett *et al*. (1979) studied the effects of Mn and Zn on versicolorin production by a mutant strain of *A. parasiticus* blocked for aflatoxin biosynthesis in a chemically defined medium. They observed increased production of versicolorin when Mn was omitted and no detectable levels of versicolorin when Zn was omitted. Davis *et al*. (1967) also found increased aflatoxin production as well as decreased mycelial growth by *A. flavus* when Mn was omitted from culture media. In the same study, *A. flavus* growth and aflatoxin production was reduced when Mg, Zn, and Fe were omitted. Conversely, Mateles and Adye (1965) reported reduced aflatoxin production when Mn

was deleted from the medium, which was attributed to reduced growth of the *A. flavus*. Thus, previous reports on the effects of Mn on aflatoxin formation are conflicting.

Peanut

Peanut or groundnut is an annual herbaceous, self-pollinating legume crop belonging to the family Leguminosae and sub-family Papillionacea. It is native to South America and is one of the world's most popular and universal crops. It is grown in diverse environments in more than 100 countries on six continents (Nwokolo, 1996; Sharma and Mathur, 2006). In 2009, the United States was the third largest producer of peanut after China and India (USDA-FAS, 2010).

Peanut is the fifth most important oil seed crop in the world due to its 36 to 54% oil content. More than half of the global crop is grown for oil (Stalker, 1997). The seeds are also nutritious and consumed in raw and prepared foods. They contain 40-50% fat, 20-50% protein, and 10-20% carbohydrate. Seeds are rich in vitamin E, niacin, folic acid, Ca, P, Mg, Zn, Fe, riboflavin, thiamine, and K (Smith, 1950). However, peanut is a poor source of fat soluble vitamins like A, D, and K. Raw peanuts also have some anti-nutritional factors such as trypsin inhibitors and various lectins (Nwokolo, 1996). Peanut is an important source of resveratrol (Burns *et al*, 2002), which has anti-inflammatory, antioxidant, and anti-infective properties. Resveratrol has potential therapeutic effects against breast cancer, prostate cancer, and neuroblastoma (Baur *et al*, 2006).

Peanut growth process

Growth of the peanut is divided into two components (vegetative growth and reproductive growth). During the vegetative growth, stem elongation and leaf development occurs. Under optimum moisture conditions, complete vegetative growth occurs by 110 days after planting. Reproductive growth involves flowering, peg, and pod development. Flowering normally begins 25 to 35 days after planting, but peak flowering occurs 4 to 6 weeks after the appearance of the first flower (Weeks *et al*., 2000). The flower opens at night, and after pollination and fertilization, the ovary develops into a stalk-like structure called the peg, which bears the ovules in its tip, grows geotropically, and penetrates the soil (Smith, 1950). In Virginia type peanuts, the time between the appearance of flowers and the entrance of the gynophores into the soil is about 14 days (Pickett, 1949). The tip of the peg enlarges to form an underground pod, which is a potentially dehiscent but functionally indehiscent legume (Smith, 1950). After the peg enters the soil, the seed requires 30 to 60 days to mature (Weeks *et al*., 2000).

Role of calcium in peanut growth/development

Peanut yield has long been known to be substantially affected by Ca levels. Wilson and Walker (1981) conducted an experiment at the Coastal Plain Experiment Station, Tifton, GA, with six peanut cultivars and four levels of gypsum: 0, 560, 1120, and 1680 kg/ha (0, 133, 265, and 398 kg/ha of supplemental soil calcium, respectively) applied at early bloom. They did not observe differences in aflatoxins among the six cultivars or among the gypsum treatments, but greater amounts of aflatoxins (130 µg/kg) were observed in peanuts without a gypsum addition compared to with gypsum. In a greenhouse trial with three different levels of added soil calcium (0, 50 and 200 mg/kg),

an inverse relationship was observed between soil calcium level and seed invasion by *A. flavus* (Bowen *et al*., 1996a). Specifically, 80% lower aflatoxin levels were detected in peanut seed from the 200 mg/kg calcium treatment compared to seed from the 50 mg/kg soil calcium treatment. These two experiments suggested that soil calcium may play a role in peanuts for protecting against aflatoxin contamination.

Calcium plays an important role in cellular structural functions, regulating membrane permeability and strengthening cell walls (Marschner, 1986). In peanut, the Ca requirement varies with the stage of pod development and is high at the start of gynophore swelling. Deficiencies at this stage result in failure of the gynophore to expand into the pod (Hartmond *et al*., 1994). Increase in the number of empty pods and decrease in the quality of seeds was reported in calcium deficient soils (Wiersum, 1951).Bledsoe *et al*. (1949) studied absorption of radioactive calcium by the peanut fruit by growing 'Dixie' runner peanut variety in sand culture with the root and fruiting zones separated from each other. Bledsoe *et al*. (1949) observed greater absorption of labeled calcium by the gynophores, shells, and seeds of the fruit when it was applied in fruiting medium compared to when applied to rooting medium as well as a decrease in calcium absorption by young pegs after a certain period. In peanut, calcium is not translocated in the phloem to the fruit, instead it is absorbed by the developing gynophore and pod from soil. Thus, extreme deficiency of calcium in the fruiting zone completely inhibits pod formation (Smith *et al.*, 1993).

Calcium is reported to affect the water status and membrane permeability of peanut leaves (Chari *et al*., 1986). Under moisture stress conditions, the loss of water in leaves of peanut plants treated with twice the recommended level of calcium was lower

compared to leaves of plants grown in calcium deficient medium. The extent of membrane damage was also lower in leaves of plants fed with higher levels of calcium, compared to leaves of plants grown with no calcium supplementation (Chari *et al*., 1986).

Drought has been reported to affect calcium uptake by peanut kernels. Gaines and Hammons (1981) conducted an experiment with four peanut cultivars (Early Bunch, Florigiant, Florunner and Tifrun) at six locations (two at Tifton GA; two at Plains, GA; one at college station, TX; one at Suffolk, VA) in the southern US. Three out of six locations were irrigated and the six locations varied in their soil calcium levels (218 ppm to 685 ppm: calculated from Gaines and Hammons manuscript). Gaines and Hammons (1981) observed significant differences in seed calcium levels (0.06% to 0.09%) due to location and also among cultivars (0.07% to 0.08%). They observed significant positive correlations between seed calcium content and total precipitation.

Constraints in peanut production

Just like any other crop, peanut production is affected by both biotic (e.g., insects, fungi, bacteria, viruses, nematodes and weeds) and abiotic stresses (e.g., physiological and environmental stresses). On a global scale, leaf spots caused by *Cercospora arachidicola* and *Cercosporidium personatum* are the most widespread diseases (Stalker, 1997). These are also the most destructive foliar diseases for peanut in Alabama. Depending on weather conditions, annual losses due to early and late leaf spot range between 5 to 10% of Alabama's total peanut crop. Other destructive diseases for peanut in Alabama include white mold or stem rot caused by *Sclerotium rolfsii*. Common soil-borne diseases include limb rot, caused by *Rhizoctonia solani*, and Cylindrocladium

black root rot, caused by *Cylindrocladium crutalariae* (Hagan, 1998a). Peanut rust (*Puccinia arachidis*) is another production constraint which occurs sporadically on peanut in Alabama (Hagan, 1998b). Peanuts are attacked by several insects and nematodes every year. Common sucking insect pests include thrips, leaf hoppers, three-cornered alfalfa hoppers, spider mites and aphids (Weeks, 1996). Burrower bugs, white fringed beetles, southern corn rootworm, lesser cornstalk borers, wireworms and cut worms are the important soil insect pests of peanuts in Alabama (Majumdar, 2009). The most serious nematode pest of peanut is the peanut root knot nematode, *Meloidogyne arenaria* race 1 (Rich and Kinloch, 2009).

Aflatoxin contamination of peanuts

Among the different biotic stresses, contamination of peanut seed by *A. flavus* and *A. parasiticus* is one of the main factors affecting peanut seed quality. Although these fungi do not directly reduce peanut yield, they produce aflatoxins, which are potent hepatotoxic and carcinogenic metabolites. Aflatoxin contamination of peanuts is common in most peanut producing regions. Peanuts contaminated with aflatoxins are heavily monitored and regulated to ensure food safety (van Egmond, 1995). This causes significant financial losses to the producers as the contaminated lots cannot be used for consumption.

Aflatoxin contamination of peanuts mostly occurs through the direct invasion of developing peanut fruit by *A. flavus* in the soil geocarposphere and eventual contamination of the kernel with aflatoxin. However, *A. flavus* may infect the developing ovary of the seed at the tip of the peg even before it enters the soil (Diener *et al*., 1987).

Aspergillus infection in peanut occurs either pre-harvest, during pod development, or post-harvest, usually in storage. Pre-harvest contamination is the major economic problem in the peanut industry (Assis *et al.*, 2005; Holbrook *et al*., 1997). Aflatoxin contamination in peanut imposes great economic loss to the U.S peanut industry, especially in the southeast (Lamb and Sternitzke, 2001). In the United States, pre-harvest aflatoxin contamination is primarily a problem in peanuts, corn, and cotton seed. All four forms of aflatoxin (B_1, B_2, G_1, and G_2) are found in peanut.

Aspergillus infection in peanuts is conditioned by three principal characteristics: the aggressiveness of the fungus, peanut genotype susceptibility, as well as soil moisture and temperature parameters (Assis *et al*., 2005). Developing crops are often resistant to infection by aflatoxigenic fungi and subsequent aflatoxin contamination unless environmental conditions favor both fungal growth and crop susceptibility (Cotty and Jaime-Garcia, 2007).

Blankenship *et al*. (1984) conducted an experiment in six environmentally-controlled plots in which soil could be heated or cooled, and with and without irrigation. In sound mature kernels from these plots, aflatoxins were found in all drought treatments and no aflatoxin was detected in the irrigated treatment with a mean soil temperature of 23.8ºC. Greatest aflatoxin contamination was observed in the drought-heated treatment with mean soil temperature of 30.5ºC.

Cole *et al*. (1985) tested mean geocarposphere temperatures ranging from 24 to 32ºC in 2ºC increments in drought-stressed peanuts for their effect on pre-harvest aflatoxin contamination. They imposed drought stress during the later part of peanut growth cycle (47 days preceding harvest) and maintained an irrigated control with a mean

geocarposphere temperature of 25.6ºC for comparison. High amounts of aflatoxins were recorded from drought stressed treatment with 29.6ºC mean soil temperature. Mean geocarposphere temperatures of 26.3 and 27.8ºC were also reported to be conducive for aflatoxin contamination of peanuts by *A. flavus*. No aflatoxins were detected in peanuts from the irrigated control.

Sanders *et al*. (1985) conducted a field study with peanut cv. 'Florunner' to determine the duration of end-of-season drought stress necessary for pre-harvest aflatoxin contamination of peanuts by *A. flavus*. Drought stress was imposed for 20, 30, 40 and 50 days before harvest at mean geocarposphere temperatures of 28-30ºC. Aflatoxin contamination was noted in peanuts stressed for 30 (19 to 3247 µg/kg), 40 (0 to 29,876 µg/kg), and 50 (408 to 17,426 µg/kg) days before harvest. Only visibly damaged peanuts contained aflatoxins (784 µg/kg) in the 20 day drought treatment, but no aflatoxins were detected in visibly undamaged peanuts and in irrigated peanuts.

Wilson and Stansell (1983) also studied the effects of irrigation regime and drought period on peanut aflatoxin contamination by imposing drought at various stages of peanut growth. Even though year to year variation occurred, in general, greater aflatoxin contamination occurred in peanuts grown with water stress in the last 40 to 75 days before harvest. A study conducted at ICRISAT (1989) evaluated four different drought stress periods (20, 30, 40 and 50 days before harvest) with mean pod zone temperature of 29-30ºC for effects on aflatoxin contamination of peanut. Even though 30 days of drought stress allowed aflatoxin contamination, 40 and 50 days of drought stress were increasingly conducive for aflatoxin contamination while 20 days of drought before harvest was insufficient for aflatoxin contamination.

Even though damage to the kernels is not a prerequisite for aflatoxin formation, mechanically or biologically damaged peanuts are predisposed to aflatoxigenic fungal invasion and frequently contain large concentration of aflatoxins (Barnes, 1971). However, visibly undamaged peanut kernels are frequently invaded by fungi and may be contaminated with aflatoxins. In peanuts with no visible damage, colonization by *A. flavus* was inversely proportional to maturity and was greatest in peanuts grown under drought conditions with elevated geocarposphere temperatures (Sanders *et al.*, 1981).

Several studies have examined the association between damage to kernels by insects and aflatoxin contamination (Bowen *et al*, 1996b; Timper *et al*, 2004; Widstrom, 1979). Damaged and developing peanut kernels are susceptible to colonization by *Aspergillus* fungi prior to harvest (Cole *et al*., 1995). When Cole *et al*. (1985) separated drought stressed peanut kernels into six different categories (jumbo, medium, No. 1, other edible, oil stock, damaged) and tested for aflatoxins , aflatoxins ranged from 10 µg/kg in the medium to 6500 µg/kg in the damaged category.

Timper *et al*. (2004) conducted field experiments to determine whether the colonization of peanut roots and pods by *Meloidogyne arenaria* affects aflatoxin levels in peanuts subjected to drought stress. In treatments without *A. flavus* inoculum, greater concentrations of aflatoxins were observed in kernels from *M. arenaria* infested plants, but in treatments that were inoculated with *A. flavus*, aflatoxin concentrations were uniformly high and were not affected by nematode infestation. These results indicate that infection of peanut kernels by *M. arenaria* increases aflatoxin contamination when peanuts are subjected to drought stress or when the conditions are not optimal for growth and aflatoxin production by *A. flavus*.

