AFLATOXIN TOLERANCE IN ELITE INBREDS; MULTIPLE BREEDING PROGRAMS, AND ADVANCED INTERCROSSED MULTI-PARENT POPULATIONS

A Dissertation

by

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ABSTRACT

Aflatoxins contaminating our food supply in preharvest maize (aka corn, *Zea mays* L.) can cause serious illnesses such as: liver damage, intestinal bleeding, and even death; produced naturally by *Aspergillus flavus* Link:Fr, aflatoxins typically contaminate food supplies in countries without appropriate infrastructure.

Maize germplasm has and should continue to be developed and identified that can tolerate or completely resist the accumulation of aflatoxin. Evaluation of elite private and public inbred and hybrid combinations along with evaluating the most critical agronomic applications is essential to help in inhibiting accumulation. Using genetic markers to help increase the efficiency in selecting and pyramiding known QTL and SNP associations into elite germplasm is also an important component in the integrated pest management to decrease aflatoxin. Using all these avenues simultaneously could help in eliminate accumulation if they work synergistically.

This study first created a common garden in which we evaluated released and pre-released public inbred lines, along with important expired Plant Variety Protection (ex-PVP) commercial lines. The best of these lines will be useful in future population development or in direct hybrid combinations. Several synthetic populations were developed using several inbred lines from multiple private and public sources. These populations were used in pyramiding known SNP's into a synthetic population which could then be used to confirm and utilize the know associated SNP. These synthetic populations were also evaluated on how certain germplasm interacts under stress and atoxigenic applications and how this germplasm tolerates aflatoxin accumulation.

Seven existing inbred lines had consistently high yield and low aflatoxin accumulation as desired. Two synthetic populations consisting of four and eight parents were developed, advanced into breeding populations and 1215 lines were selected which are currently being evaluated. Investigation into synergistic interactions between agronomic management x atoxigenic x genotype have concluded that genotypes can have better tolerance if coupled with appropriate management strategies.

This research will help in future breeding population creation along with developing insights into using multiple means to decrease or possibly eradicate aflatoxin accumulation in producers' fields.

DEDICATION

To my wife Audrey for always standing next to me and encouraging me to finish. Also, to my father and mother for raising me to be the man I am today and for Bruce Neeper and the life lesson that I learned while he drank refreshments under the shade while I was doing the manual labor.

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1. INTRODUCTION

Aflatoxins are a harmful carcinogenic mycotoxin produced by *Aspergillus flavus* Link:Fr, which limits the marketability of maize. In areas with little to no infrastructure, these mycotoxins can proceed to make their way into the local food supply. This contaminated food supply can cause liver damage, intestinal bleeding, cancer, and even death. Marketability of the commodity can also be degraded for any producer when contaminated with mycotoxins. Effective measures to combat this issue have been available, however, they have not completely eradicated the problem.

Breeding for aflatoxin tolerance is an important component of the integrated pest management to decrease aflatoxin. Inbred and hybrid development along with genomic breeding can effectively reduce aflatoxin accumulation (Warburton and Williams, 2014). Studies have shown that the use of atoxigenic strains of Aspergillus flavus can lower the contamination of mycotoxins in maize (Abbas, et al., 2006). The use of either of these approaches has lowered the contamination of aflatoxin, but has not eliminated it.

By improving inbreds, the practice of marker assisted selection, and investigation of agronomic x genotypic x environmental x atoxigenic interactions in this research, we hope to use all avenues simultaneously in order to bring aflatoxin under control. In this study we will 1.) Evaluate inbreds for aflatoxin accumulation and agronomics for better control of aflatoxin accumulation. 2.) Develop population for genotyping of known QTL and SNPs for pyramiding aflatoxin tolerance into a single germplasm. 3.) to investigate agronomic x genotypic x environmental x atoxigenic avenues in completely reducing the contamination of aflatoxin.

2. LITERATURE REVIEW

Aflatoxin, a harmful carcinogen, is highly regulated by the U.S. Food and Drug Administration. Corn samples that test over 20 ppb are prohibited from being used for human consumption (U.S. Food and Drug Administration, 2017). Animal feed may have a limit up to 200 ppb for swine (over 100 lbs) and 300 ppb for feedlot cattle. Animal feed over 300 ppb cannot be fed to any livestock but can be blended to reduce the overall amount (Park and Liang, 1993). Common commodities known to be contaminated with this toxin include *Zea mays*, *Arachis hypogaea*, and *Gossypium hirusutum* (Kilch, 2007). Corn production under stress is associated with higher production of aflatoxin, thus breeding for drought tolerance and/ or insect resistance can aid in inhibiting aflatoxin accumulations (Williams, 2006). Non-aflatoxigenic strains applied to fields have reduced aflatoxin accumulation by 83-98% through competitive exclusion (Abbas, et al., 2011). These atoxigenic strains have proven to be a useful tool in reducing aflatoxin accumulation, when simultaneously applied with genetic improvement, and indirect phenotypic enhancement. It is likely that the most influential strategy will be to develop new sources of inbred germplasm with favorable resistance traits which will further natural genetic resistance to aflatoxin accumulation (Windham and Williams, 1997). Public breeding efforts have been successful in developing partial resistance sources, but even the least susceptible genotypes still accumulate unacceptable aflatoxin levels during stressed environments and/or often yield substantially less than commercial hybrids (Wahl et al. 2016).

2.1 Cooperative tests grown to evaluate aflatoxin

Aflatoxin accumulation is severely affected by environmental conditions. In order to facilitate and procure stable germplasm, networks to test germplasm in many environments are

needed. Logistics for a single breeder to test in these differing environments are difficult due to the distances needed and the time dedicated to inoculating and subsampling, thus creating a need for cooperative tests. Creating a forum for participating breeders to join efforts can alleviate these logistics and supply an avenue for testing in multiple environments. Several cooperative trials are underway as discussed below in more detail.

Southern East Regional Aflatoxin Test (SERAT) is a cooperative test between several states including: Louisiana, Georgia, Mississippi, Illinois, and Texas. SERAT is a uniform test across different environments and inoculation methods throughout the southern corn growing regions. SERAT gives an opportunity for public breeding programs to test accumulation in other environments, allowing for detection of robust genetic sources of tolerance and allowing for a large cooperative between other scientists.

In another cooperative test to evaluate the genetics of aflatoxin susceptibility between the USDA (USDA–ARS, Corn Host Plant Resistance Research Unit, Mississippi State, MS) and Texas A&M AgriLife Research, there is a panel of 300 inbreds lines chosen to represent the majority of public material tolerant to aflatoxin accumulation and testcrossed to VA35 (Warburton, et al., 2013). This same panel of inbreds was testcrossed to Tx714 in the other heterotic group but few significant loci for aflatoxin were detected (Farfan, et al., 2015). The panel testcross hybrids with VA35 were phenotyped in seven environments, including College Station, through 2009 and 2010, using side needle inoculation (Warburton, et al., 2013). This panel's phenotypic information was used in association mapping for aflatoxin tolerance (Warburton, et al., 2015). 107 SNP associations were found that corresponded to aflatoxin accumulation in a least one or more environments. Twenty-one associations that occurred in

more than one environment are actively being validated using HIF, RILS, or transgenic lines (Warburton and Williams, 2014).

Beginning in 2012, a total of nine environments were used to screen inbred lines for aflatoxin accumulation and yield in the stressful climate of Texas where high aflatoxin pressure is often prevalent. A total of 93 inbred lines from across Southern breeding programs and ex-PVPs were evaluated for aflatoxin accumulation in grain across five growing seasons and nine environments. These locations were selected due to their southern climates, potential for higher aflatoxin accumulation and logistics. The specific objectives of this study were to evaluate elite inbred lines from diverse public programs in Texas environments in order to identify 1) trends in aflatoxin accumulation and agronomic traits; 2) the most promising inbred germplasm for genetic resistance to *A. flavus* sporulation and aflatoxin; and 3) differences between this germplasm and the ex-PVP germplasm which presumably are closely related to the elite industry material currently used today.

A very powerful hybrid trial has been ongoing since 2014 evaluates the interactions between atoxigenic applications, genotype, and environment against aflatoxin accumulation. These 8 replications by 4 environment trial allows the power to analyze many interactions. Each location has a dryland and irrigated component in both Corpus Christi, Texas and College Station, Texas. Split-plot experimental design will be utilized to investigate interactions that might occur between environment, germplasm, and atoxigenic applications

2.2 The effect of environment and other factors on aflatoxin

Environmental conditions and their interactions with genetics are a major effect in difficulties of breeding for aflatoxin resistance. Environmental conditions induce the

colonization of many pathogens, along with the production of aflatoxin in maize. Stressful conditions such as high daily and nighttime temperatures, drought, insects, mechanical damage, and limited crop inputs can increase the chance to accumulate aflatoxin in maize (Cotty and Jaime-Garcia, 2007; Odvody, et al., 1997; Widstrom, et al., 2003) . Correlation between daily temperature and aflatoxin accumulation are strong immediately after flowering, suggesting that stress during this period could increase aflatoxin accumulation (Hawkins, et al., 2008). Drought is becoming a large problem in maize production areas. Water resources are dwindling from local aquifers and river basins. These issues are greater in areas without infrastructure such as dams, canals, or wells. Increase in aflatoxin accumulation can be influenced by climate change, by either an increase in vectors transmitting the pathogen, or the increase in pathogen prolificacy (Paterson and Lima, 2010). Increasing temperatures in recent years may cancel the advancements that are currently being made in genetic and phenotypic reduction of aflatoxin accumulation. In addition to natural environmental conditions and management, other manmade causes of what statistically appear to be environmental conditions include inoculation techniques and fastidious methods of accessing aflatoxin accumulation which can be accounted for within the model by including representative variables.

Comparatively low repeatability due to high genotype by environment (GxE) interactions and experimental errors requires that the investigator evaluate multiple locations, during multiple years, to probe these interactions. Depending on the degree of interaction, many environments are often needed (Bernardo, 2002). Low repeatability often dictates how much and how often the same QTL can be found in a population tested over multiple sites. Low repeatability often dampens the ability to differentiate entries that are being evaluated, and suggests factors reducing aflatoxin are not just major disease resistance genes. In previous studies, many small effect QTL

have been identified in numerous genetic backgrounds, but few offer a substantial amount of variation. Furthermore, many of the QTL are inconsistently detected across populations and environments screened. No single QTL associated with aflatoxin accumulation to date has exhibited more than ~20% of variation explained (Willcox, et al., 2013). It is thought that stacking these small effect additive QTL into a single inbred could provide a more aflatoxin tolerant parental line that could be stable in all or most environments (Warburton and Williams, 2014).

2.3 Inoculation methods

There are numerous avenues to artificially inoculate maize with Aspergillus *flavus* and justification for each avenue. These include two major classes of methods, wounding and non-wounding (Tucker Jr, et al., 1986). Wounding inoculation techniques offer steady infection with an aspect that would mimic insect or mechanical damage thus bypassing phenotypic tolerance such as husk cover or tightness, natural insect tolerance, pericarp thickness, and erect or non-erect ears. Wounding techniques also involve repeated passes throughout the trial in order to inoculate plots at appropriate times after silk emergence. Non-wounding would not bypass these tolerances and presents both a phenotypic and genotypic evaluation of tolerance along with less passes needed for inoculation (Williams, et al., 2002; Windham, et al., 2009). Both have advantages and disadvantages so it is decided by the investigator on which avenue would work best in their program.

Kernel inoculation, a non-wounding technique, consists of spreading a carrier, usually wheat or maize kernels, colonized by *Aspergillus flavus* during flowering (Williams, et al., 2013). These kernels have been inoculated with *Aspergillus flavus* in the lab and are allowed to

sporulate before disseminating in the field. This technique is easy and mimics the natural mechanism of infection. Kernel inoculation is also used for competitive exclusion of toxigenic strains of *Aspergillus flavus* by non-toxigenic strains in products such as Aflaguard, AF36, and Aflasafe. Non-aflatoxigenic strains applied to fields have reduced aflatoxin accumulation by 83-98% through competitive exclusion (Abbas, et al., 2011).

Silk channel inoculation, a non-wounding technique, bypasses any genetic or physical resistance that could occur in the silk or husk area. Liquid inoculum is prepared in the lab by concentrating conidia of *A. flavus* and diluting to a standard aqueous suspension of 10^7 ml^{-1}. The inoculum is then inserted between the silks during flowering using a large diameter needle/syringe at three milliliters per ear. Multiple studies have used sideneedle inoculation technique which has been shown to be highly effective in differentiating genotype on aflatoxin accumulation (Henry, et al., 2010; Zummo and Scott, 1989).

