GENOTYPIC AND PHENOTYPIC CHARACTERIZATION OF RESPONSE TO AFLATOXIN AND SECONDARY TRAITS IN MAIZE

A Dissertation

by

MELANIE LOVE EDWARDS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2006

Major Subject: Plant Breeding

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Approved by:

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ABSTRACT

Genotypic and Phenotypic Characterization of Response to Aflatoxin and Secondary Traits in Maize.

(May 2006)

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One major problem facing maize producers in the southern US is contamination with the mycotoxin aflatoxin, produced by *Aspergillus flavus* (Link:fr). Aflatoxin is a serious threat to human and animal health, with no resistant commercial hybrid available.

Development of resistance to aflatoxin production has several major limitations. Aflatoxin is highly variable both across and within environments, even under inoculation, requiring several locations and replications for breeding. Additionally, there is no screening method that is reliable, rapid, inexpensive, and allows for high throughput.

Several secondary traits, such as kernel texture, kernel integrity, husk cover, and visible ear rot, have previously shown to be related to aflatoxin accumulation. These traits are easily characterized in the field

and are candidates for indirect selection if they are correlated to aflatoxin concentration.

Root lodging, a plant's inability to maintain upright stature, is another complex characteristic of root related traits that traditionally is selected for indirectly. It can greatly reduce harvestable yield. It is affected by morphological traits and environmental conditions, but its genetic components are little understood.

This dissertation comprises three studies presented in chapters II, III, and IV. Chapter II involved white and yellow hybrid maize trials as well as quality protein maize trials from several years across Texas environments. Data was analyzed both per and across location to determine repeatability of response to aflatoxin. Additionally, aflatoxin levels were correlated to several secondary characteristics (female flowering, endosperm texture, husk cover, and ear rot ratings) to determine usefulness in indirect selection.

Chapter III was a phenotypic evaluation of a recombinant inbred line (RIL) mapping population, which was derived from divergent parental inbreds Tx811 and CML176. The trials were conducted in two Texas locations, and phenotypic data for aflatoxin concentration, kernel

integrity, endosperm texture, female flowering date, and root lodging was collected. Variance components for these traits and genetic and phenotypic correlations were determined.

Chapter IV was a genotypic evaluation of the Tx811/CML176 mapping population using simple sequence repeat markers. Genotypic and phenotypic data were combined to identify quantitative trait loci (QTL) and epistatic interactions for response to aflatoxin and for root lodging.

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CHAPTER I

INTRODUCTION

Maize (*Zea mays* L.), alongside rice and wheat, is one of the world's three most important staple food crops. Over the past hundred years, plant breeders have vastly increased the yield and quality of the maize being produced. This has been accomplished through improved agronomic characteristics, better disease resistance, as well as the development of hybrid maize production. The United States is the world's largest producer and exporter of maize. About 60% of the maize produced in the US is used for livestock feed. It is produced as a hybrid crop, and the principle grain produced is yellow dent type. While the Midwest US is the predominant maize-producing region (known as the Corn Belt), maize is also a significant crop in much of the southern U.S.

One major problem facing maize producers in the southern US is contamination with the mycotoxin aflatoxin, produced by *Aspergillus flavus* (Link:fr), due to weather conditions in this region that favor its production. While maize breeders have made substantial progress in improving maize's agronomic characteristics, there is still no commercial

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This dissertation follows the style of Crop Science.

hybrid available that is resistant to aflatoxin. Aflatoxin is a serious threat to human and animal health. While many other diseases reduce yield or have other detrimental characteristics, aflatoxin is problematic because of its high toxicity. Often diseased maize can still be sold for feed, but aflatoxin contamination can either severely reduce the value of grain or make it nonmarketable altogether.

Some management techniques can help to reduce pre-harvest aflatoxin. These are the same techniques that improve overall plant health, such as planting adapted varieties, proper fertilization (particularly with appropriate nitrogen levels), weed control, insect control (specifically ear boring insects), necessary irrigation (during grain fill), and proper harvesting (Anderson *et al.*, 1975; Jones and Duncan, 1981; Lillehoj, 1983; Lisker and Lillehoj, 1991). Proper management, however, cannot eliminate aflatoxin altogether. It is therefore desirable to have genetically resistant hybrids for production in regions with conditions favorable to aflatoxin production.

