Evaluating Soil-Weather-Agronomic Practices Interactions on Aflatoxin Corn Contamination in the South

by

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ABSTRACT

Aflatoxins are potent carcinogens and contaminated corn grains if consumed can have a deleterious effect on both humans and animals. Pre-harvest aflatoxin contamination in corn (*Zea mays* L.) is a continuing concern in the Southeast United States particularly in seasons with above normal temperatures, and lower than normal precipitation; conditions that promote in-field drought. If predicting aflatoxin accumulation in grain is feasible, then contamination concerns could be minimized.

Three studies are included in this dissertation: In the first study a research was conducted to determine whether a drought index could be used to predict the risk for pre-harvest aflatoxin contamination in corn, as well as to determine risk differences in-season and among sites. Our hypothesis is that aflatoxin risk changes as the drought conditions within the growing season change. Two datasets were considered: 1) data collected from a controlled experimental site at Starkville, MS over 13 years (2000 – 2011, and 2013 – 2014), on two soil types (a silty clay loam and a loam), with three commercial hybrids with different susceptibility levels to aflatoxin contamination, and 2) data from random corn fields collected from 1977 - 2004 across fifty three Georgia counties. The Agricultural Reference Index for Drought (ARID), a generic drought index calculated on daily basis, was evaluated as an aflatoxin risk prediction tool. ARID factors were calculated for weekly windows before and after silking to evaluate the in-season changes

in aflatoxin risk. Multiple logistic regression models were used to predict aflatoxin risk as a function of the derived weekly ARID values and risk level changes were tested according to soil type and corn hybrid susceptibility. If grain contamination with aflatoxins exceeds 20 ppb, then the United States Food and Drug Administration restricts corn contamination by humans and young animals. Therefore, this threshold (20 ppb) was selected to transform the raw aflatoxin data into a binary dependent variable for the logistic model.

Results from the first study revealed: 1) aflatoxin risk might be assessed by ARID, 2) soil type and hybrid susceptibility to aflatoxin contamination were statistically significant, and 3) ARID based risk changed during the growing season. These findings could be used to minimize aflatoxin risk by adapting site-specific management strategies such as: 1) triggering irrigation during critical risk weeks, 2) altering planting dates and/or select hybrids with suitable relative maturities to reduce plant exposure to drought stress during critical growth windows, 3) based on soil type, selecting the most appropriate hybrid for a given site/location, 4) separating a field into management zones, i.e. to segregate harvest if needed, and 5) determining best harvest timing.

Weather fluctuations have an impact on the extent of aflatoxin contamination, in part by stressing the crop, and thus predisposing the host plant to *A. flavus* infection and subsequent aflatoxin contamination. Planting dates and plant densities that alleviate crop stress during critical growth stage windows are expected to reduce mycotoxin contamination.

The objectives of the second study were to: 1) assess the effect of agronomic practices (planting date and plant density) on preharvest aflatoxin contamination in

rainfed corn grown in the Coastal Plains of South and Central Alabama, 2) identify weather variables that influence aflatoxin contamination in corn, 3) determine the relative weight those variables have on corn contamination, and d) determine time windows during the growing season when weather variables are associated to corn aflatoxin contamination.

Field experiments were conducted at Fairhope, AL, and Prattville, AL, for five and two years, respectively. The experimental design was a split–split plot design, with inoculation, planting date, and plant density assigned to main plots, sub-plots, and sub-subplots. Five time windows were considered: 1) a 2 week window before mid-silk, 2) a 2-week window after mid-silk, 3) the second 2-week window after mid-silk, 4) the third 2-week window after mid-silk, and 5) a variable in length window from the end of the third 2-week window to corn physiological maturity. For each of those windows average daily minimum temperature and cumulative rainfall were calculated. Multiple regression analysis with stepwise selection was used to study the influence of weather parameters on aflatoxin contamination for the five time windows defined. Six models were developed; three from pooled Fairhope data (2010 – 2014) and three from pooled data over Fairhope (2010 – 2014) and Prattville (2013 – 2014). The response variable in each of the models was corn aflatoxin contamination; explanatory variables tested were: 1) both derived cumulative rainfall and derived average daily minimum temperature variables for the five windows defined earlier (overall model x 2), 2) derived cumulative rainfall variables only (rainfall model x 2), 3) derived average daily minimum temperature variables only (minimum temperature model x 2).

The results from the second study showed: that mid-April planting date resulted in significant (p – value < 0.05) or relative reduction in aflatoxin contamination. Plant densities tested did not influence toxin accumulation. A significant negative linear relationship was found between aflatoxin and yield for 2011 in Fairhope. The overall model developed from Fairhope data only and from Fairhope and Prattville data combined had an R^2 equal to 87 and 76%, respectively. Rainfall models alone could explain more than 50% of the observed variability. The relative weight of derived weather variables that influence corn contamination for the window around silking was determined. Daily minimum temperatures for the first and third 2 week windows following silking had the largest impact on aflatoxin contamination with partial R^2 equal to 40 and 27% (Overall Model – Fairhope). The effect direction (positive/negative) of average daily minimum temperature on aflatoxin contamination, as indicated e.g.; by the minimum temperature models, is changing through the windows considered herein. A better understanding on the influence of weather variables on the contamination process may improve pre and post-harvest management practices, assist farmers in decision making, and improve efficiency and accuracy of monitoring and prediction.

Planting dates and plant densities have an influence on corn yield and when they interact with weather conditions that can impose plant stresses yield losses for dryland corn can be significant. Optimum planting dates and optimum plant densities are location specific and their determination is needed for sound management. However, this information is usually obtained through large scale experiments that are time consuming and expensive or through modeling approaches which require data that are not always readily available. Environmental stresses result in ^{13}C discrimination (Δ), and questions

arise whether yield differences as affected by planting dates and plant densities can be reflected on ^{13}C discrimination values from corn grains harvested within and at the end of the season.

The objectives of the third study were: 1) to explore if Δ is a suitable tool to explain corn yield differences resulting from planting date and plant density practices under the environmental conditions of Coastal Plains in Alabama, and 2) explore if Δ observations from corn grains sampled within season can account for attained yield differences. Field experiments were conducted at Fairhope and Prattville, as stated earlier. Corn grain was harvested at milk (R3) and at harvest maturity and analyzed for $\delta\ ^{13}C$.

The results of the third study showed that the relationship between yield and ^{13}C discrimination in corn grains harvested at milk (R3) and at harvest maturity was not consistent between years x locations and within year x location. Δ values of grain samples reflected yield differences between mid-March and mid-April planted corn in Prattville (for both grain harvest times) and in Fairhope (for grain harvested at R3 only) in 2013 and 2014, respectively. ^{13}C discrimination in corn grain was significantly influenced by plant density only for samples harvested at milk (R3) and harvest maturity in Fairhope in 2013 and 2014, respectively. In Fairhope, lower plant densities tend to have higher Δ and lower yield per unit area compared to higher corn densities. The inconsistencies in the relationship between Δ and corn yield indicate that factors not measured in this study can influence ^{13}C discrimination in corn grain. Therefore, more research is needed to elucidate the effect of different factors under field conditions before Δ can be used as a tool to assess corn attained yield differences.

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LITERATURE REVIEW

Aflatoxin-historical background

The concept of secondary metabolism, or in other words, the synthesis of compounds not utilized by fungi, was coined and started gaining acceptance in the late 19th and early 20th centuries (CAST, 2003). By definition, secondary metabolites differ from primary metabolites because they are unnecessary for fungal growth and reproduction (Jones, 1979). The biological concept of secondary metabolites has not been elucidated yet (Smith and Moss, 1985). However, several secondary metabolites can be active against microorganisms (antibiotics), plants (phytotoxins), and animals (mycotoxins). Toxic compounds produced by fungi enumerate in thousands (CAST, 2003). Around 300 toxic secondary metabolites produced by fungi were collectively classified as mycotoxins by Cole and Cox (1981), and are related to human and animal diseases (CAST, 2003). The potential number of mycotoxins is estimated in the range of 20,000 to 300,000 unique compounds (CAST, 2003). Food safety, grain trade, and food/feed marketing could be impacted from plant-pathogenic, mycotoxin producing fungi (Blandino et al., 2008; CAST, 2003).

Etymologically the word “mycotoxin” means “a toxin produced by a fungus” (CAST, 2003; Richard, 2008). Mycotoxins that have a huge impact on agricultural commodities

include: aflatoxins, trichothecenes (nivalenol and deoxynivalenol), fumonisins, ochratoxin A, zearalenone, and ergot alkaloids (Blandino et al., 2008; Blaney et al., 2008; CAST, 2003). Among those, aflatoxins are secondary metabolites of *Aspergillus* spp. (Gong et al., 2012; Hernández-Martínez and Navarro-Blasco, 2010; Lewis et al., 2005). More specifically, aflatoxins are difuro-cumarins and they are biosynthesized through a polyketide pathway (Mishra and Das, 2003; Probst and Cotty, 2012). Contamination of food, feed, and agricultural produce with aflatoxins has been a well-recognized problem for more than 50 years (Bayman and Cotty, 1990).

An aflatoxicosis outbreak in turkey, ducklings and pheasants was observed in areas nearby London and Cheshire in England in 1960, leading to the death of 100,000 turkey poults by the end of that year with a mortality rate ranging from 50 – 90 % (Blount, 1961; Richard, 2008; Spensley, 1963). The hypothesized disease was called the "Turkey X disease", but since studies failed to reveal any evidence of infection, the possibility of poisoning was considered (Blount, 1961). The outbreaks in England were soon associated to feeds containing Brazilian groundnut meal that were produced at two feed mills located near London and at Shelby, England (Blount, 1961; Richard, 2008; Spensley, 1963). Soon it was realized that other animals (e.g.; pigs, calves) when fed the ground meal were susceptible as well. Therefore, a biological assay test using one-day ducklings was devised, to find out that when poults were fed with the suspected meal within few days a destruction of liver parenchyma cells followed along with proliferation of bile duct epithelia cells. Concentrating the toxic unknown ten-thousandfold times, revealed that the concentrates fluorescent at the blue spectra when illuminated under blue ultraviolet light (Spensley, 1963). An analytical method to detect and quantify the toxic factor was

developed, and thus, more samples could be tested in shorter time. Those tests revealed that 1) the toxic unknown factor was also present in a portion of the ground nut meal batches examined; and 2) contaminated samples were originating from numerous countries other than Brazil. This suggested the possibility for a microorganism being related to the contamination itself.

An event of duckling deaths in Kenya was associated with the consumption of a groundnut meal originating from Uganda, which was heavily contaminated with fungi (Sargeant et al., 1961). This observation allowed for the isolation and identification of the fungus as *A. flavus* (Austwick and Ayerst, 1963; Richard, 2008; Sargeant et al., 1961). Feeding ducklings with extracts from fungal cultures revealed the same symptoms described in the cases of "Turkey X disease". The toxic entity was elucidated and called aflatoxin (Richard, 2008) with the name being derived from *A. flavus* ("***A****spergillus* ***fla****vus* **toxins**") (Cotty et al., 1994). Further studies led to the isolation and identification of the four major aflatoxins B_1, B_2, G_1 and G_2 produced by *Aspergillus* spp. (Armbrecht and Fitzhugh, 1964; Richard, 2008). B aflatoxins are about two times more toxic than G toxins (Schroeder and Hein, 1967). In the following years, aflatoxins were extracted from corn and cottonseed meal as well (Mishra and Das, 2003).

Aspergillus spp. strains related to aflatoxin synthesis

Aspergillus spp. belong to the class Hyphomycetes, subdivision Deuteromycotina, and family Aspergillaceae (Mishra and Das, 2003). Generally, *Aspergillus* spp. can grow on a vast array of substrates and under different environmental conditions. As a result, most foods, feed, and agricultural commodities may be susceptible to *Aspergillus* spp.

contamination during the production, transportation, and/or storage process. Although, *A. flavus* is well established in the scientific literature as a crop pathogen, which may contaminate produce prior and after harvest, there is not a sufficient understanding of its population biology (Abbas et al., 2004a).

The genus *Aspergillus* comprises an extended family that includes several hundred species that occupy diverse ecological niches (CAST, 2003; Dyer and O'Gorman, 2012). According to CAST (2003), *Aspergillus* spp. are most abundant between the 26^{th} and 35^{th} parallels north or south of the equator. In a review article, Williams et al. (2004) mentioned that contamination of inadequately stored and dried produce is likely under risk for areas between the latitudes 40^{o} to the North and South of the equator. Generally, it is acknowledged these fungi are more common in subtropical and warm temperate climates (CAST, 2003) and aflatoxin synthesis usually occurs in the tropics and subtropics (Streit et al., 2012).

The fungi of the genus *Aspergillus* are generally considered saprophytes, and some of them can thrive under warm and dry conditions (Cotty et al., 1994). The species known to synthesize aflatoxins belong to *Aspergillus* section *Flavi* and include *Aspergillus flavus, Aspergillus parasiticus* (Schroeder and Boller, 1973), *A. nomius* (Ito et al., 1998; Kurtzman et al., 1987), *A. pseudotamarii* (Ehrlich et al., 2007; Ito et al., 2001), *A. bombycis* (Peterson et al., 2001), *A. toxicarius (Murakami, 1971), A. parvisclerotigenus (Saito and Tsuruta, 1993), A. minisclerotigenes, A. arachidicola (Pildain et al., 2008)* and *A. pseudonomius* and *A. pseudocaelatus (Varga et al., 2011).*

According to CAST (2003) review, *A. flavus* and *A. parasiticus* were considered economically more significant because they could produce aflatoxin in corn, peanut

(*Arachis hypogaea* L.), cotton (*Gossypium hirsutum* L.), and tree nuts such as almonds (*Prunus dulcis*), walnuts (*Juglans* spp.), Brazilian groundnuts and pistachio (*Pistacia vera*). It is more common for *A. parasiticus* to infect oilseeds, and *A. flavus* to be found on cereals (e.g.; corn) (Smith and Moss, 1985). Studies indicate that additional Aspergillus spp. may pose a risk for contamination in agriculturally important crops. For example, the distribution of *A. nomius* is more widespread than initially considered, and thus, could be the primary aflatoxin producing species in certain geographic locations due to its ability to produce large quantities of aflatoxins (Ehrlich et al., 2007; Varga et al., 2011). In several studies *A. nomius* was collected from soils in the Southern US and was isolated from moldy wheat, cottonseed, and Brazilian groundnut (peanut) (Egel et al., 1994; Kurtzman et al., 1987).

Diverse strains of *Aspergillus* spp. inhabit soil reservoirs and become parasitic to plants only under conducive environmental conditions. Crop infection by *A. flavus* is more common than the infection by *A. nomius* (Ehrlich et al., 2007). Yet, as indicated by studies with endemic isolates originating from soils in Thailand, the higher aflatoxin synthesis by the former compared to contamination levels imposed by *A. flavus* raise the potential importance of *A. nomius* in contamination outbreaks in that vicinity (Ehrlich et al., 2007). Nevertheless, *A. flavus*, *A. parasiticus* and *A. nomius* due to their widespread distribution and their aflatoxigenic potential, are of imminent concern to the consumers, producers and industry of agricultural crops and commodities (CAST, 2003; Ito et al., 2001).

Aflatoxin production by *Aspergillus* spp. is strain specific; there are strains characterized as aflatoxigenic and non-aflatoxigenic (Smith and Moss, 1985).

Nevertheless, aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), and G_2 (AFG_2) are usually produced by *A. parasiticus* and *A. nomius*, while *A. flavus* normally produces only aflatoxins AFB_1 and AFB_2 (Ehrlich et al., 2007; Ogundero, 1987; Payne, 1998). Several studies have indicated that some atypical *A. flavus* strains could produce AFB_1, AFB_2, as well as AFG_1 and AFG_2 aflatoxins. These atypical *A. flavus* isolates have been primarily found in regions of Africa, Argentina and Australia (Ehrlich et al., 2007). Additionally, aflatoxins M_1 (AFM_1) and M_2 (AFM_2) are found in cow's milk and are the metabolic product of ingested AFB_1 and AFB_2 toxins; though strains of *A. flavus* and *A. parasiticus* are known to synthesize the secondary metabolite AFM_1, as well (Smith and Moss, 1985; Sweeney and Dobson, 1998).

Biology of *Aspergillus* spp. and infection mode

Development of effective postharvest control strategies requires a thorough understanding of the biology of the toxigenic fungi, along with sufficient understanding of the important factors related to infection, toxin synthesis and accumulation (Payne, 1998). *Aspergillus* spp. are omnipresent, saprophytic, filamentous, soilborne fungi that survive in the soil and plant debris as mycelium or sclerotia under unfavorable environmental conditions (Abbas et al., 2009; Payne, 1998). During most of their life cycle these fungi are saprophytic, and thus, survive on plant debris and/or animal tissues (Payne, 1998). These saprophytic pseudo – pathogens can infect and contaminate a large range of plant hosts such as corn, cotton, peanut, and tree nuts (Payne, 1998; Sweany et al., 2011). Thus, despite being saprophytes, it is well known that they can also behave as weak plant pathogens.

Aspergillus spp. population depends on environmental factors such as soil temperature and soil moisture. In a review article, Payne (1998) indicated that *A. flavus* and *A. parasiticus*, will grow under a wide range of temperatures (12-48 ^{o}C) and at very low water potentials (up to -35 MPa). As a result, this semi-thermophilic and semi-xerophobic species, when faced with drought conditions resulting from high temperatures and low available moisture may outcompete other soil microorganisms and become the dominant fungi in the soil. Thus, under drought stress and high temperatures they are capable: 1) of producing abundant primary inoculum; and 2) of outcompeting other microflora for infection sites on developing corn kernels; gaining an advantage for crop colonization and subsequent aflatoxin contamination.

When environmental conditions are favorable for growth of *Aspergillus* spp., sclerotia will germinate, mycelia are produced, and sporulation occurs (Abbas et al., 2009). Sclerotia are the overwintering reproductive structures of the fungi, and the primary source of inoculum to initialize the infection cycle, along with the overwintering in plant debris mycelium (hypha) (Abbas et al., 2009; Battilani et al., 2013). The new inoculum will colonize and infect the new season crops. Moreover, it is believed that crops can be infected more readily by *A. flavus* compared to *A. parasiticus* (Sweany et al., 2011). Conidia can be dispersed by wind, rain and/or insects. It is important to note that infection of cotton and corn requires the dissemination of conidia from soil to the plant tissue either by air currents or by insect vectors (Sweany et al., 2011). Therefore, the source of inoculum may come from a remote location different from the site of interest. In peanuts, pegs are infected by the fungi that are readily present in the soil.

Corn contamination with aflatoxins

Corn infection and subsequent contamination with aflatoxins is a worldwide issue. Naturally contaminated grain was reported in several countries (e.g.; Australia, France, Mozambique, Kenya, Hong Kong, Philippines, Thailand, and Uganda) (Lewis et al., 2005; Shephard, 2008; Shotwell, 1977). However, in many countries the extent of the problem is not well documented (Payne, 1992). Aflatoxin synthesis is more likely to occur in tropical and subtropical areas (Streit et al., 2012). Although, Europe was not considered a high risk area for preharvest aflatoxin contamination, this mycotoxin was found in domestically produced corn (*Zea mays* L.) in Northern Italy for the first time in 2003. Climate change may increase the risk of aflatoxin contamination in Europe (Battilani et al., 2008a; Giorni et al., 2007; Miraglia et al., 2009). In addition, a few cases of aflatoxin contamination have been reported in Australia (Blaney et al., 2008; Chauhan et al., 2008). In the United States, preharvest aflatoxin contamination is a chronic problem. Despite corn aflatoxin contamination being more severe in the Southeast region of the US, it also occurs in the Midwestern corn belt during seasons when environmental conditions are conducive, such as, but not limited, to drought around silking and grain filling (Windham et al., 2009)

According to the literature, contamination with aflatoxins could be separated into two phases; the first phase includes infection during crop development, followed by a second phase when increase in contamination levels after crop maturation until consumption may occur (Cotty, 2001; Cotty and Jaime-Garcia, 2007; Probst and Cotty, 2012). Process of contamination can be associated to the first or the second phase; e.g.; correlated to preharvest insect damage or resulting from poor postharvest storage conditions or

handling. Events occurring during both phases contribute to the final toxin concentration in the produce (Cotty and Jaime-Garcia, 2007). Weather influences each phase in different way. A prerequisite for contamination are environmental conditions that promote fungal growth and plant susceptibility to infection. Significant infection levels are promoted by high temperature stress, drought stress, and plant wounding (e.g.; by birds, insects, mammals and hail). In addition, during the second phase, aflatoxin contamination may follow after plant maturation, storage and processing. Aflatoxin concentration could increase in harvested crop components that were infected both before and after maturation. During the second phase conducive conditions that provoke contamination include warm and humid environments. Temperature and water content of the substrate will greatly influence the final levels of toxin accumulation in the food/feedstuff.

Fungal community strain morphotype significantly influences aflatoxin concentration in crops under consideration. It is well established that different strains of *A. flavus* have different aflatoxin synthesis potentials (Joffe, 1969). In vitro studies with 1,626 *A. flavus* isolates indicated toxin production ranged from 0 to 1,500,000 μg/kg (Joffe, 1969). One way to classify *A. flavus* isolates is to characterize them as atoxigenic (non-aflatoxin producers) and toxigenic (aflatoxin producers) (Abbas et al., 2009). Furthermore, the isolates of *A. flavus* can be classified as L or S strains depending on the size and numbers of sclerotia produced in vitro (Cotty, 1989). More specifically, the L strain morphotype produced fewer but larger sclerotia (size > 400 μm) when compared to the S strain sclerotia (size < 400 μm). Sclerotial production requirements in vitro varies between the L and S strains (Cotty, 1989). More specifically, Cotty (1989) showed that although S

strains will produce massive amounts of sclerotia between 25 – 38 ^{o}C, L isolates did not produce any sclerotia at 38 ^{o}C. Furthermore, at 25 ^{o}C only 65% of *A. flavus* strain L-isolates produced sclerotia. In addition, S strain isolates displayed the ability to produce sclerotia on broader range of growth media when compared to strain L of *A. flavus.*. Cotty (1989) revealed that S strains were 10 times more toxigenic that the L strains *in vitro*. More specifically, all the S-strains produced in vitro more than 500 ng of B_1 aflatoxin per 1g of culture, whereas, 80% of Strain L isolates produced below the aforementioned threshold. This contradicts with the finding of Abbas et al. (2005) who reported that L-strain isolates were more likely to produce high levels of aflatoxins (>10,000 μg/kg) when compared to S-strain isolates. Furthermore, it has been shown that isolates which are not producing sclerotia are usually less toxigenic when compared with isolates producing large sclerotia (Abbas et al., 2005; Abbas et al., 2006).

More importantly, Cotty (1989) did not find correlation between aflatoxin production in *in vivo* and *in vitro* studies in cotton for both strains L and S. Therefore, Cotty (1989) concluded that measuring only the *in vitro* toxigenicity of an isolate is not adequate to assess the potential risk for cottonseed contamination. Notably, serial transfers of isolates grown *in vitro* (PDA) quickly let to a reduction in aflatoxin production (Sweany et al., 2011). This may explain the observed differences between *in vivo* and *in vitro* aflatoxin synthesis of an isolate. Furthermore, aflatoxin contamination levels in cotton grown at the field were comparable for both L and S strains of *A. flavus* (Cotty, 1989). Although, strain L demonstrated larger pathogenic aggression in cotton in the aforementioned study, pathogenic aggression alone was not correlated with aflatoxin production.

Factors involved in infection and contamination of the developing crop

Several factors have been correlated to *A. flavus* infection and subsequent aflatoxin contamination of developing corn kernels, including 1) weather conditions during the growing seasons; 2) plant stress; 3) *A. flavus* inoculum level; 4) hybrid variation in susceptibility to infection and/or contamination due to genetic diversity; 5) insect damage; and 6) interactions among those factors (Lillehoj et al., 1980). In this review, the focus is on the influence of weather factors, host plant stress, and management practices that may alleviate or exacerbate preharvest contamination.

Zuber and Lillehoj (1979) emphasized that a healthy non-stressed plant has a smaller probability for infection and aflatoxin contamination when compared to a stressed crop. They observed, in 1971 and subsequent years, an increased contamination of corn with aflatoxin in areas where the crops were exposed to severe drought stress, particularly from silking to late dough. On the other hand, no plant immunity to aflatoxin contamination exists when conditions are favorable. Nevertheless, corn contamination with aflatoxin is a chronic issue and its severity fluctuates from year to year and from location to location as well (Widstrom et al., 1990).

1.1 Colonization and infection by Aspergillus flavus

Given favorable environmental conditions, several successive steps are involved in corn grain contamination by *A. flavus.* Initially, *A. flavus* colonizes corn silks, grows down the silks into the ear, and subsequently colonizes the exterior surfaces of developing kernels. Important to note, kernel colonization by *A. flavus* in corn is not always associated with visible signs of fungal sporulation (Marsh and Payne, 1984).

Nevertheless, *A. flavus* demonstrated ability to rapidly colonize corn ears may indicate that fungal dissemination within the ear by insects is of less significance. Needless to say, if kernel surfaces are already colonized by *A. flavus*, then insect feeding provides an entrance for the fungus into the endosperm and embryonic tissues.

In a greenhouse study, following spray-silk inoculation, sweet corn infection was greater when the plants were grown at 32 to 38 ^{o}C than at 21 to 26 ^{o}C (Jones et al., 1980; Payne, 1986). Thus, colonization of susceptible silks and invasion of kernel tissue by *A. flavus* required high temperature and high relative humidity (Jones et al., 1980). In field studies, colonization of silks and kernel surfaces was extensive 13 days following silk inoculation (Marsh and Payne, 1984). In growth chamber studies, Marsh and Payne (1984) found extensive colonization of silks maintained at 30/34 ^{o}C temperatures. Therefore, silk colonization and downward growth of the fungus to the kernels is likely influenced by temperature.

1.2 Drought and temperature impact on aflatoxin outbreaks

Warm and humid conditions, characteristic for tropical and subtropical regions, are considered optimum for *Aspergillus* spp. growth (Hernández-Martínez and Navarro-Blasco, 2010), though many species of the genus can grow, metabolize and spoil many dry foods at very low water activities (a_w) (e.g.; minimum water activity for *A. flavus* is 0.80) (Smith and Moss, 1985). Corn kernel contamination with aflatoxin may occur when growth conditions are characterized by extended drought and high temperatures during the silking and pollination growth stage (Battilani et al., 2008a; Payne, 1992; Windham et al., 2009). In 2005, an aflatoxin outbreak in rainfed corn in Burnett district, Australia, was correlated with drought condition and high temperature occurrences during corn crop

grain filling stage (Blaney et al., 2008; Chauhan et al., 2008). In Australia, the occurrence of aflatoxin in tested corn samples has increased from less than 2% in the 1980's up to 22% in 2005. This result is in agreement with climatic observations revealing persistent dry condition and increased ambient temperatures.

Furthermore, corn plants grown under highest drought and heat stress as reflected by plant physiological responses showed the highest aflatoxin contamination in corn kernels compared to the ones grown under less stress (Kebede et al., 2012). In the same study, however, an aflatoxin resistant genotype indicating signs of high stress had the lowest level of contamination. Kebede et al. (2012) speculated that a resistant genotype has a mechanism to inhibit fungal infection, growth and/or post infection aflatoxin synthesis. In a field study, Jones et al. (1981) showed that plants exposed to drought stress had higher incidence of contamination (54.9 % aflatoxin-positive samples) compared to plants cultivated under irrigation (23.6% aflatoxin-positive samples). Irrigation decreased aflatoxin AFB_1 levels significantly, with irrigated subplots having an average contamination of 7.3 μg/kg, whereas at the non-irrigated subplots the mean contamination was 61.9 μg/kg (Jones et al., 1981). The investigators concluded that the increased infection levels observed in the non-irrigated plots were due: 1) higher inoculum levels; and 2) more susceptible silks were exposed outside the husks to airborne conidia, since drought stressed plants tend to have smaller leaf area. Also, for three out of four experimental years, irrigation or subsoiling practices resulted in reduced aflatoxin contamination by reducing drought stress (Payne et al., 1986). Thus, Payne et al. (1986) concluded that contamination could be controlled by minimizing plant water stress, either by irrigation or by breaking hard pans by subsoiling. However, they speculated that in

years conducive for contamination, not only water stress was necessary, but also, the presence of adequate amount of inoculum (disease pressure) is an important factor promoting natural infection and subsequent contamination.

Aflatoxin contamination is more severe in the Southeastern United States compared to the other regions of the country (Payne, 1992; Widstrom et al., 1990). For example, higher monthly average temperatures on June, July and August in Florida were correlated with increased incidence of aflatoxin contamination in corn, when compared to Corn Belt sites (Illinois, Iowa, Ohio), which showed lower temperatures and no contaminated samples (Lillehoj et al., 1978). Though, in several occasions aflatoxin contamination issues were reported for the Midwestern corn belt of the United States as well (Windham et al., 2009). For instance, results from studies over an 11 year period revealed an incidence of aflatoxin contamination of 2 – 3% for corn grown at Mid-West or Corn Belt with only few samples having contamination levels above 20 μg/kg (Shotwell, 1977). In contrast, studies in 1969, 1970, 1971 and 1973 indicated contamination levels greater than 20 μg/kg for 13 – 32% of tested samples. Lillehoj et al. (1978), in a study conducted over nine locations in 1976, indicated that aflatoxin occurrence during corn grain filling varied from 0 to 75% in the Corn Belt and Florida, respectively. Wallin and Minor (1986), reported that aflatoxin contamination was detected in field locations across Missouri, only during seasons when heat and drought stress occurred in the area. The authors, also, note the different levels of contamination exhibited by different hybrids; a likely indication of different susceptibility levels due to differences in genotypes. Generally, aflatoxin outbreaks are a chronic issue in the Southeastern United States, while they are sporadic in the Corn Belt of US (Payne, 1992). The latest one occurred

during growing seasons characterized by higher temperatures compared to the thirty years temperature average which, consecutively expose crops to drought stress.

Variations in reported temperatures for growth of *Aspergillus* spp. are common in the respective literature (Pitt and Hocking, 2009). Nevertheless, *A. parasiticus* and *A. flavus* can grow at temperature ranges from 10 – 12 ^{o}C to 42 – 48 ^{o}C (Pitt and Hocking, 2009; Sweeney and Dobson, 1998). The minimum temperature for growth of *A. flavus* was reported between 6 – 8 ^{o}C, and the maximum around 44 – 46 ^{o}C by (Pannasenko, 1941). The optimum growth temperature for those fungi ranges from 32 – 33 ^{o}C (Pitt and Hocking, 2009). According to other sources, optimal growth for *A. flavus* occurs between 35 and 38 ^{o}C (Windham et al., 2009; Yu et al., 2011). Abdel-Hadi et al. (2012), in a modeling study, showed that optimal fungal growth was around 27 ^{o}C and 0.98 a_w. In the same study, marginal *A. flavus* growth conditions were defined at temperatures between 20 and 35 ^{o}C and a_w greater than 0.90. When exposed to 45 ^{o}C for 5 hours conidial germination and germ tube expansion of *A. flavus* were lowered as exposure duration time increased (Abdalla, 1988).

In addition, aflatoxin production occurred under a range of temperatures between 12 – 40 ^{o}C (Sweeney and Dobson, 1998) Ciegler et al. (1966), in *in vitro* study, showed that temperature optima for aflatoxin synthesis are narrow and strain specific. Marked decline in toxin synthesis was observed for all the strains tested at temperatures above and below 25 ^{o}C. For example, at 30 ^{o}C, *A. flavus* NRRL 3000 produced smaller but still significant amount of aflatoxins, whereas strain A-13570 synthesized the toxins at barely detectable levels. Sorenson et al. (1967), showed that in solid medium strain *A. parasiticus* NRRL 2999 produced maximum aflatoxins AFB_1 and AFG_1 at 28 ^{o}C. At 32 ^{o}C comparable

amounts of AFB_1 were produced while a decline in AFG_1 synthesis was reported. Generally, the same strain, produced less aflatoxins at temperatures above 32 ^{o}C, and below 28 ^{o}C, with no aflatoxins synthesized at 8 ^{o}C. Schroeder and Hein (1968), showed in an *in vitro* study that short periods of maximum temperatures had a greater impact on depressing fungal growth and toxin accumulation than minimum temperatures. It is important to note, that the maximum temperatures in the diurnal temperature cycles examined were extremely high (40, 45, 50 ^{o}C). Based on those observations, Schroeder and Hein (1968) concluded that maximum temperature on the 24 hr diurnal cycle will be more important on aflatoxin contamination for crops harvested in summer or early fall, while average temperatures might be more significant for crops harvested late in fall. In another *in vitro* study, Schroeder and Hein (1967) indicated a positive correlation between increasing temperature between 25 and 35 ^{o}C and increased aflatoxin synthesis. There was little or no effect of the substrates (cotton seed, peanut, and rough rice) used on aflatoxin synthesis (Schroeder and Hein, 1967). In Schroeder and Hein (1967) optimal fungal growth was determined around 25 ^{o}C. In a recent system approach modeling study, it was shown that *A. flavus* strain NRRL 3357 produces aflatoxins at optimum rate at 0.98 – 0.99 a_w and 25 – 33 ^{o} C (Abdel-Hadi et al., 2012). The differences observed between studies, reflect the complex interactions between strains, substrates and temperature on aflatoxin production (Schroeder and Hein, 1968).

Some investigators suggested that the optimum aflatoxin production occurs around 30 ^{o}C (Widstrom et al., 2003; Yu et al., 2011). In contrast, the optimal temperature for corn growth is approximately at 27^{o}C. Under drought stress optimal corn growth temperature is even lower. Temperatures higher than 27 ^{o}C even for few days during the growing

season will subsequently lead to an increase in aflatoxin production. At these temperatures plant growth, grain filling capacity, and plant resistivity to fungal infection is also reduced. It is accepted that high temperatures may influence infection and subsequent contamination by impacting either the plant, or the fungus or both (Payne, 1986).

Although the relationship between temperature and aflatoxin contamination was demonstrated under controlled environments (e.g.; greenhouse) (Jones et al., 1980; Payne G. A. et al., 1988) and confirmed by several field studies (McMillian et al., 1985b; Widstrom et al., 1990), some field experiments failed to demonstrate a correlation among the two parameters (Stoloff and Lillehoj, 1981). In other works, a negative association between higher average minimum August temperature and aflatoxin incidence and severity was demonstrated (Sisson, 1986). The observation by Sisson (1986) contradicts the established theory that higher temperatures stress the plant and should result in higher contamination. It is likely on days of higher minimum temperature to have lower dew incidence that might reduce fungal growth and subsequent toxin accumulation (Sisson, 1986).