Sideneedle inoculation is a wounding technique and is used roughly 7 days after flowering by inserting 3.4-mL suspension of 3×10^8 conidia using a large diameter needle. Knife inoculation is used to mimic insect or physical damage. The inoculum of 1×10^6 mL^{-1} concentration of conidia is applied by knife to about five kernels through the husk, 20 days after midsilk. Side needle inoculation provides the highest amount of infection compared to all other techniques, however both side needle and silk channel are proven to differentiate between genotypes (Williams and Windham, 2015; Windham, et al., 2009).

Pin-bar, a wounding technique, is used 21 days after mid-silk by inserting several needles mounting onto a small bar through the side of the ear and into the husk. These needles are dipped into a conidial suspension before being pushed through the ear.

2.4 Breeding for aflatoxin tolerance

Searching exotic germplasm is critical in order to find new sources of genetic tolerance (Moreno and Kang, 1999). Fungal infection and consequent aflatoxin accumulation is sporadic and difficult to mimic for universal coverage in field trials unless some of the inoculation techniques discussed previously are employed. Inoculation avenues can affect what resistance traits are employed, for example, silk channel inoculation that bypasses any traits located in the silks. Mimicking natural infection using ground kernel inoculation methods can utilize all aspects of plant resistance: ear droop, husk tightness, and kernel characteristics (Tucker Jr, et al., 1986). In contrast, silk channel or side needle inoculation will circumvent adaptions in ear phenotypes in order to attain a deep and uniform infection. Even with adequate inoculation techniques, it is still difficult to acquire uniform infection. Heritability in maize for aflatoxin tolerance is variable, 0% - 89% (Wahl, et al., 2016). Tolerance is quantitatively inherited and highly impacted by genotype x environmental interactions. These criteria inhibit ease of breeding for increased tolerance.

Many small effect genes are expected to give the most influential tolerance towards accumulation. Breeding in order to pyramid these small affect genes using marker assisted selection (MAS) would likely be the most effective breeding method (Warburton, et al., 2009; Williams, 2006). In addition to pyramiding tolerance, it is essential that we also continually select for phenotypes that indirectly increase tolerance. Indirect traits include insect resistance, tight husk, or drought tolerance(Warburton and Williams, 2014). Some of these indirect traits are unfavorable to producers but could be beneficial in other commercial avenues.

2.5 The challenges of exotic germplasm adaptation

In recent years there has been numerous inbred germplasm releases with aflatoxin tolerant lines, available to breeders for use in other programs (in some instances under a material transfer agreement [MTA]). Many of these lines have been derived from tropical germplasm. As with any exotic derived line in the US, the lines that result from these efforts are tall, late maturing, lower yielding, and prone to lodging. A great example is Mp715 which is a late maturing and high-lodging, however, highly-tolerant inbred to aflatoxin accumulation. Mp718 and Mp719 were derived from a cross of Mp715 and Va35 as an effort to increases agronomic favorability while maintaining aflatoxin-tolerant traits. Efforts to increase agronomic favorability have generated better lines such as Mp718, Mp719, Tx739, Tx736, and Tx740 (Mayfield, et al., 2012; Williams and Windham, 2012). However, even these lines are still relatively late compared to commercially available lines on the market. The maturation of these lines can sometimes be inflated in the environments where most hybrid seed is produced in the United States, increasing the difficulty in utilizing these lines into commercial hybrids.

2.6 Multiple intermating for enhanced recombination

Recombinant inbred lines (RILs) are inbred lines that have been self-pollinated to inbreed to near homozygosity; RILs are often derived from a biparental cross. The resulting inbreds are syntheses of the two parental lines. These RILs have advantages of inheriting alleles and thus variability from each parent as well as permitting new interactions to occur from both parents' genomes. Since RILs are inbred to near homozygosity, they can be genotyped and used for future reference with little or no change in genotype. However, there are limitations to biparental RIL populations. The allelic diversity is restricted to the diversity in the parents of the original

cross. The number of effective recombination events for a biparental cross can be very low and may only identify large effect QTL, if present. A lack of power makes it difficult to find the many small effect QTL that are thought to exist. This precision is enhanced by the addition of advanced intercrossing in generations. Having multiple generations of intercrossing can break up linkage blocks and provide more accurate identification toward smaller effect QTL (Balasubramanian, et al., 2009). In maize, the intermated B73, Mo17 derived IBM population was the first to demonstrate this using modern methods. Intermating populations were shown to increase recombination frequency by 2.7 fold in the intermated B73 and Mo17 population (IBM) (Lee, et al., 2002). Multi-parent populations have additional generations of development that increase the chance of recombination and additional alleles useful for tracking these past recombination events. Multi-parent advanced intercrossed populations (MAGIC), such as Arabidopsis (Balasubramanian, et al., 2009; Kover, et al., 2009), rice (Bandillo, et al., 2013), wheat (Mackay, et al., 2014), mice (Talbot, et al., 1999; Valdar, et al., 2006) and most recently the maize MAGIC population (Dell'Acqua, et al., 2015), have offered added precision with higher density maps.

3. [1]EVALUATION OF ELITE MAIZE INBRED LINES FOR REDUCED ASPERGILLUS FLAVUS INFECTION, AFLATOXIN ACCUMULATION, AND AGRONOMIC TRAITS

Aflatoxin is a harmful carcinogenic mycotoxin produced by *Aspergillus flavus* Link:Fr, which limits the marketability of maize (*Zea mays* L.) grain and reduces the economic value for producers. Actual economic losses are difficult to measure, but are thought to be around $163 million per year in the United States for maize only, and up to $500 million annually in peanuts (*Arachis hypogaea* L.) and other crops. (Wu, 2015). Documented or suspected cases of acute aflatoxin poisoning are numerous throughout the world and result in liver damage, intestinal bleeding, cancer, and even death (Lewis, et al., 2005); especially in developing countries which lack infrastructure to test for contamination, and allow contaminated maize to flow freely in local trade. The effects of chronic exposures to aflatoxins are even more challenging to test. More than 100 countries have some type of regulations on aflatoxin concentrations (Wu and Guclu, 2012). In the United States, aflatoxin is regulated for human consumption with an upper limit of 20 (ng g^{-1}); and with a maximum feed limit of 300 (ng g^{-1}) for finishing beef, with lesser limits for swine and poultry feed (Stoloff, et al., 1991). The limits are set by the US Food and Drug Administration.

It is still unclear why *A. flavus* makes aflatoxin, but preliminary indications are that it might be to prevent insect predation (Drott, et al., 2017; Gqaleni, et al., 1997). Pre-harvest colonization of maize and the subsequent production of aflatoxins are associated with, and likely

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[1] Reprinted with permission from Evaluation of Elite Maize Inbred Lines for Reduced *Aspergillus flavus* Infection, Alftoxin Accumulation, and Agronomic Traits by Pekar, J.J., S.C. Murray, T. Isakeit, B.T. Scully, B. Guo, J. Knoll, et al. 2019. Evaluation of Elite Maize Inbred Lines for Reduced Aspergillus flavus Infection, Aflatoxin Accumulation, and Agronomic Traits. Crop Sci. doi:10.2135/cropsci2019.04.0206 in Press.

are a result of, an increase in physiological stresses of crop production (Klich, 2007). High daytime and nighttime temperatures, along with occurrences of drought and insect pressure, increase the occurrences of aflatoxin contamination (Abbas, et al., 2002). To reduce pathogen pressure and toxin accumulation, producers decrease stresses through cultural practices and management, when economical. In recent years, atoxigenic strains of *A. flavus* have become an additional management tool to reduce aflatoxins (Abbas, et al., 2011). Plant-breeding is an important component of the integrated pest management approach to decrease aflatoxin and *A. flavus* (Brown, et al., 2011). Breeding for decreased susceptibility by selecting for heritable segregating traits such as tighter, thicker and closed husk cover, or insect resistance, can reduce toxin accumulation (Widstrom, et al., 2003). A major challenge of breeding inbred lines for aflatoxin or yield is that inbred lines appear to be less robust across environments (i.e. experience more genotype x environment interactions or GxE) in a way that hybrid vigor masks in hybrids (Cole, et al., 2009; Li, et al., 2018; Schnell and Becker, 1986).

To test susceptibility of maize genotypes to *A. flavus* and aflatoxin accumulation uniformly, inoculation must be used. There are two major classes of *A. flavus* inoculation methods, wounding and non-wounding (Tucker Jr, et al., 1986). Wounding inoculation techniques offer consistent infection that mimic insect or mechanical damage, bypassing tolerances from physiological traits such as husk cover or tightness, natural insect tolerance, pericarp thickness, and erect or non-erect (drooping) ears. Non-wounding methods do not bypass other physiological tolerances and evaluate susceptibility in a way that is more relevant to farmers (Williams, et al., 2002; Windham, et al., 2009). The different types of inoculation methods have various advantages and disadvantages (Williams, et al., 2013). In this research, we chose to use a non-wounding technique, ground kernel inoculation (similar to how atoxigenic

strains are applied by producers), because it more closely mimics natural inoculation conditions. This technique also reduces the labor needed, allowing the evaluation of a larger number of genotypes and replicates for aflatoxin accumulation.

Despite a number of public breeding efforts, no genotypes have yet been identified that are completely resistant to aflatoxin, only quantitative reductions have been found. There have been few collaborative projects to date that investigate diverse public sector materials in common gardens across different environments. Public material bred for decreased susceptibility to aflatoxin in the United States is being developed by the USDA-ARS in Tifton, GA, Mississippi, and North Carolina, and by Texas A&M University (TAMU) at College Station and Texas A&M Agrilife Research at Lubbock (Table s1). The most viable example of joint testing of this material in common gardens has been in the Southeast Regional Aflatoxin Trials (SERAT), which has tested 30-40 public breeding sector hybrids for agronomics and aflatoxin accumulation each year since 2003 (Wahl, et al., 2017). The SERAT trials have shown that the best public hybrids are more tolerant than current commercial hybrids available to producers; however many, but not all, public hybrids lack the favorable agronomics and realized yield that producers demand (Wahl, et al., 2017). Perhaps more importantly, the SERAT trials demonstrated that a large portion of aflatoxin susceptibility is genetic (22%; for yield this was 19%) and heritable across very diverse but relevant environments. The SERAT trials also demonstrated aflatoxin accumulation is robust to inoculation methods, with a relatively small GxE (13%) but moderate error (39%) components (Wahl, et al., 2017). This suggests that relatively few environments are sufficient for identifying superior genetics for *A. flavus* and aflatoxin susceptibility. However, given the large influence of weather on aflatoxin accumulation (Williams, et al., 2014), many environments are needed to capture environmental conditions that

result in good genotypic separation as observed in SERAT. While SERAT trials evaluated hybrids in common gardens there have been no similar published trials of inbred evaluations from across diverse public programs' released lines.

The purpose of this research was to screen a diverse set of released and pre-released public inbred lines, along with important expired Plant Variety Protection (ex-PVP) commercial lines for low aflatoxin and high yield in Texas environments. Many of the released and to-be-released public breeding lines (Table s1) and all ex-PVP lines have not previously been screened in a common garden for aflatoxin resistance. A total of nine environments, beginning in 2012, were used to screen inbred lines for aflatoxin accumulation and yield in stressful climates of central and southern Texas where high *A. flavus* pressure and aflatoxin production is often prevalent. The specific objectives of this study were to evaluate elite inbred lines from diverse public programs in Texas environments in order to identify 1) trends in aflatoxin accumulation and agronomic traits; 2) the most promising inbred germplasm for genetic resistance to *A. flavus* sporulation that results in low aflatoxin; and 3) differences between this germplasm and the ex-PVP germplasm which presumably are closely related to elite industry lines currently used today.

3.1 MATERIALS AND METHODS

3.1.1 Germplasm

A total of 93 inbred lines from across Southern breeding programs and ex-PVPs were evaluated for aflatoxin accumulation in grain across five growing seasons. These inbreds included four Southern lines known to be susceptible based on past studies: T173 (West, et al., 2001), SC212m, Va35 (Henderson, 1976), and GA209 (Fleming, 1974) and nine lines known for, and most released because of, low-aflatoxin accumulations: GT603 (Guo, et al., 2011),

Mp313E (Scott and Zummo, 1990), Mp420 (Scott and Zummo, 1992), Mp717 (Williams and Windham, 2006), Mp718 and Mp719 (Williams and Windham, 2012), Tx740 (Mayfield, et al., 2012), Tx772 (Llorente, et al., 2004), and Tx777 (Murray, et al., 2019).