Development of resistance to aflatoxin production has several major limitations. Aflatoxin is highly variable both across and within environments, requiring inoculation for any selection to reduce susceptibility. Even under inoculation, however, aflatoxin accumulation

can be highly variable. Therefore, trials to select for resistance to aflatoxin must be carried out in several locations with several replications. Resource allocation is an important consideration for breeding programs for complex traits that require extensive testing. Additionally, there is no screening method that is reliable, rapid, inexpensive, and allows for high throughput (Payne, 1992).

Breeders do not routinely estimate genetic variances prior to choosing and creating breeding populations in advanced breeding cycles (Bernardo, 2002). Estimates of genetic variances are useful for several reasons (Dudley and Moll, 1969): predicting response to recurrent selection, allocating resources in field performance trials, constructing selection indices, and predicting single-cross performances by the best linear unbiased prediction (BLUP) method. There is little information regarding the genetic variance of response to aflatoxin, and this information would be particularly helpful for both resource allocation and for genetic correlation estimates with secondary traits.

Several secondary traits have shown to be related to aflatoxin accumulation in previous studies (Payne, 1992; Windham *et al.*, 1999; Brown *et al.*, 2001), some of which are candidates for indirect selection, such as kernel texture, kernel integrity, husk cover, and visible ear rot.

These traits are easily characterized in the field. It is desirable to know whether these traits are highly heritable and how they may be correlated to aflatoxin concentration. Correlated response, or genetic gain through indirect selection, is greatest for traits that are highly heritable and strongly correlated to aflatoxin concentration. Thus, in order to ascertain whether a trait proves suitable for indirect selection, the variance components and correlations must be ascertained.

Classic measurements of heritability of a trait involve random populations. A measurement that provides an alternative to the creation of large populations is repeatability (Cooper *et al.*, 1993). Repeatability has previously been used in wheat, which is a self-pollinated predominantly inbred crop. Maize, however, is open-pollinated and produced as a hybrid crop in the United States, and therefore it is desirable to know whether this estimation will provide a similar result in a different genetic structure. Repeatability holds the promise of providing breeders with necessary information about desired traits without the resource consumption of the production of a mapping population. Estimates of repeatability could be compared to estimates of heritability in order to ascertain the accuracy of repeatability estimates in maize breeding trials.

Root lodging, defined as a plant's inability to maintain upright stature, is another complex characteristic of root related traits that traditionally is selected for indirectly (Bruce *et al.*, 2000). Root lodging is a major problem for farmers as it can greatly reduce harvestable yield of maize (Carter and Hudelson, 1988). It is affected by several morphological traits, such as root number, root mass, angle of root growth from stalk, and others. In addition to the complex morphological traits that influence root lodging, environmental conditions greatly influence this trait. Environmental variation may be reduced when breeding for disease resistance by inoculation, but this is more difficult for a trait like root lodging. Although some means of mechanical simulation of adverse conditions have been developed (Kato and Koinuma, 1999), these means are not feasible for most maize breeding programs. The genetic components related to root lodging *per se* are little understood.

This dissertation comprises three studies presented in chapters II, III, and IV. The study in Chapter II involved white and yellow hybrid maize trials as well as quality protein maize trials from several years across Texas environments. Data from these trials was analyzed both per and across location to determine repeatability of response to aflatoxin. Additionally, aflatoxin levels were correlated to several secondary characteristics to determine potential usefulness in indirect selection.