To design strategies for reduction or elimination of pre-harvest aflatoxin contamination in peanut or any other crop, knowledge about the different factors that affect aflatoxin contamination is needed. Usually, the presence of aflatoxins in food and feed is related to climatic and other plant growth-related factors that influence fungal growth and production of these secondary metabolites. Thus, there are considerable differences between various regions of the world or different countries of the world, even within a country, relative to aflatoxin occurrence. There are also annual fluctuations in aflatoxin contamination (Kuiper-Goodman, 1995). Several scientists have studied the influence of temperature, time, moisture, stage of plant maturity, and insect damage on invasion, infection, and subsequent aflatoxin contamination of seeds by aflatoxigenic fungi, but questions remain. The goal of current research is to better understand and manage pre-harvest aflatoxin contamination in peanut. Building from the knowledge of previous literature, the objectives of current investigation were to:

1. Evaluate the effects of soluble sugar concentrations on the growth of and aflatoxin production by *Aspergillus flavus*.
2. Evaluate the effects of drought and supplemental soil calcium on aflatoxin contamination of peanut.
3. Evaluate the effects of drought and supplemental soil calcium on sugar concentrations of peanut.
4. Determine the relationship between peanut sugars and aflatoxin production.

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II. Evaluation of Sugar Concentrations for Growth of and Aflatoxin Production by *Aspergillus Flavus*

Abstract

Carbohydrate content of drought- and temperature-stressed peanut seeds is known to be higher than in non-stressed peanut seed. Glucose, fructose, and sucrose are carbohydrates that induce aflatoxin formation by *Aspergillus spp.*, and these are the primary carbohydrates in peanut seed. The objective of this study was to evaluate the effect of sugar concentrations in the range normally found in peanut seed for their effect on the growth and aflatoxin production of *A. flavus* NRRL 3357. The aflatoxigenic fungus *A. flavus* was grown in media containing 2 to 10% (w/v) soluble sugars for limited periods of time followed by analysis of toxin content by high-performance liquid chromatography (HPLC) and measurement of mycelial dry weight. Mycelial dry weight and total aflatoxin content was consistently higher with increasing sucrose concentration of the medium. However, no significant changes in mycelial dry weight and aflatoxin content were observed due to various reducing sugar levels in the medium. The effect of sugar concentration on aflatoxin production observed in this study may explain why higher pre-harvest aflatoxin contamination is found in seed produced during drought.

Introduction

Aflatoxins are highly toxic and carcinogenic mycotoxins produced as a result of secondary metabolism of four species of *Aspergillus*: *A. flavus*, *A. parasiticus*, *A. nomius,* and *A. tamarii* (Payne, 1992). Among these four species, *A. flavus* and *A. parasiticus* are the most extensively studied aflatoxigenic fungi, and *A. flavus* is the most common species involved in pre-harvest aflatoxin contamination of crops (Cary and Ehrlich, 2006). Due to their toxic and carcinogenic nature, aflatoxins are a concern throughout the world (Guo *et al*., 2008) and are regulated by many countries in order to protect consumers from harmful effects of these compounds (van Egmond *et al*., 2007). According to USDA, the maximum permissible level of aflatoxin is 20 parts per billion (ppb) in all products (except milk) destined for human consumption (Wood, 1992). The maximum permissible level of aflatoxin in milk is 0.5 ppb. Because of aflatoxins' effects on animal health, they are regulated and this has an impact on the agricultural economics associated with aflatoxin contamination (Shane, 1994).

Since their discovery in 1960, aflatoxins have been reported in several agricultural commodities (Dohlman, 2004), but they are a major concern on corn, peanuts, cottonseed, and nuts (Cotty *et al*., 1994). Aflatoxin contamination of peanut by *Aspergillus* spp. is one of the main factors affecting peanut quality (Will *et al*., 1994) and imposes great economic loss to Southeast U.S. peanut industry (Guo *et al*., 2008; Lamb and Sternitzke, 2001).

Drought stress is the major contributing factor to pre-harvest aflatoxin contamination (Guo *et al*., 2008). High temperatures and low atmospheric humidity associated with drought stress favor the growth of *A. flavus* while suppressing the growth of other microbes and giving a competitive advantage to *A. flavus* (Bhatnagar *et al*.,

2000). During drought and temperature stress, similar to the conditions under which aflatoxin contamination becomes a problem, carbohydrate content of peanut seed increases (Musingo *et al.*, 1989).

Aflatoxin contamination occurs more often in immature seed, which has high sugar content than mature seed (Manda *et al*., 2004). Pickett (1949) reported that total sugar content of Virginia type peanuts ranged from 3.6% (2.4% sucrose and 1.2% reducing sugars) to 9.6% (8.2% sucrose and 1.4% reducing sugars) dry weight (DW) in immature sound seed compared to 1.9% (1.5% sucrose and 0.4% reducing sugars) and 4.4% (4.3% sucrose and 0.1% reducing sugars) DW in mature seed over a two year study. Pattee *et al*. (2000) reported total sugar composition of Virginia and runner-type peanuts as 3.5% (3.2% sucrose and 0.012% reducing sugars) and 2.9% (2.6% sucrose and 0.007% reducing sugars) DW, respectively. Thus, total sugars in peanut range from approximately 2 to 10% DW. Manda *et al.* (2004) found significant positive correlations between peanut aflatoxin levels and sucrose, fructose, and glucose content at harvest.

Carbohydrate content in developing or drought-stressed peanut seed may play a crucial role in the production of aflatoxin. Therefore, the objective was to evaluate the effect of various sugar concentrations, in ranges which occur in peanut seed under normal irrigation and drought conditions, on the growth and aflatoxin production by *A. flavus* under *in vitro* conditions.

Materials and Methods

Fungal strain and cultural conditions: Strain NRRL 3357 of *A. flavus* was obtained from Microbial Genomics and Bioprocessing Unit, National Center for

Agricultural Utilization Research, USDA, Peoria, IL. The fungus was grown in peptone basal media (peptone 5 g, KH_2PO_4 1 g, NaCl 30 g, $MgSO_4 \cdot 7H_20$ 0.5 g, distilled water 1 L) containing different concentrations of glucose, fructose, and sucrose (described below). Fungal inoculation to the medium was done by collecting conidia from one week old *A. flavus* culture, enumerating, adjusting the conidial suspension with sterile water, and inoculating 1 ml to the medium. Flasks of inoculated media were incubated at 28ºC for specified time periods (described below). Fungal mats were separated by filtration, and media samples were assayed for aflatoxin using high-performance liquid chromatography (HPLC).

Analysis of aflatoxins: Aflatoxins were analyzed by HPLC as described by Wilson and Romer (1991) with slight modifications. The details of HPLC equipment are as follows: Nova-Pak C18 column (8x100 mm, 4 µm particle size, Waters, Milford, MA); flow rate, 1 ml min^{-1}; mobile phase, water/methanol/1-butanol (65.3:33.6:1.2 v/v); scanning fluorescence detector with 480 nm excitation wavelength and 540 nm emission wave-length. Aflatoxin extraction from the media was done with the following procedure: medium was filtered through Whatman No. 1 filter paper (GE Healthcare Inc., UK), the mycelial mat was collected and dried at 55ºC for 48 hours, and dry weights were recorded. One ml of the filtrate was added to 2 ml of 90% acetonitrile, and incubated for one hour at room temperature. After one hour, the solution was purified by passing through a Mycosep Multifunctional Cleanup Column (Romer Labs, Inc., Washington, MO). Two hundred µl of the purified solution was added to 700 µl derivatizing agent (water/trifluoroacetic acid/acetic acid; 70:20:10 v/v) and incubated at 55ºC for 30 minutes before injection into HPLC. Aflatoxins were quantified by

comparing the results with aflatoxin standards (Aflatoxin B and G mixture, Sigma Chemical Co. MO, USA) using a standard curve.

Preliminary work: Three trials were initially conducted in order to determine an appropriate inoculum dose. From these trials we found that 10^5 conidia inoculated into 25 ml media allowed detectable levels of aflatoxins.

Effects of reducing sugars and sucrose (Experiment 1). Three levels (0, 0.1, and 0.3% (w/v)) of reducing sugars (equal parts glucose and fructose), three levels of sucrose (0, 2, and 4%), and three incubation periods (7, 10 and 13 days) were evaluated. These were combined in a factorial experiment for a total of 27 treatments and 2 replications. The amount of media in each flask was 25 ml. Each of the flasks was inoculated with a conidial suspension of 10^5 and incubated at 28ºC with continuous agitation. Following removal of mycelial mats after each incubation interval, media in each flask were assayed for aflatoxins using HPLC. Mycelial mats were dried and weighed for total biomass. Total aflatoxins (sum of B_1, B_2, G_1, and G_2) and mycelial dry weights reported are reported on a per flask basis.

Effects of sucrose and incubation period (Experiment 2). Six levels of sucrose (0, 2.5, 3, 5, 10, and 20% w/v) were evaluated after each of three incubation periods (7, 12, and 17 days). Each treatment (sucrose level and incubation period) was replicated four times. Similar to experiment 1, each flask with 25 ml media was inoculated with 1 ml spore suspension (10^5 conidia) and incubated at 28ºC with continuous agitation. At each incubation period, four flasks were harvested by removing and weighing mycelial mats and analyzing aflatoxins as described above. Similar to

experiment 1, total aflatoxins (sum of B_1, B_2, G_1, and G_2) and mycelial dry weights were reported on a per flask basis.

Effects of sucrose and media modification (Experiment 3). A factorial experiment was carried out using five levels of sucrose (0, 2.5, 5, 7.5, and 10% w/v) each with 0.4% reducing sugars (equal parts glucose and fructose) and three types of media adjustments/treatments. Media with each level of sucrose were distributed into nine flasks (three for each media modification) with 100 ml per flask. Flasks, which were inoculated with 4×10^5 conidia and incubated at 28ºC, were shaken for one hour each morning and evening. Every 48 hours, 10 ml of media were removed from each flask. Flasks from the continuous (CON) media treatment were replaced in the incubator after removing the 10 ml. For the dilution (DIL) media treatment, 10 ml of sterile water was added to flasks after 10 ml were removed. In the addition (ADD) treatment, 10 ml of sugar solution, with same carbohydrate content as the original solution, was added to each flask after removing the 10 ml. The same procedure was repeated for 14 days. Collection of 10 ml from each flask and addition of water (DIL) and sugar solution (ADD) were done merely to adjust the sucrose concentrations of media. At 14 days of incubation, media remaining in the flasks were evaluated for aflatoxins and mycelial dry weights were determined. Total aflatoxin (sum of B_1, B_2, G_1, and G_2) per 25 ml of the media is reported.

Data analysis: Total aflatoxin and mycelial dry weight were analyzed by regression analysis using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) for each experiment. Regression analysis was carried out using total aflatoxin and mycelial weight data as well as aflatoxins per gram of mycelium for the second and third experiments as dependent variables and sugar levels (sucrose and total reducing sugars) as independent

variables. Regression models were considered significant if $P < 0.05$. In addition, intercepts and slopes of the regression equations in each experiment were tested for differences due to incubation intervals, and for media adjustments in experiment 3, by calculating confidence intervals. If confidence intervals did not include zero, model parameters (intercepts, slopes) were considered significantly different. If zero was included in the confidence intervals for both parameters, the models were considered similar and data were combined for determination of a single regression equation.

Results

Experiment 1. No differences in total aflatoxins due to total reducing sugars (P=0.42) nor due to the interaction of sucrose and total reducing sugars (P=0.29) were noted. Intercepts and slopes of regression equations for total aflatoxins among the three incubation periods did not differ significantly. Thus, data from all incubation intervals and levels of reducing sugars were combined for calculation a single regression equation. Aflatoxins significantly increased as sucrose concentrations rose from 0 to 4% (w/v) (P=0.0007) over all incubation intervals and reducing sugar levels (Fig. 1A). Similar to the effect that sucrose had on aflatoxins, no differences were observed in mycelial dry weights due to the levels of reducing sugars (P=0.58) nor the interaction of sucrose and reducing sugars (P=0.19). Slopes and intercepts of mycelial dry weight regression equations did not differ with incubation interval. Thus, mycelial dry weight data from all incubation intervals and levels of reducing sugars were combined. The combined regression equation showed a significant increase in mycelial dry weight as sucrose concentrations rose from 0 to 4% (w/v) (P <0.0001) (Fig. 1B).

Experiment 2. Slopes and intercepts of the regression lines describing total aflatoxins due to sucrose levels from each of the three incubation intervals tested (7, 12, and 17 DAI) did not significantly differ. When total aflatoxin data from all incubation intervals were combined, higher levels of total aflatoxins were observed with increasing sucrose concentration of the media with optimum aflatoxin (105 µg/flask) at 15% sucrose (P=0.0001) (Fig. 2A).

With respect to mycelial dry weight, slopes and intercepts of regression models for mycelial dry weight between 7 and 17 DAI were similar. However, intercepts for the 7 and 17 DAI models, and the slope for the 7 DAI model, significantly differed from the 12 DAI regression model (Table 1). Thus, mycelial dry weight data from 7 and 17 DAI were combined into one regression model and compared with the 12 DAI regression model. The intercept was higher and slope was lower at 12 DAI compared to 7 and 17 DAI. Similar to experiment 1, mycelial dry weights were positively related to sucrose concentrations of the media (Fig. 2B). Regression analysis of data on toxin per gram of mycelium with increasing levels of sucrose resulted in models that were not significant at various intervals tested (7 DAI: P= 0.12; 12 DAI: P=0.18; 17 DAI: P=0.22). This indicates that slope due to sucrose did not differ from zero.