3.1.2 Experimental design and study locations

Starting in 2012, two locations were selected for aflatoxin testing of inbreds, College Station (CS) and Weslaco (WE), Texas; only CS was used in 2016, for a total of nine environments. Limited supplementary irrigation was applied to trials as needed. College Station has an average high temperature of 33.2 °C during flowering and an average annual rainfall of 101.8 cm. Weslaco, TX has an average high temperature of 32.2 °C during flowering and an average annual rainfall of 63.2 cm. Sowing was delayed about a month longer than optimal in both locations to have higher temperatures during grain fill, which would lead to greater stress and therefore a higher potential aflatoxin contamination to differentiate genotypes. In 2013, entries increased from 13 to 38 and increased every subsequent year except 2016. A total of 57 inbred lines were only tested in one year, but in two locations. The remaining 41 were tested in multiple years with multiple locations. The experimental design consisted of a randomized complete block with four replications in 2012 and three replications in subsequent years as the number of inbred lines increased. Each replicate in CS was planted in 1-row plots 6.10m long with 0.76 m wide alleys; WE had 1-row plots 7.01 m long with 1.01m alleys.

3.1.3 Aspergillus flavus inoculation

Inoculum was prepared from stock *A. flavus* isolate NRRL 3357 (Wicklow, et al., 1998), commonly used in aflatoxin studies including many referenced here, on potato dextrose agar. Plates were incubated at 30 °C for 5 to 7 days. Once plates sporulated, they were covered with parafilm and stored at 4 °C. One square cm sample was cut from the source plate and placed in a

test tube with 15 ml sterile water plus 0.01 ml of Tween 20 and vortexed. Additional samples were plated using 0.05 ml from the parent plate and incubated for 5 to 7 days at 30 °C. Seven thousand cc of whole field corn and 3 L of distilled water were placed into a 46 cm × 38 cm × 13 cm autoclavable plastic tray and covered with aluminum foil and autoclaved for one hour. Once autoclaved, the corn was transferred into a translucent 140-quart (132 L) plastic bag. Twenty-five to 30 petri dish cultures were blended with 2 L distilled water and 1 ml Tween 20. Five hundred milliliters of this mixture was added to each bag of corn and mixed thoroughly, then placed in a room with a constant temperature of 35 °C – 38 °C, with additional mixing every 12 hours. Sporulation occurred 2 to 3 days after inoculation. The colonized kernel inoculum was applied to the plots by scattering it on the ground between the rows shortly after the majority of the plots had started silking, at an amount of 170 g per 9 linear meters of row. This was usually around 12 days after the initiation of silking.

3.1.4 Traits measured

Seven agronomic traits were also measured at College Station locations, including: 50% of plot flowering as days to anthesis (DTA) and days to silking (DTS) as well as plant height (PHT) from the ground to the tip of the tassel, and ear height (EHT) from the ground to the top ear's node attachment point. PHT and EHT were not recorded in 2012 at CS and only PHT was recorded at WE in 2012. Plots were hand harvested at or shortly after maturity, visually rated, shelled and bulked for measurement of plot weight, moisture and test weight using a Dickey-john Mini GAC moisture meter (Dickey-john, Auburn, IL). Bulked grain samples were then ground using a Romer mill (Romer Labs, Union, MO), and aflatoxin analysis was performed using a commercial quantitative assay systems (Vicam, Watertown, MA). Visual ratings were taken on all harvested ears to evaluate corn earworm (*Helicoverpa zea*) damage, *A. flavus*

sporulation, and *Fusarium spp.* sporulation during 2013, 2014, 2015, and 2016 seasons. Corn earworm damage was estimated visually as a percentage of damaged kernels. Fungal sporulation was estimated visually as a percentage of kernel surface area sporulating for *A. flavus* and 'star bursting' or whitish mycelium on the kernels for *Fusarium spp*.

3.1.5 Statistical analysis

Statistical analysis was conducted using JMP 12 software (JMP®, Version 12. SAS Institute Inc., Cary, NC, 1989-2016). In an attempt to normalize observations, aflatoxin data were transformed using the Box-Cox power transformations formula (Box and Cox, 1964)

$$x'_\lambda = \frac{x^\lambda - 1}{\lambda} \qquad \text{Eq. 1}$$

All data were first analyzed jointly to evaluate overall trends and then by individual environments (location by year). In the separate environment analysis, inbreds effects were treated as fixed while replications, range, and row effects were random. In the all-years-combined analysis (Eq. 2), inbreds were fixed while environment, replication, inbred by environment interaction, range, and row effects were random. Here range and row effects are equivalent to row and column effects, but reflect the terminology used in furrow irrigation trials. The model was fit as follows: where μ is the grand mean, g_i is the fixed effect of the genotype i, e_k is the random effect of the environment k, $(g * e)_{ik}$ is the random effect of the interaction between i and k, $(r/e)_{jk}$ is the random effect of replication j nested within environment k, $(row/e)_{lk}$ is the random effect of row l nested with environment k, $(range/e)_{mk}$ is the random effect of range m nested with environment k, ε_{ijklm} is the random residual error from genotype, environment, replication, row, and range.

$$y_{ijk} = \mu + g_i + e_k + (g * e)_{ik} + (r/e)_{jk} + (row/e)_{lk} + (range/e)_{mk} + \varepsilon_{ijklm}$$

Eq. 2

All means were compared using Fisher's Protected LSD (P = 0.05) and all aflatoxin values were then back transformed and reported as actual ng g^{-1}. Pairwise correlations were analyzed using the multivariate function in JMP software. Repeatability (h^2) was calculated as:

$$h^2 = \mathrm{G} / (\mathrm{G} + \frac{\mathrm{GE}}{\mathrm{r}} + \frac{\mathrm{Ɛ}}{\mathrm{re}})$$

Eq. 3

where G, GE and Ɛ were the variance components of genotype, genotype by environment interaction and residual error respectively, with r as number of replications and e as number of environments.

Correlations were compared using Pearson correlation coefficients. Phenotypic correlations were formed on raw data, while genotypic correlations were formed from the best linear unbiased predictor (BLUP) genotypic estimates of each trait.

3.2 RESULTS AND DISCUSSION

3.2.1 Exploratory statistics

Aflatoxin accumulation values on raw data ranged from 0 to 4500 ng g^{-1} with a grand mean of 268 ng g^{-1} across nine environments. Aflatoxin accumulation appeared higher in years where statewide drought was more prevalent (Figure 1), as expected due to higher stress. Year 2012 had the lowest accumulation means in both Weslaco and College Station at 77 ng g^{-1} and 70 ng g^{-1}, respectively. Only 13 entries were used in 2012 and no program lines were significantly better than the tolerant checks. The highest mean toxin accumulation occurred during the 2014 season in Weslaco, TX at 542 ng g^{-1}, where weather patterns favored hot and dry temperatures and low precipitation during the growing season. In 2016, timely rains during the growing season likely caused the low observed aflatoxin levels (143 ng g^{-1}), but sufficient

variation for discrimination of lines was still observed. Visual ear ratings on *A. flavus* sporulation tended to be higher in College Station than that in Weslaco, which was unexpected and possibly associated with irrigation timing during inoculation, which can increase the relative humidity and favor sporulation (Diener, et al., 1987; Hesseltine, et al., 1966).

PHT and EHT were recorded in most years with a PHT range of 76 – 249 cm and an EHT mean of 10 – 112 cm. The Mississippi program material was significantly taller for PHT than all other germplasm at 172 ± 2 and EHT 69 ± 15 cm, respectively. The remaining program materials were not significantly different as groups; however, there were significant differences between entries within each program's material, suggesting that variation and favorable alleles for desired PHT characteristics are present. In Texas and other areas in the southern United States, there have been notable correlations between PHT, EHT, and yield in diverse commercial hybrids (Farfan, et al., 2015; Liu and Wiatrak, 2011; Yin, et al., 2011), and in biparental inbred crosses (Chen et al. to be submitted). PHT appears to be an indicator trait of vigor and adaptation to hot and dry southern climates; PHT might be strongly correlated with yield because certain diverse germplasm can better overcome common stresses associated with southern growing regions (Farfan, et al., 2013). If this hypothesis is correct, then it would suggest that by selecting lines that are taller, by proxy they might also be better adapted to this growing region. Over years, the mean height and days to flowering increased, which can be explained by the increased number of entries from tropical, as opposed to temperate-derived genotypes (Table s1). Tropical derived lines tend to be later in flowering and taller in stature than many of the ex-PVP lines and susceptible checks that were first evaluated. Tropical derived germplasm also tends to be less susceptible to aflatoxins as they are usually better adapted to the hotter and dryer climates that

favor aflatoxin contamination. The changes in entries also likely contributed to the decrease of the mean and variance of aflatoxin levels from year to year.

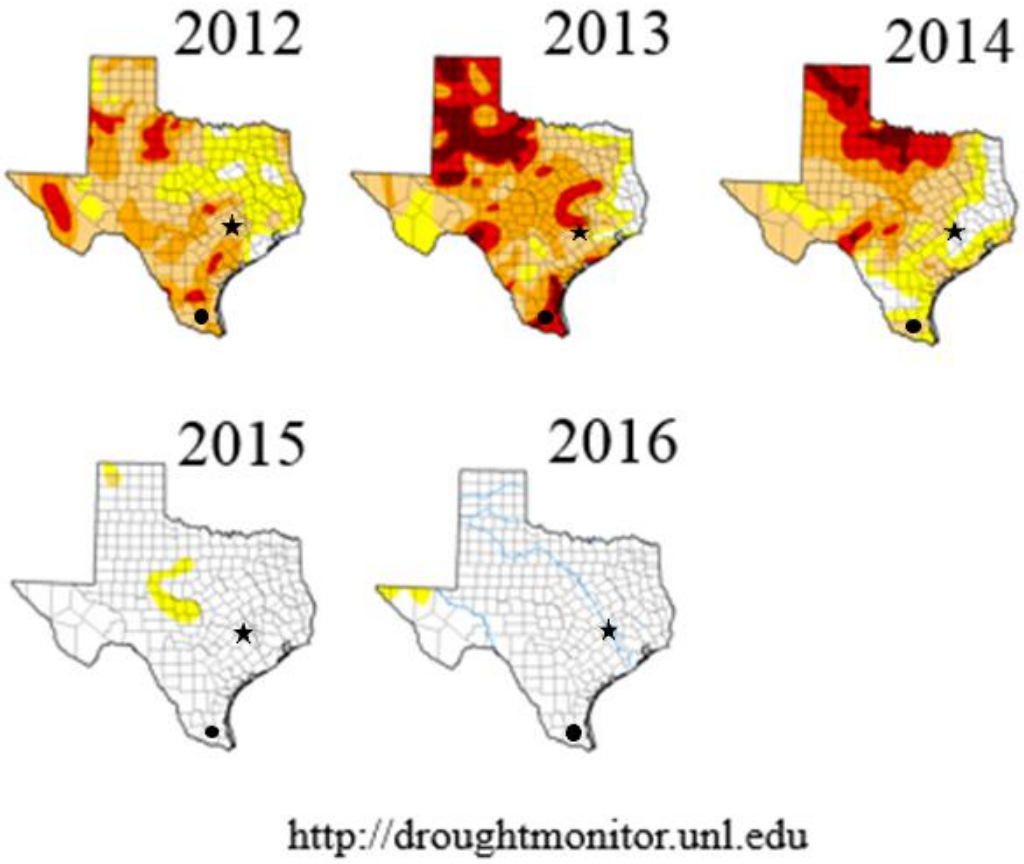

Figure 1 Drought levels in Texas during flowering time of trials in all years. Darker red colors indicate the most severe drought. College Station is designated as a star and Weslaco is designated as a circle. Reprinted from http://droughtmonitor.unl.edu.

Table 1 Summary statistics on BLUPs for back transformed aflatoxin data, visual ratings for *A. flavus* sporulation, *Fusarium spp.* star bursting, and earworm damage, yield, days to 50% anthesis (DTA), days to 50% silking (DTS), plant height (PHT), and ear height (EHT) separated by location and years. Environment is a combination of location and year. CS, College station, TX; WE, Weslaco, TX.