These secondary traits (female flowering, endosperm texture, husk cover, and ear rot ratings) were analyzed to determine the variance components and repeatability of each trait. The study in chapter III is a phenotypic evaluation of a recombinant inbred line (RIL) mapping population. This population was derived from parental inbreds Tx811 and CML176, which are divergent for several major traits. The trials were conducted in two Texas locations, College Station and Weslaco, and phenotypic data for aflatoxin concentration, kernel integrity, endosperm texture, female flowering date, and root lodging was collected. Variance components for these traits and genetic and phenotypic correlations were determined. The usefulness of secondary traits for indirect selection was examined. The final study in Chapter IV was a genotypic evaluation of the Tx811/CML176 mapping population using simple sequence repeat markers. Genotypic data was combined with phenotypic data for the population for the purpose of identifying quantitative trait loci (QTL) for response to aflatoxin and for root lodging. All marker data were compared pairwise to ascertain any epistatic interactions for these traits.

CHAPTER II

REPEATABILITY OF AFLATOXIN ACCUMULATION IN FIELD TRIALS OF WHITE AND YELLOW MAIZE HYBRIDS AND INBRED LINES IN TEXAS

INTRODUCTION

Aflatoxin (AF), a mycotoxin produced by *Aspergillus flavus*, creates serious economic and health problems. It causes economic losses due to reduced yield, loss of marketability, and the risks to both human and animal health. Preharvest aflatoxin accumulation has been shown to be a chronic problem in the southwestern United States with major losses in the 1970's and in the late 1990's particularly. The Corn Belt in the U.S. has fewer outbreaks of preharvest aflatoxin contamination; however, it has been problematic during years with drought and unusually high temperatures (Hurburgh, 1991). In the USA, grain with more than 20 ng g^{-1} of aflatoxin B1 is banned from interstate commerce and that with more than 300 ng g^{-1} cannot be used as livestock feed. There are currently no commercial maize hybrids resistant to aflatoxin.

Aflatoxin contamination is influenced by biotic factors, such as insect damage, and abiotic factors like moisture, temperature, and soil fertility. Management practices that optimize plant performance and decrease

plant stress will decrease aflatoxin concentration (Payne 1998; Widstrom 1996). These practices are the same as those that produce higher yield: planting adapted varieties, fertilization- particularly with appropriate nitrogen levels, weed control, insect control- specifically ear boring insects, necessary irrigation- most especially during grain fill, and proper harvesting (Anderson *et al.*, 1975; Jones and Duncan, 1981; Lillehoj, 1983; Lisker and Lillehoj, 1991).

Response to aflatoxin production has been shown to be under genetic control, and surveys of maize germplasm have located genetic variation for aflatoxin accumulation within breeding stock. Aflatoxin accumulation, however, is sporadic and genotype by environment (GxE) interactions have been significantly indicated by studies on the genetics of aflatoxin production in maize (Payne 1992; Brown *et al.*, 1999). As such, efforts to accurately identify tolerant genotypes are hindered by environmental effects on phenotype. Aflatoxin accumulation has long been assumed to be a low heritable trait due to the strong environmental influence. Heritability estimates are useful for breeding efforts to determine the amount of genetic variability available for selection and genetic gain. Measurements of heritability require populations of random genotypes with known genetic backgrounds.

An alternative approach to measuring the proportion of available genetic variance was suggested by Cooper *et al.* (1993) using repeatability (R) rather than heritability. Repeatability estimates in field hybrid trials can be useful to determine how much of the variation observed in aflatoxin content is associated with genetic effects of testing genotypes, as well as to assess the influence of the environment and the interaction between genotypes and environments. Additionally, identification of locations and methodologies that improved repeatability could facilitate genetic progress towards aflatoxin resistance in maize.

Objectives of the Study

(i) Determine the variation components and repeatability of response to aflatoxin in trials of white and yellow hybrids and inbreds in individual locations and across locations over six years.

(ii) Estimate repeatability of the secondary characteristics (days to silking, endosperm texture, *A. flavus* infection, and kernel integrity).

(iii) Measure correlations of secondary characteristics to response to aflatoxin.