Experiment 3. Total aflatoxins were higher with increasing sucrose in all media adjustments (Fig. 3A). Intercepts of regression models did not differ significantly among media adjustments; however, the slope of line for the ADD treatment was significantly different from both CON and DIL treatment slopes. There were no significant differences between slopes for the CON and DIL treatments (Table 2). Thus, total aflatoxins from CON and DIL were combined into one regression model and compared with the ADD

treatment model. It was found that the slope of CON and DIL model was higher than that of the ADD treatment.

Similar to the other two experiments, mycelial dry weights were significantly higher with increasing sucrose levels (Fig. 3B). Intercepts of the three regression models did not differ. There were no differences in CON and ADD treatment slopes, but both of these were significantly different from the DIL treatment. The higher value for the slope of the model demonstrates more mycelial growth with CON and ADD treatments than the DIL treatment. Regression analysis of data on toxin per gram of mycelium with increasing sucrose levels resulted in models that were not significant due to various media adjustments (CON treatment: P= 0.094; DIL treatment: P=0.32; ADD treatment: P=0.092).

Discussion

Total aflatoxin and mycelial dry weight did not differ due to differences in the concentration of total reducing sugars in the range evaluated. This might be because of the low concentrations (0.1 to 0.4% of equal parts glucose plus fructose) used in our tests compared to other *in vitro* studies that used 15% glucose (Davis and Diener, 1968) and 6% glucose and 6% fructose (Abdollahi and Buchanan, 1981). The relatively low levels of total reducing sugars that were selected for this study were based on concentrations found in peanut seed (Pickett, 1949; Ross and Kvien, 1989). With respect to the growth of aflatoxigenic fungi, Shih and Marth (1974) reported maximum growth of *A. parasiticus* with 10% glucose in a synthetic growth medium, but no report was found on the effect of fructose concentration on growth of *A. flavus* or *A. parasiticus*.

In all three experiments, aflatoxins and mycelial dry weights increased with sucrose concentration. Any increase in sucrose, such as from 2% to 4% (Experiment 1) or 5 to 10% as reported by Pickett (1949) in peanuts, resulted in increased aflatoxin levels. These results are similar to Davis *et al*. (1966) who evaluated increasing concentrations of sucrose (0, 1, 5, 10, 15, 20, 30, and 50%) in 2% yeast extract medium and noted increasing aflatoxin production with increasing sucrose concentration, with optimal accumulation at 20% sucrose content. With reference to mycelial dry weights, Davis *et al*. (1966) noted marked increases in mycelial dry weight with increases in sucrose concentration from 0 to 10% with little difference in fungal mass with greater amounts of sucrose.

There were no significant differences in total aflatoxin content due to incubation intervals between 7 and 17 DAI. This result is supported by Davis *et al.* (1966) who tested the influence of incubation time (2, 3, 5, 7, 12, 15, and 18 days) on aflatoxin production by *A. flavus*. They found maximal aflatoxin (7.3 mg/L medium) at 5 DAI and this remained constant for 7 and 12 DAI after which aflatoxin concentration declined. Similar to aflatoxin accumulation in our first experiment, mycelial dry weight differed due to increases in sucrose concentration but not due to different intervals tested (7, 10, and 13 days). This indicates that mycelia showed significant growth up to 7 days, after which little change occurred. In the second experiment, mycelial weight was higher at 12 DAI compared to 7 and 17 DAI indicating a decrease in mycelial weight from 12 to 17 DAI. Davis *et al*. (1966) also observed increases in mycelial dry weight in yeast extract sucrose medium (YES) with 20% sucrose for up to 12 DAI after which a decrease was observed (4.2 g/100 ml at 12 DAI to 3.8 g/100 ml at 15 DAI).

In the third experiment, higher levels of aflatoxins were obtained in treatments in which media were diluted (DIL) or not adjusted (CON) compared to treatments with sucrose additions (ADD). However, mycelial dry weights were higher in CON and ADD treatments compared to DIL treatment. Results are in agreement with Shih and Marth (1974) who found that aflatoxin production is maximal in conditions that are suboptimal for fungal growth (i.e., lower sucrose concentrations). In an *in vitro* study, Davis *et al*. (1966) obtained greater yields of aflatoxin with 2% yeast extract compared to 3% or 5% yeast extract when evaluated in a 20% sucrose medium with 8 days of incubation. It may be that the lower amounts of yeast extract were inadequate to support metabolic activities of the fungus for the duration of the study, and these suboptimal conditions supported greater aflatoxin production.

In experiments 2 and 3, there were no significant differences in toxin per gram of mycelium at various incubation intervals (Experiment 2) or with media adjustments (Experiment 3), even though significant increases in total aflatoxins as well as mycelial dry weight were observed. Thus, we can attribute significant increases in total aflatoxins with increases in sucrose concentrations to increases in mycelial dry weights.

In conclusion, the results of this study indicate that at sucrose concentrations found in peanuts, increase in sucrose enhances the growth of aflatoxigenic fungi and the subsequent production of aflatoxin. Since sucrose concentrations are lower in non-drought-stressed peanut seed compared to drought-stressed seed, carbohydrate accumulation might be a factor contributing to aflatoxin contamination in peanuts grown under drought stress.

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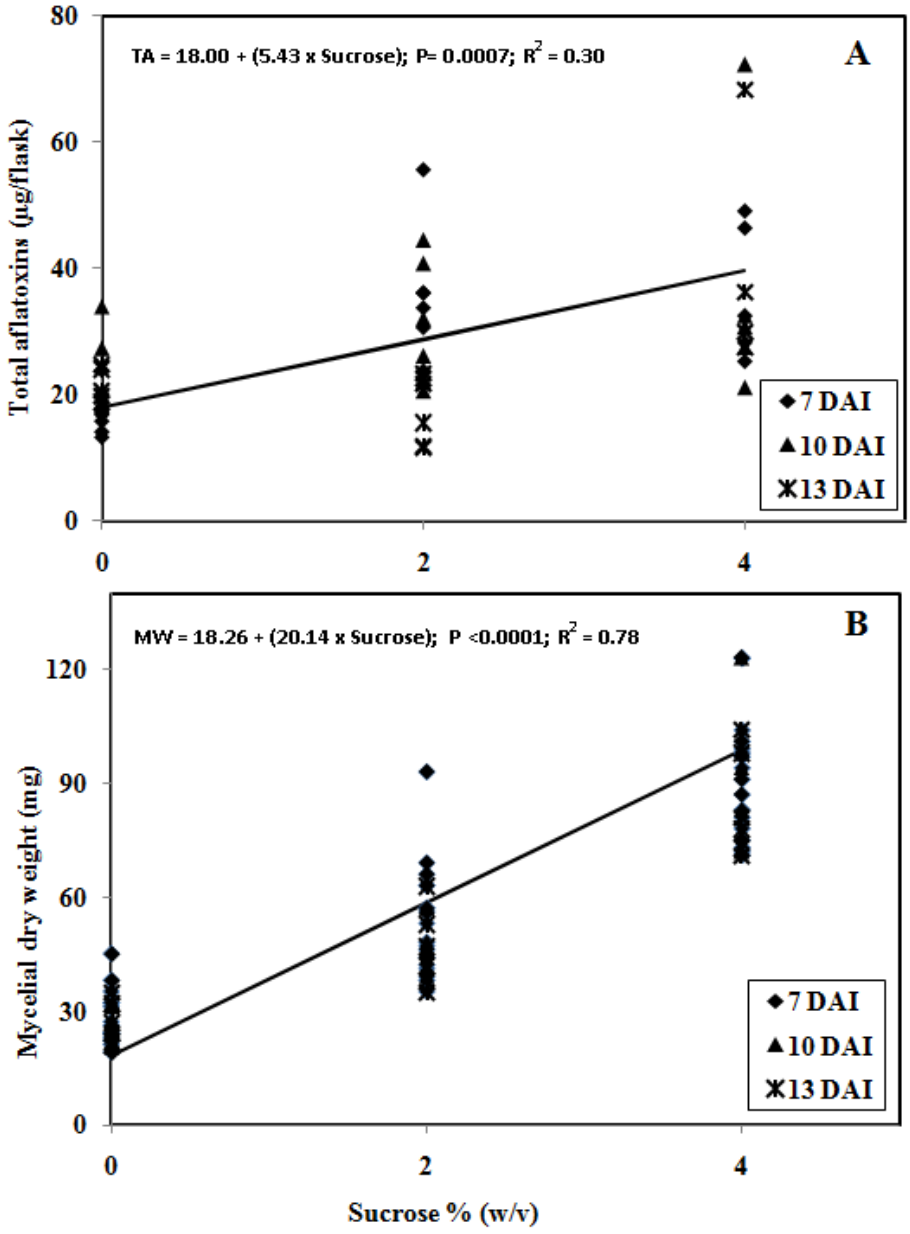

Figure 1: Effect of sucrose (0, 2, and 4% w/v) on total aflatoxins (A) and mycelial dry weights (B) of *Aspergillus flavus* NRRL 3357 in peptone basal medium after various periods (7, 10 and 13 days) of incubation at 28˚C: Experiment 1. Total aflatoxins and mycelial dry weights data from all the incubation intervals were combined into single regression model as there were no significant differences between the intercepts and slopes. DAI: days after incubation. TA: Total aflatoxins (µg/flask). MW: Mycelial dry weight (mg)

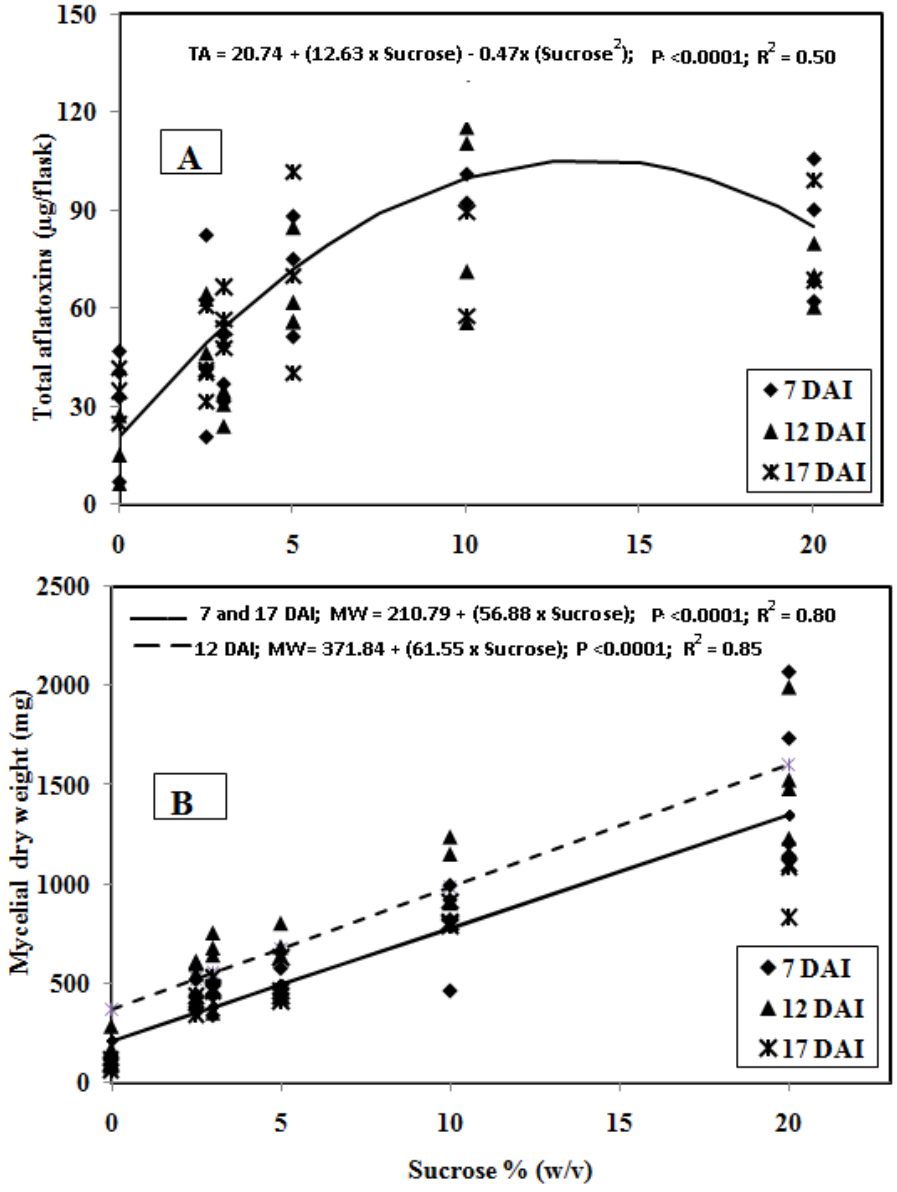

Figure 2: Effect of sucrose (0, 2.5, 3, 5, 10 and 20% w/v) on total aflatoxins (A) and mycelial dry weights (B) of *Aspergillus flavus* NRRL 3357 in peptone basal medium after various periods of (7, 12, 17 days) of incubation at 28˚C: Experiment 2. Total aflatoxin data from all the incubation intervals were combined into single regression equation because of no significant differences between intercepts and slopes. Mycelial dry weight data from 7 and 17 days after incubation (DAI) were combined into single regression model as there were no significant differences between the intercepts and slopes. DAI: days after incubation. TA: Total aflatoxins (µg/flask). MW: Mycelial dry weight (mg)