	Environment	WE12	CS12	WE13	CS13	WE14	CS14	WE15	CS15	CS16
Aflatoxins (Box-Cox)	Max	78	68	168	739	1312	667	575	686	319
	Mean	32	29	70	409	744	320	273	309	134
	Min	0.0	0.0	0.0	0.0	51	21	0.0	0.0	0
	Std Dev	25	20	40	138	328	134	152	167	82
Aflatoxins (ng g^{-1})	Max	986	994	4500	4200	2390	2310	3010	4170	1086
	Mean	77	70	136	286	542	223	347	357	143
	Min	1	1	1	1	2	2	1	1	1
	Geometric Mean	12	11	21	88	199	90	72	82	45
	Std Dev	174	188	476	593	669	345	584	620	190
Yield (T ha^{-1})	Max	3.4	4.7	4.9	6.2	4.4	5.4	5.8	3.8	3.5
	Mean	2	2.5	2.4	2.1	1.6	3	2.3	1.9	1.7
	Min	0.1	0.4	0.4	0.1	0.1	0.5	0.1	0.1	0
	Std Dev	0.7	1.4	1.2	1.3	1.1	1.3	1.1	0.9	1.1
A. flavus visual ear rot ratings (1-100)	Max	-	-	3.3	13.3	7	5.6	26.7	50.1	31.2
	Mean	-	-	0.4	3	1.5	1.3	1.3	4.4	6.4
	Min	-	-	0	0	0	0	0	0	0.4
	Std Dev	-	-	1	3.3	1.9	1.6	4.2	8.5	7.8

Fusarium spp. visual ear rot ratings (1-100)	Max	-	-	93.8	76.7	46.7	83.7	61.3	90	66.7
	Mean	-	-	23	15.9	8.5	14.6	8.9	8.8	24.4
	Min	-	-	0	0	0	0	0	0	0.3
	Std Dev	-	-	27.3	22.3	11	18.6	13.4	15.9	20.9
Earworm damage ratings (1-100)	Max	-	-	6	5.7	18.3	30	43.3	51.7	37.8
	Mean	-	-	2.9	3	7.6	9.9	6	7.3	17.7
	Min	-	-	1.3	1	0.6	1.3	0	0.9	5.9
	Std Dev	-	-	1.2	1.2	4.2	6.1	7.1	7.6	8.3
Days to anthesis	Max	71	64	-	93	-	91	-	80	80
	Mean	70	63	-	85	-	80	-	70	67
	Min	69	63	-	77	-	72	-	64	58
	Std Dev	1	0	-	4	-	5	-	4	5
Days to silking	Max	70	64	-	95	-	93	-	85	81
	Mean	70	64	-	86	-	82	-	72	69
	Min	68	64	-	79	-	74	-	66	59
	Std Dev	1	0	-	4	-	4	-	4.6	5
Plant height (cm)	Max	201	-	-	203	-	202	-	201	201
	Mean	155	-	-	157	-	160	-	155	167
	Min	134	-	-	117	-	132	-	119	128.0
	Std Dev	16.8	-	-	19.8	-	16.3	-	18.0	16.5
Ear height (cm)	Max	-	-	-	86	-	86	-	86	89
	Mean	-	-	-	59	-	58	-	58	60
	Min	-	-	-	40	-	34	-	42	43
	Std Dev	-	-	-	13	-	12	-	10	12

3.2.2 Variance components and repeatability: Aflatoxins and fungal (ear rot) damage

Variance components are useful for evaluating sources of variation in an experiment and allow repeatability to be calculated. Sufficient accumulation of aflatoxins to discriminate inbred lines occurred in all environments. Genotypic variation from the transformed aflatoxin data explained 30% overall and explained from 26.6% to 68.9% of the total variation within each individual environment. Across the full model, GxE interaction components were lowered by transforming raw aflatoxin accumulation data, which did not fall into residual error, but were distributed to other effects. Residual error variation was decreased using transformed aflatoxin data compared to raw data from 43% to 34%. Residual error was further decreased when using BLUPs compared to the raw data due to the BLUPs accounting for significant field variation (i.e. row and range effects) and replication effects.

Repeatability was used over heritability due to the lack of family structure in the genotypes. Repeatability is typically high for flowering and height, and lower for yield and aflatoxins (Farfan, et al., 2015; Wahl, et al., 2017). Across all environments, repeatability for aflatoxin accumulation data was 88% using transformed data up from 78% on non-transformed raw data (Table 2). Repeatability for aflatoxin accumulation has been moderate or low in previous studies (Wahl, et al., 2017; Warburton, et al., 2013). Repeatability for aflatoxin accumulation within individual environments was lower than for all other important agronomic traits, but still moderate to high. Logarithmic transformation of aflatoxin accumulation is represented in Table 2 in order to compared repeatability between trials. Wahl, et al., (2016) did not use the Box-Cox transformation method, thus logarithmic transformations were used for comparison. In previous studies, genotypic correlations between visual ratings and wet lab analysis have had *r*-values up to $r = 0.83$ and $r = 0.76$ for both aflatoxins and fumonisin,

respectively (Henry, et al., 2009; Horne, et al., 2016). In most environments there was very little *A. flavus* sporulation observed. However, *Fusarium* star-bursting and sporulation were consistently prevalent. Variance components for genotype were moderate to high for *Fusarium spp.* sporulation (41%), but lower for *A. flavus* sporulation (9.5%) and earworm damage (17.1%). The total percent of variance explained by GxE for *Fusarium spp.* sporulation, *A. flavus* sporulation and earworm damage were 22.6%, 28.7%, and 15.8%, respectively. That visually rating these phenotypes produced different results across different environments, would reduce the usefulness of such an observation for prediction of unknown future environments. The environment main effect explained minimal amounts of variation for *Fusarium spp.* and *A. flavus* sporulation. Variation explained by earworm damage from environment was twice the amount than that of GxE, which was explained by an overall increase of pest pressure in certain environments.

3.2.3 Variance components: yield and secondary agronomic traits

Environment, genotype, and GxE were all highly significant in the all year model for all traits with Replication, Range, and Row being intermittently significant among traits (Table 2). Genotypic variation for yield (41% overall; Table 2) was less than the variation due to environmental effects and ranged from 47.9% to 91.6% across the nine environments. Many program entries had significantly higher yields than did the tolerant and susceptible checks (Table s1). All public programs had at least one entry that yielded better than or equal to the ex-PvPs and all checks. Across all germplasm, the tropical Tuxpan derived germplasm were among the lowest yielding, while germplasm developed from the two Texas programs containing Argentinian, Bolivian, and Cuban derived germplasm had yields that were significantly higher

than that of the Tuxpan germplasm and tolerant checks. Georgia and North Carolina programs both had moderate yield. It is reasonable to expect that some of this was due to the fact the Texas lines were better adapted to the environments being tested. Because each program has diverse lines of diverse origins, the mean performance of the program should not be taken as a benchmark of the program as a whole. Identification of individual superior inbred lines is the primary goal. Within each environment, calculated from the environment x genotype model from eq. 2, variance components demonstrated the largest sources of variation came from genotype, and the least from spatial variation within field (range and row).

When separating environment into location x year, results indicated that PHT had more variation explained by location than by year. Differences between locations of PHTs is normal when planting at different times (Weslaco planted in March and College Station in April) and at different latitudes (Weslaco, 26.16° N; College Station, 30.63° N). Plants react differently to the oncoming longer days and rapid growing degree days (GDU) accruements that occur during the growing season (i.e. planting earlier results in shorter maize, than later planted maize). Flowering measurements were the opposite in that more variation was explained by the year effect than by the location effect. Flowering time (DTS) variation explained within each environment for genotype was consistently over 87% with very little residual error, meaning that flowering measurements were taken with good accuracy and had large repeatable genetic differences. Earlier varieties are favored over later maturing hybrids, thus selecting for earliness in tropical germplasm could aid in favorable hybrid agronomics (Goodman, 2005).

Table 2 Variance components and percent total for traits measured in all years and locations combined for transformed aflatoxin value (Aftrans), actual measured aflatoxin accumulation in parts per billion (afppb), yield (T ha-1), visual fusarium rating (FU), visual *A. flavus* rating (AF), visual ear worm rating (EW), days to anthesis (DTA), days to silking (DTS), plant height (PHT), ear height (EHT).

model	Aftrans	%	Afppb	%	T ha-1	%	FU	%	AF	%
Env	3403***	23	28161***	10	.18***	10	44***	9	4***	8
Genotype	4556***	30	70263***	26	.77***	41	206***	42	4*	9
Env*Genotype	1557***	10	42906***	16	.37***	20	108***	22	14***	29
Rep[Env]	86**	1	NS	1	.02*	1	6*	1	NS	0
Range[Env]	225***	1	8259***	3	.03*	1	NS	1	2**	3
Row[Env]	NS		NS	1	.05***	3	NS	0	NS	0
Residual	5105	34	115721	43	0.45	24	125	25	24	51
Total	15096	100	269730	100	1.88	100	491	100	47	100
R	0.88		0.78		0.98		0.83		0.45	

NS, not significant; **, significant at the .05 level; ***, significant at the .001 level.; R, repeatability; significance tested using Log Likelihood

Table 2. Continued.

model	EW	%	DTA	%	DTS	%	PHT	%	EHT	%
Env	26***	32	62***	75	67***	75	24***	26	11.4***	28
Genotype	14***	17	14***	17	17***	19	36***	39	17***	41
Env*Genotype	13***	16	3***	3	2***	3	12***	13	3***	8
Rep[Env]	NS	0	0.1**	0	NS	0	NS	0	NS	1
Range[Env]	NS	0	NS	0	0.3*	0	2**	2	NS	1
Row[Env]	NS	0	NS	0	NS	0	1.1*	1	NS	1
Residual	28	35	3	4	3	3	16.5	18	8.1	20
Total	80	100	82	100	89	100	93	100	41	100
R	0.72		0.93		0.95		0.88		0.92	

NS, not significant; **, significant at the .05 level; ***, significant at the .001 level; R, repeatability; significance tested using Log Likelihood

3.2.4 Correlations

The most notable of the many significant genetic correlations across all years were positive correlations between the visual ratings taken on ears for *A. flavus* infection (AF%) and ear worm damage (EW%) during processing and wet lab aflatoxin accumulation ratings (Table 3). AF% and EW% were positively significantly correlated to both non-transformed (r = 0.27, r = 0.35) and transformed aflatoxin data (r = 0.38, r = 0.30). This suggests that earworm damage was likely a contributor of aflatoxins in these inbred ears, although earworm damage has not often been associated with aflatoxins in previous hybrid studies conducted in Texas (Bibb, et al., 2018; Farias, et al., 2014; Weaver, et al., 2017). However, earworms are known to be an important component to aflatoxins, allowing the opportunistic fungi to access and infect the ear (Jones, et al., 1980; Ni, et al., 2011). Genotypic correlations for yield and aflatoxin accumulation were non-significant for non-transformed data but significant for transformed data (r = 0.20*); in contrast phenotypic correlations for yield and aflatoxins were significant for non-transformed data (r = -0.12***) but non-significant for transformed data (Table 4). Our hypothesis is that there are few or no loci that pleiotropically affect both yield (T ha^{-1}) and aflatoxins (AFppb), however some germplasm bred for resistance was not bred for yield and vice versa. This would be further confirmation that there are genetic linkages between aflatoxins and yield which could be broken through additional breeding.

Another notable correlation was related to flowering time. Transformed aflatoxin accumulation was significant and highly negatively correlated with DTS (genotypic correlation of -0.40). This increase in aflatoxin accumulation may have resulted from

favorable environmental conditions increasing later in the season. A difference in any single day could prove to increase the growth and accumulation to aflatoxin.

Correlations were vastly different for each of the programs' materials and entered checks which suggest that visual ratings used for selection would be highly dependent on varieties and populations, for instance, the MS program lines had non-significant correlations with AF% and transformed aflatoxin of r = -0.32, while the TAMU-College Station lines had a correlation of r = 0.44 and were significant at $P < 0.10$ in the same analysis (Table 4). The difference in program to program correlations was unexpected but was likely due to segregation of genetic background traits that inhibit the formation of the toxin, but not the growth of the producing fungi. This phenomenon has been noted before (Henry, et al., 2009). In addition to genetic background it could be related to the type of inoculation used for testing in the breeding program, non-wounding for TAMU-CS and wounding for MS. It is intriguing to consider that lines with high visual AF% ratings and low actual aflatoxin accumulations could have some genetic factor limiting the accrual of toxin while allowing *A. flavus* growth. If confirmed, this alternate form of decreased susceptibility could be used in pyramiding multiple physiological resistances that will decrease overall aflatoxins. The lines of interest that had high %AF but low aflatoxins, thereby deviating from the correlation included Mp14:179, GT1378, and the only two lines studied with red pericarps. The line (RedEar5-1-4-1-3/RedHybridEar-B-1-1-3)-1-1-B-B-2-B2-B15-B15 has a dark red pericarp, shown to increase phenols (Mahan, et al., 2013; Meng, et al., 2015) which can act as antioxidants. Antioxidants have been shown to reduce not only *A. flavus* growth but also to inhibit aflatoxin

biosynthesis (Nesci, et al., 2003). Further supporting this, the line (LH195\X\(RedEar5-1-4-1-3/RedHybridEar-B-1-1-3)-1-1-B-B-2-B2-B15///LH195)-B2////LH195)-1, is a BC_2F_2 red seeded version of LH195; LH195 was a consistently used exPVP line in this study which did not deviate from the %AF ratings and aflatoxin level correlation; this finding suggests it could be the red pericarp trait that contributed to overall low aflatoxins, however more investigation is needed to confirm this hypothesis.