REVIEW OF LITERATURE

Pathogen

History of Aflatoxin Research

Aflatoxin, a mycotoxin produced by the ear-rot producing fungus *Aspergillus flavus*, creates serious economic and health problems in both animals and humans. *A. flavus* has been recognized as a pathogen on corn since 1920 (Taubenhaus), but aflatoxin contamination was not considered to be problematic until the 1960s, when it was linked to poultry disease. While some studies showed preharvest infection with *A. flavus* (Barnstetter, 1927; Butler, 1947; Eddins, 1930), it was considered predominantly to be a storage problem. It was first established in field trials as a preharvest problem in corn in the 1970's (Anderson *et al.*, 1975; Rambo *et al.*, 1974). Subsequent studies were implemented for field evaluation of corn genotypes in order to identify resistance sources to *A. flavus* and/or aflatoxin accumulation (Lillehoj *et al.*, 1976; Widstrom *et al.*, 1981). There are currently no commercial corn hybrids resistant to aflatoxin. Some of the limiting factors in developing aflatoxin resistant corn are: the variation in aflatoxin accumulation that requires inoculation, more replications, and increased number of locations; the lack of a reliable, rapid, high throughput, and inexpensive screening

methodology; and the low metabolic activity of corn plants after physiological maturity (Payne, 1992).

Economic Costs of Aflatoxin

Because mycotoxins are hazardous to animal and human health, they constitute a factor for economic food production losses worldwide (Lubulwa and Davis, 1994). Early surveys addressing this issue showed a negative correlation between yield and levels of preharvest aflatoxin contamination (Duncan, 1979); however, no clear correlation between yield and aflatoxin has been established. Other economic losses are incurred due to reduced profitability as contaminated corn is worth less and farmers either cannot sell corn that tests positive for aflatoxin or receive reduced remuneration for it. When aflatoxin-contaminated corn is used for feed there is a loss of animal health and productivity. When there is a risk of aflatoxin accumulation, farmers often must implement management techniques such as increased irrigation, crop rotation, proper fertilization or pest control. Another, often overlooked economic expense is the cost of research that is necessitated for monitoring aflatoxin exposure and contamination. Finally, the economic costs associated with human health are too complex to accurately ascertain, including medical costs, loss of work or productivity, and all associated

costs involved in human livelihood. The human and social costs, of course, are incalculable.

Effect on Animals

The scientific study of mycotoxins began in 1960 when a large number of turkey poults died in England after eating contaminated foodstuff (Blount, 1961). Severe economic losses have shown to be the result of aflatoxin decreasing productivity and leading to disease in poultry (Council for Agricultural Science and Technology Report (CAST), 2004). Aflatoxins are potent liver toxins, and most animal species exposed to these mycotoxins show signs of liver disease ranging from acute to chronic. Immunosuppression is an important consideration in aflatoxin-exposed animals (CAST, 2004). Because corn is used for animal feed, the effect of aflatoxin on animals has been thoroughly documented. Coppeck *et al.* (1989) noted that aflatoxin produces weight loss, rough hair coat, anorexia, ataxia, tremors, coma, or even death in feeder pigs. In dairy cattle, it has been shown to cause tachycardia, tachypnoea, and death (Cockcroft, 1995). Aflatoxins are converted to another toxic metabolite that is excreted in milk and is important to consider in the economic aspects of aflatoxicosis in dairy cattle (CAST, 2004). In dogs, aflatoxin produces jaundice, abdominal pain, edema, anorexia,

gastrointestinal bleeding, or death (Ngindu *et al.*, 1982). While the cost to animal health can be great, the potential human health risks are even more of a concern.

Effect on Humans

Aflatoxin is associated with human liver cell carcinoma (Berry 1988; Stark 1980). Aflatoxin B1 has been classified as a probable human carcinogen by International Agency for Research on Cancer (1987). Acute aflatoxicosis is well documented in humans (Krishnamachari *et al.* 1975a, b; Ngindu *et al.* 1982; Shank 1977). Aflatoxins have been implicated with increased incidence of human gastrointestinal and hepatic neoplasms in Africa, the Philippines, and China. Aflatoxin contamination is particularly problematic in Africa, generally due to postharvest contamination. Shepherd (2003) and Bankole and Adebanjo (2003) detail the effects of aflatoxin on human health by country in Africa.