In a review article, Widstrom et al. (2003) concluded that temperature is a significant factor for both infection and subsequent corn contamination. Some of the discrepancies observed in the literature may be explained because the relationship between temperature and aflatoxin contamination is detected only during years with high contamination levels (McMillian et al., 1985b; Widstrom et al., 1990).

1.3 Rainfall effect

Low precipitation during the growing season accompanied with high temperatures is related with aflatoxin contamination severity (Widstrom et al., 2003). Despite, there was not possible to establish the hypothesis that rainfall alone is a major component in aflatoxin outbreak in dryland fields. Rather, rainfall is contributing synergistically with other climatic factors such as temperature to aflatoxin contamination severity. Preharvest aflatoxin corn contamination was influenced by rainfalls late in the season that coincided with harvest time (Jones et al., 1981). Additionally, in cotton Jaime-Garcia and Cotty (2003) showed that rainfall in July following boll opening could explain 50% of the aflatoxin concentration in cottonseed. Also, at the field level, a steady decline of *A. flavus* isolates (spores) was observed from August up to November; a time period which coincided with the rainy season in Sudan (Abdalla, 1988). The higher number of airborne isolates were observed in summer months which are characterized as hot, dry and dusty, with a peak reached in June.

Rainfall data alone, could not explain the observed differences in corn aflatoxin incident among locations extend from the Southern states to the Corn Belt region (Lillehoj et al., 1978). In all the study sites, lower than normal rainfall accumulation was reported over some timespans in the growth season. Though, distinct drought conditions in July and August in Florida (1976) might have promoted *A. flavus* infection and subsequent toxin accumulation. In another study, precipitation was not correlated with aflatoxin contamination in a five year study conducted in Georgia (Widstrom et al., 1990).

1.4 Effect of humidity, net evaporation and wind speed

Aflatoxin incidence was correlated with high humidity and high temperatures (Sisson, 1986; Widstrom et al., 2003). Specifically, July average high humidity was positively correlated with aflatoxin incidence, but, interestingly August average minimum temperature was negatively correlated with aflatoxin incidence. Additionally, Sisson (1986) showed that July average high humidity and high average temperature were positively correlated with aflatoxin contamination in corn. Though, they also monitored that aflatoxin severity was negatively associated with August average minimum temperature and July average low humidity. In a five year study in Georgia, minimum relative humidity, calculated for the 20-40 days window after full-silk, was correlated (negatively) only in one year (1986) and when the data were pooled over planting dates (Widstrom et al., 1990). In the same study, Widstrom et al. (1990) showed that aflatoxin contamination was correlated to maximum and minimum temperature, and net evaporation. Those variables were more important than humidity and precipitation for contamination to occur. Furthermore, high mean temperature and net evaporation were significantly correlated to aflatoxin contamination in grain samples at harvest (McMillian et al., 1985b; Widstrom et al., 2003). In addition, *Aspergillus* spp. hyphal fragment dispersal was negatively correlated with relative humidity, while hyphal fragment and airborne conidia dispersal were positively correlated to wind speed (Li and Kendrick, 1995). Based on results from a phytotron study, Jones et al. (1980) suggested that high humidity levels for more than 24h might be necessary for a successful kernel infection via the silks. From short time (72 hrs) incubation observations, they speculated that high humidity conditions are very likely needed only for spore germination; a preliminary step

for kernel infection. High morning dew levels, common in the Southeastern US, were suggested as a likely parameter for elevated contamination levels in corn by McMillian et al. (1985a).

1.5 Effect of soil type

Aflatoxin contamination may be influenced by soil types present where a corn crop is grown (Widstrom et al., 2003). For example, Jones et al. (1981) observed higher levels of contamination for corn grown on the sandy soils of the Coastal Plain region than for crops grown under heavier (clay) soils of the Piedmont region. Lighter soils are more prone to moisture depletion, which very likely leads to increased drought plant stress during the growing season (Jones et al., 1981). A drought stressed plant tends to develop a smaller canopy, since plant water deficit ceases leaf expansion, among others. Jones et al. (1981) suggested that drought stress predisposes corn to higher fungal infection levels by increasing the exposure of silks to airborne spores, as a result of the reduced plant canopy.

Soil type influences the microbiota present in a field as well. The source of corn infection is thought to be the inoculum of *Aspergillus* spp. found in the soil (Angle, 1986). Those spores could be carried from the soil to the infection site either by wind or insects. Angle (1986) showed that the largest population of *A. flavus* and *A. parasiticus* were found in soil under conventional tillage where the residues were incorporated in the soil, rather than with no-till systems where the corn residues remained on the soil surface. Therefore, soil type in combination with cultural practices (e.g.; conservation tillage) along with the cropping system selected may alter the population of *Aspergillus* spp. in the soil. This could influence corn infection and aflatoxin contamination. Additionally,

corn planted in May rather than April, corn harvested late in the season, and grown under low nitrogen regime showed increased aflatoxin contamination levels (Jones and Duncan, 1981). In this study, Jones and Duncan (1981) concluded that nitrogen stress could result in a crop more susceptible to aflatoxin contamination, compared to corn grown under best management practices. For corn grown on soils with substantial organic matter (ca. 6%) and ample nitrogen fertilization, potentially higher nitrogen mineralization levels might lower contamination levels when compared to sandy soils. In general, it was suggested that field selection could influence aflatoxin contamination in corn, since soil profiles have highly variable water holding capacities (Jones, 1986). Replacing corn cultivation for more drought tolerant crops (e.g.; grain sorghum (*Sorghum vulgare*)) in sandy soils or soils with shallow profiles could be an alternative to mitigate contamination levels.

1.6 Effect of management practices

The concept of reducing crop mycotoxin contamination has its origins in plant disease epidemiology (Munkvold, 2003). The principal strategy is to alter crop growth conditions in an effort to avoid infection by pathogenic fungi. Tactics that might be employed to fulfill this goal include: tillage practices, fertilization practices, crop rotation, plant densities, planting dates, irrigation, hybrid selection, disease, insect and weed control (Daves C.A. et al., 2010; Munkvold, 2003; Yu, 2012). Thus, corn infection by *Aspergillus* spp. and grain contamination with aflatoxin are affected by a number of parameters that are under farmers' control (Daves C.A. et al., 2010; Yu, 2012)**.** Cultural practices may influence preharvest aflatoxin synthesis in corn kernels because there is a relationship between drought stress, susceptibility of corn genotypes to *A. flavus*, and aflatoxin accumulation (Munkvold, 2003). Cultivation practices exposing the plant to

increased drought stress should lead to higher preharvest aflatoxin levels. For example, Wallin and Minor (1986) had noted that temperature and drought stress lead to yield reduction in corn and increased aflatoxin contamination in grain; in 1984 in Missouri. aflatoxin levels where higher at plots where yields were lower than normal.

Planting corn late in Georgia revealed lower aflatoxin concentration given that conditions for aflatoxin contamination were favorable during the growing season (Widstrom et al., 1990). According to Widstrom et al (1990) early planting dates (before 15th of April) resulted in higher aflatoxin contamination because the critical stage (20-60 days after full silk) fell between mid-June and early August. During this time of the year the temperature and net evaporation in Georgia are the highest resulting in larger plant and fungi stress due to adverse climatic conditions. Accordingly, higher levels of toxin accumulation were observed for the first two planting dates (mid-March to mid-April) compared to corn planted later (from late-April to early-May) (Smith and Riley, 1992). A significant problem with the later planting dates (June - August) was a substantial yield reduction. In North Carolina, aflatoxin incidence and level of contamination increased as corn planting date was shifted from April to May (Jones et al., 1981). Jones et al. (1981), explained the reduced aflatoxin contamination observed because the corn from pollination to grain filling was exposed to less stress when planted in April rather than in May. Inoculated (silk sprayed) short-season corn hybrid planted in mid-May had higher contamination levels than when planted at mid-April; the opposite trend was observed for the long-season hybrid (Jones et al., 1980). Mean aflatoxin concentration was greater for corn planted early in May in Georgia and Florida, when compared to plantings on early

April and early June (Lillehoj et al., 1980); though no significant toxin levels differences were found based on planting dates for Missouri and South Carolina.

In general, selection of planting dates in any region should have as a goal to minimize the exposure of the corn crop to heat and drought stress during the reproductive stages (Bruns and Abbas, 2006). The differences observed in literature between the association of planting dates and aflatoxin levels, are likely related to climate (e.g.; temperature, humidity, rainfall, among others) and insect pressure on infection and contamination (Smith and Riley, 1992). Those parameters are highly variable among locations and within seasons as well.

Planting densities were considered as one parameter that could influence corn infection by *A. flavus* and subsequent grain contamination (Jones, 1986). The optimum planting density for corn varies from location to location with a range of about 25,000 to 80,000 plants per hectare (ha^{-1}). In Mid-South, the optimum plant population was determined to be 70,000 corn plants ha^{-1} (Bruns and Abbas, 2005). Theoretically, high plant population densities induce elevated nutrient and water stress among inter row crops, and thus, predisposing corn to aflatoxin contamination (Bruns, 2003). In a study conducted in Mexico, Rodriguez-del-Bosque (1996) showed that aflatoxin contamination in corn was influenced by late planting and insect damage, but the effect of planting density was not significant. Similarly, Abbas et al. (2012) showed that planting densities were not consistently a significant factor in aflatoxin contamination for both naturally infected and inoculated corn with *A. flavus*. Furthermore, Bruns and Abbas (2005) could not establish a significant effect of planting density and nitrogen treatment on grain aflatoxin contamination. In contrast, higher population densities for corn planted in bed

plantings reduced yield and increased significantly aflatoxin concentration in the grain than lower planting densities for corn planted in furrows (Alvarado-Carrillo M. et al., 2010). The authors concluded that higher water demand under the highest planting density regime could increase plant stress, and therefore, contributed to the higher aflatoxin contamination levels.

Applying lower rates of nitrogen to soil resulted in higher aflatoxin contamination in corn (Jones, 1986; Jones and Duncan, 1981). Crops having increased nitrogen concentration in the grain and the leaf tissue tend to have lower contamination levels. In the field, corn requirements for water and nitrogen vary considerable through the season; they are minimal in the early growth stages, increase as the season progresses, and peak from flowering and grain formation (Jones, 1979). It is known that most of the nitrogen needed for plant growth reaches the rhizosphere by mass flow, and thus, nitrogen has to be carried by soil water. Therefore, nitrogen uptake and subsequent translocation in the plant could be influenced by drought stress (Jones, 1986). Among others, this might have an impact on the physiological status of the grain (C/N ratio), and could consequently alter the aflatoxin synthesis potential in the stressed kernel (Jones, 1979). Jones (1986) suggested that corn aflatoxin contamination could be minimized under balanced fertilization programs.

Several studies have evaluated the effect of irrigation on corn aflatoxin contamination (Jones, 1986). In North Carolina, irrigation and subsoiling have increased corn yield and reduce aflatoxin contamination in naturally infected treatments by reducing plant drought stress in three out of the four years the study was conducted (Payne et al., 1986). Jones et al. (1981) showed that irrigation resulted in less contaminated samples (23% positive

sample) than the non-irrigated treatment (54.9% positive samples). In the same study, irrigation reduced average contamination level tremendously and had fewer visibly infected ears compared to non-irrigation level. In a study in South Carolina, 14 out of the15 hybrids tested showed lower aflatoxin contamination when irrigated compared to the rainfed control (Fortnum and Manwiller, 1985). Despite irrigation applications to relieve drought stress in corn, greater aflatoxin levels were observed in all except one location in 1979 compared to 1980 (Stoloff and Lillehoj, 1981). Neither the drought hypothesis nor the maximum average temperature between flowering and harvest could give a plausible explanation for the observations in the aforementioned study.

1.7 Effect of pH

Several studies have examined the effect of pH on *Aspergillus* spp. growth, sporulation and aflatoxin synthesis. Moreover, *A. parasiticus* and *A. flavus* can grow under a vast array of pH values ranging from acidic (pH = 2.0) to basic (pH = 11.0) conditions (Sweeney and Dobson, 1998; Wheeler et al., 1991). Wheeler et al. (1991) illustrated that *A. parasiticus* has a low tolerance to acid conditions (pH = 2.0). In the same study, *A. parasiticus* showed optimal growth at a pH range of 3.0 to 8.0 (at optimal growth temperature of 30 ^{o}C), and was more tolerant to acidic conditions when compared to *A. flavus*. In another study, two strains of *A. flavus* showed optimum growth at 3.3 – 7.1 with lower and higher pH values where mycelium growth was observed ranging from 2.1 to 10.0, respectively (Rudolph, 1962). Optimum pH range for conidia formation ranged greatly among different *A. flavus* strains, but the production ranged from acidic (2.1 – 2.7) to basic (10.0). The sclerotia formation pH range observed was narrower that the conidial synthesis pH range detected.

In a review article, Sweeney and Dobson (1998) mentioned that pH range for aflatoxin production occurs between pH 3.0 – 8.0. Optimum aflatoxin production occurs at slightly acidic pH ≈ 6.0, but there is evidence that this may depend on the culture medium as well (Buchanan and Ayres, 1976; Sweeney and Dobson, 1998). In another study, maximum production for AFB_1, AFB_2, AFG_1, and AFG_2 toxins was observed between 25 and 30 ^oC at 5.5 and 5.9 pH (Molina and Giannuzzi, 2002). Aflatoxin production was reduced at both pH values tested (5.5 and 5.9) at 36 ^oC.

Aflatoxins regulations

The domestic and trade acceptable limits for mycotoxins contamination are established for products and not for the production processes or the treatments the products undergo along the market chains (Dohlman, 2003). Since aflatoxins are highly poisonous, several countries have established regulatory actions and are monitoring aflatoxin contamination in food, feed and derived products (Dohlman, 2003). As from 1996, there were forty-eight countries where regulatory agencies have set different acceptable total aflatoxin limits in food; the action limits range from (0 – 50 μg/kg). Twenty one countries have established tolerance levels in feedstuffs with allowable total aflatoxin levels ranging from 0 to 1,000 μg/kg.

Mycotoxin regulations in the United States have been established since 1968 (Dohlman, 2003). The United States Food and Drug Administration regulations prohibit the sale of grains with aflatoxin contamination greater than 20 μg/kg (Abbas et al., 2006; Daves C.A. et al., 2010; Windham et al., 2009). Therefore, corn grain with aflatoxin concentration above the established threshold of 20 μg/kg becomes unmarketable for

human consumption. In milk, the established level for AFM_1 aflatoxin is even lower (0.5 μg/kg) due to higher susceptibility of young animals to aflatoxin exposure (Wilkinson and Abbas, 2008; Yu, 2012).

In the European Union, the regulation limits for aflatoxins as set by the European Commission are more stringent. For example, limits for groundnuts subject to further sorting or physical treatment before human consumption are set to 8 and 15 μg/kg for AFB_1 and total aflatoxins, respectively. The upper limits for cereals, groundnuts and their processed products intended for direct human consumption are even lower (2 and 4 μg/kg for AFB_1 and total aflatoxins, respectively) (European Commission, 2006). Additionally, AFB_1 aflatoxin level in unprocessed corn intended for human consumption cannot exceed 5 μg/kg of produce (Battilani et al., 2013; European Commission, 2006). Some countries, e.g.; the Netherlands, have set a 0 limit as a maximum accepted level for aflatoxin contamination in infant foods (Aksit et al., 1997).

In Australia, regulating aflatoxins in grain food requiring toxins concentration "as low as is reasonably achievable" (Chauhan et al., 2008). Furthermore, the upper regulated limit for AFB_1 aflatoxin contamination in corn feed is established at 20 μg/kg. Additionally, in Australia trading standards for total aflatoxins (AFB_1, AFB_2, AFG_1, and AG_2) are established by the National Agricultural Commodities Marketing Association. Specifically, standards require total toxins levels in corn to not exceed 5, 15, 20 and 80 μg/kg for milling grade, prime grade, feed #1 and feed #2, respectively.

As a result, aflatoxin contamination of agricultural commodities not only imposes serious health risks for mammals (both human and animals), but it may induce tremendous economic losses to the farmers and the agricultural industry as a whole (Yu,

2012). Aflatoxin contamination of agricultural commodities has an implication on international trade as well. Though, aflatoxin contamination of food and feed is a significant issue mainly in the developing countries. This is due to lack of substantial monitoring and regulatory measures that would allow for detection of contaminated produce before it enters the food supply.

Aflatoxins: Chemistry and effects on human and animals

Aflatoxins are structurally related chemical compounds (difuro-coumarin derivatives) characterized as extremely toxic, naturally occurring carcinogens (hepatocarcinogenic), mutagenic, teratogenic, and immunosuppressive (Hernández-Martínez and Navarro-Blasco, 2010; Mishra and Das, 2003; Probst and Cotty, 2012; Widstrom et al., 2003; Wilkinson and Abbas, 2008). Furthermore, aflatoxins have been shown to be involved in stunting growth (Wilkinson and Abbas, 2008). In addition, it is estimated that species variation, sex, age, and nutritional status are among several factors that influence aflatoxin biological effects (Mishra and Das, 2003). Toxicological and carcinogenic effects may result from the metabolic activation of AFB_1 aflatoxin which results in the formation of the reactive exo-B_1-8,9-epoxide (Kelly et al., 1997; Wilkinson and Abbas, 2008). In mammalian liver the formation of the putative reactive intermediate (exo-B_1-8,9-epoxide) is determined by the presence of the P450 cytochromes. The formation of the epoxide may lead to both the toxic or detoxification pathway (Mishra and Das, 2003). In addition, mutation effects are related to the ability of the aflatoxin compound to bind to the DNA molecule and interfere with protein synthesis. Moreover, inhibiting protein synthesis and subsequently impairing differentiation processes of primordial cells may lead to teratogeneses in several species. The biological effects on a particular organism

are further influenced by the aflatoxin concentration and exposure time. More specifically, according to Mishra et al. exposure to large concentrations of aflatoxin will inhibit total biochemical processes. Lower doses may have impact on different metabolic pathways as well.

Aflatoxin effects on both animals and humans are classified as chronic or acute (Mishra and Das, 2003; Yu, 2012). Consumption of contaminated feed, food and agricultural produce results in a disease called aflatoxicosis. Research has demonstrated that consecutive aflatoxin contaminated feed consumption results in bile duct proliferation, hepatic necrosis, immune suppression, and osteosclerosis, among others. Aflatoxicosis in man due to consumption of contaminated corn is documented as well. Bhat (1989) has reported childhood liver cirrhosis and liver cancer resulting from the consumption of aflatoxin contaminated groundnut meal. Also, preliminary results indicated a potential increased lung cancer risk for workers exposed to dusts containing excessive concentrations of aflatoxins (Kelly et al., 1997).

Although, more than 20 different kinds of aflatoxins are identified, six of them (AFB_1, AFB_2, AFG_1,AFG_2, AFM_1, and AFM_2) are the ones usually observed in contaminated feed, food and agricultural produce (Hernández-Martínez and Navarro-Blasco, 2010; Mishra and Das, 2003; Sweeney and Dobson, 1998; Yu, 2012). AFB_1 and AFB_2 derived their nomenclature form the blue fluorescent color emitted under UV light on thin layer chromatography plates (Sweeney and Dobson, 1998). In addition, AFG_1 and AFG_2 will emit green fluorescent color when exposed to UV light. The subscript numbers 1 and 2 refer to major and minor compounds, respectively. *A. flavus,* produces mainly

AFB_1 and AFB_2 aflatoxins, while AFB_1, AFB_2, AFG_1 and AFG_2 aflatoxins are produced by *A. parasiticus* (Battilani et al., 2008a; Wilkinson and Abbas, 2008; Yu, 2012).

Feeding animals with AFB_1 aflatoxin contaminated feed results in the production of AFM_1 aflatoxin, a metabolite (monohydroxylated derivative of AFB_1) which is secreted then into lactating animals milk (Frobish et al., 1986; Sweeney and Dobson, 1998). Similarly, AFM_2 aflatoxin is the product of animal metabolism when fed on AFB_2 contaminated feed**.** AFM_1 has been detected in human breast milk samples from Australia and Thailand, indicating exposure of infants and mothers to AFM_1 and AFB_1 toxins, respectively (El-Nezami et al., 1995) Furthermore, AFM_1 has been detected in foodstuff such as infant milk and other related milk products (e.g.; yogurt, cheese) (Galvano et al., 1996; Sweeney and Dobson, 1998). Exposure of infants and young animals to AFM_1 is of concern because this particular aflatoxin is not inactive and its precursor (AFB_1) is carcinogenic (El-Nezami et al., 1995). Therefore, the limits for AFM_1 in infant foods are very low (0.01 – 0.05 μg/kg). The reasons for this regulation are related to the relative high consumption rates of dairy products by infants, their low body weight and their higher susceptibility to aflatoxin exposure (Aksit et al., 1997; Sweeney and Dobson, 1998).

Moreover, AFB_1 and AFG_1 are the most commonly observed (Mishra and Das, 2003). Among aflatoxins, AFB_1 is the most studied and the one considered the most dangerous due to its acute and chronic effects (Mishra and Das, 2003; Payne, 1992). Human embryonic cell growth was inhibited at 0.05 μg/mL AFB_1 concentration (Wilkinson and Abbas, 2008). Also, studies has shown fetal stunting growth in rats when the female was exposed to AFB_1 at later prenatal stages (day 16 of pregnancy) (Butler

and S., 1965; El-Nezami et al., 1995). In addition, AFB_1 demonstrated carcinogenic effects led the International Agency for Research on Cancer to classify the specific mycotoxin in class 1 among other prominent carcinogenic compounds (Battilani et al., 2013; Hernández-Martínez and Navarro-Blasco, 2010). Nevertheless, consuming foods contaminated with aflatoxin is related with the occurrence of hepatocellular carcinoma, the fifth most frequent cancer observed in human (Windham et al., 2009)**.**

Biosynthetic pathway of aflatoxins

Aflatoxin formation pathway involves the synthesis of several intermediates (Yu, 2012). A breakthrough in the understanding of aflatoxin biosynthesis followed the discovery of norsolorinic acid when an *A. parasiticus* color mutant was found that accumulates the brick-red pigment. Norsolorinic acid is the first stable aflatoxin precursor. According to the literature, the first compound involved in aflatoxin biosynthesis is a hexanoyl unit. Three different enzymes (two fatty acid synthases and one polyketide synthase) are involved in the catalysis of the hexanoyl starter unit to a polyketide. Noranthrone (norsolorinic acid synthase), a precursor of e norsolorinic acid, is produced from seven repetitive malonyl-derived ketide extensions. Although, the conversion of noranthrone to the norsolorinic acid is not well understood, it has been suggested that this may occur either through catalysis by a noranthrone oxidase, or it takes place spontaneously.

In the next step, norsolorinic acid is converted to averantin (Yu, 2012). The reaction involves the conversion of the 1'-keto group of the norsolorinic acid into the 1'-hydroxyl group of averantin. This reaction is catalyzed by ketoreductase encoded by an *aflD (nor1)*

gene. Several homologous genes of *aflD (nor-1)* such as *aflE (nor A)* and *aflF (norB)* are found in the aflatoxin pathway gene cluster. These genes may encode short chain aryl alcohol dehydrogenases which may catalyze the reaction of norsolorinic acid to averantin as well.

Following, averantin is converted to 5'-hydroxyaverantin (Yu, 2012). Research has established that there are three enzymatic steps which may contribute to the conversion of norsolorinic acid to averufin. First norsolorinic acid is catalyzed by reductase and forms averantin. Second, monooxygenase catalyzes the conversion of norsolonic acid to 5'-hydroxyaverantin. Third, a dehydrogenase enzyme catalyzes the conversion of 5'-hydroxyaverantin to averufin. Experimentation has established that 5'-hydroxyaverantin is an intermediate step on the conversion of averantin to averufin. This may occur either directly, or indirectly. In the last case 5'-hydroxyaverantin conversion to averufin is catalyzed by a cytosolic enzyme.

Furthermore, an enzyme (cytochrome P450 monooxidase), along with two genes, the *CypX* and the *afl1 (avfA)* are involved in the conversion of averufin to versiconal hemiacetal acetate (Yu, 2012). Encoded proteins from the above mentioned genes may have a role in the ring-closure step which leads to the formation of hydroxyversicolorone, which is the precursor of versiconal hemiacetal acetate (Chang et al., 2004).

The *aflJ (estA)*, an esterase gene, was identified in the aflatoxin gene cluster which is encoding for an esterase enzyme. The esterase is involved in the catalysis of versiconal hemiacetal acetate to versiconal (Chang et al., 2004; Yu, 2012). These two compounds are part of a biosynthetic grid where the steps from versiconal hemiacetal acetate to

versiconal and from versiconol acetate to versiconol are most likely catalyzed by the same enzyme.

In the following step of aflatoxin biosynthesis we have the enzymatic conversion of versiconal to versicolorin B (Yu, 2012). The *aflK (vbs)* gene is responsible for the catalysis of the above biosynthetic step. Furthermore, this part of the aflatoxin biosynthetic pathway is considered important because it leads to aflatoxin's bisfuran ring closure. This particular moiety is considered responsible for the toxicity and carcinogenicity demonstrated by the toxin.

In addition, *versicolorin B* is a critical point in the biosynthetic pathway that may lead to the formation of either AFB_1 and AFG_1 or AFB_2 and AFG_2 aflatoxins (Yu, 2012). If *versicolorin B* is not converted to *versicolorin A* through a desaturation of its bisfuran ring (reaction is catalyzed by an unstable microsomal enzyme coded in *A. parasiticus* and *A. flavus* by *aflL (verb)* gene, then the pathway leads to the formation ofAFB_2 and AFG_2. Those aflatoxins along with *versicolorin B* contain a tetrahydrobisfuran ring in their molecule. In contrast, the formation of *versicolorin A*, a compound containing a dihydrobisfuran ring, will lead to the synthesis of aflatoxins B_1 and G_1.

Furthermore, the biochemical pathway leading to the conversion of versicolorin A and B to demethylsterigmatocystin and demethyldihydrosterigmatocystin, respectively, has been studied extensively (Yu, 2012). There are several genes that are involved in the formation of intermediates that lead to synthesis of demethylsterigmatocystin and demethylhydrosterigmatocystin. For example, research indicated that the *aflM (ver1)* gene found in *A. parasiticus* is involved in the conversion of versicolorin A to an intermediate. This intermediate is believed to be a ketoreductase. Among others, genes

involved in the formation of intermediates during the conversion of versicolorin A to demethylsterigmatocystin include *stcU, stcL* and *stcS* (homologues found in *Aspergillus nidulans*). Disruption of those genes resulted in accumulation of versicolorin A indicating their importance in its conversion to demethylsterigmatocystin.

Conversion of demethylsterigmatocystin and demethyldihydrosterigmatocystin to their products (sterigmatocystin and dihydrosterigmatocystin, respectively) is catalyzed by *O*-methyltransferase I and *O*-methyltransferase II (Yu, 2012). The enzyme catalyzes the transfer of the methyl group from *S*-adenosylmethionine to the hydroxyls of demethylsterigmatocystin and demethyldihydrosterigmatocystin. The end products for these reactions are sterigmatocystin and dihydrosterigmatocystin. Encoding for the enzyme *O*-methyltransferase I is done by the gene *aflO (omtB),* which was isolated by *A. parasiticus, A. flavus* and *A. sojae.*

Furthermore, the enzyme *O*-methyltransferase A, which is encoded by the gene *aflP (omtA),* is involved in the catalysis of sterigmatocystin and dihydrosterigmatocystin to O-methylsterigmatocystin and dihydro-O-methylsterigmatocystin, respectively (Yu, 2012). In addition, the above mentioned enzyme cannot methylate demethylsterigmatocystin and demethyldihydrosterigmatocystin because of its stringent substrate-specificity. The gene that codes for *O*-methyltransferase A is the *aflP (omtA).* It has been isolated from *A. parasiticus* and *A. flavus,* butit is absent in the genome of the *A. nidulans*. Thus, *A. nidulans* is a non-aflatoxigenic species that biosynthesizes sterigmatocystin as an end-product.

In the final step of the aflatoxins biosynthetic pathway, several genes (coding for specific enzymes) seems to be involved in the conversion of O-methylsterigmatocystin to

aflatoxins AFB_1 and AFG_1 (Yu, 2012). Respectively, dihydro-O-methylsterigmatocystin is converted to aflatoxins AFB_2 and AFG_2. It was demonstrated that the gene *aflQ (ordA)* plays a role in the previously mentioned conversions, which lead to the production of both aflatoxin groups (AFB and AFG). Studies have suggested that a cytochrome P450 monooxygenase coded by the gene *cypA* is involved in the biosynthesis of the AFG_1 and AFG_2. Another cytosolic enzyme (NadA) was reported to be involved in the synthesis of an intermediate (NADA), which is located between O-methylsterigmatocystin and the two AFG group in the synthetic pathway.

Nevertheless, when aflatoxins AFB_1 and AFB_2 enter the mammalian body through the consumption of aflatoxin contaminated food and feed they are converted to the less toxic aflatoxins AFM_1 and AFM_2 (Yu, 2012). More specifically, in mammals liver cytochrome P450 enzymes will catalyze the conversion of aflatoxins to an epoxide intermediate which is more carcinogenic compared to its precursors. Alternatively, aflatoxins maybe hydroxylated to the less harmful AFM_1 and AFM_2 aflatoxins.

Methods for aflatoxin determination

Various analytical methods can be used for aflatoxin detection in produce and *in vitro* cultures, including high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), enzyme-linked immunosorbent assay (ELISA), and fluorescent polarization assay (Abbas et al., 2004b; Smith and Moss, 1985). Each of these methods has its pros and cons (Abbas et al., 2004b). For example, most of these methods can be time consuming and expensive. In this context, chromatographic methods require intermediate extraction procedures with mixtures of polar and non-polar solvents (e.g.;

water and methanol) to remove potentially interfering substances. HPLC allows for individual aflatoxins detection and quantification by coupling the instrument with sensitive detectors (e.g.; fluorescence detectors) and sophisticated data retrieval methods (Smith and Moss, 1985). Commercially available ELISA kits are relatively easy to use, and allow for a relatively fast total aflatoxin assessment and quantification (Abbas et al., 2004b). However, identification of individual aflatoxins in the analyzed sample is not feasible with ELISA.

Prediction models for aflatoxin in corn

Predicting pre-harvest risk for aflatoxin contamination in corn may improve crop management efficiency (Wu et al., 2011), thus permitting system management optimization and allowing for sound preharvest and postharvest management decisions (Battilani et al., 2013). Therefore, mitigating consumer and farm animals exposure to aflatoxins might be feasible (Battilani et al., 2013).

Based on developmental approach, models can be classified either as empirical or mechanistic (Battilani et al., 2013; Campbell and Madden, 1990). Statistical analysis of field data can reveal relationships between the response and explanatory variables, leading to the development of an empirical model. Model evaluation on experimental data follows, to check if the model is sound and to investigate relatedness of predicted and observed values. A mechanistic model usually denotes cause and effect relationships between variables, succession of processes, and might improve understanding and interpretation of the studied phenomenon (Battilani et al., 2013) by providing an insight into the behavior of a system may reveal areas where knowledge and thus understanding

is lacking and/or is incomplete (Pitt, 1993). In a mechanistic approach, the starting developmental point is rather a concept, hypothesis and/or theory; not the data *per se*, as is the case in the descriptive (empirical) model (Campbell and Madden, 1990). A model is proposed, then, experiments follow to test model's accuracy and precision. A good fit indicates that the model and thus, the theory/concept beyond it, reflects reality adequately. In contrast, a poor fit would require a reevaluation and potential revision of the theory that underlines the proposed model.

Modeling mycotoxin synthesis is a challenging task (Pitt, 1993). The difficulties arise partly because secondary metabolism regulation is not well understood. Also, the relationship between secondary and primary metabolism has not yet been elucidated. Since mycotoxins are secondary metabolites, one should expect their production curve to be parallel, but to lag when compared to a fungal growth curve (Garcia et al., 2013). Garcia et al. (2013) observed a delay of 4 – 8 days and 2 days for the initiation of AFB_1 synthesis compared to fungal growth on yellow grain maize having a_w equal to 0.90 and 0.99, respectively. The lag phase between aflatoxin synthesis and fungal growth was not observed in the maize based agar medium, suggesting behavior similar to primary metabolites (Garcia et al., 2013). Time series data on aflatoxin biosynthesis indicated a rise in toxin concentration followed by a concentration decrease (Ciegler et al., 1966). Mycelia lysis seems to be related to aflatoxin degradation (Ciegler et al., 1966), but the mechanisms is not completely elucidated adding to the challenge of mathematical modeling. Differences in hybrid susceptibility to infection and contamination, along with variations in toxigenicity levels characterizing different strains of *Aspergillus* spp., add to

the complexity and make it challenging to describe, explain, generalize and predict the mechanism of aflatoxin formation.

Several studies considered modeling of *A. flavus* growth and/or contamination prediction in the lab or field. Pitt (1993) developed a mechanistic model to describe fungal growth and aflatoxin synthesis based on temperature, water activity, pH and colony size. Garcia et al. (2013) modeled aflatoxin synthesis by *A. flavus* grown in vitro on corn agar medium and corn grain under two different water activities (0.99 and 0.90). In their approach, aflatoxin concentrations were estimated through 1) colony radius data, 2) colony surface data, and 3) fungal biomass dry weight data by using the general mixed-growth associated Leudeking-Piret model. Abdel-Hadi et al. (2012) used mixed-growth-associated product formation model to mathematically model aflatoxin production in relationship to temperature, water activity, relative expression of 10 genes, and growth rate.

Attempts to predict the in-field aflatoxin corn contamination based on environmental conditions by using empirical or mechanistic models have been recently reported (Battilani et al., 2008a; Battilani et al., 2013; Chauhan et al., 2015; Chauhan et al., 2008). Logistic regression has been used previously to assess the in-field risk of 1) gray leaf spot of corn, caused by *Cercospora zeae-maydis* (Paul and Munkvold, 2004); and 2) fumonisin contamination in corn (Battilani et al., 2008b). Battilani et al. (2008a) extended this approach to predict aflatoxin corn contamination in fields in Northern Italy by using an aridity index as an independent variable. *A. flavus* growth and aflatoxin production were predicted by applying a mechanistic model that requires hourly weather data as input variables (Battilani et al., 2013). Two mechanistic models driven by temperature

and soil moisture content reflecting drought during corn grain filling period have been recently developed in Australia to predict contamination risk (Chauhan et al., 2015; Chauhan et al., 2008).