Table 3 Significant Pearson's correlations on all data and all years, across all locations as BLUPs for DTA, DTS, PHT, EHT, yield (T ha^{-1}), visual ratings for ear worm (EW%), visual ratings for aflatoxin sporulation (AF%), visual ratings for fusarium sporulation (FU%), aflatoxins in ppm (AFppb), and transformed aflatoxin data (Aftrans).

Variable	by Variable	Phenotypic Correlation	Genotypic Correlation
AF(%)	AFppb	0.18***	0.27***
AF(%)	DTA	-0.23***	-0.21*
AF(%)	DTS	-0.22***	-0.13
AF(%)	EHT	0.07*	0.10
AF(%)	FU(%)	0.2***	0.23***
AF(%)	T ha^{-1}	-0.03	0.12
AF(%)	PHT	0.03	0.12
AFppb	DTA	0.02	-0.19*
AFppb	DTS	0.03	-0.18*
AFppb	EHT	-0.05	-0.03
AFppb	T ha^{-1}	-0.12***	-0.02
AFppb	PHT	-0.07*	0.05
AfTrans	AF(%)	0.22***	0.38***
AfTrans	AFppb	0.71***	0.79***
AfTrans	DTA	0.06	-0.40***
AfTrans	DTS	0.07*	-0.40***
AfTrans	EHT	-0.04	-0.18
AfTrans	EW(%)	0.24***	0.30***

AfTrans	FU(%)	0	0.10
AfTrans	T ha^{-1}	-0.01	0.20*
AfTrans	PHT	-0.01	-0.07
DTS	DTA	0.98***	0.95***
EHT	DTA	0.45***	0.60***
EHT	DTS	0.46***	0.63***
EHT	PHT	0.73***	0.67***
EW(%)	AF(%)	0.27***	0.22***
EW(%)	AFppb	0.24***	0.35***
EW(%)	DTA	-0.36***	-0.13
EW(%)	DTS	-0.34***	-0.10
EW(%)	EHT	0.05	-0.10
EW(%)	FU(%)	0.3***	0.28***
EW(%)	T ha^{-1}	-0.13***	-0.25***
EW(%)	PHT	0.13***	-0.05
FU(%)	AFppb	0.05	0.09
FU(%)	DTA	-0.02	0.04
FU(%)	DTS	-0.05	0.05
FU(%)	EHT	0	0.08
FU(%)	T ha^{-1}	-0.06*	-0.06
FU(%)	PHT	0.12***	0.21*
T ha^{-1}	DTA	-0.06	-0.34***
T ha^{-1}	DTS	-0.11***	-0.45***
T ha^{-1}	EHT	-0.21***	-0.28***
T ha^{-1}	PHT	-0.07*	-0.16
PHT	DTA	0.44***	0.43***
PHT	DTS	0.44***	0.46***

*, significant at the .1 level; **, significant at the .05 level; ***, significant at the .01 level.

Table 4 Pearson's correlations by germplasm group on BLUPs for DTS, yield (T ha^{-1}), visual ratings for aflatoxin sporulation (AF%), and transformed aflatoxin data (Aftrans) across all locations and all years.

		GA		LUBB		MS		Susceptible		TAMU	
Variable	by Variable	Correlation	Count	Correlation	Count	Correlation	Count	Correlation	Count	Correlation	Count
T ha^{-1}	DTS	-0.33	20	0.14	15	-0.61**	17	-0.34	5	0.19	24
AF(%)	DTS	-0.23	13	0.18	15	0.29	16	0.68	5	-0.64**	20
AF(%)	T ha^{-1}	0.13	13	0.43	15	-0.22	16	-0.18	5	0.14	20
Aftrans	DTS	-0.03	20	0.27	15	-0.09	17	-0.17	5	-0.43**	24
Aftrans	T ha^{-1}	-0.42*	20	0.42	15	0.50**	17	-0.45	5	-0.11	24
Aftrans	AF(%)	0.54*	13	0.40	15	-0.32	16	-0.62	5	0.44*	20

3.2.5 Recommendations for specific elite lines

A major goal was to identify individual lines, beyond average responses by program that appear promising for low aflatoxin accumulations and/or good yield. CY2, GT1307, Mp14:2148, Tx775, Tx777, Tx779, ANTIGO4 all consistently performed well for both high yield and low aflatoxin accumulation, but most of these were undesirably late in flowering time. Tx777 also performed well when used as a parent in the SERAT trials (Wahl, et al., 2017). Several of these inbreds were shared as a parent in the hybrid performance trial SERAT. Many of these hybrids did exhibit decreased susceptibility to the accrual of aflatoxin which could be inherited from the inbred parent. The earliest maturing of these lines was GT1307, averaging 70 days; this was considerably fewer days (-12) than the tolerant check Mp313E and similar to that of ex-PVP material. Flowering can be adjusted by selecting different parents to combine and make hybrids. However, the two lines must nic (flower at the same time) to produce sufficient seed and a split-delay planting can only be used to a point in adjusting inbred nic.

There were four susceptible checks used in testing for reduced aflatoxin accumulation, however one inbred, CY4, continually accumulated extremely high aflatoxins in multiple years. CY4 accumulated significantly more toxin than the susceptible checks in many locations with accumulations more than 1900 ng/g more than the next highest inbred. CY4 was evaluated in the South and could be classified as an improved susceptible check while still being relevant to Southern growers. CY4 could also be useful to use in a genetic linkage mapping population to better understand the mechanisms for susceptibility; increasing knowledge on how particular inbreds

accumulate aflatoxins could yield some insight on decreasing susceptibility and could be used in breeding to elucidate analysis.

3.3 CONCLUSIONS

The most robust inbred lines tested throughout this study have shown substantial reductions in aflatoxin accumulation along with favorable agronomics for hybrid production. While no complete resistance has yet been elucidated, collection of additional alleles towards decreasing susceptibility is essential in developing germplasm that could yield quantitative resistance. Testing across five years and nine environments captured high *A. flavus* infections and showed the high repeatability needed to discriminate germplasm. The common garden test performed in these trials detected trends in how each germplasm directly or indirectly tolerates infection and aflatoxin accumulation, e.g. later flowering lines had decreased *A. flavus* infection, likely due to, in part, inoculation avoidance. Using knowledge of these trends can allow effectively selecting parental lines for population development that can be pyramided into future lines; this is currently practiced in multiple public programs across the southern U.S.A. Using the knowledge gained from this study along with many other aflatoxin accumulation studies performed, lines that have better interactions with agronomic, atoxigenic, genetic, and environmental factors can be elucidated, and better germplasm selections can be made in the future.

4. CONTROL OF AFLATOXIN USING ATOXIGENIC STRAINS AND IRRIGATION MANAGEMENT IS COMPLICATED BY HYBRID DIVERSITY

Aflatoxin is a harmful carcinogenic mycotoxin produced by *Aspergillus flavus* Link:FR, which limits the marketability of maize (*Zea mays* L.) grain and reduces the economic value for producers. Actual economic losses are difficult to measure, but are thought to be around $163 million per year in the United States in maize only, and up to $500 million annually in as peanuts (*Arachis hypogaea* L.) and other crops. (Wu, 2015). Documented or suspected cases of acute aflatoxin poisoning are numerous throughout the world and result in liver damage, intestinal bleeding, cancer, and even death (Lewis, et al., 2005); especially in developing countries which lack infrastructure to test for contamination, and allow contaminated maize to flow freely in local trade. The effects of chronic exposures to aflatoxins are even more challenging to test. More than 100 countries have some type of regulations on aflatoxin concentrations (Wu and Guclu, 2012). In the United States, aflatoxin is regulated for human consumption with an upper limit of 20 (ng g^{-1}); and only maize under 300 (ng g^{-1}) can be used for finishing beef, with lower limits for swine and poultry feed (Stoloff, et al., 1991). The limits are set by the U.S. Food and Drug Administration.

It remains unclear why *A. flavus* makes aflatoxin, but it may prevent insect predation (Drott, et al., 2017; Gqaleni, et al., 1997). Pre-harvest colonization of maize and the subsequent production of aflatoxins are associated with, and likely are a result of, an increase in physiological stresses of crop production (Klich, 2007). High daytime and nighttime temperatures, along with occurrences of drought and insect pressure, have

been shown to increase the occurrences of aflatoxin contamination (Abbas, et al., 2002). To reduce pathogen pressure and toxin accumulation, producers decrease stresses through cultural practices and management, when economical. It is well known that changing management effects within environments, such as optimal fertilization and irrigation effects can impact aflatoxin accumulation, generally procedures that reduce plant stress reduce aflatoxin (Payne and Widstrom, 1992; Robens and Cardwell, 2003). In recent years, atoxigenic strains of *A. flavus* have become an additional management tool to reduce aflatoxins (Abbas, et al., 2011). These atoxigenic strains have a defective mutation in the aflatoxin pathway. The spores of atoxigenic strains are then spread throughout producers' fields on inoculated grain (wheat or sorghum) to outcompete the native aflatoxin producing strains. However, while this competitive exclusion decreases aflatoxin per se, by 40-95% (Abbas, et al., 2011; Brown, et al., 1991; Isakeit, et al., 2010; Isakeit, et al., 2011), this inoculation typically increases ear rot of the *A. flavus* fungus.

Plant-breeding is an important component of the integrated pest management approach to decrease aflatoxin and *A. flavus* (Brown, et al., 2011; Williams, 2006). Breeding for decreased susceptibility by selecting for heritable segregating traits such as tighter, thicker and closed husk cover, or insect resistance (native or transgenic), can reduce toxin accumulation (Widstrom, et al., 2003). Hybrid and inbred selections in maize have been shown to reduce pre-harvest aflatoxin accumulation by up to 90% (Abbas, et al., 2011; Brown, et al., 1991; Murray, et al., 2019; Pekar, et al., 2019; Wahl, et al., 2016). A major challenge of breeding inbred lines for aflatoxin or yield is that

inbred lines appear to be less robust across environments (i.e. experience more genotype x environment interactions or GxE) and experience more stress leading to higher levels of contamination, in a way that hybrid vigor masks in hybrids (Cole, et al., 2009; Li, et al., 2018; Schnell and Becker, 1986). To evaluate the susceptibility of maize genotypes to *A. flavus* and aflatoxin accumulation uniformly, inoculation must be used. There are two major classes of *A. flavus* inoculation methods, wounding and non-wounding (Tucker Jr, et al., 1986). Wounding inoculation techniques offer consistent infections that mimic insect or mechanical damage, bypassing tolerances from physiological traits such as husk cover or tightness, natural insect tolerance, pericarp thickness, and erect or non-erect (drooping) ears. Non-wounding methods, such as those used by farmers to apply atoxigenic strains, do not bypass other physiological tolerances and evaluate susceptibility in a way that is more relevant to farmers (Williams, et al., 2002; Windham, et al., 2009). The different types of inoculation methods have various advantages and disadvantages (Williams, et al., 2013). In this research, we chose to use a non-wounding technique, ground kernel inoculation, because it more closely mimics natural inoculation conditions and atoxigenic *A. flavus* deployment. This technique also reduces the labor needed, allowing the evaluation of a larger number of genotypes and replicates for aflatoxin accumulation.

If the aflatoxin reducing effects of improved hybrids (including insect resistance traits), improved agronomic management and deployment of *A. flavus* atoxigenic strains worked synergistically, aflatoxin should be nearly eliminated from producers' fields when these approaches are combined, however no investigation into these interactions

has yet been attempted. It is possible that these effects could be independent or antagonistic as opposed to synergistic and cumulative, which would be important in developing systems-level recommendations. A comprehensive investigation into interactions between Genotype (G) X Environment (E) X Management (M) X Atoxigenic applications (A) is labor intensive requiring a large number of replications to ensure enough statistical power to test the many interactions within a model.

Perhaps due to the complexity of looking at such interactions, most control methods have only been investigated in isolation. However, it has been previously shown that certain biological agents, such as rhizobia and arbuscular mycorrhizal fungi, have host genetic specificity allowing specific plant genotypes to allow host fungal interaction (Kloepper, 1996; Smith and Goodman, 1999). In the case of maize, it has been shown that different host genetics allow variable susceptibility to *A. flavus* (Abbas, et al., 2011; Brown, et al., 1991; Murray, et al., 2019; Pekar, et al., 2019; Wahl, et al., 2019) but this has typically been measured using aflatoxin accumulation. *A. flavus* is necessary for but insufficiently correlated to actual aflatoxin production (Henry, et al., 2009). *A. flavus* load itself is difficult to impossible to measure in plants even with tools like RT-PCR (Mideros et al. 2009). Without GxA data available, it has been necessary to assume that atoxigenic strains of *A. flavus* would act in a similar manner to the toxigenic strains. However, it is reasonable to hypothesize that certain plant phenotypes or unseen genetic interactions could induce preferential susceptibilities to infection of either the atoxigenic or the native toxigenic strains. This interaction could potentially be exploited for gains in the synergistic reduction of aflatoxin accumulation through G x A. Ignoring

this interaction could allow farmers to apply atoxigenic strains in hybrids that primarily host toxigenic strains. Similarly, it is commonly observed that some germplasm is more adapted to certain agronomic conditions than others; for instance some lines are more susceptible to water and nutrient stress than others (G x M; (Jones, et al., 1981). While we hypothesize that correctly managing G x A x M could decrease aflatoxin by more than 90%, we also hypothesize that ignoring these interactions could result in aflatoxin levels higher than expected.