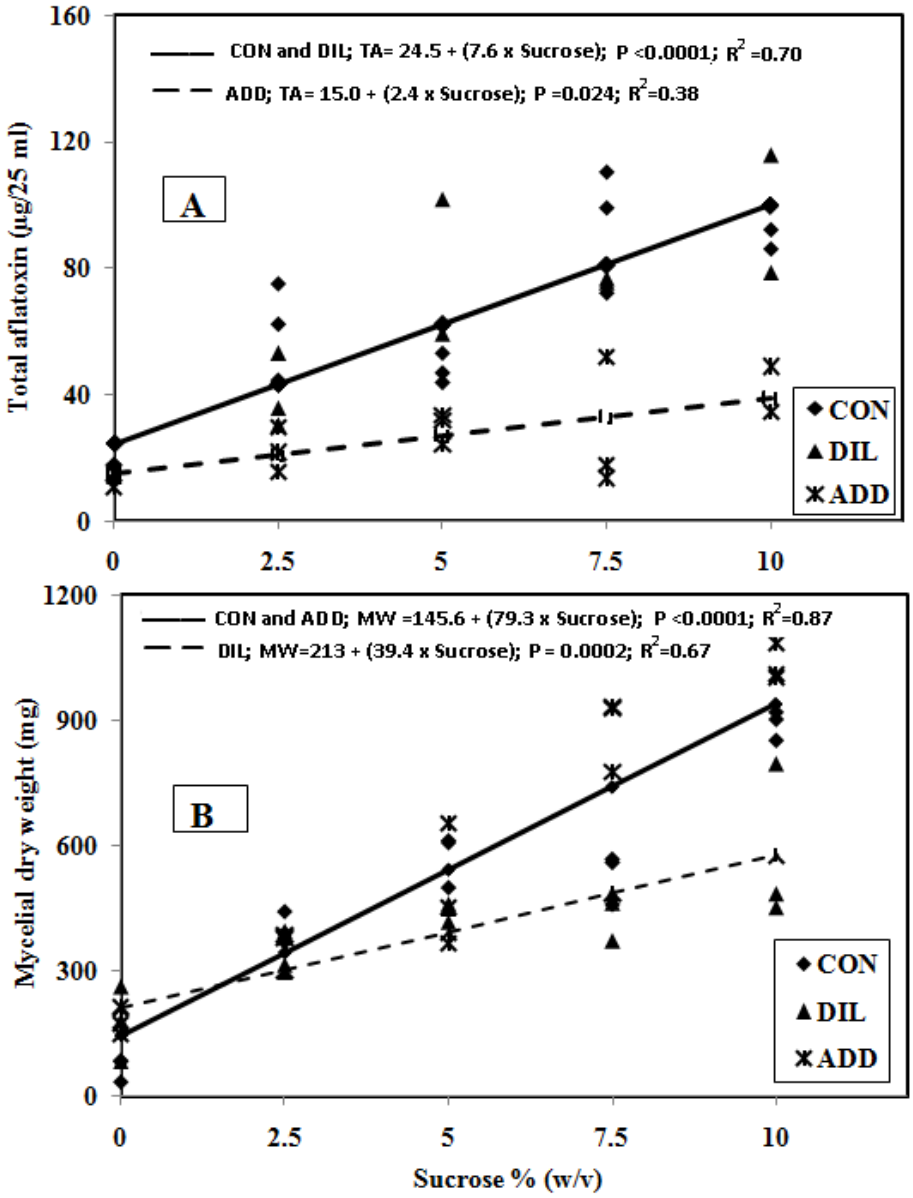

Figure 3: Effect of sucrose (0, 2.5, 5, 7.5 and 10% w/v) and media adjustments (CON, DIL, ADD) on total aflatoxin (A) and mycelial dry weights (B) of *Aspergillus flavus* NRRL 3357 in peptone basal medium after 14 days of incubation at 28˚C: Experiment 3. CON: continuous treatment where flasks were reloaded in the incubator after removing 10 ml of media once in every 48 hours for 14 days. DIL: dilution treatment where 10 ml of sterile water were added to flasks after collecting 10 ml of media once in every 48 hours for 14 days. ADD: addition treatment where 10 ml of sugar solution with same carbohydrate content as the original solution was added to each flask after collecting 10 ml of media once in every 48 hours for 14 days. DAI: days after incubation. TA: Total aflatoxins (µg/25 ml). MW: Mycelial dry weight (mg)

Table 1. Values for intercepts and slopes from individual regression lines and differences due to incubation intervals for mycelial dry weight as a function of media sucrose content: Experiment 2

Incubation time	Intercept	Compared to : 12 DAI	Compared to : 17 DAI	Slope	Compared to: 12 DAI	Compared to: 17 DAI
7 DAI	170.90	**[1]	ns[2]	67.86	**	ns
12 DAI	371.84	-	**	61.54	-	ns
17 DAI	261.31	**	-	43.02	ns	-

[1]Asterisks indicate a significant difference ($P = 0.05$) of values for regression parameters between the two incubation periods.
[2] ns indicates non-significance ($P = 0.05$).

DAI: days after incubation

Table 2. Values for intercepts and slopes from individual regression lines and differences due to media adjustments for total aflatoxin content and mycelial dry weights as a function of media sucrose content: Experiment 3.

		Compared to :			Compared to:	
Media adjustment	Intercept	DIL	ADD	Slope	DIL	ADD
Total aflatoxin						
CON[3]	28.12	ns[2]	ns	6.99	ns	**[1]
DIL[4]	20.84	-	ns	8.12	-	**
ADD[5]	15.00	ns	-	2.38	**	-
Mycelial weight						
CON	139.33	ns	ns	70.69	**	ns
DIL	213.00	-	ns	36.38	-	**
ADD	151.93	ns	-	88.00	**	-

[1] Asterisks indicate a significant difference ($P = 0.05$) of values for regression parameters among the three media adjustments.

[2] ns indicates non-significance ($P = 0.05$).

[3]CON: continuous treatment where flasks were reloaded in the incubator after removing 10 ml of media once in every 48 hours for 14 days.

[4] DIL: dilution treatment where 10 ml of sterile water were added to flasks after collecting 10 ml of media once in every 48 hours for 14 days.

[5]ADD: addition treatment where 10 ml of sugar solution with same carbohydrate content as the original solution was added to each flask after collecting 10 ml of media once in every 48 hours.

III. Greenhouse and Field Evaluation of the Effects of Drought and Soil Calcium on Pre-Harvest Aflatoxin Contamination and Sugar Allocation of Peanut

Abstract

In order to examine whether drought and supplemental soil calcium influence aflatoxin contamination and soluble carbohydrates in peanut, greenhouse and field experiments were conducted. Peanuts (*Arachis hypogaea* L.) were grown with different soil calcium levels and drought periods. In the greenhouse experiment, five levels of soil calcium (176, 681, 1255, 1563, and 1783 mg/kg) and three periods of drought (0, 35, and 60 days before harvest (DBH)) were evaluated with peanut cv. Georgia Green. Soil with low initial calcium (41 mg/kg) was used for the greenhouse study. For the field experiments peanut cv. Tifguard was used. The Wiregrass experiment was conducted with two levels of drought (0 and 55 DBH) and two levels of soil calcium (620 and 878 mg/kg). Initial soil calcium level at this site was 184 mg/kg. For the E.V. Smith experiment, only two levels of drought (0 and 55 DBH) were included, while supplemental soil calcium levels were not included since the initial soil calcium was high (407 mg/kg); a no-drought, non-inoculated treatment was included for comparison. In all three experiments, peanut plants were inoculated with *Apergillus flavus* strain NRRL 3357 at 75 days after sowing (DAS). Drought was imposed by restricting water in the

greenhouse and by rain-out shelters in the field experiments. Upon seed maturity, peanuts were harvested and assayed for aflatoxins and soluble sugars using high-performance liquid chromatography (HPLC). Calcium contents of leaves, shells, and kernels from the greenhouse experiment were estimated by inductively coupled plasma-optical emission spectroscopy (ICP-OES). Data were analyzed using mixed model and multivariate regression procedures. In all three experiments, aflatoxins were significantly higher in drought-stressed peanuts compared to non-drought-stressed peanuts. In the greenhouse study, a significant decline in total aflatoxins with increased soil calcium levels was observed; however, aflatoxin levels were low (average < 20 ppb). Significant negative correlations between calcium contents of various parts of the peanut and aflatoxins were also observed. Higher levels of total soluble sugars and sucrose were observed in drought-stressed peanuts compared to non-drought-stressed peanuts in the greenhouse and E.V. Smith experiments. However, no differences in peanut total reducing sugars were observed due to drought. No differences in sugar content of peanut seeds were found due to soil calcium levels. In these two experiments, significant positive correlations were observed between aflatoxin concentrations and total soluble sugars as well as sucrose.

Introduction

Peanut (*Arachis hypogaea*) is an important commercial crop throughout the world and is one of the crops most susceptible to *Aspergillus flavus* invasion (Guo *et al*., 2009). Aflatoxins contaminate peanuts either in the field (pre-harvest) if severe late season drought occurs or during storage (post-harvest) if improper moisture and temperature

conditions exist. Pre-harvest aflatoxin contamination is a major economic problem for the peanut industry. Although aflatoxins do not directly cause yield reduction, aflatoxins are heavily monitored and regulated because of their hepatotoxicity and carcinogenicity, to ensure food safety (van Egmond, 1995). This causes significant financial losses to producers as contaminated peanuts cannot be used for consumption.

Aflatoxin contamination of peanuts mostly occurs through direct invasion of the developing peanut pod by *A. flavus* and eventual contamination of the kernel with aflatoxin (Diener *et al*., 1987). Crops are often resistant to infection by aflatoxigenic fungi and subsequent aflatoxin contamination unless weather conditions favor fungal growth and increase crop susceptibility (Cotty and Jaime-Garcia, 2007). Drought and high temperatures are conducive to seed infection by *A. flavus* and contamination with aflatoxins (Guo *et al*., 2005a). When plants are subjected to late season drought, particularly during the 4 to 6 weeks prior to harvest, aflatoxin contamination can be a severe problem (Blankenship *et al*., 1984).

Plants undergo metabolic changes in response to water stress such as reduced photosynthetic activity (Ritchie *et al.*, 1990) and accumulation of organic acids (e.g., malate, citrate, and lactate), proline, and soluble sugars (Bohnert *et al.*, 1995; Bray, 1997; Tabaeizadeh, 1998). Calcium is involved in regulating physiological processes that influence the growth and response of plants to environmental stress. Because calcium ions influence plant defensive responses through their roles in cell membrane structure, stomatal function, cell division, cell wall synthesis, and water and solute movement, calcium availability influences the ability of the plant to repair damage from abiotic and abiotic stresses (McLaughlin and Wimmer, 1999).

Peanuts are sensitive to calcium deficiency, which is expressed as abnormal fruiting or complete inhibition of pod formation (Smith *et al*., 1993). Calcium requirements vary with the stage of pod development. Usually the calcium requirement of peanut is high at the start of peg (gynophore) swelling and deficiencies at this stage result in failure of the seed to expand into the pod (Hartmond *et al*., 1994). Pods absorb calcium directly from the soil (Smith *et al*., 1993). Thus, soil calcium content of the fruiting zone is very important for pod formation. Soil calcium deficiencies are also associated with greater seed invasion by aflatoxigenic fungi (Mixon *et al*, 1984).

The carbon source for *A. flavus* is reported to be another important factor that affects induction of aflatoxins. Soluble sugars are more favorable for aflatoxin biosynthesis (Abdollahi and Buchanan, 1981; Davis and Diener, 1968). Immature, drought-stressed peanut seeds have greater carbohydrate levels than mature, non-drought-stressed seeds (Manda *et al*, 2004; Musingo *et al*, 1989).

Aflatoxin contamination and soluble carbohydrate content of peanut might be affected by drought and soil calcium levels. Drought, which is an important predisposing factor for aflatoxin contamination, is also reported to affect soluble carbohydrate composition of peanut (Musingo *et al*., 1989). Therefore, we evaluated the effects of drought and soil calcium on aflatoxin contamination and sugar concentrations of peanut seed. Calcium accumulation by various parts of peanut (leaf, shell and kernel) was also estimated.

Materials and Methods

Greenhouse experiment: A factorial experiment with five soil calcium levels (176, 681, 1255, 1563, and 1783 mg/kg) and three periods of drought (0, 35, and 60 days before harvest (DBH)) was conducted at the Plant Science Research Center at Auburn University from February 2, 2010 to July 13, 2010. Each treatment was replicated five times. Soil with low initial calcium (41 mg/kg) was collected from the Cullars Rotation of Auburn University, Auburn, AL. The soil was loamy sand with 82.8% sand, 9.5% silt, and 7.7% clay. All soil calcium estimations were performed using a Mehlich I extract method by Auburn University Soil Testing Laboratory at Auburn, AL. The soil was dried and passed through a 2-mm sieve. Pots, 42 cm wide x 28 cm deep (16.5" x 11"), were filled with 28 kg of soil. Target soil calcium concentrations were 41 (i.e., no supplementation), 250, 500, 750, and 1000 mg/kg. Calcium supplementation was performed by the addition of gypsum to each unit of 28 kg soil followed by thorough mixing and then returning to individual pots. Following gypsum addition, soil in the pots was subjected to three watering and drying cycles and then Georgia Green peanut seeds were sown.

At 75 days after sowing (DAS) plants were inoculated with strain NRRL 3357 of *A. flavus* obtained from the Microbial Genomics and Bioprocessing Unit, National Center for Agricultural Utilization Research, USDA, Peoria, IL. Sterilized corn grits (500 g) in 1000 ml flasks were inoculated with 50 ml (1×10^5 conidia ml^{-1}) of aqueous conidial suspension from one week old *A. flavus*. Grits were incubated at room temperature and shaken twice a day for three weeks before use. Inoculation was done by sprinkling 10 g of *A. flavus*-infested corn grits per pot to the soil surface under the canopy followed by gentle manual incorporation into the top layer of soil.