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1 EVALUATING A GENERIC DROUGHT INDEX AS A PREDICTIVE TOOL FOR AFLATOXIN CONTAMINATION OF CORN: FROM FIELD TO STATE LEVEL

Abstract

Corn (*Zea mays* L.) kernel infection by *Aspergillus flavus* and subsequent aflatoxin accumulation in grain can have a deleterious effect on mammals, including both humans and animals that consume the grain. Pre-harvest aflatoxin contamination in corn is a continuing issue in the Southeastern United States especially during growing seasons characterized by extreme high temperatures, low humidity, and lower than normal precipitation; all conditions promoting in-field plant stress. Predicting the risk of this problem is challenging due to complex interactions of biotic and abiotic stress factors that govern and exacerbate the phenomenon. This study was conducted to determine whether a drought index could be used to predict the risk for pre-harvest aflatoxin contamination in corn, as well as to determine risk differences in-season and among soil types. The underlying hypothesis is that as drought conditions during the growing season are changing the risk for aflatoxin is also changing. Risk assessment was approached at: 1) field (plot) level with data obtained from an in-field controlled experiment (Mississippi study), and 2) at state level, where corn fields were sampled at a county level (Georgia study). The data used for this study consisted of historical records on aflatoxin contamination and were collected over thirteen growing seasons from 2000 to 2011; and from 2013 and 2014 at Starkville, Mississippi (1), and from random corn fields in fifty-three counties across Georgia between 1977 and 2004 (2). A controlled experiment was conducted at Mississippi with two soil types (a Leeper silty clay loam and a Myatt loam), and three commercial hybrids characterized by different susceptibility levels to aflatoxin contamination. The Agricultural Reference Index for Drought (ARID), a generic drought index for calculating drought on daily basis was used as a predictor for aflatoxin risk

prediction. ARID was evaluated as an aflatoxin risk prediction tool. Mid-silk day was selected to split each growing season into two time periods, which were further divided into positive and negative weeks representing weeks after and before mid-silk, respectively. Weekly ARID factors were calculated for all periods to evaluate the in-season alterations in aflatoxin risk. Multiple logistic regression models were used to predict aflatoxin risk as a function of the weekly ARID values in both studies. In Mississippi, risk level changes were additionally tested according to soil type and corn hybrid aflatoxin susceptibility. The United States Food and Drug Administration restricts corn grain consumption by humans and young animals if the contamination level is above 20 ppb; thus, this threshold (20 ppb) was selected to develop a binary dependent variable for the logistic model from the raw aflatoxin data. Results revealed: 1) ARID might be used as a predictive tool to assess aflatoxin risk, 2) soil type and hybrid susceptibility to aflatoxin contamination were statistically significant independent factors, and 3) critical week windows during the growing season when changes in drought conditions affect the likelihood for aflatoxin contamination were determined for both areas. These findings could be used to minimize risk by adapting site-specific management strategies such as: 1) triggering irrigation during critical risk weeks; 2) selecting the most appropriate hybrid for a given site/location based on soil type; and 3) determining best harvest time.

Keywords: *Aspergillus flavus,* infection, logistic regression, modeling, risk assessment

Introduction

Mycotoxins are toxins produced by a number of fungi that infest plants and plant material and that can impair food safety, grain trade, and food/feed marketing (Blandino et al., 2008; CAST, 2003). Of particular interest are aflatoxins, a major class of mycotoxins that can potentially contaminate food, feed, and agricultural produce. Aflatoxin contamination has a significant impact on corn grain quality (Abbas et al., 2012). Aflatoxins are difuro-cumarins biosynthesized secondary metabolites through a polyketide pathway (Fountain et al., 2014; Mishra and Das, 2003; Probst and Cotty, 2012) produced by several fungi species belonging to *Aspergillus* section *Flavi* (CAST, 2003). Among these, *A. flavus* and *A. parasiticus,* both saprophytic and weak facultative plant-parasitic fungi, are more common and of major concern because they can contaminate corn, peanuts, cotton, and tree nuts such as almonds, walnuts, Brazilian nuts and pistachio (CAST, 2003; Diener et al., 1987; Klich, 2007). The most prevalent naturally occurring forms of aflatoxins include the toxins B_1, B_2, G_1 and G_2, with types B and G being usually synthesized by *A. parasiticus* and *A. nomius*, while *A. flavus* mainly produces B_1 and B_2 aflatoxins (Klich, 2007).

Contamination by aflatoxins is a worldwide issue and their discovery followed an outbreak of Turkey “X” disease in England in 1960 (Austwick and Ayerst, 1963; Bayman and Cotty, 1990; Blount, 1961; Richard, 2008; Sargeant et al., 1961; Spensley, 1963). In many countries, the extent of aflatoxin contamination is not well known since there is a reluctance to report the problem (Payne, 1992). Nevertheless, aflatoxin synthesis is more likely to occur in areas with tropical and subtropical climates (Streit et al., 2012). In recent decades, severe aflatoxicosis outbreaks have been reported in Kenya, India, and

Malaysia (Lewis et al., 2005; Shephard, 2008). Despite Europe, including the Southern regions, not being considered at high risk for aflatoxin, significant pre-harvest corn contamination was first observed in Northern Italy in 2003 (Battilani et al., 2008a; Giorni et al., 2007; Piva et al., 2006). Climate change scenarios for the European continent predict more frequent extreme weather events (e.g.; drought, floods, warmer temperatures); as a consequence, aflatoxin risk is expected to increase (Battilani et al., 2016; Bunyavanich et al., 2003; Miraglia et al., 2009). Several cases of aflatoxin contamination in corn have been reported in Australia (Blaney et al., 2008). In the United States, corn infection and subsequent contamination is a chronic economic and health concern in the South (Davis et al., 1986; Diener et al., 1987; Payne, 1992). During years conducive for contamination, in-field contamination may occur in Midwest as well (Payne, 1992; Wallin and Minor, 1986; Zuber and Lillehoj, 1979).

Aflatoxin contamination occurs both pre-harvest and post-harvest. One tactic to mitigate contamination problems is to reduce the risk of infection prior to harvest. (Chauhan et al., 2015). This should reduce residual inoculum in harvested corn grain which is a source of further contamination under poor storage conditions. The in-field contamination is highly variable both within a particular field and among geographic areas and seasons (Battilani et al., 2008a; Hawkins et al., 2008), reflecting the effect weather conditions has on *A. flavus* incidence (Cotty and Jaime-Garcia, 2007) and plant predisposition to infection/contamination (Fountain et al., 2014).

Aflatoxin contamination is a concern due to the health impacts the toxins have on mammals and the associated economic losses (Robens and Cardwell, 2003). Aflatoxins are considered carcinogenic, mutagenic, teratogenic, and hepatotoxic compounds for both

humans and animals (Blandino et al., 2008; Blaney et al., 2008; CAST, 2003; Fountain et al., 2014; Molina and Giannuzzi, 2002). Death from acute toxicosis may result with digestion of highly contaminated food and feed (Fountain et al., 2014; Shephard, 2008). Chronic exposure may result in liver tumors (CAST, 2003). Therefore, 48 countries have established regulatory actions and are monitoring aflatoxin contamination in food and 21 countries have established tolerance levels in feedstuffs; the acceptable total aflatoxin limits in food and feed range from 0 to 50 and 0 to 1,000 μg/kg, respectively (Dohlman, 2003; Hawkins et al., 2008; Mishra and Das, 2003). The United States Food and Drug Administration (U.S. FDA) restricts consumption of corn grain by humans and young animals if contamination levels exceed 20 μg of aflatoxin/kg of grain (U.S. Food and Drug Administration, 2000). The economic burden of aflatoxins is related to: 1) direct costs incurred due to loss in crops, livestock and dairy, and 2) indirect costs associated to allocation of funds for development/establishment of quality-control programs, research and education, reduced foreign trade, increased storage and packaging costs for vulnerable products (Mishra and Das, 2003; Robens and Cardwell, 2003). Additional costs are associated with aflatoxin mitigation.

In principle, aflatoxin contamination is exacerbated in seasons characterized by higher temperatures and lower than normal rainfalls that may expose corn plants to drought stress from silking and through grain filling (Diener et al., 1987; Payne, 1992; Windham et al., 2009). Agricultural drought occurs when plant available water in the soil does not meet the atmospheric demand for evapotranspiration (Woli et al., 2012). Critical time windows when the risk is greater were identified in numerous studies and include: 1) a window extending between days 65 and 85 following planting when heat stress may

result in increased contamination (Hawkins et al., 2008), 2) a monthly bracket around silking of high atmospheric moisture demand days occur (Hawkins et al., 2008), 3) contamination process was correlated with maximum temperature, minimum temperature, and net daily evaporation 20 to 40 and 40 to 60 days after full silk (Widstrom et al., 1990), 4) depending on soil type, cumulative rainfall and maximum daily temperatures were negatively and positively correlated with aflatoxin contamination, respectively, for biweekly intervals after and/or before mid-silk (Windham et al., 2009), 5) the decadal intervals from late June to late August when drought, as quantified by an aridity index, were significantly correlated with aflatoxin contamination in Northern Italy (Battilani et al., 2008a), and 6) in a preliminary analysis, Damianidis et al. (2015) identified weekly intervals around mid-silk when the risk changed in correlation to changes in drought conditions, (e.g.; weeks four and one before mid-silk and week four following mid-silk, among others). Conclusively, drought stress around silking and during kernel development has been indicated as key risk factor for elevated corn infection and aflatoxin contamination at the end of the season (Damianidis et al., 2015; Diener et al., 1987; Luo et al., 2010; Payne et al., 1986; Windham et al., 2009).

Models have been used to answer questions related to research, crop management, policymaking, and to assess the risk associated with human and animal health (Garcia et al., 2009; Prandini et al., 2009). If aflatoxin risk could be predicted, then human/animal health concerns, and the subsequent economic losses, could be minimized. Modeling efforts to predict aflatoxin contamination have been reported in numerous *in vitro* studies. For example, Pitt (1993) developed a mechanistic model to describe fungal growth and

aflatoxin synthesis based on temperature, water activity, pH and colony size under controlled environment. Garcia et al. (2013) modeled aflatoxin synthesis by *A. flavus* grown *in vitro* on maize agar medium and maize grain under two different water activities (0.99 and 0.90). Similarly, Abdel-Hadi et al. (2012) used mixed-growth-associated product formation model to mathematically model aflatoxin production in relationship to temperature, a_w, relative expression of 10 genes, and fungal growth rate. Among others, Molina and Giannuzzi (2002) used 1) an exponential equation to model aflatoxin synthesis and degradation phases over time in an agar medium under three temperatures (25, 30, and 36 ^{o}C) and two pH regimes (5.5 and 5.9); and 2) an Arrhenius-like function to study the effect of temperature on aflatoxin synthesis at different pH levels. Although in many instances those models could predict contamination well, they have not been evaluated under field conditions (Chauhan et al., 2015; Chauhan et al., 2008). Additionally, contamination levels from *in vitro* studies do not always correlate well with *in vivo* observations (Probst and Cotty, 2012). Therefore, models developed with data generated from artificial media (*in vitro*) should be used with caution for in-field corn aflatoxin contamination assessment (Chauhan et al., 2015).

Several attempts to predict the in-field aflatoxin corn contamination based on environmental conditions have been recently reported by using empirical or mechanistic models (Battilani et al., 2008a; Battilani et al., 2013; Chauhan et al., 2015; Chauhan et al., 2008). A genetic algorithm/neutral network approach has been used to predict aflatoxin contamination in peanuts based on environmental data such as soil temperature, drought duration, and accumulated heat units (Henderson et al., 2000). Logistic regression has been used previously to assess the in-field risk of 1) gray leaf spot of corn,

caused by *Cercospora zeae-maydis* (Paul and Munkvold, 2004); and 2) fumonisin contamination in corn (Battilani et al., 2008b). Battilani et al. (2008a) extended this approach to predict aflatoxin corn contamination in fields of Northern Italy by using as independent variable an aridity index. *Aspergillus flavus* growth and aflatoxin production were predicted by applying a mechanistic model that requires hourly weather data as input variables (Battilani et al., 2013). Two mechanistic models driven by temperature and soil moisture content reflecting drought during corn grain fill have been recently developed in Australia to predict contamination risk (Chauhan et al., 2015; Chauhan et al., 2008). However, apparently, assessing in-season corn contamination with a generic drought index in the Southeastern United States has yet to be done. The hypothesis driving this study was that changes in spatial and in-season drought lead to changes in the risk for aflatoxin contamination of corn. Therefore, the aims of this study were to: 1) determine whether a drought index could be used to predict the risk for aflatoxin contamination in corn; 2) assess in-season, among soil types and among hybrids, risk differences; and 3) explore the applicability of the proposed methodology to predict the risk at regional level when minimum data are available and the uncertainties are greater.

Materials and Methods

Two different aflatoxin datasets were used in the study including aflatoxin contamination data collected from a control experiment in Starkville, Mississippi (MS); and data on aflatoxin contamination from corn samples harvested from randomly surveyed fields across fifty-three counties in South Georgia.

1.1 Mississippi field experiments dataset

Field experiments were conducted from 2000 to 2011 and 2013 to 2014, at the R. R. Foil Plant Science Research Center located at Starkville, Mississippi (Windham et al., 2009). In summary, the experimental design was a split plot design with corn hybrids assigned to the main plots, while inoculation methods (natural infection, side needle, and spray silks) were allotted to sub-plots (Windham et al., 2009). Hybrids were selected and classified into three categories based on their susceptibility to infection by *Aspergillus flavus* and subsequent aflatoxin contamination. Two of the cultivars were characterized as moderately susceptible (indicated hereafter as hybrid 1 and hybrid 2), and a third (hybrid 3) as highly susceptible to aflatoxin contamination. Starting in 2000 and up to 2005 the experiment was conducted for two soil types, a Leeper silty clay loam (Fine, smectitic, nonacid, thermic Vertic Epiaquepts) and a Myatt loam (Fine-loamy, siliceous, active, thermic Typic Endoaquults). From 2006 and after, the study was conducted only for the heavier soil type (Leeper silty clay loam). Corn ear samples were harvested from each plot, processed, and analyzed for aflatoxin contamination (μg/kg) as described in more details by Windham et al. (2009). Contamination data related to natural infection were only considered for the analysis herein.

The comprehensive database contained 240 aflatoxin contamination observations and was divided into two datasets; a model development dataset and a model evaluation dataset. Twenty percent of the data in the comprehensive database were randomly selected to create an evaluation dataset by implementing the RANUNI statement in SAS software version 9.3 (SAS, 2010), while the rest of the data (80%) were used for model development.

1.2 Georgia dataset

A second dataset with corn aflatoxin contamination data was used for this study. Corn samples were collected from random farm fields located in fifty-three counties in Georgia from 1977 to 2004. The study area was located between latitude 33.07^{o} and 30.84^{o} N and longitude 81.12^{o} and 84.89 W. Over the years, a total of 818 samples were collected. Up to the late 90's, aflatoxin contamination and identification was determined by Thin Layer Chromatography (Brown et al., 1993; Guo et al., 1995), and thereafter, the VICAM AflaTest® (VICAM, Watertown, Massachusetts) analytical method was used by following the manufacturer specifications. The database was unbalanced, since fields were not sampled from all counties every season. Samples were assigned to Georgia counties within which a sampled corn field was located. The comprehensive survey database was randomly separated into a model development dataset (containing 80% of the data) and evaluation (containing 20% of the data) dataset, as previously described.

1.3 Quantifying seasonal drought

The Agricultural Reference Index for Drought (ARID) is a simple drought index used to monitor, predict and estimate the effect of drought timing and degree on crop yields

(Woli et al., 2012). ARID, a generic drought index, reflects seasonal in-field drought conditions, requires a minimal number of site specific weather parameters, and is calculated on a daily basis. ARID values range from 0 to 1; 0 indicates no water deficit (plants transpire to their maximum potential), and 1 signifies maximum water deficit (stomata are closed).

Ideally, weather parameters required to calculate ARID include daily maximum, minimum and dew point temperatures along with precipitation, wind speed, potential evapotranspiration (ET_o) and solar radiation. However, ARID calculations could be completed even when weather parameters are missing (e.g.; daily maximum temperature, minimum temperature and rainfall will suffice). For the Mississippi data, ARID calculations were based on weather data provided by the Mississippi Agricultural and Forestry Experimental Station, while meteorological data for Georgia analysis were retrieved from: 1) DAYMET database covering the timespan from 1980 – 2004 (Thornton et al., 2014); and 2) CRONOS database for seasons 1977 to 1979 (State Climate Office of North Carolina, 2016). For Mississippi data, ET_o was estimated by FAO Penman-Monteith method (Allen et al., 2006); for Georgia, where wind speed and dew temperature data were not available for the seasons studied, ET_o was alternatively estimated by Hargreaves equation as described in details by Allen et al. (2006). Whenever the Hargreaves equation is used for ET_o estimation in a region, comparison with ET_o estimates by the FAO Penman-Monteith model is suggested (Allen et al., 2006). Univariate regression analysis indicated that for the study area in Georgia the two methods were comparable (R^2 ranged from 0.9360 to 0.9807; for the eight locations tested).

1.4 Logistic regression – concepts

Logistic regression requires the dependent variable to be formulated as a binary factor, and it can be used to estimate the probabilities for an event to occur based on preselected independent predictors. The logistic regression model can be described by the equation:

$$P(Event) = \frac{e^{\beta_o+\beta_1\chi_1+\beta_2\chi_2+\cdots+\beta_n}}{1+e^{\beta_o+\beta_1\chi_1+\beta_2\chi_2+\cdots+\beta_n}} \quad (1)$$

where *e* is the exponential constant, β_o, β_1, β_2, and β_n are the estimated coefficients, and x_1, x_2, and x_n are the independent variables. P(Event) is the probability for an event to occur; in this study to have a contaminated sample.

In binary response models (e.g.; logistic models), model assessment can be accomplished by generating the Receiver Operating Characteristic (ROC) curve and calculating the area under the ROC curve (AUC) (Damianidis et al., 2015; SAS, 2015). The AUCs of the developed and the evaluated models are then compared for equality at a preselected level of significance with the ROC curve of a model predicting by chance (model with only intercept).

The AUC provides a graphical summary to assess the predictive power of a binary model (Allison, 2012). It does not depend on an arbitrary cutpoint value needed for the construction of a classification table, which inherently has an influence on the classification of test results as events or non-events (Allison, 2012). The area under the ROC curve takes values from 0 to 1; larger values correspond to stronger associations

between predicted and observed values. A value of 0.5000 corresponds to a model with an intercept only, and thus, with no predictive power. The more the ROC curve departs from the forty five degree line the more accurate the model predicts.

1.5 Database development for both studies

Prior to conducting the logistic analysis the aflatoxin contamination data were transformed to a binary variable. The U.S. FDA restricts corn consumption by humans and young animals if grain contamination exceeds 20 μg/kg. This threshold (20 μg/kg) was used to split the raw aflatoxin data into two groups: contaminated and not contaminated. When the grain contamination level was > 20 μg/kg and ≤ 20 μg/kg, the data were assigned a value of 1 (contaminated) and 0 (not contaminated), respectively; thus, developing a new dichotomous variable for the analysis. A dependent binary variable might be used in a logistic model to predict the probabilities for an event to occur based on one or more independent input variables.

Weekly ARID factors were calculated as follows. In the first step, each growing season was divided into two time intervals surrounding mid-silk day. Thus, each season consisted of positive days and negative days indicating time periods before and after mid-silk, respectively. Positive (after mid-silk) and negative (before mid-silk) weekly ARID values were calculated and used as independent predictors in the logistic models to assess in-season risk changes in aflatoxin contamination. Mid-silk was selected as a reference day for two reasons: 1) to remove the portion of the variability related to the different growing seasons, since plant growth and development depends greatly on weather conditions that are particular for each year and do not coincide with calendar days; and 2)

as indicated in the literature, the likelihood for infection and contamination is greater around corn silking (Hawkins et al., 2008; Windham et al., 2009).

1.6 Predicting aflatoxin risk with logistic regression at field (plot) level – Mississippi study

In the current study, ARID was evaluated, as a predictive tool for pre-harvest aflatoxin contamination. Statistical analyses were carried on with PROC logistic procedure in SAS version 9.3. Multiple logistic regression was used to predict aflatoxin risk as a function of weekly ARID values, soil type and hybrid susceptibility to infection and contamination. Additionally, risk level changes were studied in their association to soil type and corn hybrids.

Inclusion of all the weekly ARID values as predictive variables in the model resulted in high multicollinearity. Multicollinearity makes the estimated coefficients more unstable, and one way to mitigate the issue is by dropping collinear variables (Allison, 2012). Thus, all potential predictors (weekly ARID values) of aflatoxin risk were initially tested at the univariable level for significance (p-value = 0.05). The Variance Inflation Factor (VIF) of the retained predictors was < 5, indicating that multicollinearity was alleviated and more robust estimates could be obtained. Logistic model development followed by including only significant weekly ARID independent variables as identified in the first step, and their association to the outcome at the multivariable level was also tested. The logistic model used for the Mississippi data set analysis is given by the following equation:

$$P(Aflatoxin) = \frac{e^{\beta_0+\beta_1 Soil+\beta_2 Hybrid+\beta_{x1} Week_{x1}+\cdots+\beta_{xn} Week_{xn}}}{1+e^{\beta_0+\beta_1 Soil+\beta_2 Hybrid+\beta_{x1} Week_{x1}+\cdots+\beta_{xn} Week_{xn}}} \quad (2)$$

where P(Aflatoxin) is the probability to have aflatoxin contamination above the selected threshold (20 μg/kg), e is the base of natural logarithm, β_o, β_1, β_2, β_{x1},...β_{xn} are the estimated coefficients, and Soil, Hybrid, $Week_{x1}$,…$Week_{xn}$ are the independent variables that were entered into the model. Stepwise selection with entry and exit criteria levels equal to 0.10 and 0.20, respectively, was employed to define significant independent predictors during the model building phase.

The predictive power of the developed model was assessed by external evaluation using the independent evaluation dataset (SAS, 2015). The estimated AUC for the developed model along with the ROC curve computed when the fitted model was applied to the independent dataset (external evaluation) were compared for equality at level of significance $\alpha = 0.05$ with the uninformative model (a model predicting by chance; AUC = 0.5000).

1.7 Predicting aflatoxin risk with logistic regression at state level – Georgia study

The data from Georgia were used to determine if ARID can be used as a tool to predict aflatoxin risk at a regional scale. Briefly, the comprehensive survey database was randomly separated into a model development and evaluation datasets, and a binary response variable was constructed from the original aflatoxin contamination data as described previously.

Assumptions needed for calculation of weekly ARID values included: 1) planting dates and 2) mid-silk day. Planting dates from the state variety trials conducted by the University of Georgia at the Coastal Plains of Georgia (Tifton, Plains, and Midville

Georgia), and by the Auburn University at Headland, Alabama were available from 1977 to 2004 (Alabama Cooperative Extension System et al., 2016; The University of Georgia CAES, 2016). For each season, the four planting dates retrieved from the aforementioned corn trial studies, were averaged, and thus, a potential planting date for each year was calculated. In a given year, all the Georgia counties with aflatoxin contamination data were assigned the calculated averaged planting date as the actual planting date. Starting from this planting date, mid-silk days were estimated based on growth degree units (GDU), calculated as [(daily maximum temperature + daily minimum temperature/2) - 10^{o}C]. It was considered that a crop had reached the mid-silking stage when 1250 – 1300 GDU were accumulated (Lee, 2016,personal communication). The estimated mid-silk day was used to split the growing season into weekly intervals following (positive) or preceding (negative) that day and weekly ARID values were calculated.

Multicollinearity as indicated by VIF < 5 for independent model parameters was not an issue in this analysis. Thus, weekly ARID values starting at week nine before mid-silk and up to week nine after mid-silk were used as predictor variables for model development. Model development and model evaluation were done as described in the previous section. The logistic model was represented by:

$$P(Aflatoxin) = \frac{e^{\beta_0+\beta_{x1}Week_{x1}+\cdots+\beta_{xn}Week_{xn}}}{1+e^{\beta_0+\beta_{x1}Week_{x1}+\cdots+\beta_{xn}Week_{xn}}} \quad (3)$$

where P(Aflatoxin) is the probability to have aflatoxin contamination above the selected threshold (20 μg/kg), e is the base of natural logarithm, β_o, β_{x1},...β_{xn} are the estimated coefficients, $Week_{x1}$,…$Week_{xn}$ are the independent weekly ARID values entered into the

model. Significant independent predictors were identified by stepwise selection having entry and exit criteria levels equal to 0.05.

Results and Discussion

1.8 Predicting the risk at a field level

Significant predictors (p-value < 0.10) for aflatoxin contamination risk in corn were 1) soil type; 2) hybrid; and 3) weeks before and after mid-silk. Odds ratio estimates indicated an increased aflatoxin risk for the highly susceptible hybrid (hybrid 3) when compared to hybrids 1 and 2 which were characterized as moderately susceptible (Table 1. 1). Additionally, crops grown in the heavier soil type (Leeper silty clay loam) showed a lower likelihood for aflatoxin contamination above the selected threshold of 20 μg/kg than corn grown in the Myatt loam. This agrees with observations from other studies indicating higher pre-harvest contamination levels for corn grown in coarse sandy soils than corn grown in finer textured soils (Davis et al., 1986; Jones et al., 1981). Due to the lower water holding capacities of the coarser textured soils, potentially, the plants are more prone to water stress through the season than when grown in heavier soil types.

The critical growing season windows, when changes in in-field drought conditions influence the risk for aflatoxin contamination, included weeks four and one before mid-silk and weeks four and eight after mid-silk day (Table 1. 1). Moreover, a 0.1 increase in in-field drought, as quantified by ARID, during weeks four and one before mid-silk and week four after mid-silk, revealed that the predicted odds for contamination to be above the preselected threshold of 20 μg/kg was 22.6, 32.5 and 22.4% higher than the odds of not having contamination. Battilani et al. (2008a) had shown that drought had an influence on aflatoxin contamination in corn in Northern Italy, and defined as critical for contamination the timespan starting the last decade (10 days) of June and the first and last decade of August. Additionally, aflatoxin occurrence have been usually associated with

higher than normal temperatures and low rainfalls around silking/pollination and grain filling period (20 – 60 days after flowering), both conditions that may expose plant to drought stress (Diener et al., 1987; Payne, 1992; Widstrom et al., 1990; Windham et al., 2009).

Interestingly, in this study, the predicted odds to have an event (contaminated sample) were 14.7% smaller than the odds to not have contamination for every 0.1 drought increase, as indicated by ARID index, during week eight after mid-silk (a near to harvest window). Rewetting events late in the season coinciding with the timespan just prior or during harvest delayed corn drying, favor unremitting aflatoxin synthesis, and thus, may increased toxin accumulation, particularly in years conducive for infection and contamination (Cotty and Jaime-Garcia, 2007; Jaime-Garcia and Cotty, 2003; Jones et al., 1981). In our preliminary work (Damianidis et al., 2015), week nine before mid-silk corresponding to planting time, was defined as a significant explanatory variable. In the current study, by including two additional seasons (2013 and 2014) in the analysis, week nine prior to mid-silk was not a significant predictor (p-value > 0.10) for aflatoxin contamination.

The relative risk for corn aflatoxin contamination above the threshold of 20 μg/kg was higher for all three hybrids when cultivated in the lighter soil type (Myatt loam; soil type 2) compared to the heavier soil type (Leeper silty clay loam; soil type 1) (Figure 1. 1 and Figure 1. 2). Increase in drought conditions during week four following mid-silk, other things equal, resulted in increased contamination risk, as well, for all the scenarios shown in Figure 1. 1. For example, the two graphs located at the lower right corner of Figure 1. 1, the predicted probabilities for contamination above the threshold of 20 μg/kg

were greater than 50% (y-axis), when a highly susceptible hybrid (hybrid 3) was exposed to moderate (ARID = 0.412, center graph) or extreme drought (ARID = 0.755, far right graph) during the week prior to mid-silk, even when no or low drought (e.g.; ARID < 0.200, x-axis) occurred on week four after mid-silk, regardless of soil type. In contrast, if the moderately susceptible hybrids (hybrid 1 and 2) were cultivated in the Leeper silty clay loam, the likelihood for contamination above the legal limit was less than 50% (y-axis) even when the crops were exposed to extreme drought conditions (ARID > 0.800, x-axis) on week four following mid-silk (four graphs in the top two rows and first two columns from left in Figure 1. 1). This observation holds when the crop cultivated in the Leeper silty clay loam was exposed to low (ARID = 0.069, far left graphs) or moderate (ARID = 0.412, center graphs) stress the week preceding mid-silk.

The impact of reduced drought late in the season on aflatoxin accumulation in corn grain was indicated in all scenarios presented in Figure 1. 2. For all the scenarios studied, late in-season drought (week eight after mid-silk) decreased the relative risk for grain contamination for both soil types, regardless of hybrid type. This finding contradicts Battilani et al. (2008a), who showed that in Northern Italy, aflatoxin contamination risk was higher when drought increased on the first and last ten day windows of August, among others. It is not mentioned in their paper what was the corn growth stage for those intervals. The eighth week after mid-silk in our study corresponds to a week before harvest, and depending on the season, ranged from the first calendar week of August to the first calendar week of September. Since crop growth and development varies considerably from season to season, any comparisons between the results of our study and the findings of Battilani et al. (2008a) could be misleading.

Drought is commonly associated with higher than normal surface temperatures, prolonged periods of no precipitation or minimal precipitation (McNab and Karl, 1991), and drier air than usual (Baldwin, 1957; McNab and Karl, 1991; Potter, 1958). Cotty (2001) suggested that aflatoxin contamination of a mature cotton seed is promoted by warm temperature, high relative humidity (above 85%) or wetting events at or after ball opening. Increased rainfall late in the season was associated with increased seed contamination levels in cotton in Texas (Jaime-Garcia and Cotty, 2003). Similarly, corn ears that were water sprayed from the third to sixth week after full silk had higher levels of contamination than non-sprayed ears (McMillian et al., 1985). Therefore, McMillian et al. (1985) concluded that heavy morning dews may promote preharvest corn contamination in the Southeast. Higher August average minimum temperature was negatively associated with aflatoxin incidence and severity in corn studies over nine locations in USA (Sisson, 1986). It was suggested by Sisson (1986) that higher night temperatures could hasten corn maturation and reduce incidence of dew formation, a factor that could be related to fungal development and aflatoxin synthesis. Our study indicates that drought conditions close to corn harvest in Mississippi can influence the aflatoxin contamination process in the opposite direction (reduce contamination levels) than in the earlier vegetative and reproductive crop stages.

The variability in drought conditions during the periods found in this study impact the extent of preharvest aflatoxin contamination in corn. Timing and the degree of drought, along with soil type and hybrid resistance on infection and subsequent toxin accumulation can significantly change the likelihood for aflatoxin contamination. Seasonal fluctuations drive the dynamic relationships in the micro-organismal

community, change the fungal community structure along with the quantity of both aflatoxigenic producers and the available primary and/or secondary inoculum in the field (Cotty and Jaime-Garcia, 2007). Plant exposure to different drought stress levels when crops are grown in different soil types with variable soil plant available water may predispose corn to *A. flavus* infection and subsequent aflatoxin contamination. In most of the genotypes tested, greater levels of contamination were observed for crops indicating the highest physiological responses to drought and heat stresses, thus revealing a relationship between aflatoxin accumulation and those stresses (Kebede et al., 2012). In the same study, an aflatoxin resistant genotype had the lowest contamination levels despite being one of the most stressed crops; this suggested that the resistance mechanism for this genotype might be more complex. All those factors are likely to influence the final toxin accumulation in the grain, and may explain contradictory reports among different studies.

1.9 Model evaluation – Mississippi analysis

Binary response models, including logistic regression models, can be assessed by calculating the AUC. The AUC for the developed model was equal to 0.8233, and was forecasting significantly better than a model predicting by chance (AUC = 0.5000; p-value < 0.0001) (Figure 1. 3). Applying the fitted model to the evaluation dataset resulted in a negligible decrease in the predictive power (AUC = 0.8092) (Figure 1. 4). A significant contrast test (p-value < 0.0001) indicated that the developed model was better than the uninformative model (AUC = 0.5000) when applied to the evaluation dataset (Figure 1. 4). Therefore, the proposed predictive model could correctly classify a corn sample as contaminated or not contaminated in nearly 82% of the cases. Thus, ROC

curve analysis showed that the developed model proposed herein could identify true positives and minimize false negatives at acceptable levels of accuracy.

As indicated by their respective ROC curves, when weekly ARID values four and eight after mid-silk along with hybrid resistance to infection and subsequent contamination are considered as individual predictors, they have the highest relative impact on the measured associations (Figure 1. 3). In contrast, sub-models with only soil type or week four before mid-silk as independent factors alone had the lowest AUC's equal to 0.5841 and 0.5673, respectively (Figure 1. 3). All the single independent variable sub-models (Figure 1. 3) had a significantly reduced predictive power when compared to the overall model (p-value < 0.0001). However, when the sub-models were compared to the uninformative model (AUC = 0.5000), their discriminative power between events and non-events was statistically significant (p-value < 0.0151) for all but the sub-model having week four before mid-silk alone as an independent variable (p-value = 0.0732).

1.10 Predicting the risk at a regional level

The logistic regression model showed that significant predictors (p-value ≤ 0.05) for aflatoxin contamination in Georgia were drought levels (ARID) observed in weeks eight, seven and three before mid-silk, along with weeks two, four and nine following mid-silk (Table 1. 2). The effect of drought, as quantified by ARID, on the likelihood of corn aflatoxin contamination changed both in magnitude and direction (positive and negative) through the season. For example, a 0.1 increase in drought conditions during week four and nine after mid-silk was estimated to increase the odds to have contamination above the 20 μg/kg legal limit by 71.8 and 77.0%, respectively. Interestingly, if the average weekly ARID value for weeks eight and three before mid-silk is increased by a value of

0.1, then the predicted odds for contamination are 3.4 and 1.3 lower than the odds of non-events (contamination below 20 μg/kg). Similarly, the model shows that a reduced drought on week two after mid-silk results in increased risk. In contrast, an increased early drought stress (week 7 before mid-silk) significantly increased the likelihood for aflatoxin accumulation above the action limit (20 μg/kg) in the grain at the end of the season.