The specific objective of this study were to 1) evaluate the aflatoxin accumulation in diverse commercial and experimental hybrids known to have varying levels of susceptibility to aflatoxin; 2) Evaluate the sources of variance from the main effects of E, M (irrigation vs. dryland) and A (atoxigenic and toxigenic inoculated vs. toxigenic only) ; and finally 3) Evaluate the proportion of variance from the interactions between main effects to determine synergistic or antagonistic relationships between aflatoxin control measures. This study will suggest how closely researchers, agricultural companies and farmers should monitor these control measures for real-world reductions in aflatoxin accumulations.

4.1 MATERIALS AND METHODS

Beginning in 2014, irrigated (IRR) and dryland (DRY) trials were initiated in Corpus Christi, TX (CC) and College Station, TX (CS). Experimental design consisted of a modified split-plot RCBD of 32 entries in 2014, and 28 entries the following years (2015, 2016 and 2017) in two locations (CC, CS) each having two management approaches (irrigated with optimal fertilization and dryland with limited fertilization;

IRR, DRY) with each having two inoculation types (atoxigenics applied -ATOX, versus no atoxigenics - noATOX) each with four replications, ensuring appropriate statistical power for investigating these interactions. In the split-split-split-plot treatments, irrigated and dryland was the main split followed by the atoxigenic application split followed by genotypes.

Fields were selected for uniformity to minimize residual error from unexplained field variation. The size of the trial was 0.70 hectares, and design was an important factor for trying to reduce residual (unexplained) error. Replications within trials were randomly allotted so that field variation was minimized. Field variation has typically been associated with range and row effects (also called row and column respectively) that we generally attribute to fertilizer and flood irrigation. In College Station, trials and replications within trials were placed so that the furrow irrigation effects were as uniform as possible. This was less of a concern in Corpus Christi due to the installation of sub-surface drip.

4.1.1 *A. flavus* inoculation

Half of the plots (ATOX) were treated with atoxigenic strain Afla-Guard (Syngenta Crop Protection, 2017) at a rate of 22.3 kg/ha. All plots were then treated with *A. flavus* isolate NRRL 3357 grown on autoclaved corn kernels. In brief, toxigenic inoculum was applied shortly after the majority of plots had started silking at an amount of 6 oz. per 9.14 linear row meters, usually around 12 days after the start of silking. Inoculum was prepared from stock *A. flavus* isolate NRRL 3357 grown on potato

dextrose agar. Plates were incubated at 30 °C for 5 to 7 days. Once plates sporulated, they were covered with parafilm and stored at 4 °C. One square cm sample was cut from the source plate and placed in a test tube with 15 ml sterile water plus 0.01 ml of Tween 20 and vortexed. Additional samples were plated using 0.05 ml from the parent plate and incubated for 5 to 7 days at 30 °C. Seven thousand cc of field corn and 3 L of distilled water were placed into a 46 cm × 38 cm × 13 cm autoclavable plastic tray and covered with aluminum foil and autoclaved for one hour. Once autoclaved, the corn was transferred into a translucent 140-quart (132 L) plastic bag. Twenty-five to 30 petri dish cultures were blended with 2 L distilled water and 1 ml Tween 20. Five hundred milliliters of this mixture were applied to each bag of corn and mixed thoroughly, then placed in a room with a constant temperature of 35 °C – 38 °C while mixing every 12 hours. Sporulation occurred 2 to 3 days after inoculation . Similar to Farfan et al. (2014) following maturity, sub-samples were taken from the research combine. These samples were then ground in a Romer mill (Romer Labs, Union, MO), and aflatoxin levels were measured via the Vicam AflaTest (VICAM, Watertown, MA).

4.1.2 Traits measured

Secondary phenotypic traits were measured at the College Station location in all years for days to anthesis (DTA), days to silking (DTS), plant height (PHT) from the ground to the tip of the tassel, and ear height (EHT) from the ground to the primary ear's node attachment point. Grain yield was measured on a plot basis with a JD 3300 modified research combine retrofitted with a Harvest Master classic grain gage for total

plot weight, moisture, and test weight (Juniper Systems, 1993-2017) and subsamples were taken from each plot during harvest. Many of the same traits were taken most years in Corpus Christi, however the ears were hand-harvested.

4.1.3 Statistical analysis

Statistical analysis was conducted using R (R Development Core Team, 2010). Assuming a non-normal distribution, as almost always the case for aflatoxin measurements, all aflatoxin data was first transformed by logarithmic transformation in an attempt to normalize the data (Betran, et al., 2002; Williams and Windham, 2015). Models were then analyzed with both raw and transformed data using lme4 R package (Bates, et al., 2015) and validated with JMP (JMP®, 1989-2019).

Data were first analyzed jointly across all years, locations and managements to evaluate overall trends and then were analyzed individually by environments (location by year). In the all-years-combined analysis (eq. 2), hybrids effects were treated as fixed while all other variables were random. In order to deduct contribution of variation from all factors, an all-random model was used for both full model and separate year analysis. Here range and row effects (equivalent to those sometimes-called row and column effects) reflect the terminology used in furrow irrigation where water and tractors drive down the row. The full model was fit as a 5-level full factorial design. All means were compared using Fisher's Protected LSD (P = 0.05). Repeatability (h^2) was calculated as:

$$h^2 = \mathrm{G} \,/\, (\mathrm{G} + \frac{\mathrm{GE}}{\mathrm{r}} + \frac{\mathrm{\mathcal{E}}}{\mathrm{re}})$$

Eq. 3

where G, GE and Ɛ were the variance components of genotype, genotype by environment interaction and residual error respectively, with r as number of replications and e as number of environments.

Correlations were compared using Pearson correlation coefficients. Phenotypic correlations were formed on raw data, while genotypic correlations were formed from the best linear unbiased predictor (BLUP) genotypic estimates of each trait.

Table 5 Summary statistics on BLUPs for transformed aflatoxin data, actual ng g-1 of aflatoxin, yield, days to 50% anthesis, days to 50% silking, plant height, and ear height separated by location and years. Environment is a combination of location and year CS

		all years	CC14	CC15	CC16	CC17	CS14	CS15	CS16	CS17
Log-transformed (Aflatoxin (ng g^{-1})+1)	Max	2.5	2.5	1.8	1.8	2.5	1	1.9	2.6	1.1
	Mean	1.1	2.1	0.8	1.4	1.7	0.5	0.6	1.2	0.4
	Min	0.5	1.6	0.3	0.7	0.8	0.1	0.2	1.7	0.4
	Std Dev	0.3	0.2	0.4	0.2	0.4	0.2	0.4	0.3	0.2
Aflatoxin (ng g^{-1})	Max	391.1	477.4	187.7	142.8	412.3	120.6	146.9	481.9	179.5
	Mean	85.9	204.3	35.7	72.6	122.9	30.5	12.8	217.7	55.4
	Min	-30.5*	67	2.4	14.3	15.4	1.9	0.9	77.3	5.9
	Std Dev	68.1	95.4	45.1	37	96.9	30.1	27	112.3	41.2
Test weight	Max	61.8	58.5	62.1	54.8		61.6	61.1	59	60.6
	Mean	57.7	54.7	59.2	56.1		58.3	58.8	55.2	58.2
	Min	53.3	51.1	54.4	53.4		54.8	56	51.9	55.2
	Std Dev	1.8	2	1.9	0.9		1.7	1.5	1.9	1.3
Yield (MT ha^{-1})	Max	9.5	8	9.6	7.4	4.8	12.1	9.7	10.5	10.3
	Mean	7.7	5.3	7.2	5.9	3.4	10.3	8.3	9	8.6
	Min	3.7	3.4	5.5	5.3	2	6.6	6.8	6.8	6.7
	Std Dev	1	1.2	1	0.5	0.6	1.3	0.7	0.8	0.8
Days to anthesis	Max	68.3	-	-	-	57.5	83.1	65.3	66.8	66

	Mean	63.3	-	-	-	54.7	78.5	61.6	61	60.8
	Min	59.2	-	-	-	51.9	75.4	57.1	58.1	57.2
	Std Dev	1.9	-	-	-	1.2	1.8	2.2	2.1	2.4
Days to silking	Max	69.5	-	-	-	59.1	82.9	67.4	67.4	67.5
	Mean	63.3	-	-	-	55.6	79.1	63.1	61.7	61.4
	Min	59.2	-	-	-	53.3	75.7	57.9	58.1	57.3
	Std Dev	1.9	-	-	-	1.5	1.8	2.8	2.3	2.9
Plant height (cm)	Max	254.9	245.5	205.2	227.3	216.3	275.1	238.6	259.7	274.2
	Mean	227.8	226.8	179.3	214.5	190.8	252.2	220.2	237.3	240.3
	Min	197.6	205.3	146.1	204.9	168.4	222.1	199.4	218.6	211.3
	Std Dev	11.5	11.2	15.4	5.3	10.5	12.4	9.7	10.9	13.9
Ear height (cm)	Max	115.1	103	65.3	97.6	93.6	113.5	94.9	123.2	117.6
	Mean	82.6	85.7	46.3	87.3	4.5	96.2	75.5	86.1	4.2
	Min	57.3	71.6	31.8	74.9	61.8	78.6	63.3	68.4	59.7
	Std Dev	10.1	6.8	8.6	5.9	8.1	9.5	8.6	12.4	14.1

*a negative BLUP value was an artifact of the model fitting all years' combined analysis.

4.2 RESULTS AND DISCUSSION

To best discriminate genotypes and other treatment effects, higher overall aflatoxin levels are useful. Here aflatoxin accumulation was sufficient to discriminate genotype sources of variation in all years. In College Station, aflatoxin accumulation was highest during the 2016 growing season, reaching levels up to 940 ng g^{-1} for an individual sample, well above the 300 ng g^{-1} upper regulatory limit. Best linear unbiased predictions (BLUPs) means in this same environment were 218 ng g^{-1}. Corpus Christi levels during the 2016 season reached levels of 1800 ng g^{-1} for an individual sample with a mean of 106.5 ng g^{-1}. In the overall analysis for Afppb, the highest variation explained was from the effect of year (40.1%), however, in transformed aflatoxin this variation returned to zero. With non-normal data, which is almost always the case with aflatoxin data, this was likely an anomaly. The treatment for ATOX was successful on average, as the mean of samples across all environments with the atoxigenic treatment applied was 11% lower than untreated. This is substantially less than the 75% to 90% that has been reported, and suggests that factors might not have synergistic effects (Abbas, et al., 2011; Brown, et al., 1991; Isakeit, et al., 2010; Isakeit, et al., 2011). There was no significant difference in aflatoxin due to irrigation. Most likely resulting from minimal soil moisture differences between dryland and irrigation treatments due to sufficient rains occurring during the 2015, 2016, 2017 growing seasons.

Hybrid grain yield means ranged from 3.4 MT ha^{-1} to 10.3 MT ha^{-1} in each environment. Yields were highest in 2015. Yield is a primary grower criterion and the

best experimental hybrid yield was 112% of the hybrid check average in the 2015 environment.

Based on 2017 data, the CC17 location flowered significantly faster at 53 DTA and 53 DTS, while the average across all recorded years was 61 DTA and 60 DTS, respectively. Later than optimal planting was conducted for all environments in hopes to maximize stress for increased aflatoxin pressure. Later planting also compressed flowering among genotypes which was beneficial to apply more uniform inoculations. A drawback to later planting and compressed flowering times was that there was less variation to determine how flowering time might have affected aflatoxin and grain yields.

PHT and EHT were recorded in all environments (Year x Location) with a BLUP mean range of 179.3cm to 252.2cm and an EHT BLUP mean range of 46.3cm to 96.2cm suggesting variation in stress between environments which limited or favored plant growth. In environments with lower PHT and EHT, yield also tended to be lower which is commonly observed in the stressful conditions of Texas (Farfan, et al., 2013; Liu and Wiatrak, 2011; Yin, et al., 2011).