Symptoms commonly associated with acute aflatoxicosis include jaundice, low-grade fever, depression, anorexia, and diarrhea, and liver damage (CAST, 2004). In the 1970's, India reported outbreaks of aflatoxin poisoning in which death rates reached as high as 25% (Krishnamachari *et al.* 1975a, 1975b). In early 2004, hundreds of deaths

from aflatoxin poisoning were reported in Kenya by the Center for Disease Control (Williams *et al.*, 2005). Aflatoxin is a serious problem, both economically and in terms of human and animal health. It has recently been registered as a bioterrorism agent. The process of aflatoxin production and management and prevention techniques are currently being studied to address this concern.

Life Cycle of Aspergillus flavus

Payne *et al.* (1992, 1998) characterized and described the infection process of *A. flavus* in corn. *Aspergillus flavus* is a soil-borne fungus that reproduces by asexual conidia. The source of inoculum for *A. flavus* is the soil, but the predominant survival structure remains unknown. Payne (1998) has suggested that the fungus survives as mycelium, conidia, and sclerotia. It has been shown in the southern United States that *A. flavus* produces sclerotia in cornfields as well as in culture (Wicklow *et al.* 1984; Zummo and Scott 1990); however, sclerotia have not been reported in the Midwest.

The conidia, which are the infecting structures, are able to colonize the ear through the silks when delivered via wind or insects. They grow into the ear and then colonize the kernels (Payne 1998; Widstrom 1996). Wounds on the kernels caused by insects may facilitate infection.

Insects are not required for aflatoxin contamination, but they have been shown to increase contamination and high levels of aflatoxin have been associated with insect injury, especially by the European corn borer, *Ostrinia nubilalis*, and corn earworm, *Heliothis zea* (Widstrom 1996). If conditions are optimal for *A. flavus* infection, the kernels and cobs may be directly infected by the fungus.

Environmental Conditions Favoring Aflatoxin Production

Aflatoxin accumulation has been shown to be highly dependent on environmental conditions as well as inconsistent in expression, even under optimal conditions when subject to natural inoculum. *A. flavus* was originally classified as storage fungi, based on studies done in temperate climates. However, in climates with hot, dry growing seasons, such as the southern United States, aflatoxin infection of corn is more likely to be a preharvest concern (Wilson and Abramson, 1992).

Temperature and moisture have been shown to be the factors that most influence the level of contamination with aflatoxin (Payne *et al.*, 1998; Widstrom, 1996). Specifically, high temperatures and drought stress resulted in high levels of aflatoxin contamination (Payne 1998). Jones *et al.* (1981) noted the role of moisture, and McGee *et al.* (1996) found that high soil temperatures increased aflatoxin accumulation. Cole *et al.*

(1995) found that neither high temperatures nor drought stress alone were sufficient for the higher levels of contamination by using field trials where soil moisture and temperature were controlled. Some have suggested that higher night temperatures are important to contamination, both in corn (Ashworth *et al.*, 1969b) and also in almonds (Doster and Michailides 1995).

A. flavus is one of the mycotoxin-producing fungi that is well adapted to grow on substrates with low moisture. Significant infection and aflatoxin contamination do not occur until the kernel moisture is below 32% (Payne, 1998). Aflatoxin can continue to be produced in kernels until the moisture reaches 15% (Payne *et al.*, 1988). When high temperatures and drought conditions are combined, particularly during kernel filling, aflatoxin levels are highest (Lisker and Lillehoj, 1991; Vincelli *et al.*, 1995).

These conditions are prevalent in the southern United States, and therefore aflatoxin is a persistent problem in that region. These conditions were also associated with a high incidence when the U.S. Corn Belt experienced higher than usual temperatures combined with low rainfall in the 1980's (Hurburgh, 1991). Other regions with these conditions that have reported preharvest aflatoxin contamination

include southern China, Southeast Asia, and Africa (Hall and Wild, 1994).