This study revealed that the risk for aflatoxin accumulation changes over the growing season. Drought conditions at particular weekly intervals relative to mid-silk, had influenced the likelihood for contamination above the legal limit set by U.S. FDA. Moreover, the estimated probability for aflatoxin contamination to exceed 20 μg/kg was defined by the level of dry/wet cycles in the preceding critical timespans. For example, if the plant was exposed to low to moderate drought on week seven (ARID < 0.492) before mid-silk, then the risk remained well below 20%, even if extreme drought occurred on week four after mid-silk (ARID = 1.000) (Figure 1. 5; three top row graphs). However, if the ARID value on week four following mid-silk was ≥ 0.6, reflecting a moderate in-field drought situation, then the probability for contamination above the legal limit exceeded 40% (Figure 1. 5; right bottom row graph). As shown in the bottom right graph of Figure 1. 6, under extreme drought conditions on week nine (ARID > 0.800) and week four (ARID = 0.998) after mid-silk, other things being equal, the likelihood for aflatoxin contamination above the action set level by U.S. FDA approached 40%. Those observations may reflect not only the complex interactions that take place between biotic and abiotic factors impacting fungal growth, sporulation, inoculum dissemination, infection, toxin synthesis and accumulation, they also indicate the interconnectivity and

the interdependence of process, that are in constant change during the growth season and occur at the host plant level, fungal level and each particular environment.

As shown in this study, predicting aflatoxin risk at field (plot) level based on soil type, hybrid and drought conditions is promising. Predicting the likelihood of contamination at a regional level might be more challenging due to multiple soil types, the very different weather/climate conditions encountered and the effect they impose on the fungus, the host and their interactions. Consequently, questions have been raised about the feasibility of the methodology proposed herein. Lack of data (e.g.; planting date, crop growth stage, soil types, hybrids) added to the overall uncertainty, and required informative assumptions (i.e. determination of potential planting dates for a given area and season) and estimations (e.g.; forecasting mid-silk day by calculating GDU). In regional studies, due to data limitations, meteorology might be the only driving factor available to assess risk, and thus, a simple predictive system might be more desirable and applicable. For all these reasons, our approach to assess the likelihood of corn contamination above the legal limit at county level was based only on minimum weather data (maximum temperature, minimum temperature, and rainfall).

1.11 Model evaluation – Georgia analysis

Contaminated and non-contaminated samples used for model training equaled to 620 (93.37%) and 44 (6.63%) samples, correspondingly. The evaluation dataset contained 154 samples, 91.56 and 8.44% were classified as non-events (had aflatoxin contamination below the 20 μg/kg threshold) and events, respectively. The AUC for the developed model was 0.9744 (p-value < 0.0001) and was predicting significantly better than the model predicting by chance (AUC = 0.5000) (Figure 1. 7). When the developed model

was applied to the evaluation dataset, the ROC curve dropped to 0.9177 (Figure 1. 8). Despite that, the ROC contrast test was significant (p-value < 0.0001), indicating that the developed model was more accurate in predicting contaminated from non-contaminated samples when compared to the uninformative model (AUC = 0.5000).

1.12 Comparing the results – field versus regional

Analyses using both Mississippi dataset and Georgia dataset indicated that drought, as quantified by weekly ARID values, is a significant driving factor that influences the risk for contamination. However, the timespans (weeks) indicated as significant varied between the Mississippi and Georgia data sets (Table 1. 1 and Table 1. 2) except for week four after mid-silk which was identified as significant by both models. Additionally, both studies are reflective of what has been observed by other researchers (Hawkins et al., 2008; Windham et al., 2009), as well; infection and subsequent aflatoxin contamination are likely influenced by environmental stresses (e.g.; drought, temperature, and moisture stresses) occurring prior to silking. Perhaps more attention and studies under controlled (greenhouse) environments considering corn vegetative growth stages might be needed to determine if those early stresses provoke physiological responses/processes at the plant and/or fungal level that may explain the variability in toxin accumulation at the end of the season.

The Georgia study has its own inherent weaknesses: 1) planting dates and mid-silk stages were estimated, thus, it is more appropriate to consider the defined weekly timespans as relative rather than absolute time windows, and 2) corn samples were collected from fields within a county but there is not information on the specific location; as a result, an error was introduced because available weather parameters for ARID

calculations were less precise. From experience, it is known that weather, and particularly rainfall amount and distribution, can be highly variable even over relatively short distances. Therefore, interpretation of odds estimates from the regional model has to be approached with extreme caution. For example, the odds estimates for week three prior to mid-silk and week two after mid-silk derived from the Georgia analysis suggest that increase in drought results in lower risk (Table 1. 2). Those suggestions contradict the principle that extended aflatoxin levels in corn are commonly encountered in seasons or at field locations associated with drought stress (Abbas et al., 2002; Abbas et al., 2004; Davis et al., 1986; Diener et al., 1987; Windham et al., 1999). Abbas et al. (2004) had shown that the incidence of *A. flavus* propagules recovered from corn grains was greater in a field site that had received supplemental water compared to other field sites. The opposite trend was observed for aflatoxin contamination levels; moreover, no association between the latest one and colonization levels was detected. Thus we may conclude that the odds ratios for week three prior to mid-silk and week two following mid-silk, as suggested by the Georgia model, are likely erroneous.

We consider the field model more robust, since the data obtained were derived from a controlled in-field experiment. Hence, potential strategies to mitigate aflatoxin contamination should rely more on the information derived from the controlled (Mississippi) experiment which is in agreement with the principle knowledge of the phenomenon. Therefore, the Georgia results should be rather considered as a preliminary work illuminating the potential of the proposed methodology to assess the risk over a larger regional area; however, a more detailed georeferenced database will be necessary to address the limitations and contradictions observed herein. Additionally, agronomic

information such as hybrid type, growth stages (at least planting dates and/or mid-silk) may add to the robustness of a future regional model.

1.13 Potential strategies to mitigate pre-harvest contamination

Site-specific management strategies could be adapted at the beginning or through the season to minimize host plant stress, which may reduce aflatoxin risk. Use of irrigation during critical risk weeks may reduce the risk, but irrigation amount and timing should be based on crop needs, atmospheric conditions, and soil water holding capacity. Planting date adjustment, within-field planting density and/or selection of hybrids with suitable relative maturities to reduce plant exposure to drought stress during critical growth timespans should be considered, as well. A grower could consider selecting of the most appropriate hybrid for a given site/region based on soil type and drought risk assessment in a particular locality. Separation of the field into management zones based on risk stratification criteria (e.g.; soil texture, plant available water holding capacity, electrical conductivity, and/or soil organic matter content) could be considered, as well. Separation of the field into management zones will allow to: 1) plant appropriate hybrid type per zone; 2) segregate harvest if necessary; and 3) apply variable rate irrigation/fertigation at different zones as needed. Determination of best harvest timing may be based on the predicted contamination risk for a particular season and location. Therefore, decisions for early harvest, subsequent grain drying, and proper grain storage aiming to reduce/cease further infection and toxin accumulation, might be an option, but the additional associated cost needs to be accounted for. Grain storage segregation based on risk prediction for different harvested produce lots could be one more option.

In addition, risk maps by county or by regions within a single state or regional risk maps could be generated using ARID data. Those regional aflatoxin risk maps might be useful for adaption or implementation of risk mitigation strategies including: 1) selection of drought tolerant corn hybrids in high risk areas, 2) cultivating cover crops for soil moisture conservation on high risk areas, 3) shifting in planting dates, and 4) applying variable rate irrigation and seed to minimize plant water stress. Moreover, the logistic model used herein to predict aflatoxin risk in corn, could be incorporated into decision support systems, and develop on-line tools to predict the risk earlier in season based on changes in drought conditions during corn growth and development. This may allow for more informative, effective, and efficient crop management decisions by the producers and the agri-business sectors.

Conclusions

Results from the control experiment (Mississippi) analysis indicated that ARID could be used as a predictive tool for aflatoxin risk assessment. Hybrid susceptibility to infection/contamination, along with soil type contributed significant to predicting aflatoxin occurrence. Additionally, this work identified significant weeks during the growing season when changes in drought had an influence on the likelihood of aflatoxin contamination. This study illuminated that the critical timespan for infection and subsequent contamination extend both prior and beyond mid-silk. Time windows, as indicated by the Mississippi study when changes in drought have the greatest influence on aflatoxin risk, included weeks four prior and after mid-silk, among others. Additionally, the highly susceptible hybrid grown in lighter soil showed a higher risk for aflatoxin contamination with changes in drought conditions during critical week widows when compared to the moderately susceptible hybrids grown in the heavier soil. A preliminary work is presented here in, as well, where the proposed methodology was extended from field (plot) level to a regional scale (Georgia study). Both predictive models were externally assessed on independent datasets and showed high accuracy in classifying samples as contaminated above or below the preselected threshold (20 μg/kg). Identifying critical weeks influencing the risk for contamination early in the season may allow farmers, researchers and extension specialists to monitor changes of aflatoxin risk with in-season drought changes, and thus, make more informative management decision in an effort to mitigate the problem. This is true particularly during years characterized by conducive to toxin accumulation conditions. Finally, this work emphasizes the effect drought timing and drought severity has on pre-harvest corn aflatoxin risk alterations

during the season and further illuminates the impact drought has on contamination levels under different environments.

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Table 1. 1: Odds ratio estimates and profile-likelihood confidence intervals for statistically significant independent variables for Mississippi data analysis as determined from the logistic regression model via stepwise selection with entry and exit values set to $\alpha = 0.10$ and $\alpha = 0.20$, respectively.

Effect	Unit	Odds Ratio Estimates	90% Confidence Limits	
Mod resistant 1 vs susceptible	1	0.127	0.057	0.285
Mod resistant 2 vs susceptible	1	0.195	0.091	0.418
Silty clay loam vs loam	1	0.198	0.095	0.411
Week_4	0.1	1.226	1.044	1.440
Week_1	0.1	1.325	1.151	1.525
Week 4	0.1	1.224	1.079	1.390
Week 8	0.1	0.853	0.758	0.960

Week_4, Week_1 are weeks before mid-silk.
Week 4, Week 8 are weeks following mid-silk.
Mod resistant 1 & Mod resistant 2 are moderately susceptible hybrids.
Susceptible is highly susceptible hybrid.
Silty clay loam = Leeper silt clay loam.
Loam = Myat loam

Table 1. 2 : Odds ratio estimates and profile-likelihood confidence intervals for statistically significant independent variables for Georgia data analysis as determined from the logistic regression model via stepwise selection with entry and exit values set to $\alpha = 0.05$.

Effect	**Unit**	**Odds Ratio Estimate**	**95% Confidence Limits**	
Week_8	0.1	0.295	0.182	0.430
Week_7	0.1	3.167	1.978	5.884
Week_3	0.1	0.749	0.576	0.955
Week2	0.1	0.729	0.574	0.907
Week4	0.1	1.718	1.360	2.245
Week9	0.1	1.77	1.403	2.308

Week_8, Week_7 and Week_3 are weeks before mid-silk.
Week2, Week4, and Week9 are weeks after mid-silk

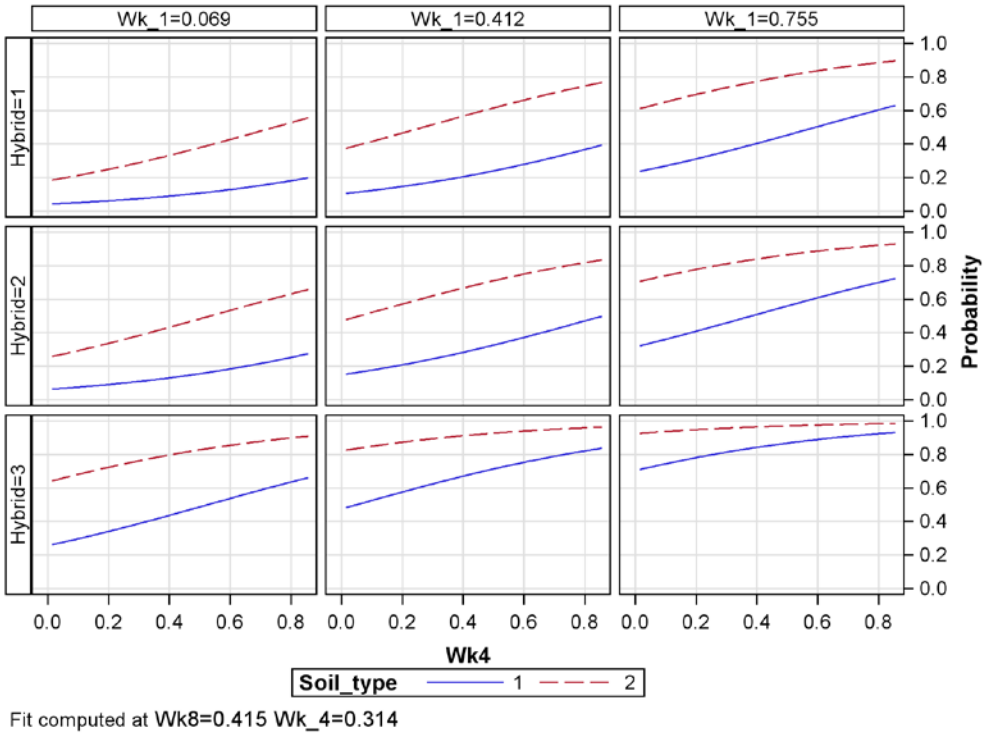

Figure 1. 1: Predicted probabilities (Mississippi analysis) for having aflatoxin contamination above the threshold of 20 µg/kg with changes in drought conditions on the fourth week after mid-silk, given that moderately (Hybrids 1 & 2) and highly (Hybrid 3) susceptible hybrids are cultivated under Leeper silty clay loam (Soil type 1; solid line) and Myatt loam (Soil type 2; dashed line). Three scenarios (columns) are presented herein for each hybrid (rows): 1) low (ARID=0.069), 2) medium (ARID=0.412) and 3) severe (ARID=0.755) drought conditions for the week prior mid-silk. Week four before mid-silk and week eight after mid-silk were set fixed to their respective mean values.

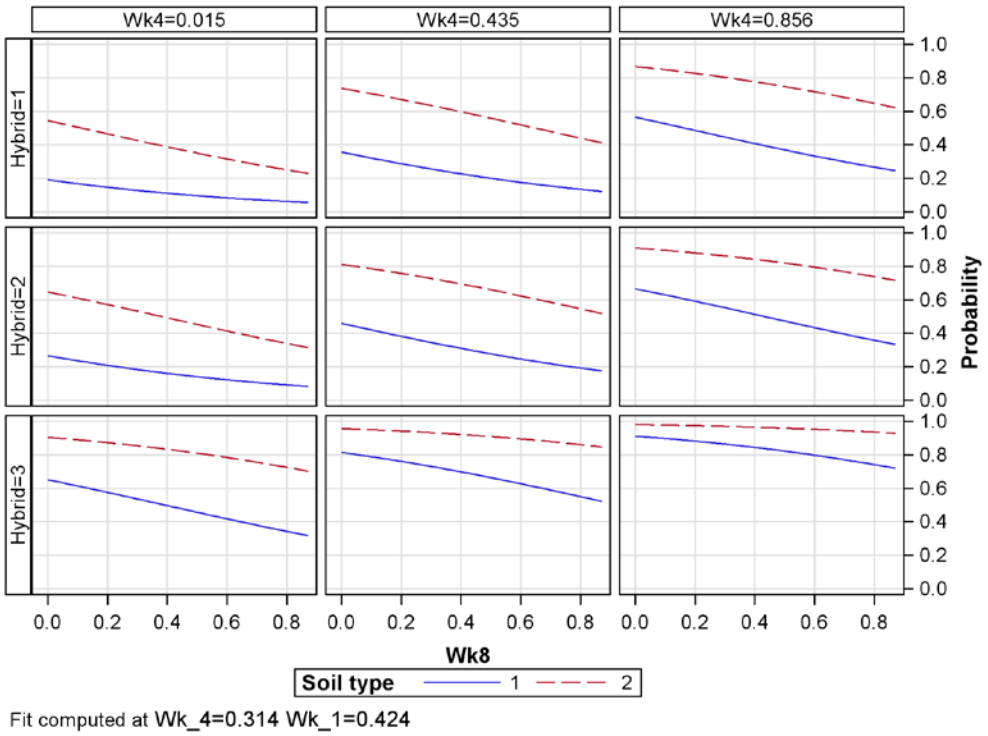

Figure 1. 2: Predicted probabilities (Mississippi analysis) for having aflatoxin contamination above the threshold of 20 µg/kg with changes in drought conditions on the eighth week after mid-silk, given that moderately (Hybrids 1 & 2) and highly (Hybrid 3) susceptible hybrids are cultivated under Leeper silty clay loam (Soil type 1; solid line) and Myatt loam (Soil type 2; dashed line). Three scenarios (columns) are presented herein for each hybrid (rows): 1) low (ARID=0.015), 2) medium (ARID=0.435) and 3) severe (ARID=0.856) drought conditions for the week four after mid-silk. Week four and one before mid-silk were set fixed to their respective mean values.

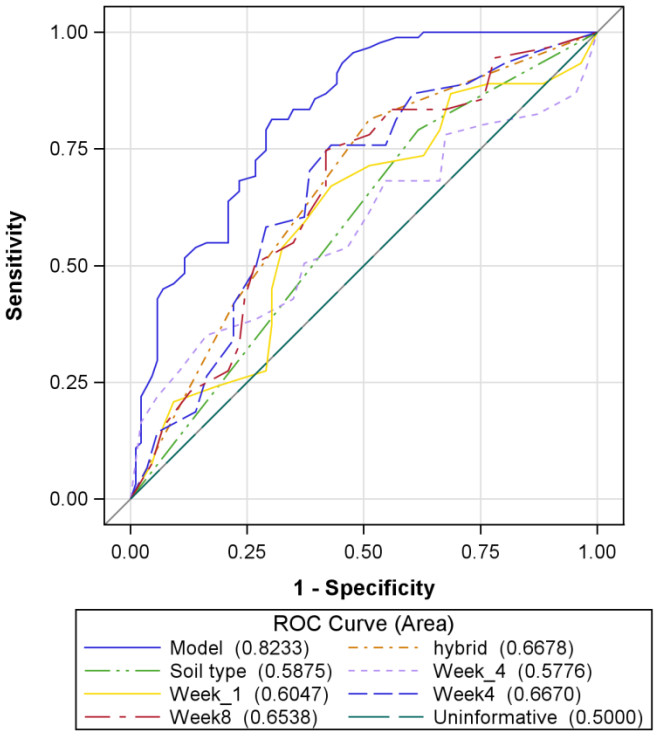

Figure 1. 3: Receiver Operating Characteristic curves for the fitted model (Model), and for each significant variable as identified in the logistic model, indicating the relative weight of each predictor variable on the studied association (Aflatoxin predicted probabilities vs. predictor variates). Uninformative is the model with no predictive power (predicting by chance). Values in parentheses correspond to area under the curve (AUC) calculated for each particular model tested; Mississippi analysis.

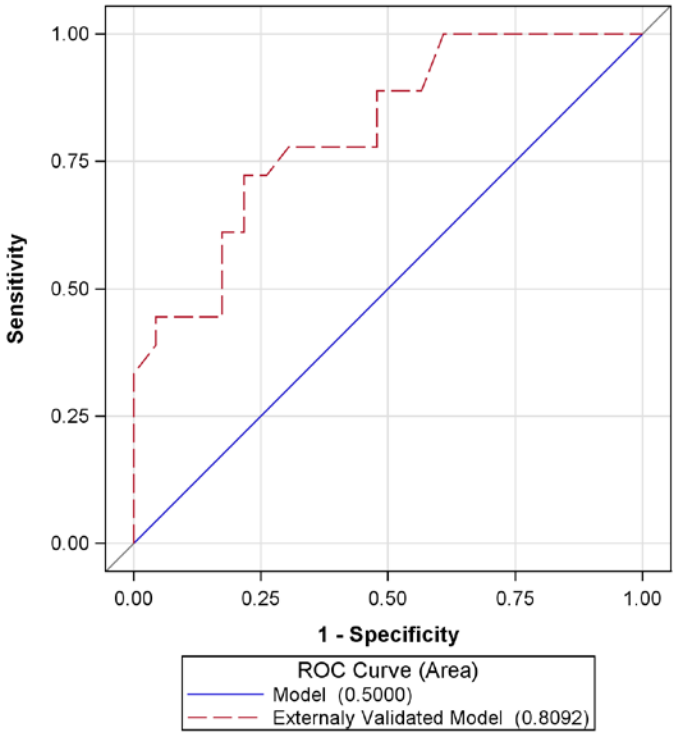

Figure 1. 4: Receiver Operating Characteristic curve from applying the fitted model (Externally Validated Model) to the validation dataset, compared to the AUC (Area Under the Curve) of the uninformative model (Model). Values in parentheses correspond to calculated AUC; Mississippi analysis.

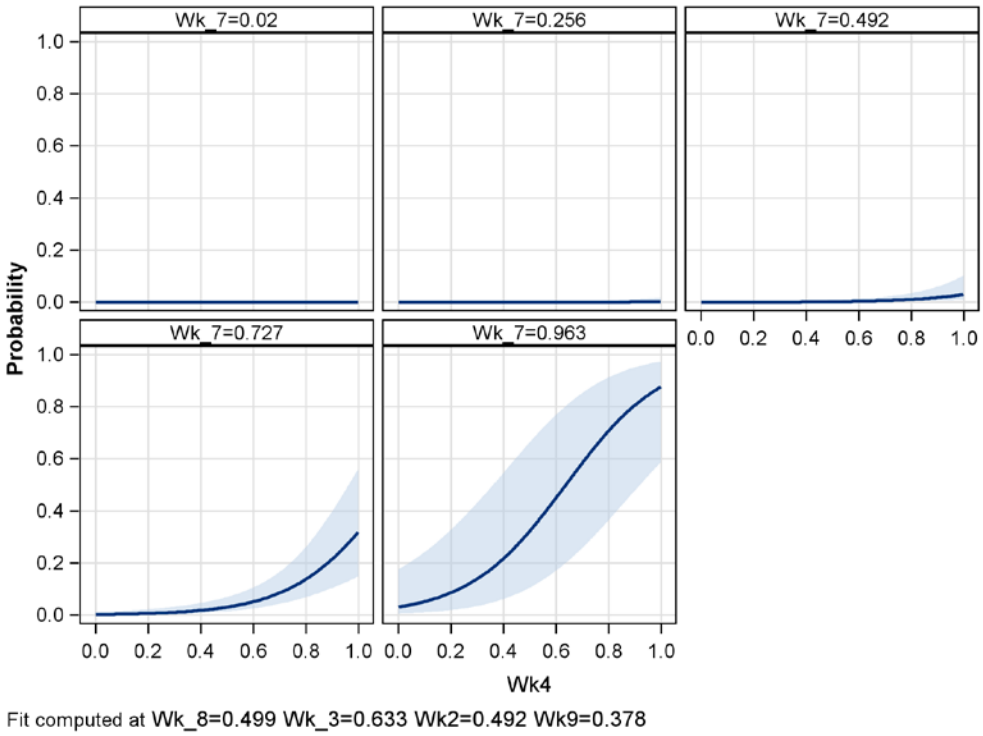

Figure 1. 5: Predicted probabilities (Georgia analysis) plot panel indicating aflatoxin risk change when drought conditions are changing on week four after mid-silk for five different aridity scenarios early in the seasons (week 7 before mid-silk). ARID values for the rest of the weeks were set equal to their mean. Shaded band represent confidence limits at $\alpha = 0.1$.

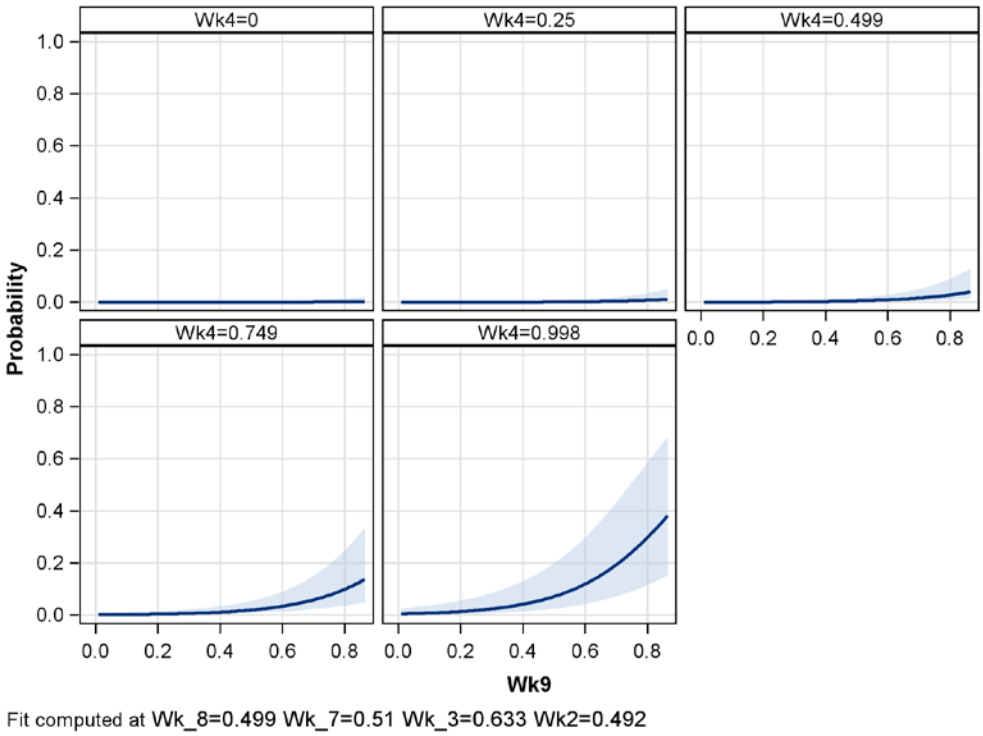

Figure 1. 6: Predicted probabilities (Georgia analysis) plot panel indicating aflatoxin risk change when drought conditions are changing on week nine after mid-silk for five different aridity scenarios for week four following mid-silk. ARID values for the rest of the weeks were set equal to their mean. Shaded band represent confidence limits at $\alpha = 0.1$

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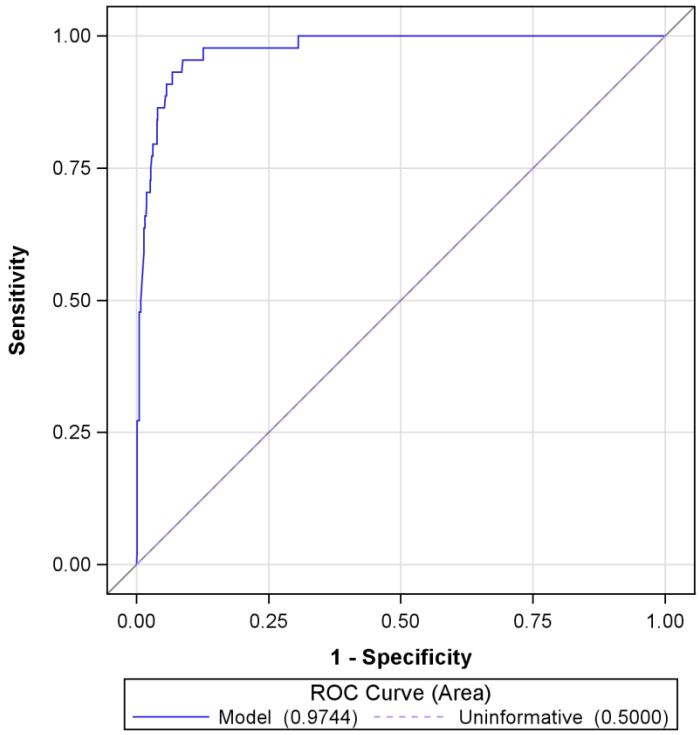

Figure 1. 7: Receiver Operating Characteristic curve for the fitted model (Model). Uninformative is the model with no predictive power (predicting by chance). Values in parentheses correspond to calculated area under the curve (AUC); Georgia analysis..

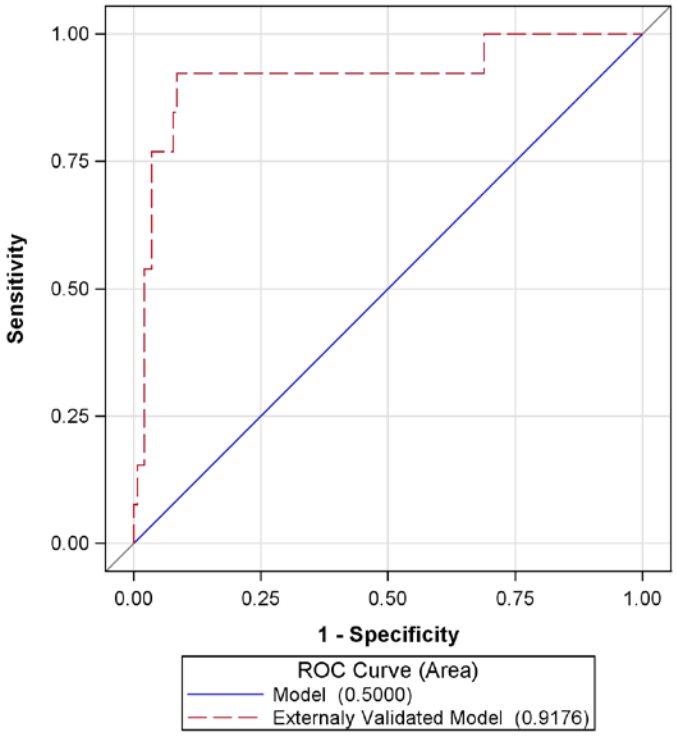

Figure 1. 8: Receiver Operating Characteristic curve from applying the fitted model (Externally Validated Model) to the validation dataset, compared to the AUC of the uninformative model (Model). Values in parentheses correspond to calculated AUC; Georgia analysis.

2 CORN AFLATOXIN CONTAMINATION LEVELS AS INFLUENCED BY AGRONOMIC MANAGEMENT IN ALABAMA

Abstract

Aflatoxin contamination of corn (*Zea mays* L.) following infection by *Aspergillus flavus* (*A. flavus*) is a chronic issue in the Southeast United States, and agronomic practices (i.e. planting date and population density), climatic conditions, and their interaction may have an influence on toxin accumulation. In principal, alleviating crop stress during critical growth stages should improve yields and lessen contamination. The objectives of this study were to: a) asses the effect of agronomic practices (planting dates and plant densities) on preharvest aflatoxin contamination in rainfed corn grown in the Coastal Plains of South and Central Alabama, b) identify weather variables that influence aflatoxin contamination and determine the relative weight those variables have on corn contamination, and c) determine time windows during the growing season when weather variables are associated to corn aflatoxin contamination. Field experiments were conducted in Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014). Multiple linear regression was used to assess the influence of daily average minimum temperature and cumulative rainfall over five time windows around mid-silk. The time windows considered were: 1) a 2-week window before mid-silk, 2) a 2-week window after mid-silk, 3) the second 2-week window following mid-silk, 4) the third 2-week window after mid-silk, and 5) a variable in length window ending at physiological maturity). For each time window average daily minimum temperature and cumulative rainfall were calculated. Multiple regression analysis with stepwise selection was used to study the influence of weather parameters on aflatoxin contamination for the five time windows defined. Six models were developed; three from pooled Fairhope data (2010 – 2014) and three from pooled data over Fairhope (2010 – 2014) and Prattville (2013 – 2014). The

response variable in each of the models was corn aflatoxin contamination; explanatory variables tested were: 1) both derived cumulative rainfall and derived average daily minimum temperature variables for the five windows defined earlier (overall model x 2), 2) derived cumulative rainfall variables only (rainfall model x 2), 3) derived average daily minimum temperature variables only (minimum temperature model x 2). Univariate regression analysis was used to explore the relationship between aflatoxin contamination in corn and grain yield. The effect of planting date (mid-March vs. mid-April) plant density (44,480, 54,360, 64,250, and 74,130 plants ha-1) on aflatoxin contamination in corn was tested as well. The results of this study showed that mid-April planting date resulted in significant ($p - value < 0.05$) or relative reduction in aflatoxin contamination. Plant densities did not have an influence in toxin accumulation. A significant negative linear relationship was illustrated between aflatoxin and yield for 2011 in Fairhope. The overall model developed from Fairhope data had an $R^2 = 87$ %. The R^2 for the minimum temperature model developed form Fairhope and Prattville pooled data was 60%. The influence and effect direction (positive/negative) e.g. of average daily minimum temperature on aflatoxin contamination changes over the time window considered in this study. A better understanding of the influence of weather variables on corn contamination may lead to better crop management, and development of more accurate prediction systems.

Keywords: *Aspergillus* spp., Densities, Planting dates, Rainfall, Temperature

Introduction

Corn is a major crop in the Southern United States contributing significantly to the economy, since it is used in crop rotations, as animal feed, in the fermentation industry and for direct human consumption (Abbas et al., 2002). Aflatoxin contamination is highly detrimental to corn grain quality. As a result of its high toxicity and the long time stringent regulation guidelines set by United States Food and Drug Administration (U.S. FDA), the toxin is associated with the greatest losses and management costs among mycotoxins. (Fountain et al., 2014; Robens and Cardwell, 2003). Food, feed, and agricultural produce contaminated with aflatoxins have been recognized as a problem for more than 50 years (Bayman and Cotty, 1990). The toxins are deleterious to both human and animals, known for their immunosuppressant, mutagenic, teratogenic, carcinogenic effects, and as such, also have a significant impact on the corn grain industry (Hernández-Martínez and Navarro-Blasco, 2010). Therefore, balancing the economic importance of corn with the public health risk associated with contaminated food consumption is crucial, and a 20 μg kg^{-1} upper limit has been set by U.S. FDA for corn oriented toward human consumption (U.S. Food and Drug Administration, 2000)

Weather and climate fluctuations influence corn infection by *A. flavus* and have an impact on the extent of contamination (Cotty and Jaime-Garcia, 2007). In the United States, preharvest aflatoxin contamination is a constant concern, especially during growing seasons characterized by high temperature, low humidity and drought (Abbas et al., 2012; Abbas et al., 2006; Abbas et al., 2002; Damianidis et al., 2015). Infection by *Aspergillus flavus* and subsequent kernel contamination is more severe in the Southeast of the U.S., however, it might also occur in the Midwestern corn belt when environmental

conditions are conducive (Payne, 1992; Windham et al., 2009). The problem is commonly exacerbated in seasons characterized by above normal temperatures and below average precipitations around anthesis and for the duration of grain filling (Hawkins et al., 2008; Widstrom et al., 1990; Windham et al., 2009).

Among other factors, heat and drought stress results in poor plant growth, reduced yield, and promotes fungal infection, which in the case of several *Aspergilli* spp. may lead to aflatoxin accumulation, and, thus, economic losses (Abbas et al., 2012; Ferrigo et al., 2014; You and Chan, 2015). Plant resistance to fungal infection and toxin synthesis is a quantitative trait involving numerous genes and is highly influenced by genotype x environment interactions (Fountain et al., 2014). On the one hand, reactive oxidative species are produced as plant response to biotic (i.e. pathogen attack) and abiotic stresses (i.e. heat and drought) (Torres et al., 2006; Vellosillo et al., 2010; You and Chan, 2015). On the other hand, fungi exposure to oxidative stress may be a prerequisite for aflatoxin biosynthesis, (Fountain et al., 2014). These observations support studies showing correlations between aflatoxin contamination, weather conditions imposing stress on the plant and/or the fungus, and poor corn yields (Hawkins et al., 2008; Jones et al., 1981; Rodriguez-del-Bosque, 1996; Widstrom et al., 1990; Windham et al., 2009).