Table 6 Variance components and percent total for traits measured in all years and locations combined for transformed aflatoxin value (Aftrans), actual measured aflatoxin accumulation in parts per billion (Afppb), yield (T ha-1), plant height (PHT), ear height

model	Aftrans	%	Afppb	%	T ha-1	%
Env*Year*IRR*INOC*Gen	1.93E+02	0.8%	6.33E-11	-	1.67E-09	-
Year*IRR*INOC*Gen	1.34E+02	0.6%	-	-	4.00E-09	-
Env*IRR*INOC*Gen	-	-	4.61E-03	0.4%	-	-
IRR*INOC*Gen	-	-	-	-	1.18E-08	-
Env*Year*INOC*Gen	4.83E+02	2.0%	1.37E-10	-	-	-
Env*Year*IRR*Gen	3.68E+02**	1.6%	2.60E-02	2.5%	6.29E-02	0.4%
Env*INOC*Gen	-	-	1.26E-03	0.1%	3.50E-07	-
Year*INOC*Gen	1.28E+01	0.1%	3.71E-09	-	3.95E-08	-
Year*IRR*Gen	-	-	2.32E-09	-	1.03E-06	-
Env*IRR*Gen	1.96E+02	0.8%	4.86E-04	-	-	-
Row*Loc	-	-	8.80E-03	0.8%	2.24E-01***	1.4%
INOC*Gen	-	-	2.09E-03	0.2%	-	-
IRR*Gen	-	-	-	-	-	-
Env*Year*Gen	1.79E+03	7.6%	9.94E-06**	0.0%	1.08E-01**	0.7%
Env*Gen	1.35E+03	5.7%	2.60E-02	2.5%	7.06E-02	0.4%
Range*Loc	2.66E+02*	1.1%	2.84E-03***	0.3%	2.18E-01***	1.3%
Gen	9.71E+02**	4.1%	3.90E-02	3.7%	6.70E-01**	4.1%
Rep*Loc	5.32E+02***	2.2%	8.91E-03***	0.8%	3.14E-02**	0.2%
Env*Year*IRR*INOC	-	-	9.53E-10	-	-	-
Year*IRR*INOC	-	-	-	-	-	-
Env*Year*INOC	1.24E+03	5.3%	4.95E-03	0.5%	3.71E-08	-
Env*Year*IRR	1.30E-05*	-	2.90E-01	27.4%	5.49E-01	3.4%

Env*IRR*INOC	-	-	1.07E-10	-	1.14E-08	-
Year*INOC	3.35E-05	0.6%	2.85E-08	-	-	-
Year*IRR	1.40E+02	0.6%	2.36E-07	-	2.08E-01	1.3%
Env*Year	1.29E+03	5.5%	4.24E-01	40.1%	5.54E-02	0.3%
IRR*INOC	-	-	5.39E-11	-	-	-
Env*INOC	-	-	2.32E-02	2.2%	-	-
Env*IRR	-	-	-	-	-	-
Year	5.76E+03	24.3%	4.39E-03	0.4%	6.23E-01	3.8%
INOC	1.08E+03	4.6%	2.68E-02	2.5%	-	-
IRR	7.00E+01	0.3%	4.95E-08	-	4.09E-01	2.5%
Env	-	-	1.72E-06	-	1.23E+01**	75.5%
Residual	7.79E+03	32.9%	1.65E-01	15.6%	7.68E-01	4.7%
Total	2.37E+04	100.0%	1.06E+00	100.0%	1.63E+01	100.0%
R	73.21%		88.33%		95.61%	

NS, not significant; *, significant at the .1 level; **, significant at the .05 level; ***, significant at the .001 level.; R, repeatability; significance tested using Log Likelihood

Table 6. Continued.

model	PHT	%	EHT	%
Env*Year*IRR*INOC*Gen	-	-	-	-
Year*IRR*INOC*Gen	-	-	-	-
Env*IRR*INOC*Gen	-	-	-	-
IRR*INOC*Gen	#######	-	-	-
Env*Year*INOC*Gen	-	-	-	-
Env*Year*IRR*Gen	-	-	#######	0.80%
Env*INOC*Gen	-	-	-	-
Year*INOC*Gen	-	-	-	-
Year*IRR*Gen	-	-	-	-

Env*IRR*Gen	-	-	-	-
Row*Loc	8.49E+00***	0.60%	#######	0.70%
INOC*Gen	#######	-	#######	0.40%
IRR*Gen	#######	0.20%	#######	0.50%
Env*Year*Gen	7.30E+00**	0.50%	#######	1.60%
Env*Gen	#######	0.60%	2.60E+01**	7.50%
Range*Loc	2.15E+01***	1.60%	5.89E+00***	1.70%
Gen	1.09E+02***	8.20%	6.35E+01***	18.20%
Rep*Loc	4.62E+00**	0.30%	1.76E+00*	0.50%
Env*Year*IRR*INOC	-	-	-	-
Year*IRR*INOC	-	-	-	-
Env*Year*INOC	-	-	-	-
Env*Year*IRR	-	-	-	-
Env*IRR*INOC	-	-	#######	-
Year*INOC	-	--	-	-
Year*IRR	#######	1.10%	#######	2.60%
Env*Year	#######	12.10%	#######	-
IRR*INOC	-	-	-	-
Env*INOC	-	-	-	-
Env*IRR	#######	3.30%	#######	3.70%
Year	#######	-	#######	21.70%
INOC	-	-	#######	-
IRR	-	-	-	-
Env	#######	65.40%	#######	10.10%
Residual	#######	6.10%	#######	30.30%
Total	#######	100.00%	#######	100.00%
R	97.33%		94.59%	

NS, not significant; **, significant at the .05 level; ***, significant at the .001 level; R, repeatability; significance tested using Log Likelihood

Table 6. Continued.

model	DTS	%	DTA	%
Env*Year*IRR*INOC*Gen	-	-	-	-
Year*IRR*INOC*Gen	-	-	4.28E-10	-
Env*IRR*INOC*Gen	6.05E-08	-	7.38E-09	-
IRR*INOC*Gen	7.93E-08	-	3.21E-09	-
Env*Year*INOC*Gen	2.99E-08	-	1.95E-02	-
Env*Year*IRR*Gen	-	-	3.40E-08	-
Env*INOC*Gen	6.59E-09	-	1.65E-08	-
Year*INOC*Gen	3.25E-08	-	2.97E-02	-
Year*IRR*Gen	-	-	6.46E-09	-
Env*IRR*Gen	8.08E-08	-	3.29E-07	-
Row*Loc	2.51E-01***	0.2%	9.18E-02***	0.1%
INOC*Gen	1.17E-02	-	-	-
IRR*Gen	1.53E-02	-	3.89E-06	-
Env*Year*Gen	1.61E-01**	0.1%	1.41E-01**	0.1%
Env*Gen	1.63E+00***	1.5%	1.29E+00***	1.3%
Range*Loc	4.39E-01***	0.4%	1.85E-01***	0.2%
Gen	3.49E+00***	3.2%	2.24E+00**	2.2%
Rep*Loc	6.88E-03**	-	1.29E-01***	0.1%
Env*Year*IRR*INOC	3.47E-08	-	-	-
Year*IRR*INOC	-	-	-	-
Env*Year*INOC	1.73E-08	-	3.79E-10	-
Env*Year*IRR	1.28E-01*	0.1%	4.57E-02	-

Env*IRR*INOC	5.48E-08	-	4.06E-09	-
Year*INOC	1.36E-07	-	9.66E-09	-
Year*IRR	-	-	-	-
Env*Year	4.83E+00	4.5%	1.21E+01	12.0%
IRR*INOC	4.72E-06	-	1.29E-10	-
Env*INOC	7.11E-03	-	-	-
Env*IRR	-	-	4.62E-02	-
Year	8.46E+01	78.6%	7.43E+01	74.1%
INOC	-	-	1.20E-08	-
IRR	1.20E-06	-	2.37E-08	-
Env	1.07E+01	9.9%	8.74E+00	8.7%
Residual	1.34E+00	1.2%	9.15E-01	0.9%
Total	1.08E+02	100.0%	1.00E+02	100.0%
R	98.53%		98.36%	

NS, not significant; **, significant at the .05 level; ***, significant at the .001 level; R, repeatability; significance tested using Log Likelihood

1

4.2.1 Variance components and repeatability: Aflatoxin

Variance components are useful for evaluating sources of variation in an experiment and allow repeatability to be calculated. Env x Year and the Env x Year x Irrigation interaction variables for Afppb explained 40.1% and 27.4%, respectively. However, when aflatoxin data was transformed the major sources of variation fell into Env alone and Env x IRR, 32.9% and 24.3%, respectfully. These major sources of variation for aflatoxin data are interesting, however, are not sources that a farmer would have much control over. Total genotypic variation was 23% for AfTrans and 9% for Afppb in the full model (all collected data), suggesting genotype was a minor to moderate driver of aflatoxin variation in this experiment, however it is one a producer can easily manipulate. This lower genotypic variation also reflects a generally less-susceptible set of varieties tested here. Env x Year x IRR x Gen for transformed aflatoxin explained 1.6% variation and this four-way interaction would be useful in selecting reduced aflatoxin susceptible genotypes for different environments such as dryland in Corpus Christi vs Irrigated in College station. While mean comparisons showed that atoxigenic (ATOX) applications reduced aflatoxin, this main effect explained only 2.4% variation in aflatoxin levels for non-transformed aflatoxin data. When total variation of inoculation is summed, the variation explained was 14%. This demonstrates that for this experiment selecting resistant genotypes would have a larger effect than applying atoxigenic strains. On transformed aflatoxin data, Env x INOC x Gen and Env x Year x INOC x Gen explained 1.6% and 2% variation, respectively. Demonstrating the effectiveness of atoxigenic control applications depended on the different locations and genotypes examined. Due to logistics and knowledge of flowering it was difficult to ensure that

the atoxigenics were applied at the same developmental stage in each environment, which could have led to some of this variation. Gen x INOC explained 2% of total variation, demonstrating that atoxigenics had different effectiveness for different genotypes. However, when aflatoxin data was transformed in log(aflatoxin+1), 0% of the variation was explained, making it possible that this was a statistical anomaly of non-normal data. The full model for transformed aflatoxin data explained 76.7% variation with only 23.3% variation remaining as unexplained residual error.

A substantial interaction of 27.4% was seen between Env x Year x IRR probably brought forth by irrigation or rainfall timing events during inoculations which may have increased or decreased the amount of aflatoxin accumulation across different environments. However, when aflatoxin data was transformed in log(aflatoxin+1), 0% of the variation was explained, making it possible that this was a statistical anomaly of non-normal data. No defined pattern towards higher aflatoxin accumulation in dryland or irrigated splits was observed.

Repeatability was used over heritability due to the lack of family structure in the genotypes. Repeatability is typically high for flowering and height, and lower for yield and aflatoxins (Farfan, et al., 2015; Wahl, et al., 2017). Across all environments, repeatability for aflatoxin accumulation data was 73% using transformed data up to 88% on non-transformed raw data (Table 6). Repeatability for aflatoxin accumulation has been moderate or low in previous studies (Wahl, et al., 2017; Warburton, et al., 2013). Repeatability for aflatoxin accumulation within individual environments was lower than for all other important agronomic traits, but still moderate to high.

4.2.2 Variance components: grain yield and secondary agronomic traits

Within the full model, the significant variation explained for grain yield was from Env (76%) with only 4% explained by the Year. Only 3% of the variation was explained by the irrigation regime. The reduced variation from irrigation could be explained by rainfall amounts during most seasons that limited moisture stress from occurring. Environment explained 64% variation for PHT, similar to grain yield, however only 4% of the variation for EHT was explained by environment. In EHT the variation was more attributable to genetics (35%) whereas it only explained 3% of the variation for PHT.

Flowering variation was mostly explained by Env x Year interaction (DTS = 79%, DTA = 59%). Indicating that flowering time was mostly dependent on the location of the trial. Residual error for both DTS and DTA was 5% and 4%, respectively. Comparing across all agronomic traits, the environment had the greatest effect on creating experimental variability than nearly any other source. This was surprising given these two locations and the three years were relatively similar to each other. This is important because environment is what farmers have the least ability to control, suggesting that for yield and agronomic traits, genetics and management can only meet farmers needs so much.

4.2.3 Correlations

Phenotypic correlations (figure 2) differed between transformed aflatoxin and Afppb compared to yield. Transformed aflatoxin was significant ($p< 0.01$) and correlated negatively with grain yield at r = -0.33*** and Afppb at r = -0.15***. These negative correlations show that better yielding material is less aflatoxin accumulating overall, supporting the hypothesis that adaptation may be more important than resistance per se across diverse elite hybrids. PHT and EHT were significant ($p < 0.01$) and positively correlated with yield at r = 0.57 for PHT and r =

0.24 for EHT. This positive correlation between plant height and grain yield is very similar to that found by Farfan et al. 2013 (r=0.61) across over 10,000 plots of elite commercial hybrids. Positive correlations between PHT and aflatoxin also support the hypothesis that more vigorous material is less aflatoxin susceptible. As well as DTA and DTS which were highly correlated and significant with grain yield ($p < 0.01$) at $r = 0.58$ and $r = 0.61$, respectfully. Later flowering material accumulating higher grain yield is somewhat surprising, because these plants would be subjected to hotter and more stressful conditions during grain fill, however, later material is also more likely to be adapted to sub-tropical environments such as Texas.