Plants were irrigated regularly until 90 DAS. After 90 days, plants were subjected to various watering schedules to reflect different drought scenarios. Fungicides and insecticides were applied as recommended by Alabama Co-operative Extension System. Upon maturity, peanuts were harvested, shelled, and ground into meal. Representative soil samples from each treatment were collected at harvest for soil calcium analysis. Final soil calcium values were higher than the targeted values indicating some error in initial soil calcium level estimations. Thus, final soil calcium concentrations are used as treatment values. At the highest calcium additions, soil calcium values are higher than typical field values. Soil moisture was monitored by soil moisture probes (Watchdog Series 1000, Spectrum Technologies Inc., East-Plainfield, IL); greenhouse temperatures were also monitored (Table 1).

E. V Smith experiment: From May 20, 2010 to October 23, 2010, a field experiment was conducted at E.V. Smith Research Center (Shorter, AL). The experiment was a randomized complete block design with two drought (0 and 55 DBH) treatments replicated four times. An additional non-inoculated and no-drought treatment was included with four replications for comparison. Four rows were included in each replicated plot with two outer border rows. Plot sizes were 5.5 x 2.7 m (18 x 9 ft) with 0.9 m (3 ft) spacing between rows. Soil type at this site was a sandy loam with 76.3% sand, 16.3% silt, 7.5% clay, and 407 mg/kg soil calcium. Five to six peanut seeds were sown per 0.3 m (one foot). The peanut cultivar used in this experiment was ‘Tifguard’. Multiplication of the inoculum was done as previously described for the greenhouse study and plants were inoculated with 180 g of infested corn per row at 75 DAS by sprinkling over the foliage then manually dislodging particles from foliage to the soil

surface. Rain-out shelters (3.7 x 2.7 m (12 x 9 ft)) were installed over the three rows of drought plots at 100 DAS. Rain-out shelters consisted of plastic sheeting stretched over arched polyvinyl chloride (PVC) pipes. Fungicides and pesticides were applied as recommended by Alabama Co-operative Extension System. At maturity, plants were mechanically dug by tractor and pods were handpicked from turned plants. In the case of the drought treatments, peanuts were collected from the center portion of middle row that was covered by rain-out shelters. Harvested peanuts were dried for three days in ambient laboratory conditions, then shelled and ground. Weather data were compiled for the drought stress period from AWIS weather services, Inc. (Table 1). Precipitation data for the growth season is presented in Fig. 1.

Wiregrass experiment: A field experiment was conducted at the Wiregrass Research and Extension Center, Headland, AL from May 22, 2010 to October 25, 2010. The plot sizes were 4.6 x 6.4 m (15 x 21 ft), consisting of eight rows at 0.9 m (3 ft) spacing. Peanut cv. 'Tifguard' seeds were sown at the rate of 5 to 6 per 0.3 m (one foot). Two border rows were included on each side leaving the center four rows for sampling. The experiment was a factorial experiment with two factors (soil calcium and drought) each at two levels and replicated four times. The soil was loamy sand with 83.8% sand, 11.3% silt and 5% clay content and 184 mg/kg initial soil calcium level. Targeted soil calcium treatment levels were 184 mg/kg (no additional Ca) and 368 mg/kg calcium (184 mg/kg of Ca addition or 5 kg of gypsum per plot). The two drought treatments were no-drought (0 DBH) and drought for 55 DBH. *A. flavus* inoculum was produced as described above. Plants were inoculated by sprinkling 150 g of infested corn per row at 75 DAS.

Rain-out shelters were installed over the drought treatment plots at 100 DAS. The dimensions of the rain-out shelters were 3.7 x 2.4 m (12 x 8 ft). Each shelter was placed over drought treatment plots in such a way that it would cover 1.8 m (6 ft) lengthwise of each calcium level and widthwise three rows at 0.9 m (3 ft). Thus, one rain-out shelter was used for two calcium treatments in a single drought plot. Fungicides and pesticides were applied as needed. At maturity, peanut pods were harvested, then dried, shelled, and ground into meal. Under rain-out shelters, peanuts were collected only from the middle row. At the time of harvest, soil samples were collected from each plot for determination of soil calcium content. Final soil calcium values were higher than the targeted values indicating some error in initial soil calcium level estimations. Thus, final soil calcium levels (620 and 878 mg/kg) were used as treatment values. Weather data were compiled for the drought stress period from AWIS weather services, Inc. (Table 1). Precipitation data for the growth season is presented in Fig. 1.

HPLC analysis of total aflatoxins: Using ground peanut meal, aflatoxins were extracted using the following procedure: 50 g of ground peanut meal was mixed with 100 ml of 90% acetonitrile in 250 ml bottles. The peanut meal with acetonitrile was shaken for one hour on a wrist action shaker then filtered through Whatman No. 1 filter paper (GE Healthcare Inc., UK). The filtrate was purified by passing through Mycosep Multifunctional Cleanup Columns (Romer Labs, Inc., Washington, MO). Two hundred µl of the purified solution was added to 700 µl derivatizing agent (water/trifluoroacetic acid/acetic acid; 70:20:10 v/v/v) and incubated at 55°C for 30 minutes before injection into the HPLC. Aflatoxins in peanut were assayed by HPLC as described by Wilson and Romer (1991) with slight modifications. The details of HPLC equipment are: Nova-Pak

C18 column (8 x100 mm, 4µm particle size, Waters, Milford, MA); flow rate, 1 ml min^{-1}; mobile phase, water/methanol/1-butanol (65.3:33.6:1.2 v/v); scanning fluorescence detector with 365 nm excitation wavelength and 418 nm emission wave length. A commercially available Aflatoxin B and G mixture (Sigma Chemical, St. Louis, MO) served as standard for total aflatoxins quantification (sum of B_1, B_2, G_1, and G_2).

HPLC analysis of peanut soluble sugars: Extraction and analysis of soluble carbohydrates from peanut kernels was performed as outlined below (Basha, S. M personal communication, 2010, October). Approximately 1 g of peanut meal was ground with 5 ml of HPLC grade hexane with a mortar and pestle, and the homogenate transferred to 15 ml centrifuge tube and covered with aluminum foil. Tubes were placed on a horizontal shaker (Barnstead Lab-Line Model: Max-Q 2508) and left overnight at room temperature with 40% reciprocal shaking. The homogenate was sonicated for 10 minutes (Branson, model 5510 Branson Ultrasonic Corporation, Danbury, CT) and clarified by centrifugation at 10,000 *g* for 15 min at 4ºC. Clarified supernatant was decanted and the pellet was retained and re-extracted with an additional 5 ml of hexane. The supernatant was removed and the presence of oil was determined by pipetting an aliquot of supernatant on to Whatman No. 1 filter paper. If the presence of oil was detected, then an additional hexane defatting step was repeated. Alternatively, in the absence of oil, the pellet was air dried and further extraction steps were performed. For carbohydrate extraction, approximately 0.5 g of dried defatted ground peanut pellet was added to 5 ml of 80% HPLC grade ethanol in a Corex™ centrifuge tube and sonicated for 30 minutes followed by a one minute vortex. Centrifuge tubes were shaken on horizontal shaker for 30 minutes followed by centrifugation at 10,000 *g* at 4°C for 15 minutes.

Clarified supernatant was collected and the extraction procedure repeated with an additional 5 ml of 80% HPLC ethanol. The supernatants were combined and retained with the pellet discarded. Finally, the combined supernatant was filtered through 0.2 µm nylon membrane filter (VWR, Batvia, IL) into chromatography vials (Agilent technologies, Inc. Santa Clara, CA), and analyzed immediately for soluble sugar content. Sucrose, glucose, and fructose were determined by HPLC (Simhadzu Corporation, USA) with a Bio-Rad Aminex HPX-87P 300 x 7.8 mm prepacked HPLC carbohydrate analysis column with 9 µm particle size (Hercules, CA); flow rate, 0.6 ml min^{-1}; isocratic de-ionized water mobile phase; 85°C column temperature; and detection with a refractive index (RI) detector.

ICP-OES estimation of calcium levels: Leaf, shell, and kernel samples from the greenhouse experiment were analyzed for calcium using ICP Optima 7300 DV (Perkin Elmer Inc., Waltham, MA, USA). For the sample preparation, leaf, shell, and ground peanut samples were dried at 55ºC for 45 minutes, then 10 mg of dried tissue was placed in two ml Eppendorf tubes and digested with 150 µl of trace metal grade 40% nitric acid at 95ºC for 40 minutes. Samples were subsequently diluted to 600 µl with double-distilled water for analysis. Commercially available mixed-metal standards including calcium were used for the construction of standard curves. Sample blanks were prepared by digesting 150 µl of nitric acid without plant material and diluting to 600 µl with double-distilled water.

Data analysis: Data transformations were performed whenever there was non-normality in the data. Data analysis was carried out with SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA). After statistical analysis, means were back-transformed for presentation

herein. Data on total aflatoxins and calcium levels of leaves, shells, and kernels from the greenhouse experiment were analyzed by multivariate-regression analysis for each drought period. Regression analysis was performed with total aflatoxins and calcium levels of tissues as dependent variables with soil calcium at the time of harvest as the independent variable; linear and quadratic models were fit to data. Regression models were considered significant if $P < 0.05$. In addition, intercepts and slopes of the regression equations were tested for differences due to drought levels by calculating confidence intervals. If confidence intervals did not include zero, model parameters (intercepts, slopes) were considered significantly different. If zero was included in the confidence intervals for both parameters, the models were considered similar and data were combined for determination of a single regression equation. Data on peanut sugars were analyzed by mixed-model analysis and differences due to drought, final soil calcium, and the two-way interaction of drought and final soil calcium were estimated. Relationships between total aflatoxins and peanut sugars were evaluated by correlation analysis. Calcium levels of leaves, shells, and kernels were also correlated with total aflatoxins.

Data from field experiments on total aflatoxins and soluble sugars were analyzed by mixed-model analysis and differences due to drought, soil calcium, and their interaction were determined. Mean separations were done by least significant difference (LSD) test. Dunnett's test was used to compare data on total aflatoxins, and soluble sugars with no-drought, non-inoculated treatment in E.V. Smith experiment.

Results

Greenhouse experiment: Significant differences in total peanut aflatoxin levels were observed in plants treated under normal or drought (P <0.0001) conditions and with different amounts of soil calcium levels (P=0.02). When all three drought treatments were compared, no differences were found between slopes and intercepts for total aflatoxins in 60 and 35 DBH treatments. However, intercepts of regression models for both the drought treatments and the slope of the regression model for the 60 DBH treatment are each different from 0 DBH treatment regression model. Thus, aflatoxin data from both the drought treatments were combined into one regression model and compared to the 0 DBH treatment model where the intercept and slope were higher for drought-treatment compared to non-drought treatment. Regression analysis shows that as final soil calcium levels increased, aflatoxin levels decreased (Fig. 2).

Peanut leaf calcium levels differed due to soil calcium levels (P <0.0001) but not among various drought treatments (P=0.44). Slopes and intercepts of regression models describing the relationship between soil calcium and peanut leaf calcium levels at different drought periods were also not significantly different. Thus, data from all the drought treatments were combined. Peanut leaf calcium content was found to be positively related to soil calcium levels up to about 1400 mg/kg soil calcium (Fig. 3).

Shell calcium levels differed significantly among various soil calcium (P <0.0001) and drought (P= 0.02) treatments. Intercepts and slopes of regression models describing the relationship between calcium content of peanut shells and soil calcium levels at various drought treatments differed significantly. With no drought (0 DBH) treatment, there were increasing shell calcium levels as soil calcium rose. Similar trend was seen with 60 DBH treatment, but shell calcium was consistently lower than with no drought

treatment. Shell calcium with the 35 DBH treatment was consistently higher than with other water regimes up through about 1250 mg/kg soil calcium level beyond which greater soil calcium had a negative effect on shell content (Fig. 4).

Similar to shell calcium, kernel calcium levels also differed significantly due to drought (P=0.005) and soil calcium levels (P <0.0001). When intercepts and slopes of regression models describing kernel calcium content and soil calcium levels at various moisture regimes were compared, no significant differences were observed between 0 and 35 DBH treatments. However, both of these were significantly different from the 60 DBH regression model. Thus kernel calcium data from 0 and 35 DBH treatments were combined and compared with 60 DBH treatment. From the regression lines, it can be inferred that higher kernel calcium accumulation occurred in 0 and 35 DBH drought treatments as compared to the 60 DBH drought (Fig. 5), especially with up to 1563 mg/kg soil calcium. Correlations between total aflatoxins and calcium content of peanut leaves (r = -0.42; P=0.0006), shells (r = -0.39; P=0.002) and kernels (r = -0.33; P=0.009) were significant (Fig. 6).

Total soluble sugar content of peanut kernels was higher in drought-stressed compared to non-drought-stressed plants (P=0.008). Highest total sugar content was observed in peanut kernels from the 60 DBH treatment which was similar to the 35 DBH treatment, and lowest total sugars were observed in 0 DBH treatment (Table 1). No differences in sugar content was noted due to final soil calcium (P=0.85) or the interaction of drought and supplemental soil calcium (P=0.14).

Like total sugars, significant differences in sucrose contents of peanut kernels were observed due to drought (P=0.02) but not due to soil calcium (P=0.63) or the interaction

of drought and calcium (*P*=0.12). Peanut kernels from both drought treatments had higher sucrose compared to non-drought-stressed peanuts. No differences in sucrose concentrations were observed between 35 and 60 DBH drought treatments. No significant differences in total reducing sugars were observed due to drought (*P*=0.21), soil calcium (*P*=0.13), or the interaction of drought and soil calcium (*P*=0.97).

In the greenhouse experiment, total aflatoxins were positively correlated with total soluble sugar content (r=0.22; *P*=0.08) and sucrose (r = 0.23; *P*=0.07) of peanut kernels at the 10% level of significance. No significant negative or positive correlations were observed between total aflatoxins and total reducing sugars.