Management practices that reduce corn stress (i.e. plant density, planting dates, nutrition, and insect damage control) in an effort to mitigate in-field aflatoxin contamination have been reported (Abbas et al., 2007; Bruns, 2003; Bruns and Abbas, 2005; Jones and Duncan, 1981; Jones et al., 1981; Payne, 1992; Rodriguez-del-Bosque, 1996). The optimum plant population for corn in the Midsouth U.S. was determined to be around 70,000 plants ha^{-1} (Bruns and Abbas, 2005). Theoretically, high population

densities induce elevated nutrient and water stress among inter row crops, and thus, predispose corn to aflatoxin contamination (Bruns, 2003). Additionally, altering planting dates can impact plant exposure to heat and drought stress during the reproductive stage. Therefore, by altering the aforementioned management practices, and thus, exposing the crop to a minimum possible stress throughout the critical windows in crop growth and development, it is expected to lessen aflatoxin contamination levels. Since environmental conditions are highly variable from place to place, local field studies are needed to elucidate how management practices and the associated weather patterns at critical crop growth stages influence toxin biosynthesis and accumulation in a given area, and thus providing the baseline for recommendations to growers.

The hypothesis driving this study is that management practices reducing corn plant stress lower preharvest aflatoxin contamination levels. The objectives herein were to a) assess the effect of agronomic practices (planting dates and plant densities) on preharvest aflatoxin contamination in rainfed corn grown in the Coastal Plains of South and Central Alabama, b) identify which weather variables are influencing aflatoxin contamination in corn, c) determine the relative weight of significant weather variables on contamination in corn for South and Central Alabama, and d) determine time windows during the growing season that weather variables are associated to corn aflatoxin contamination.

Materials and methods

2.1 Experimental sites and cultivation practices

Field experiments were conducted at Gulf Coast Research and Extension Center, in Fairhope, AL (30^{o} 32'N, 87^{o} 52W) from 2010 – 2014, and at Prattville Agricultural Research Unit, near Prattville, AL (32^{o} 25'N, 86^{o} 26'W) from 2013 – 2014. The soil series at Fairhope and Prattville were Malbis fine sandy loam (Fine-loamy, siliceous, subactive, thermic Plinthic Paleudults) and Lucedale sandy loam (Fine-loamy, siliceous, subactive, thermic Rhodic Paleudults), respectively. Cultivation practices varied over season and location depending on equipment availability, but did not differ from the recommended agronomic practices in the state of Alabama. Briefly, a field area was strip-tilled before planting at Fairhope in each season (2010 – 2014), while at Prattville in 2013 and 2014 a conventional tillage and paratill were applied, correspondingly. Generally, corn was planted each year following soybean or cotton. During winter the land was either left to fallow (Prattville, 2013), or planted to a wheat or rye cover crop prior to corn cultivation (Prattville 2014; Fairhope 2010 – 2014). Fertilization and soil pH adjustment followed the recommendations of Auburn University Soil Test Laboratory. Nitrogen was applied twice during the growing season with total nitrogen application rates ranging from 145 – 182 kg ha^{-1}.

In each growing season the same corn hybrid (Pioneer 31P42) was planted in four row plots 9.1 m long. Rows within an experimental unit (plot) were spaced 0.96 and 0.90 m apart in Fairhope and Prattville, respectively. The experimental design at both locations was a split-split plot design with six blocks. The inoculation treatment was assigned to whole plots, while planting date and plant density factors were allocated to

sub plots and sub-sub plots, respectively. Inoculation techniques included: 1) non-inoculation (control), to resemble natural infection and subsequent contamination; and 2) artificial contamination, to ensure uniform kernel exposure to *Aspergillus flavus* spores, allowing for potential aflatoxin accumulation at levels sufficient to reveal possible effects of planting date and/or plant density on aflatoxin synthesis regardless of environmental conditions (Abbas et al., 2002). There were two planting dates: 1) a standard planting date (approximately mid-March) and 2) a late planting date (approximately mid-April) (Table 2. 1). Plant density treatments consisted of four levels representing standing crop populations of approximately 44,480, 54,360, 64,250, and 74,130 plants ha^{-1} (densities D1, D2, D3, and D4, respectively). In the 2010 growing season, only the first three densities (D1, D2, and D3) were included in the study.

2.2 Inoculum preparation and inoculation technique

2.2.1 Seasons 2010 – 2012

In 2010 – 2012 growing seasons, cracked corn was inoculated with *A. flavus* and was used as the inoculant carrier. Briefly, 7.5 kg of cracked corn were soaked overnight with 2390 mL of water, bagged, and autoclaved for 55 min at 121 ^{o}C. Two stock solutions to control bacterial and fungal growth were utilized; the first stock solution contained chlorotetracycline and streptomycin (10 g each, diluted with 1000 mL water), while the second solution contained Botran (0.3 g mixed into 900 mL acetone). Fifty and 20 mL of stock solutions one and two, respectively, were added and mixed to 7.5 kg autoclaved corn. Cultures of field-collected toxigenic *A. flavus* isolates were started and grown on acidified potato dextrose agar (APDA) on 9 cm petri dishes at room temperature (approximately 25 ^{o}C) for 5 – 7 days. Conidia were rinsed and collected from the plates

with autoclaved distilled water containing 0.01 % Tween® 20. Prepped corn (456 g) was placed in small plastic bags, inoculated with 20 mL of spore suspension, homogenized by manual shaking every 24 h, and incubated for 3 days at room temperature (approximately 25 ^{o}C). Inoculated corn was scattered by hand in the middle two rows onto the soil at a rate of 1,057 kg ha^{-1} a week prior silking (R1).

2.2.2 Seasons 2013 – 2014

Data analysis from the first three seasons indicated that spreading inoculated corn on the ground was not effectively promoting infection and subsequent contamination (Table 2. 2). Therefore, side needle wounding, using an Idico tree-marking gun fitted with a 14 – gauge hypodermic needle (Idico Products Co., New York), was adapted (Abbas et al., 2002; Windham et al., 2009; Zummo and Scot, 1989). Inoculum preparation was carried on as described in detail by Abbas et al. (2002) and Windham et al. (2009). To ensure adequate infection, modifications included: 1) the use of four native Alabama aflatoxigenic *A. flavus* isolates (E316.1, PV11027, SM310.3, and TV203.1) collected, isolated and preserved by Dr. K. L. Bowen (Plant Pathology Laboratory, Auburn University) (Cambell and White, 1994; Walker and White, 2001), and 2) four inoculum solutions standardized to 9.0 x 10^{6} conidia / mL (Zummo and Scot, 1989) prepared from each toxigenic isolate and mixed at equal volumes (v/v/v/v) to produce the final inoculum solution. The primary (top) ear from corn in the middle two rows (30 plants per row) was inoculated approximately 7 days after mid-silk by injecting approximately 2 mL of solutions into one to two corn kernels.

2.3 Harvest and aflatoxin contamination assessment

Ten top corn ear samples were hand-harvested at harvest maturity from the two middle rows from each treatment plot. Ears were machine shelled, and the sample grain weight obtained per plot ranged from 0.92 – 1.83 kg / plot (10.0 – 13.8 % of total plot yield, respectively). Grains originating from the same experimental unit were manually mixed and a subsample (250 g) was obtained for aflatoxin assessment (Jaime-Garcia and Cotty, 2003). Kernels were ground to < 2 mm with a Thomas – Whiley Laboratory Mill, model 4 (Swedesboro, NJ). Plots were harvested with a grain combine. Yield per plot was added to hand harvested corn grain weight and reported at 15.5 % moisture content (Bowen et al., 2014). Important growth stage days per growing season and location for different planting dates are given in Table 2. 1

Total aflatoxin quantitative assessment was run on 10 g of ground corn per treatment (Veratox® test, Neogen Corp., Lansing, MI) following manufacturer instructions. Veratox® is a competitive direct enzyme-linked immunosorbent assay (ELISA) test with detection limit and quantitation range equal to 1.4 and 5 – 50 ppb, respectively. Therefore, all assays exceeding the upper quantitation threshold (50 ppb) were diluted as needed and reassayed (Bowen et al., 2014). Duplicates were run for more than 10% of the samples to verify aflatoxin content. Samples from 2014 season were analyzed by running high-performance liquid chromatography (HPLC) at the Biological Control of Pests Research Unit, USDA-ARS, Stoneville, MS as described by Abbas et al. (2015). HPLC and ELISA methods were found comparable (Abbas, H. K., personal communication).

2.4 Weather parameters

Air temperature and rainfall were measured on both experimental sites for 2013 and 2014 seasons with a HOBO Pendant® Temperature / Alarm Data Logger, model 64k – UA-001-64 (Onset Computer Corporation, Bourne, MA), and a tipping bucket rain gauge manufactured by RainWise Inc (Trenton, ME). Both sensors were installed at each field at the beginning of the growing season. Additional weather data were collected by the on-farm weather stations located at the Gulf Coast Research and Extension Center, in Fairhope, AL, and the Prattville Agricultural Research Unit. Data were obtained through the Alabama Mesonet Weather Data network, and monthly summaries are provided in Table 2. 3.

Maximum and minimum daily air temperatures were averaged, while cumulative rainfall was considered, as well, for consecutive time periods starting two weeks before mid-silk (R1) and extending up to physiological maturity (R6). Thus, five time windows were defined: 1) fourteen days before, and 2) fourteen days after mid-silk, 3) from day 15 to day 28 after silking, 4) from day 29 to day 42 following mid-silk, and 5) a variable day- length window starting on day 43 after mid-silk and extending up to physiological maturity day (ranging from 0 – 14 days, depending on planting date and growing season). Therefore, a total of fifteen weather variables were evaluated (3 weather variables x 5 time windows) for their association with end of season aflatoxin contamination. Mid-silk was designated as reference day for two reasons: 1) to take out part of the variability intrinsically related to different planting dates and growing seasons, since crop development and maturity is influenced by environmental conditions that are particular for each planting date x location x year combination, and thus, do not coincide with

calendar days; and 2) previous work indicates a critical time for corn infection and contamination the time span around and beyond mid-silk (Betrán and Isakeit, 2004; Hawkins et al., 2008; Payne et al., 1988; Windham et al., 2009).

2.5 Statistical analysis

Aflatoxin concentrations (ppb) were log-transformed (log10 (ppb+1)) to stabilize the variance, and all the analyses thereafter were implemented on transformed data. Aflatoxin values reported herein are the geometric means (antilogarithm of the logarithmic mean). Yield data met normality assumptions and therefore no transformation was needed. Generalized linear mixed model analyses in SAS version 9.3 using PROC GLIMMIX procedure (SAS, 2010) were performed. Fixed effects means were compared at level of significance $\alpha = 0.05$ with least-square means differences adjusted with Tukey's test to control for experiment wise error rate. Pooling aflatoxin data (i.e. over each location and seasons 2013 and 2014), with year and location considered fixed, revealed the effects as being significant (p – values < 0.05, Table 2. 4), thus justifying separate analysis by year and location. Analyses for each response variable were performed separately by year and location with block, block x inoculation, and block x inoculation x planting dates considered as random effects (PROC GLIMMIX, SAS version 9.3).

Multiple linear regression analyses were performed to investigate potential relationships between aflatoxin contamination due to natural infection and the calculated weather variables for five time periods before and after silking. In all models assessed, aflatoxin content, averaged over six replicates for distinct environments defined as year x location x planting date x density, was the dependent variable. In an initial step, a group

of five rainfall or five temperature derived variables were considered separately as explanatory variates in the regression models. Then, five rainfall and five minimum air temperature variables were entered together into the linear model and tested as explanatory factors. Because of poor associations with aflatoxin levels, maximum temperature factors were excluded from the latest analysis. Significant independent variables for all models were determined by employing stepwise selection (PROC REG, SAS version 9.3) with entry and exit criteria levels equal to 0.1. Additionally, linear relationships between aflatoxin contamination and yield were evaluated by running univariate linear regression analyses with PROC REG procedure (SAS version 9.3), as well. Aflatoxin contamination data ($\log_{10}$(ppb+1)) and yield were used as response and explanatory variables, respectively, after being averaged by distinct environments (i.e. 6 replicates x inoculation x planting date x density).

Results

2.6 Weather conditions

Year 2010 in Fairhope was generally hotter with mean maximum temperatures being greater than the historic average observed from April through August (Table 2. 3). For the same season, rainfall was well below the historic average on April and July, while it exceeded historic average rainfall in May. The 2011 growing season at Fairhope was extremely dry with grain filling period coinciding with spells of hotter than the historic average maximum and minimum temperatures. Seasons 2012 and 2013 in Fairhope, were cooler in June and July since maximum temperatures were below the historic average. June and July corresponds to the typical corn grain filling period in Alabama. Season 2012 in Fairhope was drier than the historic average, while 2013 and 2014 were wetter than the historic average. In Fairhope 2014, higher daily temperatures than the historic average were observed for June, July and August; minimum monthly temperatures were greater than the historic monthly averages with the exception of March and July. Despite 2014 being a wet season, August was extremely drier in Fairhope than the historic average for that month. In Prattville, the 2013 growing season had lower maximum and minimum temperatures, and received less precipitation when compared to the historic average. Drier conditions than the historic average were observed in Prattville in 2013 in March, May, and June. Season 2014 in Prattville was wetter than the historic average, but less precipitation was received in July and August compared to the historic average for the area.

2.7 Treatment effects on aflatoxin contamination

Spreading infested cracked corn on soil at Fairhope (2010 – 2012) did not result in significantly higher aflatoxin levels (p – value > 0.05) compared to natural infection (Table 2. 2). In contrast, side needle inoculation method induced aflatoxin contamination at significantly higher levels (p – value < 0.05) compared to non-inoculated in 2013 – 2014 at both study locations. At Fairhope, only in 2011 and 2012, natural infection resulted in contamination levels greater than the action level (20 μg of aflatoxin per kg of grain oriented for human consumption) set by the U.S. FDA (U.S. Food and Drug Administration, 2005). Aflatoxin synthesis and concentration, due to natural infection, were well below the action set limit (20 μg kg^{-1}) at Prattville for both growing seasons (2013 – 2014). Both inoculation treatments resulted in aflatoxin levels greater than 20 μg kg^{-1} only for Fairhope 2011; an extremely dry year (Table 2. 2 and Table 2. 3). If year and location were considered fixed, analysis over pooled data for 2013 and 2014, showed significant inoculation effect (p – value < 0.0001) (Table 2. 4).

Planting date effect on aflatoxin contamination varied among years (Table 2. 2). Mid-March planting generally resulted in greater aflatoxin contamination at the end of the season compared to late planting (mid-April), but was significant (p – value < 0.05) only for at Fairhope in 2011, 2013 and 2014. Analysis of pooled data for both locations and seasons 2013 – 2014 indicated significant planting date effect (p – value < 0.0001) (Table 2. 4), with least square means estimates for aflatoxin contamination being 35.8 and 20.8 μg kg^{-1} for mid-March and mid-April planting, respectively.

Plant density effect on aflatoxin concentration was non-significant (p – values > 0.05) when individual years per location were considered (Table 2. 2). A similar trend was

observed for pooled data analysis (p – value = 0.6834, Table 2. 4). Significant two way interactions at $\alpha = 0.05$ were observed for inoculation x planting date, and planting date x plant density effects for Fairhope, 2013 (Table 2. 2).

2.8 Relationship of weather variables with aflatoxin contamination

2.8.1 Southern Alabama (Fairhope)

Multiple linear regression analysis with stepwise technique (SAS, version 9.3) indicated that rainfall alone could explain 71.5% of the variability in aflatoxin contamination due to natural infection at Fairhope in 2010 – 2014 (Table 2. 5). Cumulative rainfall occurring for the 2-week interval before and the 2-week interval after silking altogether accounted for 48.4% of the observed variability. Additionally, the rainfall model suggested that cumulative rainfall for the variable in-length window (including day 43 following silking and up to physiological maturity) was also significant (p – value < 0.0001).

The R^2 for the minimum air temperature model was 82.4% (Table 2. 5). The most important variable influencing aflatoxin accumulation at harvest maturity was minimum average air temperature for the 2-weeks following silking (partial R^2 = 44.0%). Other significant variables selected were minimum average air temperatures for the 2-weeks before silking and the third 2-week interval following mid-silk, which explained 15.4 and 23.0% of the overall variability, respectively.

In the fitted overall model, the most important variables impacting pre-harvest aflatoxin contamination in corn were average minimum air temperatures for the first and

third 2-week periods following silking, having partial R^2 equal to 40.0 and 27.0%, respectively (Table 2. 5).

2.8.2 Two locations: South & Central Alabama (Fairhope & Prattville)

Multiple linear regression analysis with stepwise selection procedure (SAS, version 9.3), when applied to combined natural infection data from Fairhope (2010 -2014) and Prattville (2013 – 2014), revealed that cumulative rainfall and daily average minimum air temperature taken together and alone could explain a significant portion of the observed pre-harvest aflatoxin variability (Table 2. 6). The R^2 for the rainfall, the minimum temperature and the overall model was equal to 50.0, 60.2, and 76.0%, respectively. All the fitted models were significant (p – values < 0.0001).

As the rainfall model suggests, the influence of cumulative rainfall per se on pre – harvest aflatoxin contamination changes over the season. More specifically, cumulative rainfall several days before physiological maturity could explain 25.2% of aflatoxin concentration at harvest. Also, amassed precipitation over the two weeks before and the 2-week interval of weeks three and four after silking, were included in the model having a partial R^2 equivalent to 18.9 and 6.0%, correspondingly (Table 9).

If only the minimum air temperature factors were evaluated as explanatory variables, then the regression analysis indicates that preharvest aflatoxin contamination is greatly influenced by minimum temperature on the first 2-week window after silking (partial R^2 = 31.0%, Table 2. 6). Additionally, minimum temperature variables corresponding to the 2-week period before silking and the second 2-week window after mid-silk were also significant (p – value < 0.1).

Evaluating average minimum air temperatures and cumulative rainfall variables altogether as response variates indicated that aflatoxin contamination was influenced by cumulative rainfal and average minimum temperature for the timespan among days 43 following silking and up to physiological maturity(variable in length time window) (Table 2. 6). Rainfall, for the two weeks prior to mid-silk, as well, was significant at α = 0.1.

2.9 Relationship between aflatoxin contamination and yield

Univariate regression analysis (PROC REG, SAS version 9.3) was used to evaluate the linear relationship between average aflatoxin contamination (log(ppb+1)) and mean yield data under different environments (Figure 2. 1). A strong negative relationship between aflatoxin concentration and yield was detected for Fairhope, 2011 (model p-value = 0.0006, RMSE = 0.2517, coefficient of variation = 13.37) (Figure 2. 1A). Individual regressions for each of the remaining season x location x inoculation x planting dates x densities environments resulted in non-significant relationships (data not shown). Regression analysis with aflatoxin concentration (log(ppb+1)) as dependent variable and yield as explanatory variable, showed strong negative linear relationship for Fairhope 2010 – 2012 with R^2 = 0.8827 (model p – value < 0.0001, RMSE = 0.2528, coefficient of variation = 24.69) (Figure 2. 1B). If only natural infection data were included in the analysis, the fitted linear model for Fairhope 2010 – 2014 could explain 60.6% of the variability and the model was significant (model p – value < 0.0001, RMSE = 0.3913, coefficient of variation = 53.05) (Figure 2. 1C).

Discussion

In the current study we show that there is an association between aflatoxin contamination and several of the derived averaged minimum temperature and cumulative rainfall variables considered for the timespans starting two weeks before silking and extending to physiological maturity. When both averaged minimum temperature and cumulative rainfall were considered as explanatory variables, then multiple linear regression analysis could explain from 76 and up to 87% of the observed variability. In the overall model for Fairhope, minimum average temperature for the 2-week interval after silking had the greatest impact on contamination (Partila R^2 = 39.96%). Both overall models ranked the relative importance of minimum temperature variables on aflatoxin contamination as greater than the rainfall variables considered herein. However, when only cumulative rainfall factors were considered as explanatory variables, then 50 and 71% of the observed aflatoxin variability could be explained for the pooled data analysis in Fairhope and Prattville, and Fairhope analysis alone, respectively. The relative importance and the direction (positive or negative) of the effect of minimum temperature and cumulative rainfall on the contamination for the 2-week windows starting before silking and ending with a variable length window at physiological maturity changes. Our analysis shows that the critical time for infection and contamination starts before mid-silk. Depending on the season, aflatoxin concentration was numerically or significantly greater for mid-March planted corn than for Mid- April planted corn. Planting densities were not significant under all the environments studied. A negative linear relationship was detected between aflatoxin contamination and yield under the extremely dry season of 2011 in Fairhope.

Increased plant stress during the growing season, and particularly around mid-silk and kernel maturation, may predispose plant to infection and subsequent toxin accumulation (Abbas et al., 2007; Bruns, 2003; Jones et al., 1981; Lillehoj et al., 1980; Payne, 1992). At mid-silk and subsequent grain filling, aflatoxin accumulation level may be affected by the level of plant stress (Abbas et al., 2012). In-field crop management practices are of eminent importance at this point, since several cultural components including but not limited to fertilization, irrigation, tillage, insect and weed control, may reduce contamination and can be managed by the producer (Widstrom et al., 2003). Planting dates and densities have been studied relative to their role in aflatoxin contamination (Abbas et al., 2012; Abbas et al., 2007; Bruns, 2003).

In theory, reducing competition between adjacent plants for water and nutrients, and thus reducing crop stress, should reduce aflatoxin contamination. Under a certain environment, different population stands, during a critical stage of crop development, should influence aflatoxin synthesis and may reduce in-field aflatoxin contamination by altering plant stress. In this line, Zuber and Lillehoj (1979) suggested that reduced plant stress from anthesis to late dough should lessen infection and subsequent contamination. However, in the current study, plant densities did not have significant effect on aflatoxin contamination. These results confirm the findings of Rodriguez-del-Bosque (1996), Abbas et al. (2004), and Bruns and Abbas (2005) that no information exists to support the aforementioned hypothesis. This might be indicative of plant stress being overestimated as a factor influencing infection by *A. flavus* and subsequent contamination for corn in Alabama.

Modern hybrids, which possess an erect up-right leaf architecture, can more efficiently withstand the plant to plant competition for limited resources than did hybrids cultivated before the 90's (Bruns, 2003). As a result, under prevailing environmental conditions, it is possible the herein tested plant densities failed to differentiate stress levels over a hypothetical threshold necessary to induce a significant effect on aflatoxin synthesis in the grain. This hypothesis is supported by the density effect on yield data (Table 3. 4). As plant standings increased, in five out of seven environments except in Fairhope 2010 and 2011, a relative and/or significant ($p - value < 0.05$) positive yield effect was observed.

Altering planting date is another management practice studied extensively for its potential effect on aflatoxin contamination in corn (Bruns, 2003; Bruns and Abbas, 2006; Jones and Duncan, 1981). The overall concept is to optimize planting time in an effort to minimize plant exposure to heat and drought stress during the reproductive stage (Bruns and Abbas, 2006). In the current study, mid-March planting date (normal corn planting time for Alabama) consistently showed higher aflatoxin contamination than planting late (mid-April). Although, the effect was significant ($p - value < 0.05$) only in 2011, 2013, and 2014 in Fairhope, the relative trend in all environments was that earlier planting had higher levels of contamination compared to later planting.

In accordance to this study, Lillehoj et al. (1980), showed that planting dates affected aflatoxin synthesis and concentration in naturally infected corn. Corn grown in 1977 in Georgia and Florida, had significantly lower levels of contamination when planted in early (1^{st} April) than late (1^{st} May). Furthermore, late planting in the spring season in Mexico, exposed plants to highest minimum temperatures during reproductive stage and

resulted in highest aflatoxin concentrations (Rodriguez-del-Bosque, 1996). In contrast, a three year study in Arkansas revealed a non-significant reduction in aflatoxin content for corn planted in mid-April compared to later in early-May (Abbas et al., 2007). Similarly, grain contamination was not significantly affected by planting dates (early vs late April) in a study conducted at Mississippi from 2002 to 2004 (Bruns and Abbas, 2006).

Literature and our findings confirm the variability and the locality characterizing optimum planting dates for corn across the US (Bruns, 2003) due to differences in climatic conditions and plant growing season lengths. Those differences validate the necessity for additional local studies on the effect of planting dates on yield and aflatoxin contamination, since they may shed light on environmental factors which prevail in space and time for a specific area. This may allow mitigating grain toxin contamination more effectively in the vicinity of interest. The differences observed in this current study in aflatoxin levels among seasons and between locations within seasons, indicate the potential effect weather factors may have on the processes of infection and contamination.

Aflatoxin outbreaks, in naturally infected kernels, are usually associated with drought conditions typically accompanied with higher than normal air temperatures and more sporadic precipitation events (Abbas et al., 2002; Bruns, 2003; Diener et al., 1987; Windham et al., 2003). Among others, heat and drought stresses, are considered to diminish corn resistance and, thus predispose crops to *A. flavus* infection and subsequent in-field contamination (Bruns, 2003; Damianidis et al., 2015; Fountain et al., 2014; Hawkins et al., 2008; Windham et al., 2009). Frequently, elevated temperature and low precipitation around and/or beyond mid-silk have been positively correlated with

aflatoxin concentration at harvest. However, Abbas et al. (2007) indicated a negative correlation between heat stress and aflatoxin contamination detected in preharvest corn, thus demonstrating the complex relationships and interactions between biotic and abiotic factors contributing to the phenomenon. Among others, the high variability in aflatoxin contamination among seasons and locations might be partially explained by the complexity of host resistance to *A. flavus* and aflatoxin contamination, and the interaction between genotype x environmental conditions strongly influences the expression of this trait (Fountain et al., 2014).

In the current study, regression analysis with temperature and rainfall as independent variables calculated for the period of two weeks before silking and the grain fill window revealed an association between aflatoxin contamination and several of the weather parameters tested. Minimum temperature and rainfall models could explain from 50 - 76% and up to 87% of the observed aflatoxin variability when data from both or one location alone were considered, respectively. The importance and direction of rainfall and minimum temperature impact is changing over plant growth and development. In both scenarios examined, the critical time for infection and contamination starts at least two weeks before silking and extends over the grain filling period.

Both rainfall models calculated herein indicate that precipitation variables are negatively associated with aflatoxin contamination. Additionally, the precipitation models tested herein, point out that reduced rain for the 2-week period before silking will result in increased toxin levels in the grain. Windham et al. (2009), showed that moisture stress 21 – 42 days prior to inoculation was negatively correlated with aflatoxin contamination in plant in a loam but not at silty clay loam. Thus, they suggested that

moisture stress prior to silking may have a significant effect on aflatoxin synthesis, as well. Widstrom et al. (1990), in a five year study at Tifton GA, did not find significant correlation between average precipitation over 20 to 40 and 40 to 60 day time periods following silking and aflatoxin concentration in corn grown on loamy sand. Our analysis shows that with corn grown in sandy loam soils, moisture stress two weeks before silking may significantly increase aflatoxin contamination. Interestingly, the pre-silking 2-week precipitation variable accounted for more than 30% of the variability explained by the Fairhope (partial $R^2 = 0.26$) and Fairhope and Prattville (partial $R^2 = 0.19$) rainfall models. For the Fairhope rainfall model, cumulative rain for days 1 – 14 after mid-silk and from day 43 and up to physiological grain maturity were also negatively associated with aflatoxin biosynthesis. Remarkably, when Prattville data were included in the analysis, cumulative rainfall for the second 2-week period following mid-silk becomes significant, while the 2-week period just after mid-silk was not ($p - value < 0.01$). Among others, differences in weather patterns with the subsequent stresses imposed on the host and the fungi, and different soil types, insects and micro-organismal communities between the regions studied, may have contributed to this observation.

Temperature stress has been correlated to aflatoxin contamination in numerous studies (Hawkins et al., 2008; Mc Millian et al., 1985; Widstrom et al., 1990; Windham et al., 2009). *A. flavus*, a thermal-tolerant fungus, can thrive under environmental conditions that result in drought and heat stress (Diener et al., 1987; Widstrom et al., 2003). Optimal growth of fungus occurs at high temperatures (35 – 38 ^{o}C), which may be related to the maximal infection rates observed for corn grown under 34/30 ^{o}C day/night temperatures (Diener et al., 1987; Payne G. A. et al., 1988). The optimal temperature for

aflatoxin synthesis is around 30 ^{o}C, while the range temperature for rapid toxin production and accumulation is 20 – 35 ^{o}C (Schroeder and Hein, 1967; Sorenson et al., 1967). It is well documented that aflatoxin outbreaks are usually observed in years characterized by above normal temperatures (Diener et al., 1987). Our study indicated that maximum temperature, under specific environmental conditions, could explain only a small portion of the toxin variability observed (R^2= 0.3606 for Fairhope alone, and R^2 = 0.1614 when both locations considered). The potential importance of elevated minimum temperatures on aflatoxin contamination in corn was suggested by several researchers (Abbas et al., 2002; Rodriguez-del-Bosque, 1996; Widstrom et al., 1990). However, Abbas et al. (2002) were not able to draw conclusions on the relative importance of day vs. night temperature on aflatoxin synthesis, since both were elevated during the conducive experimentation year.

In this study, minimum temperature was closely associated with aflatoxin contamination observed in the non-inoculated treatments. Both minimum temperature models considered, indicated that increased minimum temperatures during the 2-week interval following midsilk can explain more than half of the total variability accounted for by the models. This interval coincides with the timespan when silks are green – yellow (freshly pollinated) and yellow – brown. Previous work has shown that colonization of silks and kernel infection was significantly greater when inoculum was applied at yellow – brown rather than brown silks (Marsh and Payne, 1984). Silk colonization rate depends on the physiological stage of the tissue, temperature, humidity and moisture, with higher day/night temperatures promoting colonization of senescing silks and the downward growth of the fungi through the silk channel (Diener et al., 1987;

Marsh and Payne, 1984). Furthermore, minimum temperature variables for the second (Fairhope and Prattville analysis) and third (Fairhope analysis alone) post-silking 2-week intervals were also positively associated with preharvest contamination levels. These intervals commonly will include milk (R3) and dent (R5) stages. Marsh and Payne (1984) showed that *A. flavus* was readily isolated through the season from the silks with the greatest incidence observed at milk, while, internal grain infection was observed at dent stage. It is likely that our findings reflect colonization and infection processes for the afore-mentioned timespans. Furthermore, since, the temperature range for rapid production and toxin accumulation is between 20 – 35 ^{o}C (Schroeder and Hein, 1967) those, periods may be, additionally, indicative of increased aflatoxin synthesis resulting from minimum temperature upsurges at night.

Interestingly, the minimum temperature models herein indicated that night (minimum) temperatures for the 2-week prior mid-silk period was negatively associated with observed aflatoxin contamination. A drop in minimum temperature may result in a significant increase in diurnal temperature variation, and, thus, dew formation (FAO, 2012). Fungus growth, development and successive conidia production could be promoted under wet conditions, resulting, therefore, in an increase of primary and/or secondary inoculum in the soil, plant debris and/or plant canopy. Thus, a higher minimum temperature just before flowering should have the opposite effect (drier conditions) leading to less airborne *A. flavus* inoculum available for silk colonization, infection and subsequent aflatoxin contamination later in the season.

Additionally, in southern Alabama, as suggested by the overall model for Fairhope location, a significant portion of the grain aflatoxin variation observed can be partially

explained (approximately 10 %) by an increase in precipitation during the second and third 2-week post – silking intervals. This indicates that rewetting events may increase contamination before grain maturation is reached. Jones et al. (1980) showed that four-week-old ears remained susceptible to infection through silks when kept humid and temperatures were high. Nevertheless, the overall model, when both locations were considered, suggested that increased rainfall before silking and late in grain maturation had a negative association with aflatoxin synthesis. The inclusion of a second location in the analysis, not only shifted the time periods when a particular weather variable had an influence on contamination, but also altered the degree of effect that rainfall and minimum air temperature had on the toxin synthesis indicating the complex and dynamic nature of the phenomenon.

A better managed crop grown under reduced weather extremes, and thus, less stressed, will commonly produce higher yields and would be expected to have lower levels of aflatoxin contamination (Jones et al., 1981; Rodriguez-del-Bosque, 1996). The most adverse conditions for corn production in this study occurred in Fairhope 2011, when high air temperatures, limited and mostly poorly rain distribution before, on, and after silking, led to conditions of potentially prolonged drought stress. In concurrence to this, poor yields were achieved in this year, and aflatoxin contamination, resulting from both natural infection and non-wounding artificial inoculation, were above the action level (20 mg/kg $^{-1}$) set by U.S. FDA. The likely more stressed corn (PD1) accumulated significantly more aflatoxins in the grain than the crop planted late (PD2).

In 2012, a relatively cool and wet year when good crop stands were achieved, natural infection lead to contamination levels greater than 20 mg kg^{-1}, as well. The earlier

planting date yielded significantly less than PD2, and had relatively increased aflatoxin concentration compared to late planted corn (see planting date and inoculation x planting date effects; Table 2. 2). Since 2012 followed a dry, conducive to aflatoxin synthesis year (2011), we assume soil-borne inoculum was present in the area in adequate concentrations for natural infection and subsequent contamination to exceed the 20 mg kg^{-1} threshold. This follows the findings of Shearer et al. (1992), who showed a gradual decrease in *A. flavus* populations isolated from soil in the two years that followed an aflatoxin outbreak in 1988 in Iowa fields.

Although a correlation does not necessarily reveal a cause and effect relationship, linear regression analysis illuminated a negative linear association between aflatoxin concentration and yield. The relationship was noted for Fairhope 2011 ($p - value < 0.05$), but was not significant ($p - value > 0.05$) for others seasons alone. Evidently, as indicated by seasonal yields and weather data summaries, corn crops should experience less stress in Fairhope for 2010, 2012, 2013, and 2014 when compared to Fairhope 2011. The same conclusion could be drawn for corn grown at Prattville on both seasons when compared to the extremely dry 2011 year in Fairhope. Therefore, we suggest, for individual seasons, the relationship to be obtainable on hot, dry years that commonly lead to drought stress during critical time windows influencing yield set and aflatoxin contamination levels.