Genotypic correlations (figure 3) for transformed aflatoxin accumulation and flowering time were highly significant ($p < 0.01$) and moderately correlated at $r = -0.49$. This decrease in aflatoxin accumulation may have resulted from favorable environmental conditions increasing later in the season or again due to the adaptation of the later material from sub-tropical environments. A difference in any single day could prove to increase the growth and accumulation to aflatoxin or that the later flowering more tropical material tends to be less susceptible to accumulation. This is similar to studies such as: SERAT (Wahl, et al., 2017), (Betran, et al., 2006), and another study directly aimed at maturity and aflatoxin accumulation (Betran and Isakeit, 2004).

In contrast to the phenotypic correlations shown above, genotypic correlations for yield were only lightly positively correlated and significant for PHT ($r = 0.23^*$, $P< 0.10$). Correlations for PHT and yield have been observed in several studies and help to prove that yield is closely correlated with PHT in some growing regions and can be used as an important predictor in plant yield (Farfan, et al., 2015; Katsvairo, et al., 2003; Yin, et al., 2011).

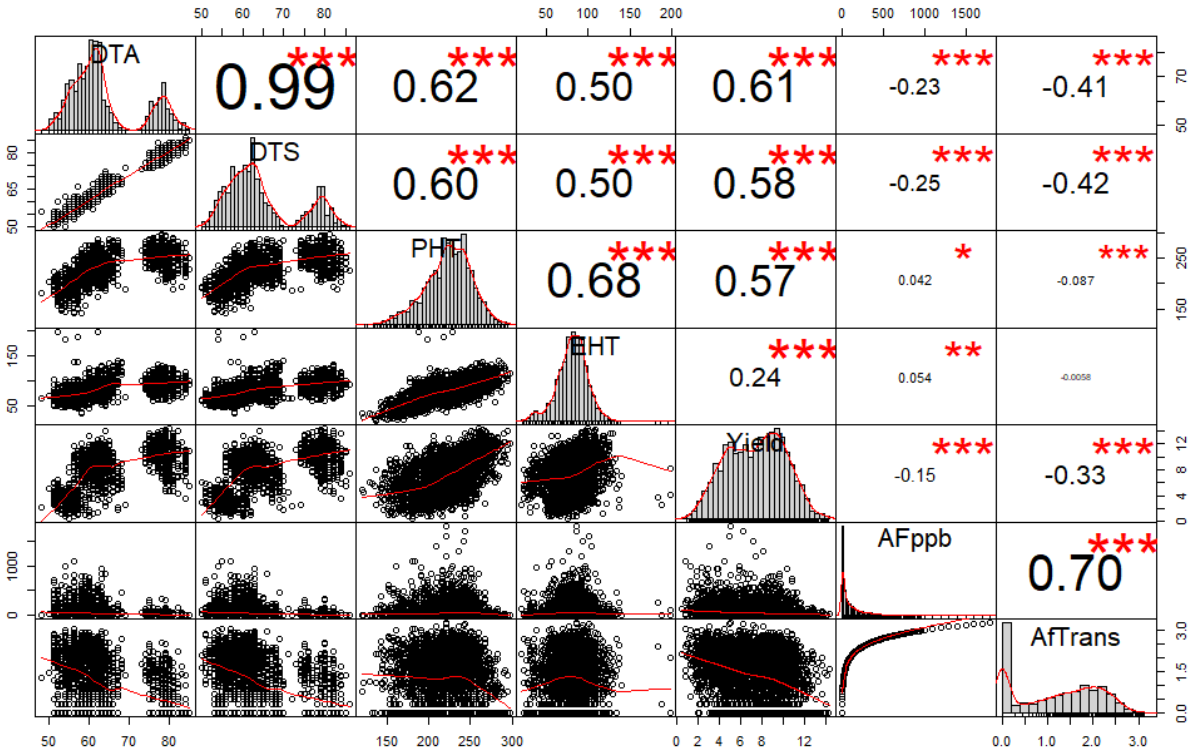

Figure 2 Phenotypic correlations on all raw data and all years, across all locations for DTA, DTS, PHT, EHT, yield (T ha-1), aflatoxins in ppm (AFppb), and transformed aflatoxin data (Aftrans).

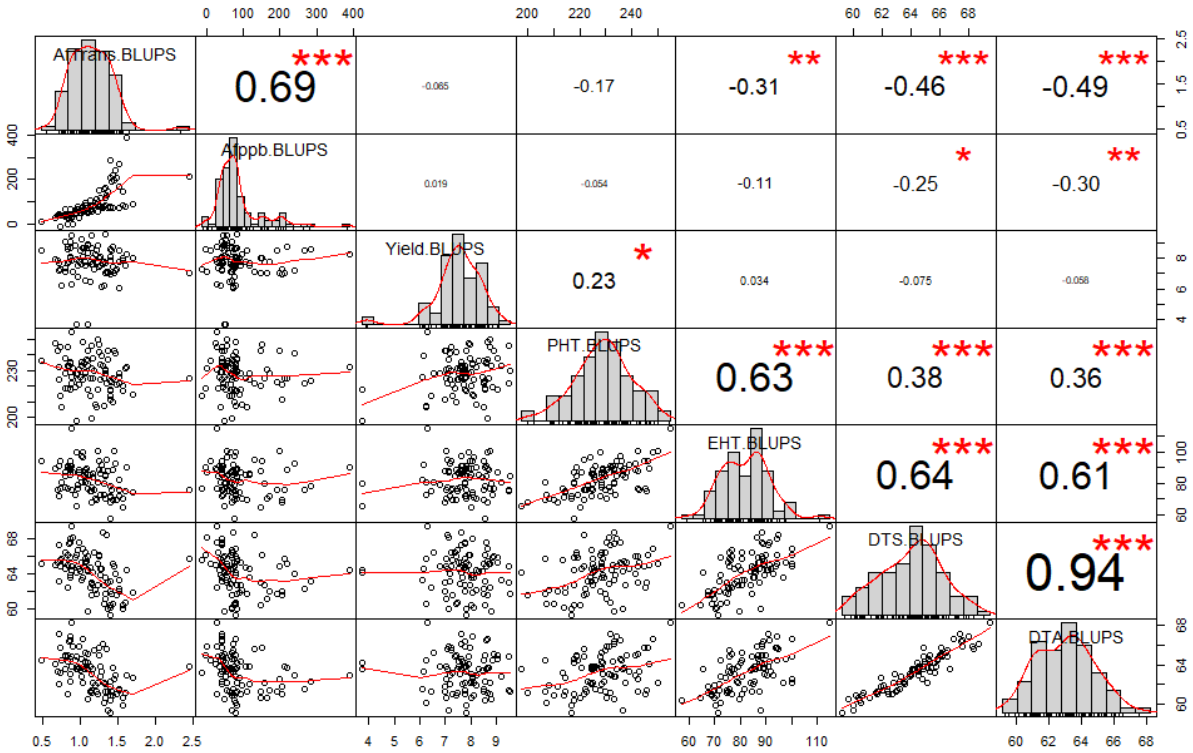

Figure 3 Genotypic correlations on all data and all years, across all locations as BLUPs for DTA, DTS, PHT, EHT, yield (T ha^{-1}), aflatoxins in ppm (AFppb), and transformed aflatoxin data (Aftrans). Larger font indicates a larger correlation.

4.2.4 Recommendations for specific hybrid lines

The Best Linear Unbiased Estimators (BLUES) for each hybrid were obtained across all environments, within specific environments and under specific management conditions. Over the entire experiment hybrids: LH195/Tx777, LH195/Tx779, LH195/(((CML 326/B104)x(CML285/B104))-2-2-B-B-B-B/(CML288/NC300)-B-9-B1-B-B-B-B-B)-B-2-B-B-2-1, TR8145RR2/(((CML 326/B104)x(CML285/B104))-2-2-B-B-B-B/(CML288/NC300)-B-9-B1-B-B-B-B-B)-B-2-B-B-2-1, TR8145RR2/Tx779, and Tx781/Tx777 were estimated to be the best for both lowest aflatoxin and highest grain yield. The Texas by Texas cross Tx781/Tx777 is especially interesting as it is a complete public hybrid and contains tropical-derived germplasm in both parents. Increasing the

interest in this hybrid is that it out yielded the check average for yield in 2015 by 107% and in 2016 by 5%. In addition to the high yield from this line, it accumulated 62% less aflatoxin than the average checks in 2015, while in 2016 it accumulated 15% less aflatoxin than the average checks. Further encouraging the hypothesis that highly adapted sub-tropical hybrids developed for the central-South Texas growing region can reduce total aflatoxin accumulation and benefit the local producers.

Several hybrids did well in both dryland and irrigated regimes (Table 7). Most notable were the hybrids consisting of Bolivian material (Tx777 and Tx779) several hybrids tested shared one of these parents with coded testers. The most interesting is, again, the Tx781/Tx777 hybrid, which seem to be performed well in both dryland and irrigated regimes.

Table 7 Notable hybrids for yield and low aflatoxin accrual.

Year	Pedigree	BLUPs	%Check average	Log_{10}(aflatoxin) BLUPS	%Check average
2014	LH195/(((CML 326/B104)x(CML285/B104))-2-2-B-B-B-B/(CML288/NC300)-B-9-B1-B-B-B-B-B)-B-2-B-B-2-1	126.93 ± 46.09	93.65%	3.1 ± 1.29	-19.06%
	TR8145RR2/(((CML 326/B104)x(CML285/B104))-2-2-B-B-B-B/(CML288/NC300)-B-9-B1-B-B-B-B-B)-B-2-B-B-2-1	126.04 ± 46.09	92.99%	3.27 ± 1.29	-14.62%
2015	NP2643GT/Tx777	135.36 ± 15.87	112%	1.22 ± 0.45	-38%
	Tx779/LH195 (ex-PVP)	134.67 ± 15.9	112%	1.12 ± 0.45	-43%
	Tx781/Tx777	130.48 ± 15.87	108%	1.3 ± 0.45	-34%
	TR8145/Tx777	129.39 ± 15.87	107%	1.1 ± 0.45	-44%
	LH195 (ex-PVP)/Tx777	129.11 ± 15.87	107%	1.14 ± 0.45	-42%
2016	LH195 (ex-PVP)/Tx777	122.5 ± 27.81	103%	3.82 ± 0.95	-10%
	Tx781/Tx777	122.43 ± 27.81	103%	3.81 ± 0.95	-10%
	LH195 (ex-PVP)/Tx779	121.23 ± 27.98	102%	3.71 ± 0.96	-12%
	NP2643GT/Tx777	121.17 ± 27.81	102%	3.87 ± 0.95	-8%

	GP474GT/Tx780	119.84 ± 27.81	101%	3.86 ± 0.95	-9%
2017	TR8145RR2/Tx779	102.97 ± 46.91	103%	2.48 ± 0.45	-39%
	GP474?BGTCBLL (glyphosate+liberty link+corn borer)/Tx779	102.06 ± 46.91	102%	3.5 ± 0.46	-13%
	Tx794/Tx779	97 ± 46.91	97%	3.34 ± 0.45	-17%
	BMP-2/Tx777	96.9 ± 46.91	97%	2.74 ± 0.45	-32%

4.3 CONCLUSIONS

This study demonstrated the complex interactions between different technologies that have been proven to reduce aflatoxin when examined in isolation. Genetics, agronomic management and atoxigenic strains all substantially influenced aflatoxin. While historically studied in isolation, this study showed some variation explained from interactions between genotype by atoxigenic inoculation in differing environments, however these were not found to be significant.

Breeding is an important component of integrated pest management for aflatoxin tolerance. However, full resistance to aflatoxin accumulation has not been found. It has been determined that genotypic variation is significant and can be manipulated by the producer. However small, this can help producers in choosing lower-accumulating varieties. Managing how such varieties are grown agronomically will contribute to an overall decrease or possible complete reduction of aflatoxin accumulation in maize. This study shows that decreases in aflatoxin accumulation can be achieved through selecting genotypes that do better in dryland or irrigated regimes, by inoculating with atoxigenic strains , and the possibility of selecting genotypes that are synergistic with atoxigenic strain applications in reducing toxins. There were no significant interactions between genotype and atoxigenic inoculation, however trends were seen that showed lowered aflatoxin accrual in certain genotypes.

Twelve hybrids did considerably well compared with checks for both aflatoxin accumulation and yield and the majority of entries were of TAMU x TAMU hybrids. This bias will inherently skew the trend to favor the TAMU x TAMU hybrids for tolerance to aflatoxin accumulation.

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