Genetics of Response to Aflatoxin Contamination

Lower levels of aflatoxin production in some corn genotypes have been found in public sources through germplasm screening studies (Campbell and White, 1995a; Darrah *et al.*, 1987; Scott and Zummo, 1988, 1990; Thompson *et al.*, 1984; Widstrom *et al.*, 1987).

Additionally, some inbred lines of corn (Brown *et al.* 1998; Campbell and White 1995a; Huang *et al.,* 1997; Widstrom 1996) with low levels of resistance to aflatoxin accumulation *per se* have been identified. These sources, however, have yet to produce a commercial hybrid that accumulates aflatoxin at acceptable low levels. The genetic component of aftlatoxin tolerance is tempered by genotype x environment interaction (GxE) effects. When environmental conditions in the field are optimal for aflatoxin production, the effectiveness of genetic tolerance is limited.

However, research has established that tolerance to aflatoxin production is partially genetically based and quantitatively inherited, with low broad-sense heritabilities. Additive gene effects have been shown to be more important than dominance gene effects (Campbell and White,

1995b; Campbell *et al.*, 1997; Hamblin and White, 2000). Open pollinated cultivars of maize grown in the southeastern United States before the routine use of hybrid corn production were more susceptible to preharvest aflatoxin contamination than hybrids (Zuber *et al.*, 1983). Despite this, there remain no commercial hybrids with acceptable levels of tolerance to aflatoxin. In breeding for reduced aflatoxin contamination in hybrid production, general combining ability effects are more important than specific combining ability effects (Zuber *et al.*, 1978; Widstrom *et al.*, 1984; Gardner *et al.*, 1987; Gorman *et al.*, 1992; Naidoo *et al.*, 2002).

Traits in Maize Affecting Aflatoxin Accumulation

Chemical methods of resistance that are under genetic control have been identified. The enzyme b-1-3-glucanase may have a role in the inhibition of *A. flavus* growth on the grain when present in maize kernels (Lozovaya, 1998). Huang *et al.* (1997) have identified two other kernel proteins that appear to confer resistance. One inhibits aflatoxin production with no effect on fungal growth, while the other protein inhibits the growth of the fungus. Chen *et al.* (1998) also suggested a trypsin inhibitor in kernels that may confer resistance when present in high concentrations. Tubajika and Damann (2001) also implicated a trypsin inhibitor for increased resistance to aflatoxin contamination.

Additionally, physical methods of resistance are known to exist, such as kernel pericarp wax and husk covering over the ear. Wax and cutin layers on the surface of maize kernels have been indicated in conferring resistance to aflatoxin accumulation (Guo *et al.*, 1995; Russin *et al.*, 1997). Thicker pericarp layers may prevent initial contamination by *A. flavus* conidia in undamaged kernels (Tubajika and Damann, 2001). Other research has focused on indirect protection of kernels by breeding for better husk coverage (McMillian *et al.*, 1985; Lisker and Lillehoj, 1991). Tighter husk coverage may reduce insect susceptibility, and therefore fewer damaged kernels for *A. flavus* infection.

Genotype by Environment Interaction

While the expression of some traits is completely under genetic control, other traits are influenced by environmental factors. In breeding, environmental effects must be accounted for and removed in order to accurately assess genetic differences and select superior genotypes. If the environmental influence affects all genotypes similarly, this effect does not influence genotypic differences or selection. When the environment affects some genotypes differently than others, GxE is significant (Fehr, 1991). This interaction complicates breeding efforts, and requires more extensive evaluation over multiple years and

environments in replicated trials. GxE interactions have been significant in several studies on the genetics of aflatoxin production in corn (Payne, 1992; Brown *et al.*, 1999). Efforts to accurately identify genotypes that accumulate lower levels of aflatoxin are hindered by these interactions, since part of the phenotypic variation is not due to genotypic variation. In general, when environmental conditions are optimal for aflatoxin production, genotypic differences are displayed and selection is possible. When conditions do not favor aflatoxin production, however, not only is selection power diminished because phenotypic variance is lower, but what minimal phenotypic variance that is exhibited may be due to genotype by environment interactions rather than evidence of a superior genotype.