After, pooling data and analyzing 1) for 2010 – 2012 by including both inoculation levels, and 2) for 2010 – 2014 for the non- inoculated treatment only, negative linear relationships between aflatoxin contamination and yield were observed, (Figure 2. 1B and Figure 2. 1C). This relationship was masked when side-needle technique was used,

since high levels of aflatoxins were attained constantly even for the wetter than the historic average years (Fairhope 2013, Fairhope 2014, and Prattville 2014). Leaf elongation, and thus leaf area index, is dramatically reduced when plants experience drought stress and/or heat stress (Denmead and Shaw, 1960). It is well documented that drought and/or heat stress increases the likelihood for corn contamination (Damianidis et al., 2015; Kebede et al., 2012). Jones et al. (1981) suggested that smaller leaf area may expose the silks to increased concentration of airborne inoculum, and thus amassed infection levels and subsequent contamination. This may be part of the explanation of the significant negative relationship between aflatoxin synthesis and yield detected in this and several other studies, as well (Betrán and Isakeit, 2004; Jones et al., 1981; Rodriguez-del-Bosque, 1996)

Wounding and non-wounding inoculation methods to study aflatoxin contamination had been reviewed by Windham et al. (2003). Natural infection and subsequent kernel contamination are sporadic occurrences, and commonly vary among locations and seasons. An artificial inoculation method may overcome those inconsistencies and may permit distinguishing the effect of management practices in pre- harvest aflatoxin contamination. Thus, for short term studies, selecting an appropriate inoculation technique for a given location might be of interest.

For this study, spreading infested cracked corn on the soil did not induce consistently high levels of aflatoxin contamination (Fairhope 2010 – 2012). The method was effective only under the extremely dry conditions encountered in 2011, when the mean estimated contamination level for the inoculated corn exceeded four times the action limit (20 μg kg-1) as set by U.S. FDA. Olanya et al. (1997) suggested that conidia production and

dispersal are influenced by weather conditions. Thus, it is likely that conidial dispersal from the inoculum source to susceptible silks was strongly favored during the prevailing weather conditions at silking and grain filling in 2011, but not in 2010 and 2012. Additionally, plants with reduced drought stress (seasons 2010 and 2012) are expected to be more resistant to infection and toxin accumulation.

As anticipated, natural infection was not a dependable approach to induce high contamination levels (Windham et al., 2009), since aflatoxin mean estimates ranged from 0.4 up to 47.5 mg kg^{-1} across all years and locations. Furthermore, natural infection did not result in uniform infection in treatments either. These results agree with findings of Windham et al. (2009) who showed that natural infection is more influenced by maximum temperature and rainfall parameters around silking/pollination than is side needle inoculation. Soil-borne populations of *A. flavus* is considered the main source of inoculum in the corn agroecosystem in Iowa (Olanya et al., 1997), and tends to decline when conditions are not favorable for aflatoxin outbreaks (McGee et al., 1996; Shearer et al., 1992). This may explain the reduction in aflatoxin levels observed for the non-inoculated treatments for subsequent years following the 2011 growing season in Fairhope, and the low levels of contamination observed at Prattville, as well. Additionally, a less stressed plant tends to develop a more robust canopy, which may act as a physical barrier to hinder more efficiently silks from airborne inoculum (Jones et al., 1981) than a crop grown under stress. Therefore, declined infection rates should be plausibly anticipated.

Side-needle inoculation technique resulted in high and uniform aflatoxin concertation across treatments for both seasons and locations tested (Table 2. 2). Wounding

inoculation methods introduce conidia directly to kernels bypassing potential plant resistance mechanisms to infection, and they are likely less sensitive to environmental influences than natural infection and non-wounding inoculation methods (Windham et al., 2003; Windham et al., 2009). Interestingly, in 2013 in Prattville, artificial inoculation resulted in three to five fold higher toxin concentrations than the toxin levels observed in 2014 and 2013 for the same treatment in Fairhope, respectively. Those observations might be explained: 1) May 2013 was extreme dry in Prattville and 2) silking occurred on June (Prattville, 2013), which was characterized by below the historic average precipitation and higher day / night temperatures. Additionally, precipitation on July and August were above the historic average (Table 3. 7), as well, a condition that is believed to increase aflatoxin synthesis late in the season since it delays corn drying (Jaime-Garcia and Cotty, 2003; Jones et al., 1981).

Conclusions

The most important findings of this study are: 1) an association between aflatoxin contamination and several of the weather parameters tested was established, 2) minimum temperature and rainfall models derived could explain from approximately 50 up to 87% of the observed aflatoxin variability, 3) minimum temperature weather variables could explain more of the variability than maximum temperature factors (analysis not shown), under the conditions encountered in this investigation, 4) the importance and direction of rainfall and minimum temperature impact on the phenomenon is changing over the timespan considered herein, and 5) all regression models suggested that the critical time for infection and contamination starts at least two weeks before silking and extends to periods covering parcels of the grain filling window as well.

Additionally, this study confirmed that under the environmental conditions in South and Central Alabama agronomic practices had influence on corn yield and aflatoxin contamination. Depending on the year, planting earlier in the season (mid-March) resulted in a significant or a relative increase in aflatoxin accumulation in corn grain compared to levels obtained for corn planted later (mid-April). Planting dates had a significant effect on final yield with the exception of 2014 in Prattville, but the direction of the effect was highly variable across season, reflecting the variability characterizing local and seasonal weather patterns. Planting densities did not influence aflatoxin accumulation in corn, but, increasing standing population had positive influence on yield. A significant negative linear relationship was illustrated between aflatoxin and yield for the extremely dry 2011 season in Fairhope, and when data from Fairhope were pooled over the 2010 – 2012, and 2010 – 2014 years, as well.

Natural and non-wounding infection (spreading cracked corn) approaches proved unreliable, and both were relatively ineffective in inducing uniform and consistent contamination in the fields of Alabama. This confirms that *A. flavus* is a weak parasite, which makes relevant short term studies problematic and highly dependent on seasonal weather conditions. Side-needle inoculation method consistently induced high contamination levels in corn, even under conditions that were not conducive for infection and subsequent toxin synthesis. Thus, this technique might be the first choice for experiments conducted in Alabama when kernel wounding does not interfere with experimental objectives, and uniform crop exposure to *A. flavus* is desirable and/or is required.

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Table 2. 1: Planting, silking, physiological maturity, and harvest dates for planting date treatment per location and growing season.

Location	Year	Treatment[a]	Planting date	Silking[b]		Physiological maturity[c]		Harvest[d]	
Fairhope	2010	PD1	19-Mar	29-May	(71)	11-Jul	(43)	N/A	
		PD2	15-Apr	10-Jun	(56)	22-Jul	(42)	N/A	
	2011	PD1	17-Mar	29-May	(73)	11-Jul	(43)	N/A	
		PD2	12-Apr	13-Jun	(62)	26-Jul	(43)	N/A	
	2012	PD1	20-Mar	25-May	(66)	20-Jul	(56)	7-Aug	(74)
		PD2	13-Apr	15-Jun	(63)	2-Aug	(48)	25-Aug	(71)
	2013	PD1	14-Mar	1-Jun	(79)	23-Jul	(52)	13-Aug	(73)
		PD2	18-Apr	21-Jun	(64)	5-Aug	(45)	24-Aug	(64)
	2014	PD1	21-Mar	7-Jun	(78)	28-Jul	(51)	11-Aug	(65)
		PD2	14-Apr	19-Jun	(66)	6-Aug	(48)	11-Aug	(53)
Prattville	2013	PD1	15-Mar	9-Jun	(86)	29-Jul	(50)	22-Aug	(74)
		PD2	16-Apr	21-Jun	(66)	7-Aug	(47)	3-Sep	(74)
	2014	PD1	22-Mar	8-Jun	(78)	29-Jul	(51)	15-Aug	(68)
		PD2	21-Apr	22-Jun	(62)	8-Aug	(47)	4-Sep	(74)

[a] PD1 and PD2 are normal and late planting dates
[b] Numbers in parenthesis are days from planting date to silking.
[c] Numbers in parenthesis are days from silking to physiological maturity.
[d] Numbers in parenthesis are days from silking to hand harvest (harvest maturity, % moisture content 18-22 %).

Table 2. 2: Treatment effects on aflatoxin contamination for Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014).

	Fairhope, AL Year					Prattville, AL Year	
	2010	**2011**	**2012**	**2013**	**2014**	**2013**	**2014**
Effect[b]	**Aflatoxin Least Squares Mean Estimates (ppb)[a]**						
Pr>F							
IM	*0.6134*	*0.0654*	*0.5133*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*
PD	*0.6749*	*0.0009*	*0.4428*	*<.0001*	*0.0209*	*0.4502*	*0.4623*
D	*0.9794*	*0.1589*	*0.2619*	*0.5972*	*0.1390*	*0.6661*	*0.3274*
IM x PD	*0.807*	*0.238*	*0.6976*	*<.0001*	*0.9825*	*0.0956*	*0.1821*
PD x D	*0.9984*	*0.8578*	*0.9114*	*0.0411*	*0.054*	*0.5661*	*0.3104*
IM x D	*0.7603*	*0.0888*	*0.8036*	*0.9367*	*0.3586*	*0.5340*	*0.9725*
I x PD x D	*0.0145*	*0.8772*	*0.8274*	*0.7961*	*0.9689*	*0.0460*	*0.9274*
IM							
I	5.1	88.6	18.6	141.2 a	202.9 a	673.2 a	147.0 a
No-I	7.0	47.5	37.2	3.3 b	0.4 b	3.4 b	3.9 b
PD							
PD1	6.5	115.4 a	28.0	49.5 a	19.8 a	57.9	28.3
PD2	5.4	36.3 b	24.9	11.2 b	12.4 b	49.2	24.0
IM x PD							
I x PD1	5.3	191.4	14.3	553.6 a	253.6	878.0	185.2
I x PD2	4.9	40.7	24.2	35.4 b	162.3	516.2	116.6
No-I x PD1	8.0	69.5	54.0	3.6 c	0.7	2.9	3.6
No-I x PD2	6.0	32.4	25.6	3.1 c	0.1	3.9	4.3
D							
D1	5.6	51.3	51.7	25.2	18.1	57.4	21.3
D2	6.2	40.3	22.7	27.0	16.4	56.1	26.8
D3	6.1	107.9	26.7	19.3	18.3	55.8	32.5
D4		79.2	15.2	24.7	11.1	45.3	24.8

	Fairhope, AL Year					Prattville, AL Year	
	2010	2011	2012	2013	2014	2013	2014
Effect[b]	**Aflatoxin Least Squares Mean Estimates (ppb)[a]**						
(Continues)							
s							
IM x D							
I x D1	4.9	112.7	28.2	162.3	193.7	723.3	117.5
I x D2	4.2	70.5	17.1	155.4	237.3	810.5	158.4
I x D3	6.3	141.5	14.8	107.2	277.5	481.8	186.9
I x D4		54.6	16.7	146.9	132.8	727.5	134.0
No-I x D1	6.4	23.1	94.4	3.2	0.9	3.7	3.2
No-I x D2	8.8	22.9	30.0	4.0	0.3	3.0	3.9
No-I x D3	6.0	82.3	47.5	2.8	0.3	3.4	5.0
No-I x D4		114.6	13.9	3.5	0.1	3.4	3.9
PD x D							
PD1 x D1	6.2	106.0	81.5	40.5 ab	34.0	56.9	29.9
PD1 x D2	6.7	67.2	15.3	84.3 a	17.3	69.6	28.8
PD1 x D3	6.7	216.8	34.6	42.4 ab	20.6	43.5	30.1
PD1 x D4		114.7	13.7	41.4 ab	12.5	65.2	24.9
PD2 x D1	5.0	24.6	32.7	15.5 bc	9.4	57.9	15.1
PD2 x D2	5.7	24.0	33.5	8.2 c	15.6	45.1	25.0
PD2 x D3	5.6	53.5	20.5	8.5 c	16.2	47.2	35.0
PD2 x D4		54.6	16.9	14.5 bc	9.9	47.7	24.7

[a] Least squares means in the same column followed by different letter are significantly different at $Pr \leq 0.05$ (Tukey test).

[b] IM = Inoculation method, PD = Planting date, D = Plant density. I and No-I refer to artificial inoculation and natural infection, respectively. PD1 and PD2 represent early and late planting dates. D1, D2, D3, and D4 correspond to plant densities of 44,480, 54,360, 64,250, and 74130 plants ha-1, correspondingly

Table 2. 3: Average maximum, minimum temperatures and total rainfall per month for Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014) growing seasons and historic normal.

	Fairhope, AL						Prattville, AL		
	2010	**2011**	**2012**	**2013**	**2014**	**Historic average**	**2013**	**2014**	**Historic average**
Mean Monthly Maximum Air Temperature (^oC)									
March	18.6	22.2	24.2	18.9	19.2	21.3	18.3	19.4	21.0
April	25.8	25.7	24.7	24.3	23.9	25.0	25.2	25.1	25.0
May	29.7	28.3	29.4	26.4	28.6	28.8	28.4	29.7	28.8
June	32.5	32.8	30.1	31.0	31.6	31.4	32.9	32.8	32.3
July	33.0	31.8	31.2	31.2	32.7	32.1	30.9	32.9	33.4
August	33.0	33.7	30.9	31.5	32.8	32.1	31.4	33.9	33.3
Mean Monthly Minimum Air Temperature (^oC)									
March	7.5	10.1	13.9	5.7	7.2	9.5	4.5	6.0	7.7
April	13.2	14.7	13.8	12.3	13.4	13.2	12.7	12.3	11.3
May	20.6	15.6	17.9	15.6	17.5	17.3	15.3	16.2	16.0
June	23.5	23.1	20.1	22.2	22.3	21.0	21.5	21.3	20.2
July	24.7	23.5	22.5	22.1	22.2	22.4	22.1	21.1	22.1
August	25.2	23.0	19.3	22.2	22.5	22.1	21.6	21.7	21.8
Sum Rainfall (mm)									
March	120.9	103.1	56.1	36.3	163.1	144.1	68.6	153.4	154.8
April	47.5	23.1	35.8	103.4	512.1	120.4	115.3	200.2	106.0
May	182.1	20.3	186.2	224.8	227.6	122.9	0.0	122.9	99.8
June	109.5	98.8	254.3	191.5	194.6	160.7	51.8	100.8	99.9
July	47.5	202.4	167.4	445.0	160.3	203.1	237.5	109.2	132.7
August	218.2	44.7	274.3	180.6	6.4	166.7	134.1	90.9	95.8

[a] Historic normal for Fairhope, AL from 1950 – 2008.
[b] Historic normal for Prattville, AL from 1970 – 2014.

Table 2. 4: Type III test effects for aflatoxin contamination and yield when pooled over locations and seasons 2013 -2014.

	Aflatoxin	Yield
Effect[a]	**Pr > F[b]**	
Year	0.0003	0.1096
Location	<0.0001	0.0001
Year x Location	0.2182	0.4834
Inoculation	<0.0001	0.0125
Year x Inoculation	0.8148	0.6501
Location x Inoculation	0.8852	0.693
Planting dates	<0.0001	0.0001
Year x Planting dates	0.0319	<0.0001
Location x Planting dates	0.0013	<0.0001
Inoculation x Planting dates	<0.0001	0.0728
Densities	0.6834	<0.0001
Year x Densities	0.0625	0.0055
Location x Densities	0.5959	<0.0001
Inoculation x Densities	0.8197	0.5364
Densities x Planting dates	0.2427	0.002

[a] Up to two way fixed effects interactions presented herein.
[b] Effects are significant at $\alpha = 0.05$

Table 2. 5: Linear regression models fit statistics for aflatoxin contamination with weather variables for different environments; Fairhope, AL, seasons 2010 – 2014.

Independent Variable	Estimated Coefficient	Standard Error	R^2	Adjusted - R^2	Root Mean Squared Error	Coefficient of Variation	Pr > |t|	Variance Inflation Factor
Overall Model								
Model			0.8720	0.8499	0.2383	32.81	<0.0001	
Intercept	-14.8604	1.6979					<0.0001	0.00
Rain(II)	0.0013	0.0005	0.0631				0.0237	1.27
Rain(III)	0.0015	0.0006	0.0302				0.0150	1.47
Temp min (-I)	-0.2969	0.0273	0.1123				<0.0001	2.14
Temp min (I)	0.4055	0.0445	0.3996				<0.0001	2.08
Temp min (III)	0.5427	0.0698	0.2668				<0.0001	1.55
Rainfall Model								
Model			0.7147	0.6871	0.3441	47.37	<0.0001	
Intercept	1.9932	0.1758					<0.0001	0.00
Rain(-I)	-0.0071	0.0009	0.2618				<0.0001	1.22
Rain(I)	-0.0062	0.0017	0.2222				0.0010	1.20
Rain(IV)	-0.0097	0.0019	0.2307				<0.0001	1.20
Minimum Temperature Model								
Model			0.8243	0.8073	0.2701	37.18	<0.0001	
Intercept	-11.7746	1.5990					<0.0001	0.00
Temp min (-I)	-0.2705	0.0296	0.1540				<0.0001	1.96
Temp min (I)	0.3928	0.0490	0.4398				<0.0001	1.96
Temp min (III)	0.4086	0.0641	0.2304				<0.0001	1.01

Linear regression models fit statistics for combined data for Fairhope, 2010 – 2014. Rainfall and minimum air temperatures (Temp. min) were averaged over 14 days intervals around silking; (-I) = 14 days prior silking, (I) = 14 days after silking), (II) = days 15 – 28 after silking, (III) = days 29 – 42 after silking, (IV) = remaining days following time window (III) up to physiological maturity. Dependent variable was aflatoxin content y = log10(ppb+1) averaged for six replicates for different environments specified as: Non – Inoculated x Planting dates x Densities. Multiple regression models were built with stepwise procedure (SAS, version 9.3); significant levels for a variable to enter and stay in the models were set to $\alpha = 0.1$.

Table 2. 6: Linear regression models fit statistics for aflatoxin contamination with weather variables for different environments; Fairhope, AL, seasons 2010 – 2014 and Prattville, AL, seasons 2013 – 2014.

Independent Variable	Estimated Coefficient	Standard Error	R^2	Adjusted - R^2	Root Mean Squared Error	Coefficient of Variation	Pr > |t|	Variance Inflation Factor
Overall Model								
Model			0.7596	0.7443	0.2592	36.55	<0.0001	
Intercept	-6.2577	0.7083					<0.0001	0.00
Rain(-I)	-0.0053	0.0006	0.1886				<0.0001	1.16
Rain (IV)	-0.0027	0.0016	0.2739				0.0922	1.66
Temp min (IV)	0.3177	0.0311	0.2971				<0.0001	1.66
Rainfall Model								
Model			0.5005	0.4687	0.3736	52.68	<0.0001	
Intercept	1.4035	0.1189					<0.0001	0.00
Rain(-I)	-0.0048	0.0008	0.1886				<0.0001	1.13
Rain(II)	-0.0011	0.0006	0.0596				0.0646	1.02
Rain(IV)	-0.0090	0.0019	0.2524				<0.0001	1.14
Minimum Temperature Model								
Model			0.6024	0.5770	0.2701	37.18	<0.0001	
Intercept	-3.7375	1.0060					0.0005	0.00
Temp min (-I)	-0.1528	0.0351	0.1385				<0.0001	2.11
Temp min (I)	0.1611	0.0660	0.3099				0.0185	2.56
Temp min (II)	0.1810	0.0424	0.1540				<0.0001	1.51

Linear regression models fit statistics for combined data for Fairhope, 2010 – 2014 and Prattville, 2013 - 2014. Rainfall and minimum air temperatures were averaged over 14 days intervals around silking; (-I) = 14 days prior silking, (I) = 14 days after silking), (II) = days 15 – 28 after silking, (III) = days 29 – 42 after silking, (IV) = remaining days following time window (III) up to physiological maturity. Dependent variable was aflatoxin content y = log10(ppb+1) averaged for six replicates for different environments specified as: Location x Non – Inoculated x Planting dates x Densities. Multiple regression models were built with stepwise procedure (SAS, version 9.3); significant levels for a variable to enter and stay in the models were set to $\alpha = 0.1$

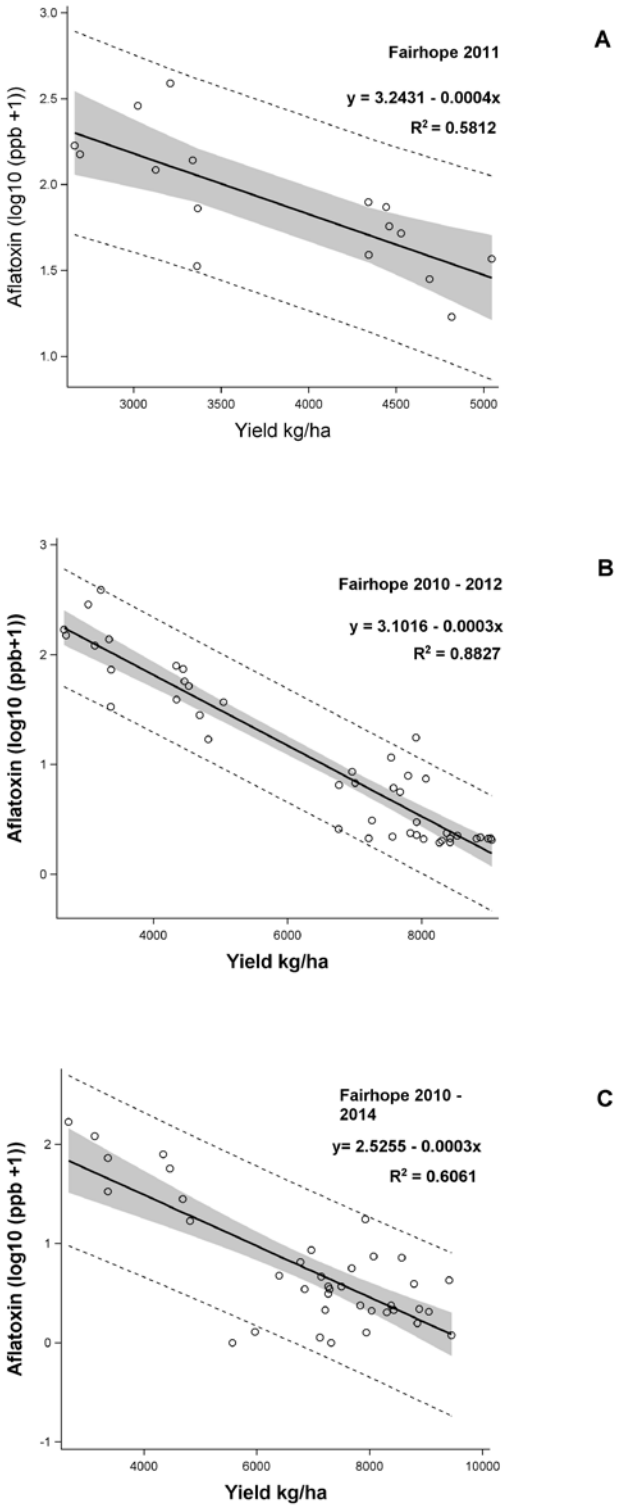

Figure 2. 1: Relationship between aflatoxin contamination and yield: A) Fairhope (2011): Environments defined as Inoculation x Planting date x Plant density; B) Fairhope (2010 – 2012): Environments defined as Inoculation x Planting date x Plant density. C) Fairhope (2010 – 2014),

only data from the non – inoculated treatment included. Environments defined as Planting dates x Plant densities. Each point is an average of six replicates. In 2010, there were three densities studied. In 2011 – 2014, four density levels were tested. Shaded area and area enclosed by the upper and lower dotted lines represent 95% confidence and prediction limits, respectively.

3 EXPLORING ^{13}C DISCRIMINATION INDEX AS A TOOL TO ASSESS CORN YIELD DIFFERENCES DUE TO PLANTING DATE AND PLANT DENSITY IN THE COASTAL PLAINS OF ALABAMA

Abstract

Planting dates and plant densities influence corn yield and interact with weather conditions to impose plant stresses that result in significant yield reductions for dryland corn. Optimum planting dates and optimum plant densities are location specific and sound management decisions rely on this information. However, this information is usually obtained through large scale experiments that are time consuming and expensive or through modeling approaches which require data that are not always readily available. Environmental stresses result in ^{13}C discrimination (Δ), and question arise if yield differences as affected by planting dates and plant densities can be reflected on ^{13}C discrimination values from corn grains harvested within and at the end of the season. The objectives of this study were: 1) to explore if Δ is a suitable tool to explain corn yield differences resulting from planting date and plant density practices under the environmental conditions of Coastal Plains in Alabama, and 2) explore if Δ observations from corn grain sampled within season can be used to assess potential yield losses. Field experiments were conducted at Fairhope and Prattville. The experimental design was split plot with planting dates (mid-March and mid-April) assigned to main plots and four densities (44,480, 54,360, 64,250, and 74,130 plants ha^{-1}) assigned to sub-plots. Corn grain was harvested at milk (R3) and at harvest maturity, and analyzed for ^{13}C concentration. The association between yield and Δ in corn grains harvested at milk (R3) and at harvest maturity was not consistent between year x locations and within year x location. Δ values of grain samples reflected yield differences between mid-March and mid-April planted corn in Prattville (for both grain harvest times) in 2013 and in Fairhope (for grain harvested at R3 only) in 2014. Δ in corn grain was significantly influenced by

plant density only for samples harvested at milk (R3) and at harvest maturity in Fairhope in 2013 and 2014, respectively. In Fairhope, lower plant densities tend to have higher Δ and lower yield per unit area compared to higher corn densities. The inconsistencies in the relationship between Δ and corn yield indicate that factors not measured in this study can influence Δ values in corn grain. Therefore, more research is needed to elucidate the effect of different factors under field conditions before Δ can be used as a tool to assess corn attained yield differences.

Introduction

Management practices such as planting date and plant density have an influence on corn (*Zea mays* L.) grain yield and yield losses for dryland corn can be significant when theyinteract with weather conditions that impose plant stresses (e.g.; drought). Understanding the associations between factors that limit corn yield is necessary when studying and recommending management practices for a specific location. However, management recommendations to optimize yield are commonly based either on large experiments that can be expensive and time consuming, or on modeling approaches that usually require information that is not readily available. ^{13}C discrimination (Δ) has been used as a tool to evaluate water stress in corn (Clay et al., 2005; Dercon et al., 2006) because plant available water, and thus water stress conditions may significantly influence the concentration of isotopic ^{13}C in plant tissues (Van Kessel et al., 1994).

To understand why Δ can be used as tool to assess water stress some theoretical background is provided (Clay et al., 2001; O'Leary, 1993). Isotope composition is expressed as the ratio (R) of the heavier to the lighter isotope ($R= {}^{13}C/{}^{12}C$). Commonly R is expressed as either stable isotope composition (δ^{13}) or as ^{13}C discrimination (Δ):

$$\delta^{13}C = \left[\frac{R_s}{R_{st}} - 1\right] * 1000 \qquad (1)$$

where, R_s and R_{st} are the $^{13}C/^{12}C$ ratios of the sample and the standard (limestone from the Pee Dee formation in S. Carolina) (Clay et al., 2001; Clay et al., 2005; O'Leary, 1993),

$$\Delta = \frac{\delta^{13}C_a - \delta^{13}C_p}{1 + \frac{\delta^{13}C_p}{1000}} \quad (2)$$

where, δ^{13}Ca and δ^{13}Cp represent the δ^{13}C values of air (-8‰) and the plant sample measured values, respectively.

In C4 plants discrimination against ^{13}C during photosynthesis is described by the following equation (Farquhar, 1983):

$$\Delta = a + (b_4 - \varphi(b_3 - s) - a)\frac{C_i}{C_a} \quad (3)$$

where C_i/C_a is the ratio of the intracellular to the ambient CO_2 concentration, α (4.4‰) is the discrimination due the diffusion of CO_2 from the atmospheric air into the leaf intracellular space through stomata, b_4 (-5.7‰) is the net discrimination due to dissolution of HCO_3^- and its fixation by phospoenolpyruvate carboxylase (PEPC), b_3 (29‰) is the discrimination by RUBISCO during the carboxylation of CO_2 in the bundle sheath, and φ stands for the ratio describing the rate CO_2 leaks out of the bundle sheath to the rate of PEP carboxylation, and s (1.8‰) is the discrimination due to CO_2 leakiness from the bundle sheaths back to mesophyll cells. Equation 3 predicts that as stomata close Δ increases (Clay et al., 2001; Farquhar, 1983).

Previous research on corn, a C4 plant, revealed that water stress conditions resulted in greater Δ value in corn plant tissues (Clay et al., 2001; Clay et al., 2009; Monneveux et al., 2007). Hansen et al. (2013) has shown that corn plants sampled from a moderately yielding field zone had higher Δ value, an indication of higher plant stress, when

compared to plants from a higher yielding zone that had lower plant tissue Δ values. However, other studies indicated that although the relationship between Δ observations and wheat grain yield were significant, the direction (positive or negative) of the correlations was not consistent (Heng et al., 2005).

Corn growth and development is affected by factors such as temperature, rainfall, and soil available water (Tsimba et al., 2013). Dryland corn yields are mostly limited by low precipitation and high temperatures (Norwood, 2001b) and it is important for non-irrigated corn to utilize efficiently the water that is found in the soil profile. Since those factors vary temporarily and spatially corn planted on different dates and different densities should experience dissimilar environments and can be exposed to varying stress levels. Therefore, the impact of climatic conditions on yield is expected to vary among different planting dates and different plant densities.

Optimum corn planting date varies among regions and can have an impact on attainable yield (Bruns and Abbas, 2006; Van Roekel and Coulter, 2011). Optimum corn yield declines if planting is delayed after the optimum planting window (Bruns and Abbas, 2006). Nielsen et al. (2002), showed that cumulative days and thermal requirements to reach physiological maturity of regionally adapted corn hybrids were reduced by 9 days and 144 growth degree days, respectively, for corn planted late (early June) versus early planted corn (early May). Short growing seasons, as a result of late planting, are usually associated with yield losses (Van Roekel and Coulter, 2011). Coulter (2010) indicated that in Minnesota corn planted in late May yield up to 80% less than corn planted at late April. In Southern Wisconsin, over 12 environments (4 seasons x 3 locations), an optimum planting date for short and long season hybrids was identified

between the 1st and the 7th of May. For each day planting was delayed a yield decrease of 0.5 to 1.1 % per day was observed for the 2-week window starting on the 8th of May (Lauer et al., 1999). Rates of yield reduction were accelerated for each day planting was delayed over the next two 2-week windows, and ranged from 1.3 to 1.9 and from 2.0 to 2.8%, respectively. In the same study, the optimum planting date for corn in North Wisconsin ranged from 8 to 14 May. Similarly, in Northern Wisconsin yield declined at an accelerated rate as planting dates were delayed (e.g.; yield declined by 0.2 to 1.7% when corn was planted over the 2-week window following the optimum planting time). Studies of irrigated corn at Tifton Georgia showed a 50% yield reduction at late May or early in June compared to the normal March planting date (Lee, 2016). In general, the corn planting date should be selected to minimize plant exposure to heat and drought stresses particularly at the reproductive stage to minimize yield losses (Bruns and Abbas, 2006). Therefore, based on the climatic conditions of an area, it is crucial to select a planting window that will minimize the risk for crop failure and minimize yield losses.

Plant density is another agronomic practice that can greatly impact corn yield, and thus identifying optimal corn density population is crucial for achieving high yields and to minimize production risks (Assefa et al., 2016). Compared to other grasses, corn is more responsive to plant density changes (Sangoi, 2001). However, using high corn population increases interplant competition for light, soil nutrients and soil available water (Lee, 2016; Norwood, 2001a). Environmental conditions, particularly drought, can increase the risk for corn crop failure (Allen, 2012). In Georgia, in years with adequate and well distributed rainfall during the growing season dryland corn population densities above 49,500 plants ha^{-1} will result in high yields; however, in dry years the risk for yield

losses increases significantly above the aforementioned density population threshold (Lee, 2016).

One of the most limiting yield factors in dryland farming is soil moisture (Heng et al (Heng et al., 2005; Mask and Mitchell, 1988). Improving corn crop productivity in drought-prone areas requires farmers, researchers, extension specialists, and extension agents to better recognize best-suited management alternatives to optimize the use of limited natural resources such as soil water availability (Heng et al., 2005). Altering planting dates and plant densities can alleviate or impose more stress on corn during the critical vegetative and reproductive plant growth stages and can change the risk for yield losses.It is expected that corn planted on different planting dates and at different plant densities will experience different levels of field stresses. Therefore, influence of planting dates and plant densities on corn yield differences may be reflected on observed Δ values derived from corn grain tissues collected within and at the end of the season. However, information on the use of Δ as a tool to assess yield differences in corn related to management and its interaction with environment in the Southern Coastal Plains is limited. Therefore the objectives of this study were: 1) to explore if Δ is a suitable tool to explain in-field yield differences in corn resulting from planting date and plant density under the environmental conditions of Coastal Plains in Alabama, and 2) explore if Δ observations from corn grains sampled within season can be used to assess potential yield losses.

Materials and Methods

3.2 Research sites and cultivation practices

This research was conducted at Gulf Coast Research and Extension Center, in Fairhope, AL (30^{o} 32'N, 87^{o} 52W) from 2010 – 2014, and at Prattville Agricultural Research Unit, near Prattville, AL (32^{o} 25'N, 86^{o} 26'W) from 2013 – 2014. The soil texture at Fairhope site was fine sandy loam, while at Prattville was sandy loam. The soil series were Malbis (Fine-loamy, siliceous, subactive, thermic Plinthic Paleudults) and Lucedale (Fine-loamy, siliceous, subactive, thermic Rhodic Paleudults) for Fairhope and Prattville, respectively. Cultivation practices varied over season and location depending on equipment availability, but did not differ from the recommended management practices for the area. Briefly, the experimental field was strip-tilled before planting at Fairhope in each season (2010 – 2014), while at Prattville in 2013 and 2014 a conventional tillage and paratill were applied, respectively. Generally, corn was planted each year in rotation following soybean or cotton. During winter, the land was either left to fallow (Prattville, 2013), or planted to wheat or rye cover crop prior to corn cultivation (Prattville 2014; Fairhope 2010 – 2014). Fertilization and lime application for soil pH adjustment followed the recommendations of Auburn University Soil Test Laboratory. Nitrogen was applied twice during the growing season with total nitrogen application rates ranging from 145 – 182 kg ha^{-1}. Approximately one third of total nitrogen was applied at planting and two thirds of the total nitrogen were applied when corn was at V6.

Pioneer 31P42 corn hybrid was planted in all years and both locations. Each experimental plot had a length of 9.1 m and consisted of four rows. Inter-row spacing was 0.96 and 0.90 m in Fairhope and Prattville, respectively. The experimental design

was split-plot, with planting date treatment assigned to the whole plots and plant density effect assigned to the sub plots. Each treatment was replicated six times. Two planting dates were tested: 1) the recommended planting date for the area of study (mid-March (PD1)) and 2) weather permitting, a month later planting (mid-April (PD2)). Plant densities tested included standing populations of approximately 44,480 (D1), 54,360 (D2), 64,250 (D3), and 74,130 (D4) plants ha^{-1}. In 2010 D4 was not included in the experiment. The middle two rows of each individual plot were combine harvested at harvest maturity (grain moisture content ranged from 18 – 22%). Combine grain weight was reported at 15.5% moisture.