E.V. Smith experiment: Mixed-model analysis showed significant differences in total aflatoxins due to drought (*P*=0.04). Total aflatoxins were higher in drought-stressed (54.16 ppb) peanuts compared to non-drought-stressed (7.34 ppb) peanuts (Fig. 7). No aflatoxins were detected in peanuts from the non-inoculated, non-drought treatment.

Significantly higher total soluble sugars (*P*=0.007) and sucrose levels (*P*=0.006) were found in peanut kernels that had been exposed to drought compared to non-stressed kernels. No significant differences (*P*=0.29) in reducing sugars were observed between non-drought-stressed and drought-stressed peanuts (Table 1). Total sugars, sucrose or total reducing sugars in peanuts from the non-inoculated, non-drought treatment also did not differ from the inoculated non-drought treatment. However, total sugars and sucrose levels in the non-inoculated, non-drought treatment are numerically lower than from the inoculated-drought treatment.

Total aflatoxins were significantly and positively correlated to sucrose (r=0.65; *P*=0.055) and total sugars (r=0.64; *P*=0.06) at the 10% level of significance. No

significant correlations were observed between aflatoxins and total reducing sugars of peanut.

Wiregrass experiment: Mixed-model analysis was performed on peanut aflatoxin data from the two soil calcium (620 and 878 mg/kg) and two drought (0 and 55 DBH) treatments. Aflatoxin levels differed among treatments due to drought (P=0.0002); however, no differences were observed due to soil calcium (P=0.803) or due to the interaction of drought and soil calcium (P=0.503). Peanuts in the drought-stress treatments showed higher levels of aflatoxins than non-drought-stressed peanuts (Fig. 7).

Mixed-model analysis failed to detect differences in levels of total sugars (P=0.27), sucrose (P=0.39), or total reducing sugars (P=0.68) from drought-stressed versus non-stressed peanut kernels (Table 1). Soil calcium did not impact total soluble sugars (P=0.94), sucrose (P=0.66), or total reducing sugars (P=0.92). The two-way interaction of drought and soil calcium also showed no differences for total sugars (P=0.50), sucrose (P=0.63), and total reducing sugars (P=0.77) in peanut kernels. No significant correlations were noticed between total aflatoxins and peanut sugars in this experiment.

Discussion

In all experiments drought-stressed peanuts had significantly higher levels of aflatoxins compared to non-drought-stressed peanuts. Plants have been reported to be resistant to infection by aflatoxigenic fungi and subsequent aflatoxin contamination unless environmental conditions favor both fungal growth and crop susceptibility (Cotty and Jaime-Garcia, 2007). Drought stress coupled with high soil temperatures predispose peanuts to enhanced infection by *A. flavus* and aflatoxin contamination (Blankenship *et*

al., 1984; Cole *et al*., 1982; Manda *et al*., 2004; Payne, 1998). Thus, our results are in agreement with previous reports.

In the greenhouse experiment, significantly higher aflatoxin levels were observed in 35 and 60 DBH drought treatments compared to no-drought. From these results and previous experiments by Sanders *et al*. (1985) and Wilson and Stansell (1983), it can be concluded that 30 to 40 days before harvest might be a critical stress period for triggering pre-harvest aflatoxin contamination of peanuts. In our greenhouse study, low levels of aflatoxins (<20 ppb) were detected compared to our field experiments at E.V Smith (up to 55 ppb) and Wiregrass (up to 1600 ppb). Low levels of aflatoxins in the greenhouse trial might be attributed to controlled temperatures and relatively mild drought stress conditions. The variation in aflatoxin values of our field experiments might be explained by the weather and soil data. At E.V. Smith, the average soil minimum and maximum temperatures after installation of rain-out shelters (or during the drought period) were 25.4 and 32.5ºC, respectively. Soil temperatures at Wiregrass during the drought period were 29.4ºC (average minimum) to 31.5ºC (average maximum). Therefore, mean soil temperatures at E.V. Smith and Wiregrass were 28.9ºC and 30.5ºC, respectively. Furthermore, the high aflatoxin content of the no drought treatment at the Wiregrass site may be due to the extremely dry 2010 growing season. Blankenship *et al*. (1984) found that high levels of aflatoxins occurred in peanuts grown in drought-heated soil with a mean temperature of 30.5ºC but detected no aflatoxins in irrigated plots with 24ºC mean soil temperature. Blankenship *et al*. (1984) also reported that 25.7 to 27ºC mean geocarposphere soil temperatures were marginal for aflatoxin contamination of peanuts. Thus, while soil temperatures at both field locations were favorable for aflatoxin

contamination, the higher temperatures at Wiregrass may have been slightly more conducive than those at E. V. Smith.

Aflatoxin contamination of peanuts varies significantly by soil type (Codex Alimentarius Commission, 2004). Particularly under dry conditions, sandy soils favor aflatoxigenic fungal growth and aflatoxin production compared to heavier soils with higher water holding capacity (Guo *et al*., 2005b). The soil at our E.V. Smith site was a sandy loam, while the soil at Wiregrass was loamy sand; soil moisture holding capacity of loamy sand is generally less than that of sandy loam (Scherer *et al*., 1996). Therefore, we can attribute lower amounts of aflatoxins at E.V. Smith to lower geocarposphere temperatures and higher water holding capacity of the soil.

In our greenhouse study, total aflatoxin content decreased with higher soil calcium levels. Bowen *et al*. (1996) also reported significantly lower levels of aflatoxin in peanut seeds grown with 200 mg/kg calcium supplementation compared to 50 mg/kg calcium supplementation. These results support observations by Wilson and Walker (1981), who noted high aflatoxin content in peanuts produced without additional calcium, but low and similar aflatoxin in peanuts treated with 560, 1120 or 1680 kg/ha gypsum. In our Wiregrass experiment, no significant differences in aflatoxins were observed between 620 mg/kg and 878 mg/kg calcium treatments. This may be due to the high soil calcium levels, since peanuts are considered to have adequate calcium when soil test values (Mehlich I extractant) are >250 mg/kg in the fruiting zone (ACES, 2010). Further, results from the Wiregrass study are also similar to those of Wilson and Walker (1981). From these results, it can be inferred that aflatoxin contamination is not managed with supplemental soil calcium in situations of drought and excessively high soil temperatures.

In the greenhouse study, calcium levels of shells and kernels were significantly affected by drought. Calcium levels were significantly higher in shells and kernels from no and minimum drought treatments compared to the prolonged, 60 day drought treatment. These results are similar to those of Gaines and Hammons (1981) who found a significant positive correlation between kernel calcium content and total precipitation. In the greenhouse study, peanut kernel calcium levels were in the range of 0.05 to 0.18% (or 0.55 µg/mg dry weight to 1.83 µg/mg dry weight), which are also similar to the ranges observed by Gaines and Hammons (1981).

In the greenhouse and E.V. Smith experiments, sucrose and soluble sugar contents of peanuts were higher in drought-stressed compared to non-drought-stressed peanuts. Similar results were observed by Musingo *et al.* (1989) who found 3 to 6% increases in total carbohydrates in drought-stressed peanuts compared to irrigated, non-stressed peanuts. They also noticed an association of greater aflatoxin contamination with drought stress. Significant positive correlations were observed between peanut total soluble sugars and sucrose to aflatoxins in our two experiments. Manda *et al.* (2004) also found a significant positive correlation between aflatoxin and sucrose content kernels of Virginia type peanuts as well as greater aflatoxin in immature seed compared to mature seed. The current study differs from Manda *et al*. (2004) in that they also found a positive correlation between the content of reducing sugars (glucose and fructose) and aflatoxin content of kernels, which we did not observe. In addition, they worked with Virginia-type peanut cultivars, while we used currently available runner-type peanuts. Pattee *et al*. (2000) consistently found lower reducing sugar contents in runner-types than in Virginia-types, which may partially explain the reason, why our results differed from Manda *et al*.

(2004) with respect to the relation between reducing sugar and aflatoxin contents of peanut kernels.

Sugar concentrations of peanuts showed some variation among our three experiments. However, sucrose concentrations were higher compared to glucose and fructose and accounted for more than 80% of total sugars in all three experiments in all treatments. Our results are consistent with previous studies which reported sucrose as predominant sugar in Virginia-type (Oupadissakoon *et al*., 1980; Pattee *et al*., 2000; Pickett, 1949) and runner-type (Pattee *et al*., 2000) peanuts comprising >80% of peanut soluble sugars.

The results of our studies confirm the significant role of drought stress as well as increasing accumulation of sucrose or total soluble sugars in kernels in predisposing peanuts to aflatoxin contamination. A positive correlation of peanut soluble sugars with aflatoxins occurred even with only a 1 to 2% increase in sugar content. Thus, peanut soluble sugars, which have been proven to readily support aflatoxin biosynthesis *in vitro,* may also have a significant role in aflatoxin contamination of peanuts *in vivo.* Increases in peanut sugars under drought stress may be a reason for higher aflatoxin contamination in drought year compared to non-drought years. In addition, significant negative correlations were observed between aflatoxins and calcium contents of leaves, shells, and kernels in our greenhouse experiment under mild drought stress. Under high soil temperatures and severe moisture stress (e.g., our field studies), however, no significant decline in aflatoxins due to increased soil calcium was observed. Thus, during drought years, irrigation is the only consistent means of reducing the risk of aflatoxin

contamination. However, irrigating peanut throughout the season is not economically feasible for many peanut producers.

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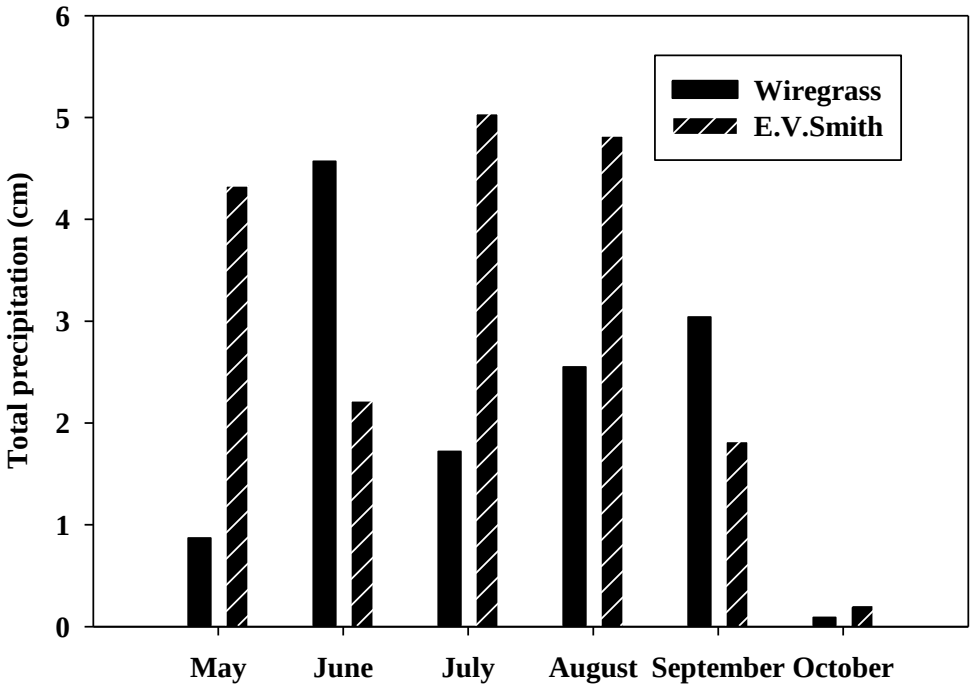


Figure 1: Growth season (from sowing to harvest) precipitation data for Wiregrass and E.V. Smith Research Centers collected from AWIS (Agricultural Weather Information Service)

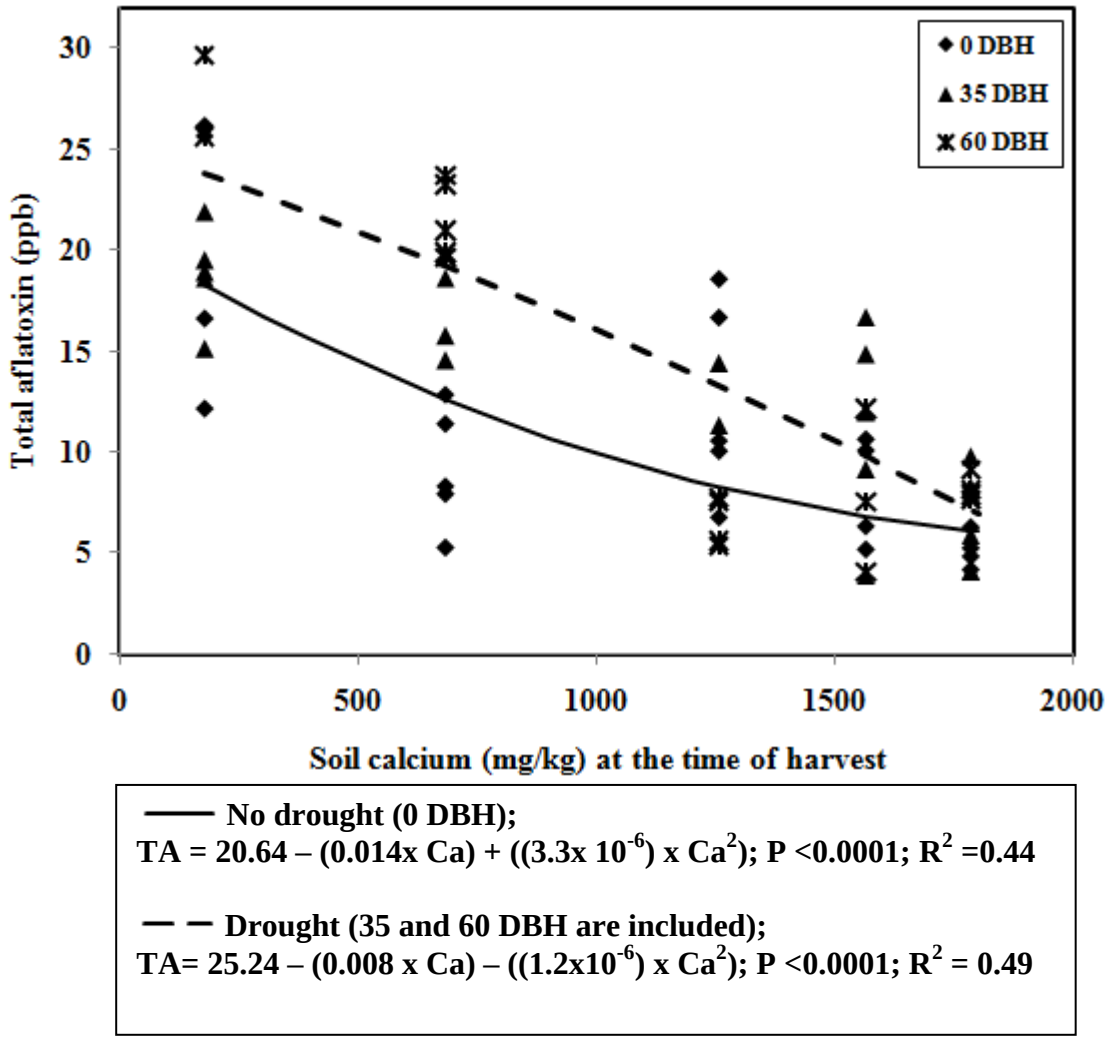


Figure 2: Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut aflatoxin content during drought or normal greenhouse conditions. Aflatoxin data from 35 and 60 DBH treatments were combined into single regression model as there were no significant differences between intercepts and slopes of regression models and compared with 0 DBH regression model.