Management Techniques

Bruns *et al.* (2003) maintain that any genetically resistant material will be inadequate without proper crop management practices. Reduction of postharvest aflatoxin accumulation will continue to require sound management practices during harvest, handling and storage in order to avoid losses due to mycotoxins.

While genetic resistance is desired, there are management practices that have been shown to reduce incidence of preharvest aflatoxin.

Management practices that maximize plant performance and decrease plant stress will decrease aflatoxin contamination (Cole *et al.*, 1995; Jones *et al.*, 1981; Michailides, 1996; Payne, 1998; Widstrom, 1996). These practices are the same as those that produce higher yield: planting adapted varieties, proper fertilization- particularly with appropriate nitrogen levels, weed control, insect control specifically ear boring insects, necessary irrigation most especially during grain fill, and proper harvesting (Anderson *et al.*, 1975; Jones and Duncan, 1981; Lillehoj, 1983; Lisker and Lillehoj, 1991).

When harvesting, there are two major considerations regarding aflatoxin prevention. Jones *et al.* (1981) have shown that delayed harvest can result in higher aflatoxin levels. Early harvesting followed by drying is recommended, although it is not always economically feasible. In order to prevent postharvest infection, combines should be adjusted to minimize damage to kernels and to prevent damaged kernels, which may have higher levels of contamination, (Malone *et al.*, 1998; Munkvold and Desjardins, 1997; Widstrom, 1996). These and other management techniques for minimization of aflatoxin production are extensively reviewed by Bruns *et al.* (2003). Use of management techniques alone has been inadequate, however, for controlling aflatoxin contamination.

Traditional Breeding to Reduce Aflatoxin

Breeding Options

It is desirable to have a commercial hybrid that is genetically resistant to aflatoxin accumulation. This could be achieved one of several ways. Resistance to fungal infection could be based on plant x pathogen genetic interaction. There could be morphological or physiological traits that prevent fungal growth or infection. Finally, the corn could have resistance to mycotoxin production rather than to fungal infection and development. Whichever path of resistance is pursued, traditional breeding improves the genetic makeup of the corn by making selections based on the phenotype evidenced. Traditional plant breeders conduct experiments designed to identify the best genotype and estimate environmental effects on the phenotype. Accurate, facile, rapid, and inexpensive screening is necessary to achieve desired results from field experiments, as well as to obtain an understanding of the underlying genetic mechanisms at work. Additionally, when breeding for pathogen resistance, the exposure to the disease must be such that escapes are prevented, yet those with tolerance or resistance can still be identified. In some situations, natural inoculum in the field is enough to screen for resistance, while in others artificial inoculum is needed for accurate screening. While natural inoculum of *A. flavus* is present in most fields

in the southeastern U.S., the spatial variation and the sporadic expression of aflatoxin production indicate the need for inoculation.

Heritability

In order to make progress through breeding, there must be genetic variation within breeding material that is identifiable and selectable by breeders. In complex traits, identification of genetic variation can be complicated by GxE. Additionally, the number of genes as well as their mode of action needs to be characterized. Genetic variation may be due to additive, dominant, or epistatic effects. The portion of genetic variation that is available to accumulate genetic gain through selection is termed narrow-sense heritability. This is a measurement of the proportion of additive genetic variance to total phenotypic variance. With lower heritability traits, breeders have less selection power, and environmental effects affect genetic gains. Classic measurements of heritability are estimated by evaluating random genotypes in several environments. Cooper *et al.* (1993) utilized a measurement of repeatability that estimates the genetic variation available for breeding in fixed genotypes. Response to aflatoxin contamination has long been considered to be lowly heritable due to the large environmental effect and the GxE.