3.3 Carbon 13 discrimination for plant stress assessment

In the 2013 and 2014 growing seasons, ^{13}C discrimination was used to evaluate water stress in corn (Clay et al., 2001; Dercon et al., 2006). Corn ears were harvested twice during the growing season. Two samplings per season per location were selected because the relationship between Δ and grain yield as reported in previous studies was contradictory (Dercon et al., 2006; Heng et al., 2005) and under drought the relationship (Δ versus grain yield) depends greatly on environmental conditions, and the sampling time (Monneveux et al., 2007). Ten top corn ears per plot were randomly hand harvested from three replications at milk stage (R3) (Table 3. 1) from the two side plot rows (rows 1 and 4), and again at harvest maturity from the two middle rows. At milk (R3) stage corn ears were harvested from the side plot rows because the two middle plot rows were used to assess corn grain yield per unit area. Grains from the lowest 1/3 of the cobs were hand shelled. The grain from the lowest 1/3 of the cob was dried at 80 ^{o}C for 48 h (Monneveux et al., 2007) or until constant weight was reached. The grain from the lower

1/3 of the cob was selected for ^{13}C analysis because under heat stress (which can lead to drought stress) distribution of assimilates in the cob was altered; preferentially assimilates were distributed towards the lowest 1/3 of the cob in cost of the upper $2/3^{ds}$ of the cob (Suwa et al., 2010). If this is true, one could expect the water stress history experienced by the plant to be reflected in the basal part than the upper $2/3^{rds}$ of the cob. A grain sub-sample from the basal 1/3 of the cob (50 g) was milled to talc powder size with a Miller Cyclotec 1093 Sample Mill (UDY Corporation, Colins, CO). Approximately 1 mg of the corn grain powder per treatment was enclosed in a tin capsule and analyzed for $\delta^{13}C$. Briefly, samples were combusted at 1020 ^{o}C with He carrier flow rate of 100 mL/min in a Carlo Erba NA1500 elemental analyzer, feeding a ThermoFinnigan Delta^{PLUS}XL isotope ratio mass spectrometer via a Conflo III interface (Jonathan Karr, personal communication). Raw $\delta^{13}C$ values were normalized with a combination of international and calibrated internal reference materials versus Vienna Pee Dee Belemnite. Weight% C was calculated against an acetanilide reference. Precision of $\delta\ ^{13}C$ at one standard deviation was approximately 0.1 per mil. Ratio of number of samples to standards was approx. 7:1. Duplicates were run for every tenth sample. The analysis for $\delta\ ^{13}C$ was carried at the Duke Environmental Stable Isotope Laboratory, Nicholas School of the Environment, Division of Earth and Ocean Sciences, Durham, NC. Results of the carbon isotope composition analyses were expressed as $\delta^{13}C$ (‰) (eq. 1).

The top 2/3 of the ears was machine shelled. The total grain weight from the second hand harvest was expressed as the sum of the weight of grains shelled from the basal and the top part of the cob and was reported at 15.5% moisture content. Total plot yield was

derived as the sum of the grain weight from the combine and the grain weight from the second harvest.

3.4 Weather data

Air temperature and rainfall were measured on both experimental sites for 2013 and 2014 seasons with a HOBO Pendant® Temperature / Alarm Data Logger, model 64k – UA-001-64 (Onset Computer Corporation, Bourne, MA), and a tipping bucket rain gauge manufactured by RainWise Inc (Trenton, ME). Both sensors were installed at each field at the beginning of the growing season. Additional weather data were collected by the on-site weather stations located at the Gulf Coast Research and Extension Center, in Fairhope, AL, and the Prattville Agricultural Research Unit. Data were obtained through the Alabama Mesonet Weather Data network. Monthly summaries and historic average values of maximum temperature, minimum temperature and cumulative rainfall for Fairhope (1950 – 2008) and Prattville (1970 – 2014) are provided in Table 3. 8.

Maximum and minimum daily air temperatures were averaged, while cumulative rainfall was considered, as well, for consecutive time periods starting two weeks before silking (R1) and extending up to physiological maturity (R6) for each planting date (Figure 3. 1 & Figure 3. 2). Thus, five time windows were defined: 1) the 2-week time period before silking, 2) the 2-week time period after silking, 3) the second 2-week window after silking, 4) the third 2-week window following silking, and 5) a variable day-length window following the end of the third 2-week window after silking and extending to physiological maturity day (ranging from 0 – 14 days, depending on planting date and growing season) (Table 3. 1 & Table 3. 2). The derived weather variables along with two indices, the Shannon diversity index (SDI) (Bronikowski and

Webb, 1996) and the abundant and well-distributed rainfall (AWDR) index (Tremblay et al., 2012) were used to explain yield differences between planting dates. SDI takes values between 0 – 1. The closer SDI is to 1 the more evenly the rainfall is distributed in a given time period. AWDR does not have upper boundaries; a larger value represents abundant and well distributed rainfall for a given time period (Table 3. 2).

3.5 Assessment of soil moisture dynamics

Change in soil moisture status were assessed at the top 30 cm of the soil profile at each experimental site for the 2013 and 2014 growing seasons. Soil moisture was measured with EC-5 volumetric water content sensors (Decagon Devices, Inc., Pullman, WA) at the depths of 15 and 30 cm every four hours during the growing season.

Undisturbed soil samples were collected with a drop hammer assembly at soil depths of 15 and 30 cm at both locations in 2013. The soil samples were used for estimation of soil moisture retention curves at the Soil Physics Laboratory at Auburn University (data not shown). The undisturbed soil samples were rest on a ceramic plate which was enclosed in pressure cell. In-house pressure was passed through the pressure cells at pressures of 0, 5, 10, 20, 50, 120, 200, 400, 500 cm of H_2O pressure head and the volumetric soil water content corresponding to each pressure was determined. Field capacity (θ_{fc}) corresponds to the amount of water remaining in the soil after free drainage has ceased. At θ_{fc} the matric potential is between -0.1 to -0.33 bars. Based on the soil types at Fairhope and Prattville we considered that θ_{fc} was reached at matrix potential of $\approx$ - 0.12 bars (120 cm H_2O head).Volumetric water content at permanent wilting point for the soil types at both locations and both depths was determined with the pressure plate

method, where saturated disturbed soil samples were placed under 15 bars pressure for two consecutive days (Richards and Fireman, 1943).

The percent depletion of the soil available water in the top 30 cm of the soil profile was measured by modifying the equation from Panda et al. (2004) as:

$$Depletion\ (\%) = 100 * \sum_{i}^{n} \frac{FC_i - \theta_t}{FC_i - WP_i} * n_i \quad (4)$$

where, FC_i, and WP_i represent the volumetric water content at field capacity and the volumetric water content at wilting point respectively at the two depths of 15 and 30 cm as determined in the laboratory, θ_t is the daily average of the volumetric soil moisture content as measured by the EC-5 sensors installed at 15 and 30 cm, n_i is a derived weight factor. Soil moisture in the top 0-20 cm was expected to fluctuate more due to precipitation and evapotranspiration effect than soil moisture at the deeper 20-30 cm layer. Readings from sensors installed at 15 cm were expected to reflect more accurately the soil dynamics in the upper 0-20 cm than sensors installed in the lower depth (30cm). Therefore, daily soil moisture percent depletion calculated from the moisture content at 15 and 30 cm was multiplied by a weight factor (n_i) of 2/3 and 1/3 respectively.

Daily percent depletion values of the soil available water for the top 30 cm of the soil profile were averaged by weekly intervals starting 2-weeks before silking and extending to corn grain physiological maturity. The average weekly soil moisture variables derived were used to study the correlation between potential plant water stress (as reflected by soil moisture depletion) and Δ.

As soil moisture is removed due to evapotranspiration the percent depletion in the soil profile is increased. When soil moisture percent (%) depletion reaches a certain level corn will experience moisture stress and Δ values observed in corn grains should change. To examine how potential soil moisture stress is affecting Δ three different scenarios were explored (Table 3. 7). In the first scenario, weekly soil available water percent depletion was calculated as the average of all daily soil available water percent depletion data ($\theta_{\text{Mean depletion}}$). In the second and the third scenario, weekly soil available water percent depletion was calculated by excluding days when percent soil moisture depletion was less than 10 ($\theta_{\text{depletion} > 10\%}$) and 30% ($\theta_{\text{depletion} > 30\%}$), of the plant available water in the top 30 cm of the soil profile, respectively.

3.6 Statistical analysis

The effect of planting date, plant density and planting date x density interaction on yield and Δ were analyzed by using the PROC GLIMIX procedure of SAS version 9.3 (SAS, 2010). Pooling yield data together, with year and location considered fixed, indicated the effects as being significant (p-values < 0.05, Table 3. 3). Therefore yield analysis for separate year x location was justified. Analyses for yield and Δ values were conducted separately by year x location with planting date and plant density considered fixed (Table 3. 4 & Table 3. 5). The effects of block and block x planting dates were treated as random. Means of fixed effects were compared at level of significance $\alpha = 0.05$. Tukey test was used to adjust least-square means differences and control for Type I experiment wise error rate.

Spearman's rank correlation coefficients were calculated by using PROC CORR SPEARMAN procedure in SAS version 9.3. Correlation coefficients were considered

statistically significant when p-value < 0.05 but all generated p-values are presented herein. Spearman correlation coefficients were calculated between yield and Δ values with both being averaged by Year x Location x Planting dates x Densities (Table 3. 6). Spearman correlation analysis was conducted between the weekly percent depletion of soil available water and the Δ index. For this analysis Δ was averaged (Δ_{Mean}) over distinct environments defined as Year x Location x Planting dates x Densities.

Results

3.7 On site weather conditions

Climatic conditions in Fairhope in 2010 and 2011 were hotter and drier than the historic average (Table 3. 8). Monthly cumulative rainfall from March to May was below the historic average in Fairhope in 2012, but cumulative rainfall during the grain filling exceeded the historic average. In the 2013 and 2014 growing seasons, the climate in Fairhope was wetter and cooler compared to the historic average. In 2013 Fairhope received 30.8 and 241.9 mm more rainfall than the historic average in June and July, respectively. Fairhope in 2014 received more cumulative rainfall in all months but July and August compared to the historic monthly cumulative rainfall averages for the area. At Prattville 2013 growing season was cooler and drier than the historic average, while in 2014 it was wetter than the historic average.

3.8 Influence of planting date and plant density on ^{13}C discrimination

Δ differences observed in corn grains with respect to planting date treatment were not consistent over locations, years and harvest time (Table 3. 5). Planting date had a significant effect (p-value < 0.05) on Δ in corn grain samples harvested at milk (R3) stage (HV1) in 2014 season in both Fairhope and Prattville (Table 3. 5). In 2013 and 2014 seasons in Prattville, planting date had a significant influence (p-value < 0.05) on Δ values in corn grains harvested after physiological maturity (HV2) (Table 3. 5). In Fairhope in 2014, grains from mid-March planted corn (PD1) had lower Δ compared to grains harvested from mid-April planted corn (PD2). In Prattville in 2013 and 2014, corn grains from both harvests had higher Δ mean estimates for mid-March grain samples than

for mid-April grains. However, the relationship was only significant for the grain tissues collected at milk (R3) (HV1) in 2014, and for the grains sampled at physiological maturity (HV2) both in 2013 and 2014.

Δ differences observed in corn grains with respect to plant density treatment were not consistent over locations, years and harvest time (Table 3. 5). In Fairhope in the 2013 and 2014 seasons, plant density treatment had a significant influence on Δ values in the corn grains harvested at milk (R3) and at physiological maturity, respectively (Table 3. 5). For grain harvested at milk (R3) (HV1) in 2013 in Fairhope, Δ observations were significantly higher for D1, D2, plant densities than for the D3 level (Table 3. 5). In 2014 in Fairhope, the grain samples at HV2 for the higher corn population density (D4) had significantly (p-value < 0.05) lower Δ values than density D1 (Table 3. 5). In general, in Fairhope we observed that Δ in corn grain tend to increase at low plant densities, however the relationship was not always significant and was not observed in Prattville (Table 3. 5). The two way interaction between planting date and plant density on grain Δ was significant only for samples collected at physiological maturity in Fairhope in 2013 (Table 3. 5).

3.9 Influence of planting date and plant density on yield

Moderate to highly significant (p-value < 0.05) negative correlation was detected between corn yield and Δ index for grains harvested at milk (R3) (HV1) stage in Fairhope 2014 and for corn grain harvested on both milk (R3) (HV1) and harvest maturity (HV2) stages in Prattville in 2013 (Table 3. 6). The effect of planting date on yield was significant (p-value < 0.05) in both locations and for all seasons but Prattville in 2014 (Table 3. 4). In Fairhope in 2010, 2013 and 2014 seasons, mid-March planted corn

yielded significantly more than mid-April corn (Table 3. 4). The opposite, higher yield from mid-April planted corn compared to mid-March planting was observed in Fairhope in 2011 and 2012 seasons and in Prattville in 2013 (Table 3. 4). Plant density effect was significant (p-value < 0.05) in all environments (year x location) studied except Fairhope 2010 (Table 3. 4). In general, as plant density increased greater yield per unit area was attained (significantly or numerically) as well. However, this was not the case in 2010 and 2011 seasons in Fairhope were the highest density level tested yielded 175 (numerical difference) and 587 (significant at p-value < 0.05) kg ha^{-1} less than densities D1 and D2, respectively (Table 3. 4).

3.10 Correlations between soil moisture depletion and ^{13}C discrimination

For the $\theta_{\text{Mean depletion}}$ and the $\theta_{\text{depletion} > 10\%}$ scenarios for HV1 corn grains, the weekly percent soil moisture depletion values for all the four weeks considered (two weeks before silking and two weeks after silking), were positively and significantly (p – value < 0.10) correlated to the Δ_{Mean} (Table 3. 7). From the corn samples tested at physiological maturity, Δ_{Mean} was positively correlated with the soil moisture percent depletion data in 7 out of 10 and 8 out of 10 weeks considered for the scenarios $\theta_{\text{Mean depletion}}$ and $\theta_{\text{depletion}> 10\%}$, respectively (p-value < 0.10) (Table 3. 7). Average soil moisture percent depletion on week 7 after silking for the $\theta_{\text{Mean depletion}}$ barely miss significance at $\alpha = 0.10$ (p-value = 0.1283). When the percent soil moisture depletion weekly average excluded depletion values lower than 30% ($\theta_{\text{depletion}> 30\%}$), a positive correlation between Δ_{Mean} assessed at milk stage and the soil moisture percent depletion was found on 3 out of the 4 weeks surrounding silking. For corn grains at physiological maturity and the $\theta_{\text{depletion}> 30\%}$ scenario, 5 out of 10 weeks considered herein indicated significant (p – value < 0.10)

positive correlation between average soil moisture percent depletion and Δ_{Mean} data. The results of the third scenario are questionable. In several weeks sample size had been reduced (e.g.; $n < 30$) and therefore the estimates of the correlation are expected to be more “noisy”. The findings from the first two scenarios indicated that there is a weak to moderate positive correlation between soil moisture percent depletion in the top 30 cm of the soil profile and the Δ_{Mean} values determined in corn grain at milk (R3) (HV1) and at harvest maturity (HV2) for the vast majority of the weeks tested.

Discussion

Significant negative correlation has been detected between Δ and yield in 2014 in Fairhope for grains harvested at milk stage, and in Prattville in 2013 for corn grains harvested both at milk and physiological maturity stages. However, the relationship between Δ and yield was not consistent and was not observed under all the environments (year x location) the experiment was conducted. It might be that during wet years when cumulative rainfall over the growing season was larger than the historic average, the correlation between yield and Δ did not hold (Prattville in 2014 and Fairhope in 2013) or was not consistent between grains harvested at different growth stages (Fairhope, 2014). Contrasting to Fairhope, the relationship between yield and Δ holds for both sampling dates (milk and physiological maturity) in Prattville in 2013 which was a drier year compared to the historic average. We conclude that for the seasons where the relationship between yield and Δ was not detected or was inconsistent between grain sampling times, either corn was not exposed to a strong level of water stress for the correlation to be detected or other factors not measured in this study influence the observations.

One of the major limitations of dryland corn production in the Coastal Plains of Alabama is soil moisture, and water stress has been correlated with Δ in corn (Clay et al., 2009; Dercon et al., 2006). In this study we showed that weekly average soil moisture percent depletion from the top 30 cm of the soil profile was positively correlated to Δ in corn grain. This hold true for grains harvested at milk stage for the $\theta_{\text{Mean depletion}}$ and $\theta_{\text{depletion } > 10\%}$ scenarios studied; in both scenarios the positive correlation holds for each of the considered four weeks around silking (Table 3. 7). A positive correlation between Δ and percent soil moisture depletion in the top 30cm was also found for most of the

weeks tested herein when corn grain samples were harvested at the end of the season. As percent depletion increases, corn plants are potentially more stressed and they respond to water loss by closing stomata which is expected to increase Δ (Clay et al., 2005). However, the strength of the correlation between Δ and the average percent soil moisture depletion varied among weeks and in most of the cases the strength of the correlation ranged from weak to moderate. Variation in Δ in corn, a C4 plant, depends on C_i/C_a (the ratio between intracellular and atmospheric CO_2 concentration) and φ, the proportion of leakiness of CO_2 out of the bundle sheath during photosynthesis. (Farquhar, 1983; Monneveux et al., 2007). An increase in φ as indicated by equation 2 should result in higher Δ. Bowman et al. (1989) showed that φ in corn was increased with increasing water stress, and that φ varied diurnally and influenced Δ values. This could explain the correlation between percent soil moisture depletion and Δ in corn grains in our study.

Within and between season differences in yields obtained among planting dates illustrate the influence that different weather conditions may have on results. Research has shown that temperature and moisture stress during both late vegetative and reproductive stages influences corn yield losses (Çakir, 2004; Denmead and Shaw, 1960; Ethan and Peter, 2015; Eyshi Rezaei et al., 2015). Denmead and Shaw (1960) showed that moisture stress alone 30 days before silking, at silking, and 30 days beyond silking, negatively impacted corn yield by 25, 50 and 21 %, respectively. Additionally, corn is sensitive to elevated temperature during silking and grain filling period (Ethan and Peter, 2015; Sánchez et al., 2014). In a review study, optimum temperatures for anthesis and grain filling were calculated being equal to 30.5 ^{o}C (± 2.5 °C) and 26.4 °C (± 2.1 °C), respectively (Sánchez et al., 2014). Therefore, temperatures above those thresholds are

expected to stress corn and impact yield. Our maximum temperature summaries (Table 3. 2) indicate that during grain filling corn for both planting dates experienced temperatures above 30 ^{o}C indicating that heat stress is responsible for yield losses and yield differences.

In 2010 in Fairhope, mid-March planted corn yielded 893 kg ha^{-1} more than mid-April planted corn. In 2010, PD1 (mid-March) corn received 80.5 mm more cumulative rainfall for the time period starting two weeks before silking and ending at physiological maturity compared to PD2 crop (PD1 = 252.5 mm and PD2 = 175 mm). The SDI index indicated that rainfall was more evenly distributed during the late vegetative stage to physiological maturity for mid-March planted corn than for the mid-April planted corn. Those observations, along with the 0.8 ^{o}C lower average maximum temperatures for the time period from late vegetative stage to the end of the reproductive stage observed for mid-March (32.1 ^{o}C) versus mid-April (32.9 ^{o}C) planted corn, may explain the higher yields attained by the PD1 planted crop. This is in accordance with several studies that showed heat and moisture stresses around flowering were associated with corn grain losses (Denmead and Shaw, 1960; Eyshi Rezaei et al., 2015).

The lowest yields obtained in this study with seven environments (year x location) were observed for PD1, followed by PD2 at Fairhope 2011. For both planting dates, the average maximum temperatures remained above 32 ^{o}C following silking with the exception of the latest grain filling period (defined as time III and IV herein) for PD2. A temperature drop late in the season, followed by a series of well-distributed precipitation events, coincided with late grain filling for PD2 plants. For example, the SDI index during the late grain filling period (time windows III and IV) for mid-March and mid-

April planted corn was equal to 0.360, and 0.693, respectively, whereas the AWDR for the same period was 5.3 (PD1) and 103 (PD2). This may have alleviated some of the earlier mid-April planted corn stress, thus, resulting in low, but still significantly (p – value < 0.05) higher yield compared to earlier planted corn.

For Fairhope 2012, average maximum temperatures (PD1 = 30.3 ^{o}C and PD2 = 30.8 ^{o}C) and cumulative precipitation (PD1 = 396.2 and PD2 = 393.2 mm) during the time window from late vegetative stage to the end of grain filling alone cannot explain the improved yield observed for PD2 compared to PD1. However, rainfall for the overall time window discussed here (from –I to IV) was more abundant-well distributed and more evenly distributed for PD2 than for PD1 as indicated by AWDR and SDI indices, respectively, which corresponds with the yield differences observed for corn planted on the two different days.

In 2013 in Fairhope, cumulative rainfall received from silking to physiological maturity was approximately the same for both planting dates (PD1 = 540 mm and PD2 = 547 mm). However, mid-April planted corn received 94.5 mm of more rain than mid-March planted corn during the 2-week time period before silking. For the first 2-week period considered in this study, SDI index indicated that the rainfall events were more evenly distributed for mid-April than for mid-March planted corn (SDI for PD2 = 0.675; SDI for PD1 = 0.129). Potential moisture stress that occurred at the late vegetative stage for mid-March planted corn, may be reflected by the higher Δ values (p – value = 0.0726) for PD1 corn grains harvested at milk stage compared to observed Δ values for PD2 corn (Table 3. 5). Despite that, moisture alone cannot explain the yield differences for this season since yield was significantly higher for mid-March than mid-April planted corn.

Average maximum and minimum temperature experienced from mid-April corn during the time between the two weeks before silking and the physiological maturity were 1.2 ^{o}C and 1.0 ^{o} C higher than for mid-March corn. Temperatures above 32 ^{o}C reduces pollen germination, interferes with tasseling and silking synchronization which results in poor pollination, and increases kernel abortion (Sánchez et al., 2014). As a result, high temperature stress during and around anthesis is expected to reduce yield, which is in accordance with the differences in attainable yield between PD1 and PD2 planted corn observed in this study. Additionally, during the IV time window the average temperature for PD2 corn was 34.2^{o}C, 2.8 ^{o}C above the temperature PD1 corn experienced when at late grain filling. Higher temperature stress at the end of the late reproductive stage could impact on grain weight accumulation. Therefore, yield for the mid-April planted corn should be affected more (further reduced) at the end of the season, as well, when compared to mid-March planted corn yield. Generally, when corn is grown under higher temperatures regimes, growth and development is hastened. As a result, corn growing period is shortened and yield penalties occur. In this study, PD1 corn reached physiological maturity 52 day after silking, while PD2 corn had a grain filling window of 45 days. In 2013 in Fairhope higher temperatures experienced by mid-April planted corn during the critical late vegetative stage and most of the grain fill timespan compared to temperatures encountered by mid-March planted corn maybe related to the lower yield attained by PD2 corn compared to PD1 corn.

In the last (2014) season in Fairhope, overall rainfall for PD1 corn during the 2-week interval before silking and the grain maturation was 112 mm higher than the precipitation received by PD2 corn (PD1 = 357 mm and PD2 = 469 mm). Reduced rainfall combined

with hotter weather conditions, as illustrated by the higher average maximum temperature (31.9 vs 32.6 ^{o}C for PD1 and PD2 planted corn, respectively) 2-weeks before and during grain filling for mid-April planted corn, may partially explain yield differences in 2014. Higher Δ values for samples collected three weeks after silk (around milk stage), indicated that PD2 corn was significantly ($p - value < 0.05$) more stressed than PD1 plants. This agrees with the lower yields observed for the later planted corn. For the two weeks around silking, rainfall was more abundant and well distributed for mid-March corn compared to mid-April corn. Water stress around these stages can account for significant yield losses (Çakir, 2004; Denmead and Shaw, 1960; Shaw and Newman, 1991). However, at the end of the season, PD1 corn was more stressed as indicated by higher Δ values compared to corn planted on mid-April. Unusually severe rust infection was observed in 2014 in Fairhope approximately 10 and 21 days before PD1 and PD2 corn reached physiological maturity, respectively. Late planted corn was more severely infected than PD1 (field observations), and the disease was severe earlier in its growth stage compared to the earlier planting crop (the mid-April planted crop was in the late dough to early dent stage). Severe rust disease during grain fill may reduce yield by diverging assimilates from the crop to the fungus (Wise, N/A). Additionally, epidermal leaf tissue could be ruptured by rust pustules and this may interfere with stomata water loss regulation, and thus affecting vapor and CO_2 diffusion in and out of the leaf. Obviously, if a heavily infected C4 plant could not close stomata efficiently as response to water stress, Δ values should be decreased, compared to a healthy or less infected plant (Clay et al., 2005). This reasoning may explain the discrepancy observed between Δ

analysis for the second in-season sampling time and yield performances in the Fairhope 2014 season.

Observed Δ values in Prattville in 2013 for both grain harvests indicated the late planted corn was less moisture stressed compared to corn planted in mid-March. This is in accordance with the significant ($p - value < 0.05$) yield increase, and the higher cumulative precipitation received by PD2 corn (PD1 = 284 mm and PD2 = 330 mm) at the late vegetative stage and at grain filling time.

In 2014 in Prattville mid-March planted corn yielded 536 kg ha^{-1} more than mid-April planted corn; however the difference was not significant at $\alpha = 0.05$. Δ values for both grain harvests were higher for mid-March planted corn than for mid-April planted corn ($p - value < 0.05$). This indicates that PD1 corn was exposed to higher environmental stresses compared to PD2 corn for the time interval considered herein. Cumulative rainfall received from late vegetative stage to physiological maturity for corn planted on the two different planting dates, as well as, rainfall distribution as indicated by SDI and AWDR indices could explain the differences observed in yield but not in Δ values. However, PD2 corn received more abundant and well distributed rainfall for the time windows –I, I, and II than PD1. Weather summaries indicate that PD1 corn was more stressed than PD2 from late vegetative stage to mid grain fill, which agrees with Δ values observed in corn grains collected at HV1. During the time of late grain fill (III and IV) PD1 corn received 107 mm of rainfall while PD2 corn received only 30 mm. Hot weather at the end of the growing season exposed PD2 corn to extremely higher temperatures than PD1 corn. From dent to corn physiological maturity, severe stress can interfere with grain mass accumulation and can be responsible for the yield differences between PD1 and

PD2 corn (Nielsen, 2016). However, these late season stresses as indicated by the weather summaries in time windows III and IV were not reflected in Δ values for grains collected at harvest maturity. Δ variation is driven by complex interactions between the intracellular to atmospheric CO_2 partial pressure ratio (C_i/C_a) or the φ ratio (Monneveux et al., 2007). The duration and the intensity of the stress determines the degree to which changes in Ci/Ca and φ are reflected in Δ (Monneveux et al., 2007). PD2 corn had a shorter grain fill time window compared to PD1 corn, and maybe early in-season stresses are more readily reflected on Δ observed in corn grains at harvest maturity. This may explain why the correlation between yield and Δ was non-significant in Prattville in 2014.

When seasons were wetter than the historic average both in Fairhope (2012, 2013, and 2014) and in Prattville (2014), the highest attained corn yield (numerical difference) was observed for the highest (D4) density studied. In seasons drier than the historic average lower corn plant densities out yield the higher plant density. However, the yield differences between different planting densities tested were not always significantly different (e.g.; Fairhope, 2010). Planting density is one agronomic practices that can significantly influence corn yield (Sangoi, 2001). Plant density can alter plant architecture, can change corn growth and development, influence carbohydrates synthesis, and partition of assimilates among plant organs. Optimum corn population density to maximize the utilization of available resources with the goal to achieve maximum attainable yields vary among agronomic systems and environments. One of the main factors than highly suppress corn productivity is soil available water (Mask and Mitchell, 1988; Sangoi, 2001).

When plant density increases above the optimum, grain yield is expected to decline because interplant competition for limited resources such as soil nutrients, and soil moisture is more severe at high rather than low plant stands (Tollenaar et al., 1997). Our data for wetter than historic average seasons indicate that optimum plant density was approached at D4 level, since attained yield per unit area increased as plant population density increased. However, yields at D3 and D4 were not significantly different. Among others, if extreme soil moisture stresses around silking and the early grain filling did not occur, then detrimental effect on attained yield due to increased plant densities should not be observed (Westgate, 1994).

The influence of plant densities on Δ values was significant in Fairhope in 2013 for corn grain harvested at milk (R3) stage, and in 2014 for the second harvest (grains harvested at harvest maturity). In 2014 in Fairhope, the effect of density on Δ for grain samples collected at harvest maturity barely miss significance at $\alpha = 0.10$ (p-value = 0.0999). In Fairhope, there was a trend for Δ to increase as plant density decreased; a trend opposite to the effect of plant density on yield. The same trend was observed by Clay et al. (2009) who showed that corn planted at 149,000 plants ha^{-1} under irrigated and non-irrigated regimes yielded more and had lower Δ value than the lower plant density (74,500 plants ha^{-1}) considered at that study. The increase in yield per unit area as corn density increase s can be attributed to increased resource use efficiency (e.g.; soil moisture uptake) (Clay et al., 2009). Under crowded conditions, the reflected light from the plant canopy has a lower red/ near infrared ration (NIR). As a result, phytochrome formation in its inactive red form (Pr) is favored over the active infrared form (Pfr). This triggers the shade avoidance response with plants developing narrower and longer leaves,

taller stems, delayed or incomplete grain fill, and ultimately reduced yield. This was not measured. However, modern corn hybrids, due to breeding and selection can produce high yields under relatively high plant densities. Despite that, downregulation of C-metabolism enzymes such as, phosphoenolpyruvate carboxylase, had been shown by Clay et al. (2009) for high density corn. This observation was suggested to be an adaptive compensation for a lower red/NIR ratio of light for corn plants grown under dense compared with lower densities, which could result in unchanged or even lower Δ values for the higher densities than the lower density treatments.

Conclusions

In this study we explored the use of Δ as a tool to explain attained yield differences for dryland corn cultivated at different planting dates and plant densities. Δ discrimination in corn grain was significantly influenced by plant density only for samples harvested at milk (R3) and harvest maturity in Fairhope in 2013 and 2014, respectively. In Fairhope, higher plant densities tended to have lower or unchanged Δ values in grain and higher yields in wet years than lower densities. Planting dates had a significant impact on corn yield in all seasons except Prattville in 2014, but no constant trend was observed reflecting the influence of seasonal weather events on yield. Normal planting date in Alabama may favor higher yields than a month later planting, but does not guarantee higher yields every year. Δ values of grain samples collected were significantly influenced by planting date at both harvests in Prattville in 2013 and in Fairhope in 2013 for grain harvested at physiological maturity only and in 2014 at both harvests.

The relationship between yield and Δ in corn grains harvested at milk (R3) and at harvest maturity was not consistent between years x locations and within year x location. In Prattville in 2013, negative relationship between Δ and corn yield was observed for grain samples harvested on both milk (R3) and harvest maturity. A significant negative correlation between yield and Δ was observed in Fairhope in 2014 for grain sampled at milk stage but not for the second harvest at the end of the season. In years wetter than the historic average (Fairhope 2013, Fairhope 2014, and Prattville 2014) the relationship between yield and Δ does not hold or the information related to early in-season environmental stresses could be masked as aging effect may play a role. For the drier

than the historic average year (Prattville, 2013), the relationship between yield and Δ in corn grain hold on both harvest times, indicating that Δ method might be a potential tool in dry years to assess yield differences. However, before any concrete conclusion can be made about the utility of Δ as a tool to assess yield differences due to planting date and plant density further studies are needed.

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Table 3. 1: Planting, growth stages, and harvest days for planting date treatments per location and growth season.

Location	Year	Treatment[a]	Planting date	Silking[b]		Milk[c]		Dent[d]		Physiological maturity[e]		1st Harvest[f] (HV1)		Week[g]	2nd Harvest[h] (HV2)	
Fairhope	2010	PD1	19-Mar	29-May	(71)					11-Jul	(43)				N/A	
		PD2	15-Apr	10-Jun	(56)					22-Jul	(42)				N/A	
	2011	PD1	17-Mar	29-May	(73)					11-Jul	(43)				N/A	
		PD2	12-Apr	13-Jun	(62)					26-Jul	(43)				N/A	
	2012	PD1	20-Mar	25-May	(66)	15-Jun	(21)	29-Jun	(35)	20-Jul	(56)				7-Aug	(74)
		PD2	13-Apr	15-Jun	(63)	29-Jun	(14)	20-Jul	(35)	2-Aug	(48)				25-Aug	(71)
	2013	PD1	14-Mar	1-Jun	(79)	27-Jun	(26)	9-Jul	(38)	23-Jul	(52)	27-Jun	(26)	(4)	13-Aug	(73)
		PD2	18-Apr	21-Jun	(64)	9-Jul	(18)	23-Jul	(32)	5-Aug	(45)	9-Jul	(18)	(3)	24-Aug	(64)
	2014	PD1	21-Mar	7-Jun	(78)	21-Jun	(14)	11-Jul	(34)	28-Jul	(51)	26-Jun	(19)	(3)	11-Aug	(65)
		PD2	14-Apr	19-Jun	(66)	11-Jul	(22)	16-Jul	(27)	6-Aug	(48)	16-Jul	(27)	(4)	11-Aug	(53)
Prattville	2013	PD1	15-Mar	9-Jun	(86)	28-Jun	(19)	26-Jul	(47)	29-Jul	(50)	28-Jun	(19)	(3)	22-Aug	(74)
		PD2	16-Apr	21-Jun	(66)	12-Jul	(21)	26-Jul	(35)	7-Aug	(47)	12-Jul	(21)	(3)	3-Sep	(74)
	2014	PD1	22-Mar	8-Jun	(78)	22-Jun	(14)	2-Aug	(55)	29-Jul	(51)	30-Jun	(22)	(4)	15-Aug	(68)
		PD2	21-Apr	22-Jun	(62)	11-Jul	(19)	5-Aug	(44)	8-Aug	(47)	17-Jul	(25)	(4)	4-Sep	(74)

[a] PD1 and PD2 are normal (mid-March) and late (mid-April) planting dates for South and Central Alabama.
[b] Numbers in parenthesis are days from planting date to silking.
[c] Numbers in parenthesis are days from silking to milk stage.
[d] Number in parenthesis are days when plant was from early up to late dent stage depending on season and location.
[e] Numbers in parenthesis are days from silking up to physiological maturity.
[f] Numbers in parenthesis are days from silking up to 1st hand harvest.
[g] Week after silking when 1st harvest corn samples were collected.
[h] Numbers in parenthesis are days from silking to 2nd hand harvest

Table 3. 2: Summaries of maximum temperature, minimum temperature, cumulative rainfall, Shannon diversity index (SDI), and abundant and well distributed rainfall index (AWDR) for the time period two weeks before silking to corn physiological maturity for Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014).