DBH: denotes the number of days of drought before harvest

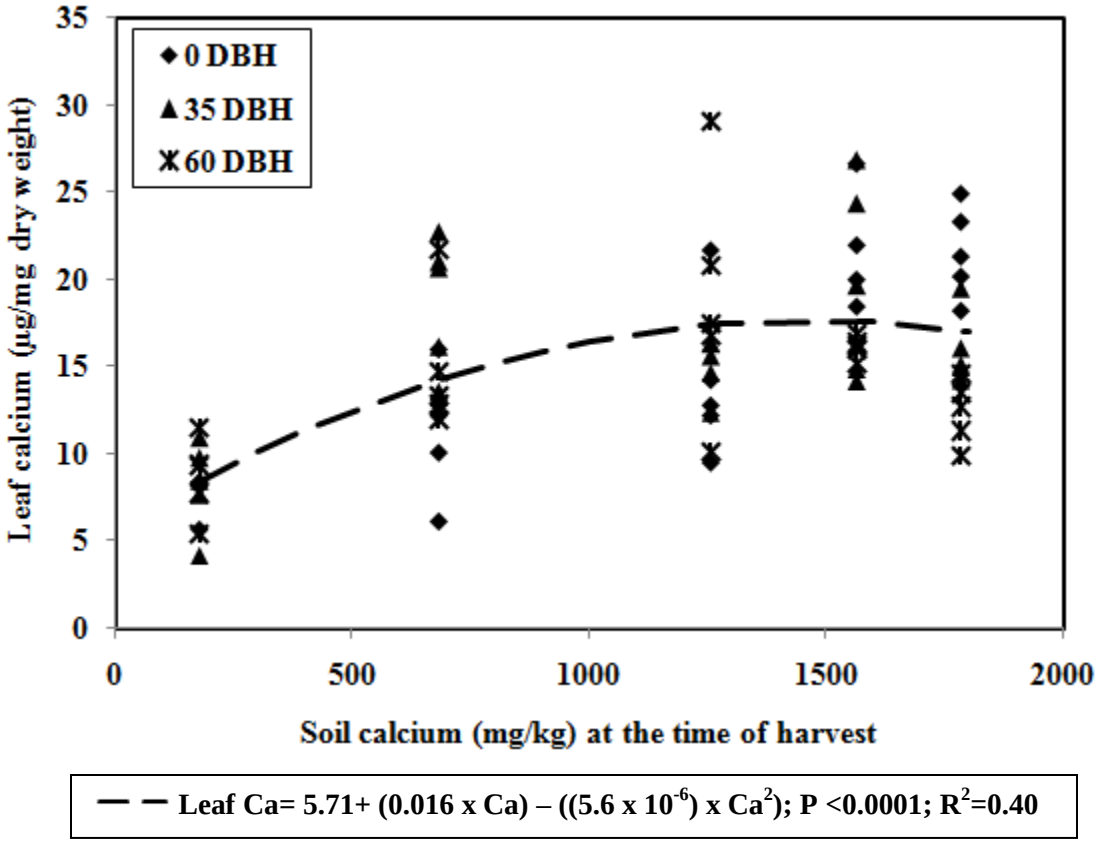
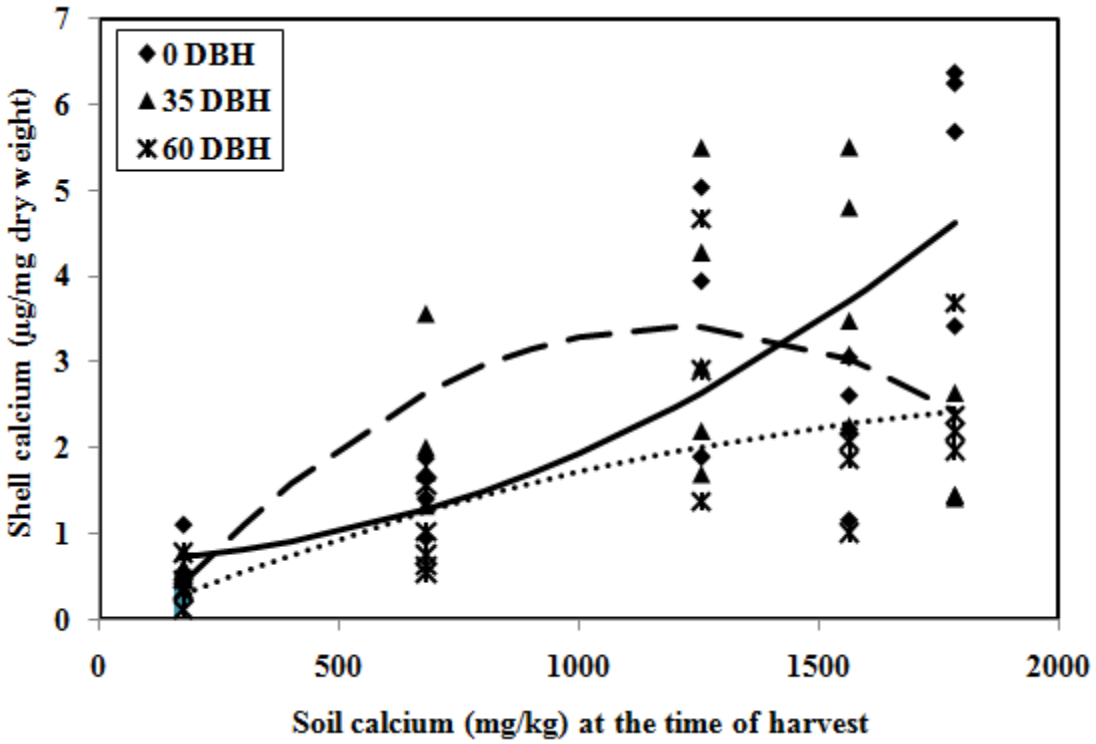


Figure 3: Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut leaf calcium content under greenhouse conditions. Intercepts and slopes of regression models for leaf calcium did not differ under various moisture regimes (0, 35 and 60 DBH treatment), Leaf calcium data from all the drought treatments were combined into single regression model.

DBH: denotes the number of days of drought before harvest



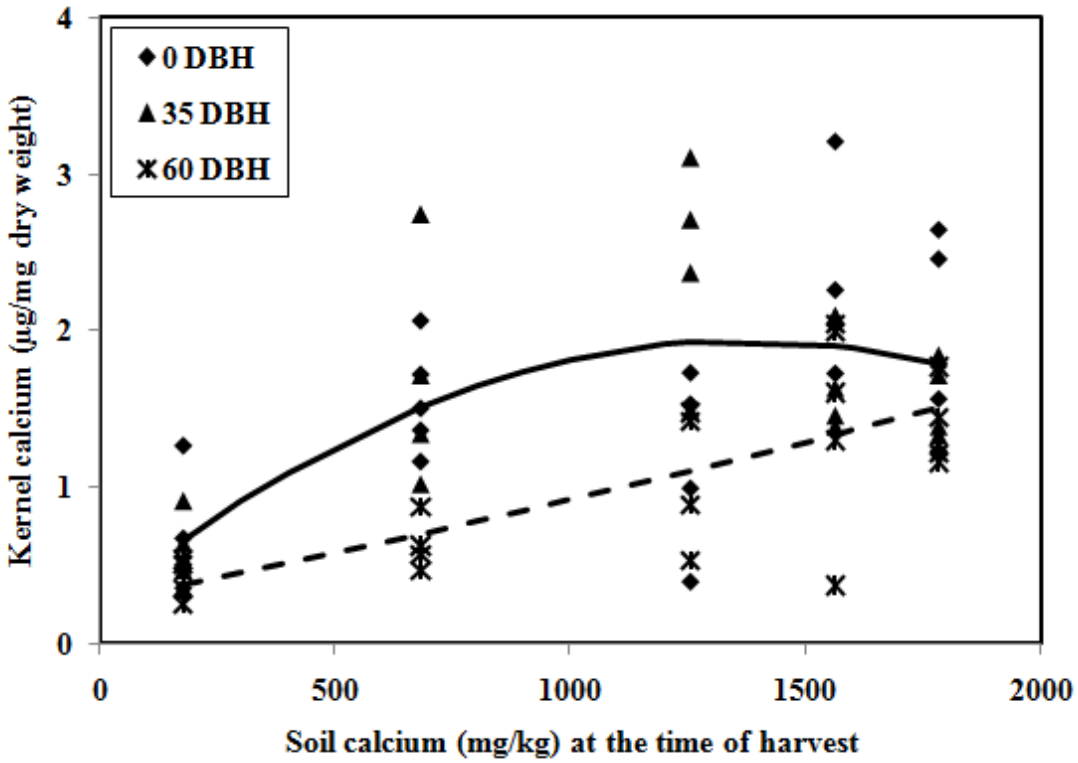
0 DBH;
Shell Ca= 0.71- ((6.4 x 10^{-6}) x Ca) + ((1.2 x 10^{-6}) x Ca^2); P=0.0002; R^2=0.61

35 DBH;
Shell Ca = 0.70 + (0.007 x Ca) – ((2.8x 10^{-6}) x Ca^2); P=0.001; R^2=0.49

60 DBH;
Shell Ca = 0.09 + (0.002 x Ca) – ((5.1 x 10^{-7}) x Ca^2); P=0.001; R^2=0.51

Figure 4: Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut shell calcium content under drought (35 and 60 DBH) or normal greenhouse conditions

DBH: denotes the number of days of drought before harvest



0 and 35 DBH;
Kernel Ca = 0.25+ (0.0024x Ca) – ((9 x 10^{-7}) xCa^2); P <0.0001; R^2 =0.42

60 DBH;
Kernel Ca = 0.28+ ((5.8 x 10^{-4}) x Ca) + ((5.8 x 10^{-8}) x Ca^2); P=0.0009; R^2=0.53

Figure 5: Comparison of effect of soil calcium levels (176, 681, 1255, 1563 and 1783 mg/kg) on Georgia Green peanut kernel calcium content under drought or normal greenhouse conditions. Kernel calcium data from 0 and 35 DBH were combined into single regression model as there were no significant differences between intercepts and slopes and compared with 60 DBH regression model