			Mean maximum Temperature (°C)		**Mean minimum Temperature (°C)**		**Cumulative Rainfall (mm)**		**SDI**		**AWDR**	
Location	**Year**	**Time[a]**	**PD1[b]**	**PD2[c]**	**PD1**	**PD2**	**PD1**	**PD2**	**PD1**	**PD2**	**PD1**	**PD2**
Fairhope, AL	2010	-I	30.5	31.0	21.0	22.1	93.5	98.0	0.473	0.704	44.2	69.1
		I	31.0	33.5	22.3	24.1	98.0	29.5	0.704	0.453	69.1	13.4
		II	33.7	32.0	24.1	23.8	29.5	31.5	0.453	0.530	13.4	16.7
		III	31.9	33.1	23.8	25.0	31.5	16.0	0.530	0.429	16.7	6.9
		IV	33.3	35.0	25.0	22.8	0.0	0.0	.	.	.	.
		Total	32.5	33.4	23.8	23.9	159.0	77.0	0.562	0.471	99.1	36.9
	2011	-I	28.1	33.4	15.9	21.5	0.5	45.0	0.000	0.254	0.0	11.4
		I	33.3	32.3	21.0	24.5	45.0	40.9	0.254	0.249	11.4	10.2
		II	32.4	33.0	24.3	23.6	40.9	14.7	0.249	0.360	10.2	5.3
		III	32.9	31.4	23.3	23.2	14.7	147.1	0.360	0.664	5.3	97.7
		IV	32.9	30.6	26.7	23.3	0.0	7.6	.	0.722	.	5.5
		Total	32.9	31.8	23.8	23.7	100.6	210.3	0.288	0.499	26.9	118.7
	2012	-I	29.0	29.0	17.4	19.8	6.1	224.5	0.194	0.616	1.2	138.3
		I	31.3	31.0	20.1	19.9	84.3	1.3	0.453	0.255	38.2	0.3
		II	28.9	31.0	19.0	21.4	184.2	38.4	0.509	0.401	93.7	15.4
		III	32.1	31.2	21.6	23.1	18.8	106.5	0.150	0.549	2.8	58.4
		IV	30.2	31.8	21.6	24.3	102.8	22.5	0.498	0.110	51.2	2.5
		Total	30.6	31.2	20.6	22.2	390.1	168.6	0.402	0.329	185.9	76.6
	2013	-I	29.3	30.7	18.5	22.3	9.1	103.6	0.1	0.7	1.2	70.0
		I	30.1	32.1	21.4	22.6	103.1	94.0	0.563	0.555	58.0	52.2
		II	31.7	30.5	22.9	21.9	87.1	295.4	0.561	0.512	48.9	151.4
		III	30.8	31.9	22.0	22.6	294.4	154.2	0.509	0.728	149.7	112.2
		IV	31.4	34.2	22.0	22.7	54.9	3.0	0.699	0.490	38.3	1.5
		Total	31.0	32.2	22.1	22.5	539.5	546.6	0.583	0.571	295.0	317.2

Location	Year	Time[a]	Mean maximum Temperature (°C)		Mean minimum Temperature (°C)		Cumulative Rainfall (mm)		SDI		AWDR	
			PD1[b]	PD2[c]	PD1	PD2	PD1	PD2	PD1	PD2	PD1	PD2
(Continues)	2014	-I	29.8	31.8	21.3	22.2	133.4	116.3	0.644	0.391	85.9	45.5
		I	31.9	32.8	22.3	23.2	125.5	73.9	0.462	0.640	57.9	47.3
		II	32.7	32.6	23.0	22.0	51.3	46.5	0.474	0.576	24.3	26.8
		III	32.3	32.4	21.7	22.4	79.5	112.8	0.594	0.415	47.2	46.8
		IV	32.9	33.5	23.5	21.5	79.5	7.4	0.293	0.472	23.3	3.5
		Total	32.4	32.8	22.6	22.3	335.8	240.5	0.455	0.526	152.7	124.4
Prattville, AL	2013	-I	32.2	32.5	20.3	21.6	4.8	0.0	0.441	.	2.1	.
		I	32.6	32.6	21.8	21.6	0.0	72.6	.	0.617	.	44.8
		II	31.6	30.2	21.7	22.4	110.2	102.4	0.699	0.587	77.1	60.1
		III	31.5	32.2	22.4	22.3	69.9	115.3	0.522	0.617	36.4	71.1
		IV	31.4	33.8	22.1	22.1	99.3	39.9	0.555	0.000	55.1	0.0
		Total	31.8	32.2	22.0	22.1	279.4	330.2	0.592	0.455	168.6	176.0
	2014	-I	31.7	31.0	20.6	22.1	62.5	98.0	0.551	0.704	34.4	69.1
		I	33.2	33.0	21.1	21.1	29.2	60.7	0.379	0.420	11.1	25.5
		II	33.0	32.9	21.1	21.5	60.7	80.3	0.420	0.409	25.5	32.8
		III	32.9	32.6	21.5	21.3	80.3	26.7	0.409	0.083	32.8	2.2
		IV	33.0	34.8	22.2	22.0	26.7	3.3	0.095	0.372	2.5	1.2
		Total	33.0	33.3	21.5	21.5	196.9	170.9	0.326	0.321	71.9	61.8

[a] - I = 2-week time period before silking, I = 2-week time period after silking, II = second 2-week time period after silking, III = third 2-week time period after silking, IV = variable in length time period starting after the III window and extending to physiological maturity.
[b] PD1 = mid-March planting (normal planting time)
[c] PD2 = mid-April planting (late planting time)

Table 3. 3: Type III test of fixed effects for corn yield when data were pooled for season 2010 – 2014 (1st analysis), and when data were pooled over 2011 – 2014 (2nd analysis).

	Yield	
	1st analysis	2nd analysis
Effect	**Pr > F[b]**	
Year	<0.0001	<0.0001
Location	<0.0001	<0.0001
Year x Location	0.3598	0.4583
Planting dates	0.3681	0.0938
Year x Planting dates	<0.0001	<0.0001
Location Planting dates	<0.0001	<0.0001
Year x Location x Planting dates	0.0336	0.0154
Densities	<0.0001	<0.0001
Year x Densities	<0.0001	<0.0001
Location x Densities	0.1125	0.0248
Year x Location x Densities	0.3297	0.4719
Planting_dates x Densities	0.6472	0.5649
Year x Planting dates x Densities	0.4223	0.3366
Location x Planting dates x Densities	0.5772	0.3155
Year x Location x Planting dates x Densities	0.7908	0.5378

[b]Effects are significant at α =0.0

Table 3. 4: Treatment effects on corn yield in Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014).

	Fairhope, AL								**Prattville, AL**			
	Year								**Year**			
	2010		**2011**		**2012**		**2013**		**2014**		**2013**		**2014**	
Effect[b]	**Yield Least Squares Mean Estimates (kg ha^{-1})**[a]													
Pr>F														
PD	0.0001		0.0016		0.0217		0.0051		<0.0001		0.0235		0.2325	
D	0.5484		0.0240		0.0003		0.0002		<0.0001		0.0008		<.0001	
PD x D	0.3845		0.8404		0.8345		0.2142		0.9297		0.2561		0.7712	
PD														
PD1	7889	a	3096	b	7962	b	8564	a	8380	a	7846	b	8850	
PD2	6996	b	4603	a	8538	a	6913	b	6426	b	9368	a	8314	
D														
D1	7592		4013	ab	7573	b	6949	b	6294	b	7992	b	8205	b
D2	7319		4089	a	8206	ab	7710	a	6953	b	8857	a	8077	b
D3	7417		3793	ab	8544	a	8022	a	7982	a	8763	a	8998	a
D4			3502	b	8677	a	8274	a	8382	a	8814	a	9050	a
PD x D														
PD1 x D1	7921		3232		7303		7500		7288		7342		8575	
PD1 x D2	7681		3362		8032		8569		7937		8027		8319	
PD1 x D3	8065		3126		8209		8783		8845		8184		9146	
PD1 x D4			2664		8305		9406		9448		7830		9361	
PD2 x D1	7263		4794		7843		6399		5300		8641		7835	
PD2 x D2	6956		4817		8380		6850		5969		9688		7834	
PD2 x D3	6769		4460		8880		7262		7119		9343		8849	
PD2 x D4			4340		9050		7142		7315		9798		8739	

[a] Least squares means in the same column followed by different letter are significantly different at Pr ≤ 0.05 (Tukey test).

[b] PD = Planting date, D = Plant density. PD1 and PD2 represent normal (mid-March) and late (mid-April) planting dates. D1, D2, D3, and D4 correspond to plant densities of 44,480, 54,360, 64,250, and 74130 plants ha^{-1}.

Table 3. 5: Treatment effects on plant stress as indicated by 13C discrimination (Δ) analysis on grain samples at milking and physiological maturity for Fairhope, AL (2013 – 2014) and Prattville, AL (2013 – 2014).

	Fairhope, AL				**Prattville, AL**			
	Year				**Year**			
	2013		**2014**		**2013**		**2014**	
Effect[b]	**HV1**[c]	**HV2**[d]	**HV1**	**Hv2**	**HV1**	**HV2**	**HV1**	**HV2**
	^{13}C discrimination (Δ) index (‰)[a]							
Pr>F								
PD	0.0726	0.1946	0.0105	0.1636	0.2983	0.0136	0.0004	0.0477
D	0.0027	0.0999	0.5865	0.041	0.7042	0.2309	0.1989	0.4247
PD x D	0.3762	0.0309	0.3286	0.2005	0.2200	0.3057	0.7972	0.3033
PD								
PD1	3.44	3.45	3.11 b	3.44	4.08	4.06 a	3.88 a	4.17 a
PD2	3.35	3.39	3.23 a	3.40	3.63	3.67 b	3.62 b	3.83 b
D								
D1	3.45 a	3.49	3.21	3.48 a	3.89	3.84	3.65	4.10
D2	3.44 a	3.42	3.14	3.44 ab	3.79	3.87	3.74	3.97
D3	3.31 b	3.40	3.19	3.41 ab	3.83	3.94	3.76	3.93
D4	3.37 ab	3.38	3.15	3.35 b	3.90	3.83	3.83	3.99
PD x D								
PD1 x D1	3.52	3.53 a	3.18	3.47	4.04	4.06	3.82	4.38
PD1 x D2	3.50	3.44 ab	3.13	3.50	3.95	4.03	3.84	4.07
PD1 x D3	3.34	3.42 ab	3.09	3.40	4.19	4.18	3.90	4.11
PD1 x D4	3.39	3.49 a	3.05	3.39	4.13	3.98	3.95	4.11
PD2 x D1	3.38	3.42 ab	3.25	3.48	3.74	3.62	3.48	3.82
PD2 x D2	3.38	3.38 ab	3.15	3.38	3.64	3.70	3.65	3.88
PD2 x D3	3.29	3.42 ab	3.29	3.42	3.47	3.69	3.62	3.76
PD2 x D4	3.34	3.27 b	3.24	3.31	3.67	3.68	3.72	3.87

[a] Least squares means in the same column followed by different letter are significantly different at Pr ≤ 0.05 (Tukey test).

[b] PD = Planting date, D = Plant density. PD1 and PD2 represent normal (mid-March) and late (mid-April) planting dates. D1, D2, D3, and D4 correspond to plant densities of 44,480, 54,360, 64,250, and 74,130 plants ha^{-1}, correspondingly.

[c] HV1 = First harvest. Corn ears harvested 18 – 27 days after silking (approximately at milk stage (R3)).
[d] HV2 = Second harvest. Corn ears samples harvested after physiological maturity (harvest day at the end of the growing season).

Table 3. 6: Spearman correlations between yield and ^{13}C discrimination (Δ) index of the corn grain samples

	Yield vs. ^{13}C discrimination (Δ) index							
	Fairhope				**Prattville**			
	2013		**2014**		**2013**		**2014**	
Harvest	**r[a]**	**p-value**	**r**	**p-value**	**r**	**p-value**	**r**	**p-value**
HV1[a]	0.3095	0.4556	-0.7619	0.0280	-0.9286	0.0031	0.3333	0.4198
HV2[b]	0.3571	0.3851	0.0476	0.9108	-0.7130	0.0465	0.0476	0.9108

[a]HV1 corresponds to grain samples collected around milk (R3) stage.
[b]HV2 corresponds to grain samples collected at corn harvest maturity.
Spearman correlation coefficients and p-values between corn yield and Δ values for corn grain samples collected at milk stage and harvest maturity in Fairhope, AL and Prattville, AL for 2013 and 2014. Yield and Δ have been averaged by Year x Location x Planting dates x Densities.

Table 3. 7: Spearman correlation coefficients between average soil moisture depletion for the top 30 cm of the soil profile and averaged 13C discrimination (Δ) index of the corn grain samples.

		Average soil moisture depletion (%) vs. Average ^{13}C discrimination (Δ) index								
		1st scenario[f]			**2nd Scenario[g]**			**3nd Scenario[h]**		
Harvest	**Time[c]**	**r[d]**	**p-value**	**n[e]**	**r**	**p-value**	**n**	**r**	**p-value**	**n**
HV1[a]	Week-2	0.7698	<0.0001	32	0.6675	<0.0001	32	0.6766	<0.0001	31
	Week-1	0.3296	0.0655	32	0.4472	0.0117	31	0.3712	0.0566	27
	Week1	0.5059	0.0031	32	0.4103	0.0270	29	0.2021	0.3121	27
	Week2	0.5532	0.0010	32	0.4682	0.0079	31	0.5250	0.0049	27
HV2[b]	Week-2	0.5763	0.0006	32	0.5337	0.0017	32	0.5161	0.0030	31
	Week -1	0.1617	0.3767	32	0.2641	0.1511	31	0.2827	0.1531	27
	Week 1	0.4131	0.0188	32	0.3374	0.0734	29	0.1771	0.3770	27
	Week 2	0.5513	0.0011	32	0.4774	0.0066	31	0.4347	0.0235	27
	Week 3	0.3915	0.0267	32	0.5084	0.0049	29	0.5800	0.0037	23
	Week 4	0.4740	0.0061	32	0.6271	0.0003	29	0.5148	0.0101	24
	Week 5	0.1976	0.2784	32	0.2434	0.2034	29	0.4947	0.0266	20
	Week 6	0.6617	<.0001	32	0.5470	0.0057	24	-0.0677	0.8034	16
	Week 7	0.2746	0.1283	32	0.4446	0.0260	25	0.1297	0.6586	14
	Week 8	0.4471	0.0825	16	0.6783	0.0153	12	0.4000	0.6000	4

[a]HV1 = 1st harvest; corresponds to grain samples collected around milk (R3) stage.

[b]HV2 = 2nd harvest; corresponds to grain samples collected at corn harvest maturity.

[c] Weekly time intervals starting two weeks before silking (R1) (Week- 2) and extending to corn physiological maturity (R6). Weeks marked with negative numbers are weeks before silking; weeks marked with positive numbers are weeks after silking. Week8 represent a variable length time window (range = 0 – 3 days) starting at the end of the 7th week after silking (R1) end ending on corn physiological maturity stage (R6). Daily % depletion soil moisture data were averaged for each of the weekly and the variable length (Week8) windows (where applicable). ^{13}C discrimination (Δ) index was averaged for three replicates representing environments defined as Location x Year x Planting date x Density treatments.

[d] Pearson correlation coefficients

[e] number of observations

[f] Three different scenarios were evaluated. In the 1st scenario ($\theta_{\text{Mean depletion}}$)weekly % soil moisture depletion was derived as the average of all the available daily % soil moisture depletion values; In the 2nd ($\theta_{\text{depletion > 10\%}}$) and 3nd ($\theta_{\text{depletion > 30\%}}$) scenarios weekly % soil moisture depletions were derived by excluding daily % depletion soil moisture data if soil moisture depletion in the top 30cm of the soil profile was less than 10% and 30%, respectively.

Table 3. 8: Average maximum temperature, average minimum temperature, and cumulative rainfall per month for Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014) growing seasons and historic average.

	Fairhope, AL						Prattville, AL		
	2010	**2011**	**2012**	**2013**	**2014**	**Historic average[a]**	**2013**	**2014**	**Historic average[b]**
Mean Monthly Maximum Air Temperature (^oC)									
March	18.6	22.2	24.2	18.9	19.2	21.3	18.3	19.4	21.0
April	25.8	25.7	24.7	24.3	23.9	25.0	25.2	25.1	25.0
May	29.7	28.3	29.4	26.4	28.6	28.8	28.4	29.7	28.8
June	32.5	32.8	30.1	31.0	31.6	31.4	32.9	32.8	32.3
July	33.0	31.8	31.2	31.2	32.7	32.1	30.9	32.9	33.4
August	33.0	33.7	30.9	31.5	32.8	32.1	31.4	33.9	33.3
Overall Average	*28.8*	*29.1*	*28.4*	*27.2*	*28.1*	*28.5*	*27.8*	*29.0*	*29.0*
Mean Monthly Minimum Air Temperature (^oC)									
March	7.5	10.1	13.9	5.7	7.2	9.5	4.5	6.0	7.7
April	13.2	14.7	13.8	12.3	13.4	13.2	12.7	12.3	11.3
May	20.6	15.6	17.9	15.6	17.5	17.3	15.3	16.2	16.0
June	23.5	23.1	20.1	22.2	22.3	21.0	21.5	21.3	20.2
July	24.7	23.5	22.5	22.1	22.2	22.4	22.1	21.1	22.1
August	25.2	23.0	19.3	22.2	22.5	22.1	21.6	21.7	21.8
Overall Average	*19.1*	*18.3*	*17.9*	*16.7*	*17.5*	*17.6*	*16.3*	*16.4*	*16.5*
Cumulative Rainfall (mm)									
March	120.9	103.1	56.1	36.3	163.1	144.1	68.6	153.4	154.8
April	47.5	23.1	35.8	103.4	512.1	120.4	115.3	200.2	106.0
May	182.1	20.3	186.2	224.8	227.6	122.9	0.0	122.9	99.8
June	109.5	98.8	254.3	191.5	194.6	160.7	51.8	100.8	99.9
July	47.5	202.4	167.4	445.0	160.3	203.1	237.5	109.2	132.7
August	218.2	44.7	274.3	180.6	6.4	166.7	134.1	90.9	95.8
Overall Sum	725.7	492.5	974.0	1181.5	1263.9	917.8	607.3	777.5	688.9

[a]Historic average for Fairhope, AL from 1950 – 2008
[b]Historic average for Prattville, AL from 1970 – 2014

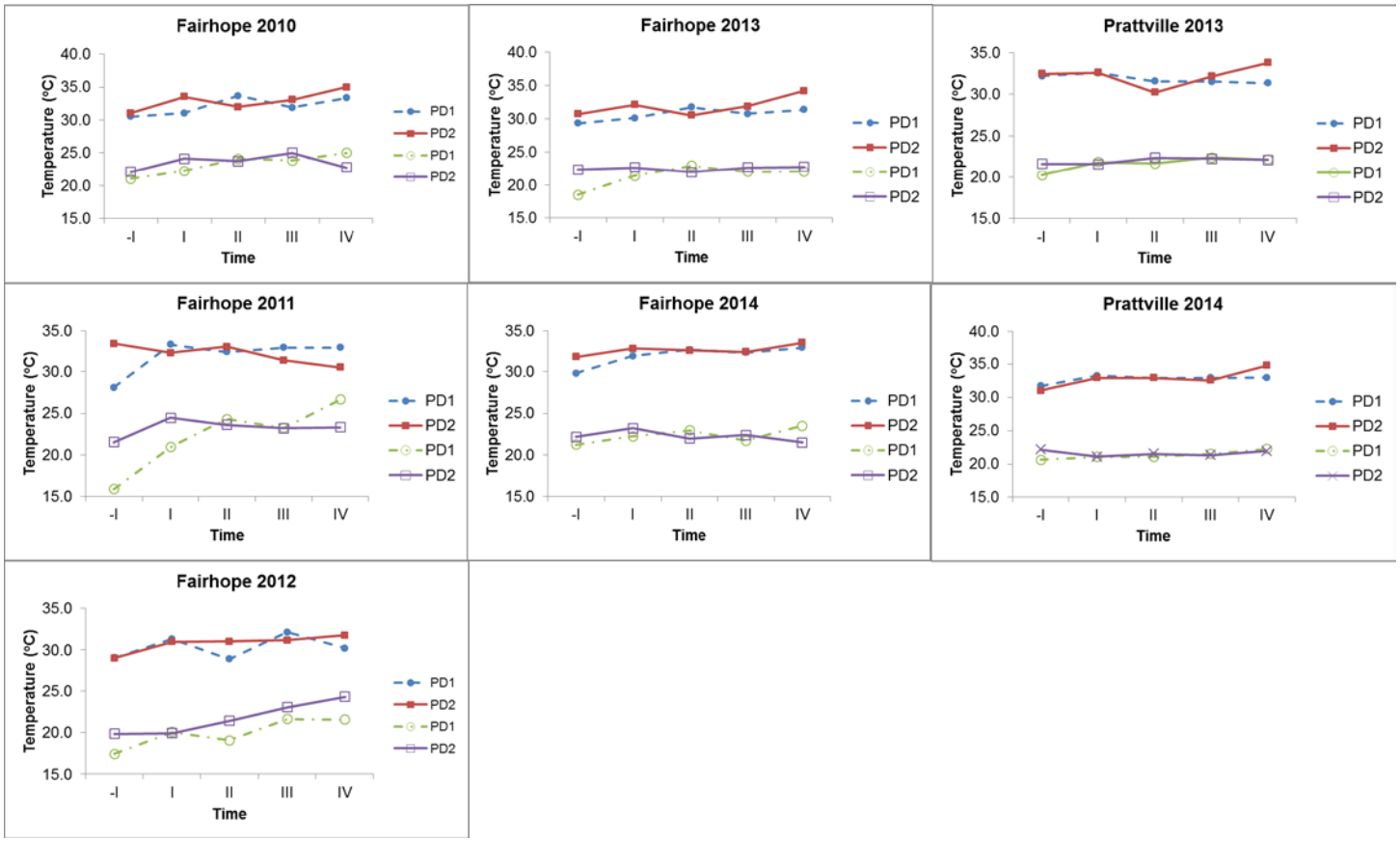

Figure 3. 1: Average minimum (empty markers) and maximum (solid markers) air temperature for mid-March (PD1; dashed lines) and mid-Aprl (PD2; solid lines) planting dates for Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014). Time window presented herein starts two weeks before silking (-I) and extends to corn physiological maturity (end of interval IV).-I = 2-week window before silking, I = 2-week window following silking, II = the second 2-week window after silking, and III = third 2-week window after silking, IV = variable in length window ending at physiological maturity.

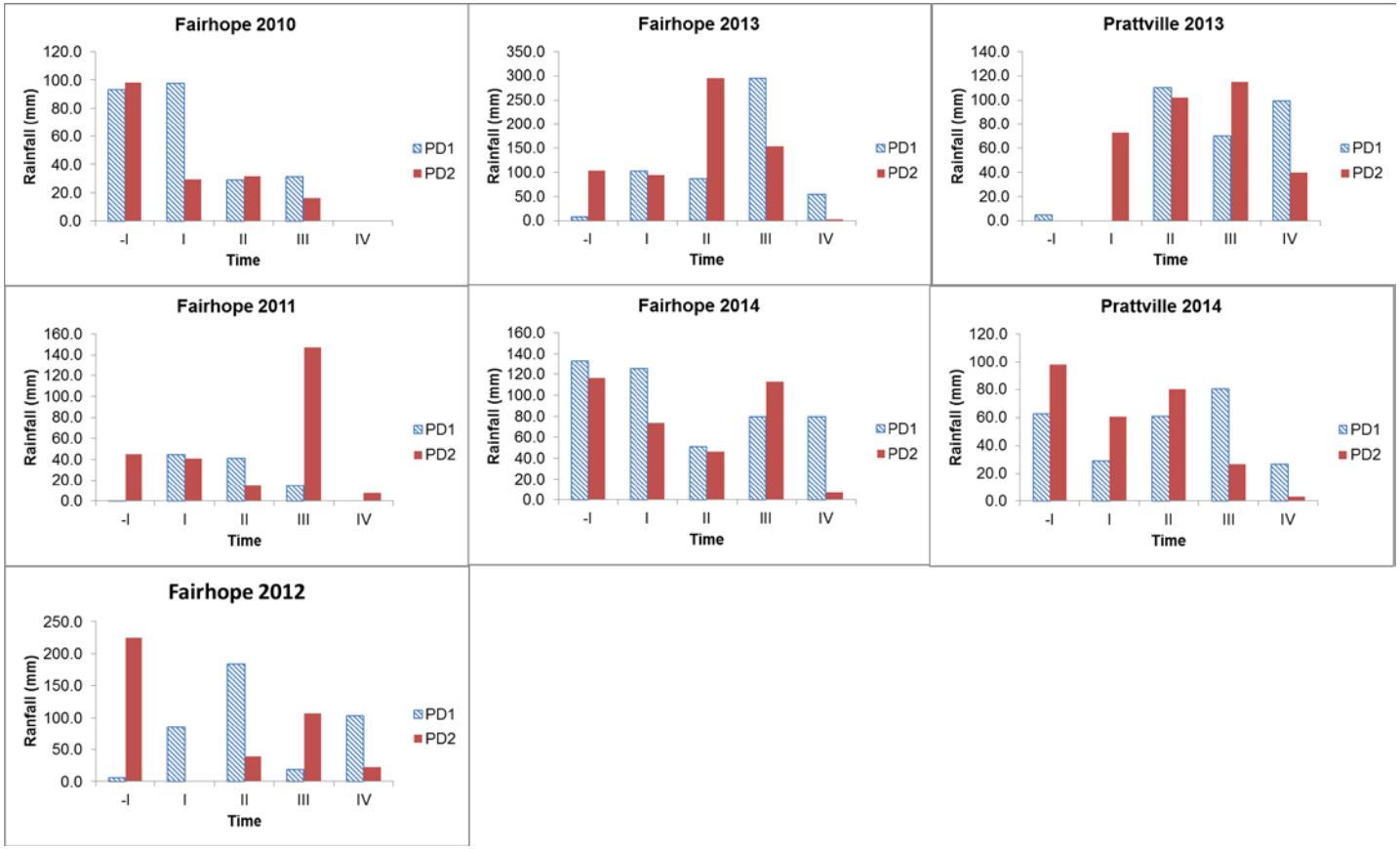

Figure 3. 2: Cumulative biweekly rainfall for mid-March (PD1) and mid-Aprl (PD2) planting dates for Fairhope, AL (2010 – 2014) and Prattville, AL (2013 – 2014). Time window presented herein starts two weeks before silking (-I) and extends to corn physiological maturity (end of interval IV).-I = 2-week window before silking, I = 2-week window following silking, II = the second 2-week window after silking, and III = third 2-week window after silking, IV = variable in length window ending at physiological maturity.

4 SUMMARY

Pre-harvest aflatoxin contamination is of concern due to potential economic losses and health impacts on human and domestic animal using corn and its byproducts in their diet. Aflatoxins are secondary metabolites synthesized by *Aspergillus* spp. that belong to a broader group of fungal compounds called mycotoxins. Predicting the risk of aflatoxin contamination is challenging because the phenomenon is influenced by the interaction of biotic and abiotic factors, and it is exacerbated during seasons characterized by higher than normal temperatures, lower than normal rainfalls, and low humidity; all conditions that may result in drought stress for the host plant. If predicting the risk for aflatoxin contamination is possible, then the risks can be minimized.

This dissertation focused on the evaluation of a simple drought index (ARID) as a tool to predict aflatoxin contamination in corn fields, and the evaluation of the effect weather parameters and management practices can have on the phenomenon. Additionally, we explored the use of Δ as a tool to predict corn yield losses due to corn exposure to within season water stresses resulting from changes in planting date and plant density practices. The objectives of the first study were to: 1) determine whether a ARID could be used to predict the risk for aflatoxin contamination in corn; 2) assess in-season, among soil types and among hybrids, risk differences; and 3) explore the applicability of the proposed methodology to predict the risk at regional level when minimum data are available and the uncertainties are greater. The objectives of the second study were to: 1) assess the effect of agronomic practices (planting date and plant density) on preharvest aflatoxin contamination in rainfed corn grown in the Coastal Plains of South and Central

Alabama, 2) identify which weather variables are influencing aflatoxin contamination in corn, 3) determine the relative weight of significant weather variables on contamination in corn planted in South and Central Alabama, and 4) determine time windows during the growing season that weather variables are associated to corn aflatoxin contamination. The objectives for the third study were to: 1) explore if Δ is a suitable tool to explain in-field yield differences in corn resulting from planting date and plant density under the environmental conditions of Coastal Plains in Alabama, and 2) explore if Δ observations from corn grains sampled within season can be used to assess potential yield losses.

Results from the control experiment (Mississippi) of the first study indicated that ARID could be used as a predictive tool for aflatoxin risk assessment. Hybrid susceptibility to infection/contamination, along with soil type contributed significantly to predict aflatoxin occurrence. Additionally, this work identified significant weeks during the growing season when changes in drought had an influence on the likelihood of aflatoxin contamination. This study indicated that the critical timespan for infection and subsequent contamination extend both prior and beyond mid-silk. Time windows, as indicated by the Mississippi study when changes in drought have the greatest influence on aflatoxin risk, included weeks four prior and after mid-silk, among others. Additionally, the highly susceptible hybrid grown in lighter soil showed a higher risk for aflatoxin contamination with changes in drought conditions during critical week widows when compared to the moderately susceptible hybrids grown in the heavier soil. The proposed methodology was extended from field (plot) level to a regional scale (Georgia study). Both predictive logistic regression models were externally assessed on independent datasets and showed high accuracy in classifying samples as contaminated

above or below the preselected threshold (20 μg/kg). Identifying critical weeks influencing the risk for contamination early in the season may allow farmers, researchers and extension specialists to monitor changes of aflatoxin risk with in-season drought changes, and thus, adjust crop management decisions in an effort to reduce aflatoxin risk. This is true particularly during years characterized by conducive to toxin accumulation conditions. Finally, this work emphasizes the effect drought timing and drought severity has on pre-harvest corn aflatoxin risk alterations during the season and further illuminates the impact drought has on contamination levels under different environments.

Results from the second study indicated that there is an association between aflatoxin contamination and weather conditions. Average minimum temperature and cumulative rainfall changes were found strongly related to aflatoxin contamination. The minimum temperature and rainfall regression models derived could explain from approximately 50 up to 87% of the observed aflatoxin variability. The importance and effect of rainfall and minimum temperature on the phenomenon is changing over the timespan considered. All regression models suggested that the critical time for infection and contamination starts at least two weeks before silking and extends to periods covering parcels of the grain filling window as well.

Additionally, the second study confirmed that under the environmental conditions prevailing in the Coastal Plains of Alabama agronomic practices had influence on corn yield and aflatoxin contamination. Depending on the year, planting earlier in the season (mid-March) resulted in a significant or a relative increase in aflatoxin accumulation compared to levels obtained for corn planted later (mid-April). Planting dates had a significant effect on final yield with the exception of 2014 in Prattville, but the direction

of the effect was highly variable across season, reflecting the variability characterizing local and seasonal weather patterns. Planting densities did not influence aflatoxin accumulation in corn, but, increasing plant population had a positive influence on yield. A significant negative linear relationship was found between aflatoxin and yield for the extremely dry 2011 season in Fairhope, and when data from Fairhope were pooled over the 2010 – 2012, and 2010 – 2014 years, as well.

The results from the third study indicated that Δ discrimination in corn grain was significantly influenced by plant density only for samples harvested at milk (R3) and harvest maturity in Fairhope in 2013 and 2014, respectively. In Fairhope, higher plant densities tended to have lower or unchanged Δ values in grain and higher yields in wet years than lower densities. Planting dates had a significant impact on corn yield in all seasons except Prattville in 2014, but no constant trend was observed reflecting the influence of seasonal weather events on yield. Standard planting date (mid-March) for dryland corn in Alabama may favor higher yields than a month later planting (mid-April), but does not guarantee higher yields every year. Δ values of grain samples collected were significantly influenced by planting date at both harvests in Prattville in 2013 and in Fairhope in 2013 for grain harvested at physiological maturity only and in 2014 at both harvests.

The relationship between yield and Δ in corn grains harvested at milk (R3) and at harvest maturity was not consistent between years x locations and within year x location. In Prattville in 2013, negative relationship between Δ and corn yield was observed for grain samples harvested on both milk (R3) and harvest maturity. A significant negative correlation between yield and Δ was observed in Fairhope in 2014 for grain sampled at

milk stage but not for the second harvest at the end of the season. In years wetter than the historic average (Fairhope 2013, Fairhope 2014, and Prattville 2014), the relationship between yield and Δ does not hold or the information related to early in-season environmental stresses could be masked as aging effect may play a role. For the drier than the historic average year (Prattville, 2013), the relationship between yield and Δ in corn grain hold on both harvest times, indicating that Δ method might be a potential tool in dry years to assess yield differences. However, before any concrete conclusion can be made about the utility of Δ as a tool to assess yield differences due to planting date and plant density further studies are needed.

Results from these studies could be used by farmers, extension agents, researchers and the corn industry to assess aflatoxin risk for corn grains based on a simple predictive system that uses yearly weather data to reflect field drought conditions. In the future, the predictive system developed herein could be incorporated into decision support tools to predict pre-harvest aflatoxin contamination in corn. Alternatively, a more complex predictive system could be developed based on the simple logistic regression model presented in this study. Shading light on weather influence on infection by *A. flavus* and subsequent corn aflatoxin contamination may allow for the development of better management practices to control the problem, target monitoring efforts to critical in season time windows, and efficient allocation of funds to geographic areas which are more prone to contamination risk. The positive correlation between corn grains Δ and weekly soil moisture % depletion indicates that potential corn water stress could be effectively reflected in Δ observed in corn grains within season. Since fluctuation in weather and drought conditions during the season impact the extent of contamination by

influencing both the fungus and the host plant, potentially Δ in grains could be used as a tool to monitor drought stress within season and to assess the effect different water conditions have on yield and aflatoxin contamination. However, more research is needed to evaluate if Δ is correlated to aflatoxin contamination at harvest and to identify the best within season sampling time to early detect this relationship.