DBH: denotes the number of days of drought before harvest

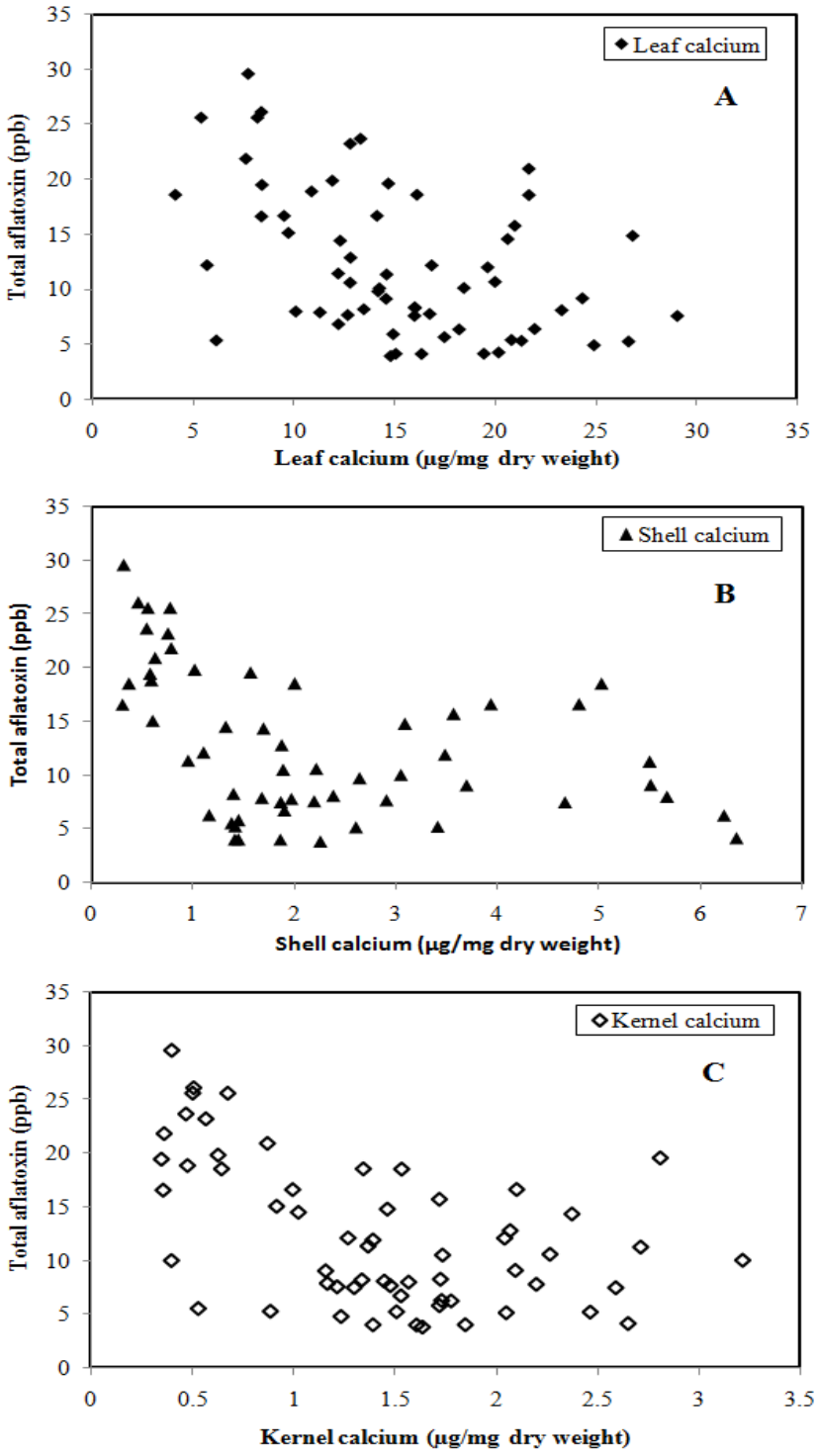


Figure 6: Relationship between total aflatoxin and leaf (A), shell (B) and kernel (C) contents of Georgia Green peanut under greenhouse conditions.

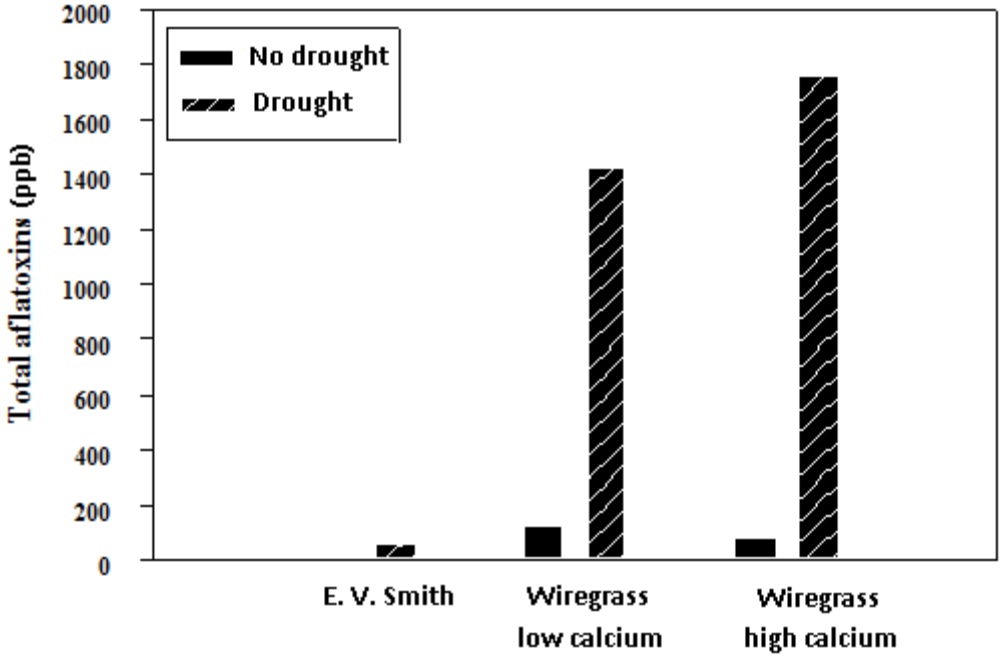


Figure 7: Aflatoxin content of peanut cv. Tifguard grown without and with drought stress (0 and 55 DBH) at E.V. Smith and Wiregrass Research Centers, 2010. Wiregrass low calcium: 620 mg/kg of soil calcium. Wiregrass high calcium: 878 mg/kg of soil calcium.

DBH: denotes the number of days of drought before harvest

Table 1: Total aflatoxins, soluble sugars, and weather parameters from the period of initial drought-stress to peanut harvest: Greenhouse, E.V.Smith and Wiregrass experiments

Treatment (DBH)*	Total aflatoxins (ppb)	Total soluble sugars (%)	Sucrose (%)	Total reducing sugars (%)	Average air temp. (ºC)	Average soil temp. (ºC)	Average soil moisture** (%)		Total precipitation (cm)***
							No-drought	Drought treatment	
Greenhouse experiment									
0	10.81b	4.00b	3.57b	0.47a					
35	15.21a	4.96a	4.58a	0.49a	27.3	NA****	11%	2%	-
60	17.00a	5.20a	4.56a	0.70a					
E.V.Smith experiment									
0	7.34b	6.32b	5.30b	1.01a	23.0	28.9	2.9%	1.1%	6.1
55	54.16a	9.03a	7.76a	1.26a					
Wiregrass experiment									
0	98.49b	8.81a	7.18a	1.30a	27.2	30.5	NA	NA	8.0
55	1587.63a	7.01a	5.96a	1.16a					

Figures in each column for each experiment followed by same alphabet do not differ significantly.

* DBH: denotes the number of days of drought before harvest.

**Due to equipment failures, soil moisture data were recorded only for 10 and 3 days before harvest respectively for greenhouse and E.V. Smith experiment.

*** Total precipitation is total rainfall received during the period of initial drought-stress to peanut harvest by plots that were not covered by rain-out shelters

****NA: Not available

IV. Summary

This project was initiated with the goal of better understanding the effects of drought and soil calcium on peanut sugars and aflatoxin. The main objectives of this investigation were to: 1) evaluate peanut sugar concentrations for the growth of and aflatoxin production by *Aspergillus flavus*; and 2) in greenhouse and field trials, evaluate the effects of drought and supplemental soil calcium on aflatoxin contamination and sugar concentrations of peanut seed. For objective 1, various sugar concentrations in the ranges that occur in peanut were evaluated for their effect *in vitro* on growth and aflatoxin production by *A. flavus* strain NRRL 3357. Media with varying concentrations were incubated for limited periods of time and, following removal of mycelial mats, were assayed for aflatoxins by high-performance liquid chromatography (HPLC). Mycelial mats were dried and dry weights determined.

Three preliminary studies were conducted to determine an appropriate inoculum dose (10^5 conidia per 25 ml of media) for obtaining detectable levels of aflatoxin. After the preliminary trials, three more trials were carried out. Experiment 1 was a factorial experiment with three levels of total reducing sugars (0, 0.1, and 0.3%) and three levels of sucrose (0, 2 and 4%). In this experiment, media were harvested at three different times (7, 10 and 13 days after inoculation (DAI)). In experiment 2, six different levels of sucrose (0, 2.5, 3, 5, 10 and 20%) were evaluated at three different time periods (7, 12 and 17 DAI). Experiment 3 consisted of five levels of sucrose (0 to 10% at 2.5%

increments), each with 0.4% reducing sugars, and three media adjustments (continuous (CON), dilution (DIL) and addition (ADD)).

In all experiments, significant increases in mycelial dry weights and total aflatoxins were observed with increases in sucrose concentrations, even when sucrose content was as low as found in peanut seed. Total reducing sugars in the media did not significantly influence total aflatoxins or mycelial dry weights. In experiments 1 and 2, where various incubation intervals were included, no differences in aflatoxins due to incubation intervals were found. With respect to mycelial dry weight, no differences were observed due to incubation interval in experiment 1, indicating significant growth up to 7 days after incubation (7 DAI). In experiment 2, mycelial dry weights were higher at 12 DAI compared to 7 or 17 DAI indicating a decrease in mycelial dry weights from 12 to 17 DAI. In experiment 3, higher levels of aflatoxins were obtained in treatments where media were diluted or not adjusted compared to treatments with sucrose additions. However, mycelial dry weights were higher in CON and ADD treatments compared to DIL treatment. Media in the dilution treatment might have become depleted of nutrients resulting in stress and thereby more aflatoxins, supporting the concept that aflatoxins are produced by aflatoxigenic fungi as a result of stress or in the conditions that are sub-optimal for fungal growth. The addition treatment with sucrose supplementation (i.e., without nutritional stress) and continuous or maintenance treatment provided for optimum fungal growth and resulted in reduced aflatoxin production in the media.

Results of the current study demonstrate the significance of sucrose levels in the ranges that occur in peanut in supporting aflatoxin production *in vitro*. As drought-stressed peanuts are reported to have more sugars than non-drought-stressed peanuts,

results of our study may provide a rational for higher aflatoxin contamination in peanut seeds produced under drought.

The effects of drought and supplemental soil calcium on total aflatoxins and sugar concentrations of peanut were also evaluated in greenhouse and field trials. Peanuts from these studies were analyzed for aflatoxin and sugar contents. In our greenhouse experiment, total aflatoxins were generally low (<20 ppb). However, significant differences in aflatoxins were observed due to drought, soil calcium, and the two-way interaction of drought and soil calcium. Peanuts from both drought treatments (35 and 60 days before harvest (DBH)) were contaminated with significantly higher levels of aflatoxins compared to those from the no-drought (0 DBH) treatment. From these results and previously published studies, it can be concluded that drought-stress occurring 5 or more weeks before harvest may result in greater aflatoxin contamination of peanuts. In the greenhouse study, total aflatoxins significantly declined with increasing soil calcium levels from 176 to 1783 mg/kg. Calcium contents of leaves, shells, and kernels were also estimated after harvest and calcium levels rose significantly with increases in soil calcium levels. Except for leaves, calcium levels of shells and kernels were significantly higher in 0 and 35 DBH treatments compared to 60 DBH treatments, indicating decreased calcium uptake by shells and kernels when peanuts were grown under prolonged drought stress. Significant negative correlations between total aflatoxins and calcium contents of peanut leaves, shells, and kernels were also observed. These results reinforce the importance of soil calcium in reducing peanut aflatoxin in the absence of substantial drought stress. Higher levels of total soluble sugars and sucrose in drought-stressed peanuts compared to

non-drought-stressed peanuts were observed. Significant positive correlations were also observed between total soluble sugars, sucrose, and total aflatoxins.

From the experiment at E.V. Smith Research Center, with two drought treatments (0 and 55 DBH), significantly higher levels of aflatoxins were found in drought-stressed peanuts compared to non-drought-stressed peanuts. An additional no-drought, non-inoculated treatment was included for comparison, in which no aflatoxins were detected. Similar to the greenhouse experiment, total soluble sugars as well as sucrose levels were higher in drought-stressed compared to non-drought-stressed peanuts and significant positive correlations between sucrose, total soluble sugars with total aflatoxins were found.

The field experiment at Wiregrass Research and Extension Center, with two levels of soil calcium (620 and 878 mg/kg) and two drought treatments (0 and 55 DBH), had results similar to the preceding experiments with higher aflatoxin levels in drought-stressed peanuts compared to non-drought-stressed peanuts. No significant differences in total aflatoxins were observed between 620 and 878 mg/kg soil calcium treatments in both drought and non-drought treatments. No significant differences in total soluble sugars, sucrose, and total reducing sugar levels were observed due to drought or soil calcium or their interaction. Peanut sugars and total aflatoxins in this experiment were not correlated.

Aflatoxins levels in the greenhouse were lower than those in the field studies. This might be because of lower temperatures in the greenhouse. We also observed variation in aflatoxin levels between the two field experiments. Aflatoxin levels were very high (up to 1600 ppb) in peanuts from the Wiregrass experiment compared to those

from the E.V. Smith experiment (up to 55 ppb). This difference can be attributed to high mean soil temperatures and a lower water holding capacity of soil at Wiregrass compared to E.V. Smith. Even though a significant decline in total aflatoxin values with increases in soil calcium levels in the greenhouse at all the drought levels tested was noted, the same pattern was not observed in the Wiregrass field study. It could be that the role of soil calcium on aflatoxin contamination is overwhelmed by the effect of other factors such as drought and soil temperatures.

The results of these studies reinforce the significant role of drought stress in predisposing peanuts to aflatoxin contamination. In our greenhouse and E.V. Smith experiments increased accumulation of total soluble sugars and sucrose in drought-stressed-peanuts compared to non-drought-stressed peanuts occurred. A positive correlation of peanut soluble sugars with aflatoxins was observed even with only 1 to 3% increase in sugar content. Thus, peanut soluble sugars, which are proven to be good sources for supporting aflatoxins biosynthesis *in vitro*, may also have a significant role in aflatoxin contamination of peanuts *in vivo.* Increases in sugars in peanut plants under drought stress may indicate a reason why fungal invasion and subsequent contamination of peanuts with aflatoxins are higher during drought years compared to non-drought years. Thus, during drought years, avoiding drought stress and associated high soil temperatures by providing supplemental irrigation is the only consistent means of reducing aflatoxin contamination.

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