Inoculation Methods

One way to increase screening precision, and thus selection power for breeding is to employ sound inoculation such that escapes are minimized. There are several inoculation techniques that have been used to research the response of corn genotypes to *A. flavus* and aflatoxin production. Inoculation techniques are classified as either wounding or non-wounding. Wounding inoculation techniques include the knife (Widstrom *et al.*, 1981, 1982, 1986, 1996), pinbar (Campbell and White, 1994; King and Scott, 1982; Tubajika and Damann, 2001; Tubajika *et al.*, 2000), pinboard (Campbell and White, 1994, 1995a, b; Naidoo *et al.*, 2002; Olanya *et al.*, 1997; Walker and White, 2001), side-needle (Scott and Zummo, 1994; Windham and Williams, 1998, 2002; Zummo and Scott, 1989), toothpick (Wicklow *et al.*, 1994; Zhang *et al.*, 1998), and punch drill/pipe cleaner (Wicklow *et al.*, 1994) techniques.

Non-wounding techniques include spraying of the silks with a suspension of *A flavus* conidia (Cardwell *et al.*, 2000, Jones *et al.*, 1980; Payne *et al.*, 1988, Windham and Williams, 1999, Windham *et al.*, 1999), silk channel injection (Zummo and Scott, 1989), and granular material application (Odvody *et al.*, 1996; Olanya *et al.*, 1997).

Windham *et al.* (2003) have compiled a comprehensive review of inoculation methods and comparison of these methods in different environments. Recommendations are given for particular environments contrasting the inoculation needs for research in the southern United States with those of the Corn Belt.

Quantification of Aflatoxin Contamination

The World Health Organization has set the maximum residue level for aflatoxin in human foods at 20 parts per billion (ppb) (CAST, 1989). Food products that exceed this level cannot be marketed, with intervention levels in Europe set even lower (Miller, 1996). Accurate, rapid, and inexpensive quantification of aflatoxin content is important both in commodity sales and for research purposes. Testing for aflatoxin involves obtaining an adequate sample, preparing the sample, and finally conducting the analytical procedure. Once an adequate sample has been taken and prepared, the toxin must be extracted and the extract purified of any contaminants. The purified extract must be analyzed to determine the presence and quantity of aflatoxin present in the sample. Several quantification procedures may be used, including thin-layer chromatography (Singhe *et al.*, 1991), high-performance liquid chromatography, gas chromatography, fluorometry, or immunologically based tests such as enzyme-linked immunosorbent assay (Campbell and

White, 1995a). VICAM (Watertown, MA) produces Aflatest, which utilizes immunoabsorbent columns that can be used to separate aflatoxins (B1, B2, G1, G2) at concentrations as low as 1 ng/g with smaller samples. Regardless of the overall testing procedure used, including sampling, sample preparation, and analysis, variability at each step must be minimized. The greatest variability has been shown to occur in the sampling step, which must be undertaken in such a manner as to maximize uniformity and reduce error.

Limitations of Breeding for Resistance

There are several ways in which progress in breeding for lower aflatoxin accumulation has been hindered. While careful experimental design can help to lower the amount of environmental error that potentially biases selection decisions, the optimal design often requires too many trials in different years and locations or too many genotypes for screening to be viable based on the resources available. Additionally, even under inoculation, variations in aflatoxin accumulation due to genetic differences may be difficult to identify due to sporadic expression in the field. Finally, precise aflatoxin screening is costly and labor and time demanding. This limits the number of trials/ and genotypes that a breeding program can feasibly analyze.

MATERIALS AND METHODS

Plant Material

The aflatoxin trials analyzed for repeatability within and across locations were white and yellow hybrids, their parental inbred lines, and a set of quality protein maize (QPM) lines. The white hybrids tested were predominantly for food corn purposes. The yellow hybrids were predominantly intended for animal feed. The QPM lines were selected for their hard endosperm and lower rates of ear rot infection, both characteristics having been related to lower aflatoxin infection.

Commercial white food hybrids used as checks differed for each year, but included in these trials were from Pioneer (P32H39, P30G54), Asgrow (RX901W, RX921W, RX949W, RX951W) and/or Triumph (1851W, 1910W). No commercial checks were included in 2000 trials. Commercial yellow corn hybrids used for checks were from Pioneer (P3223, P31B13, P32R25), Dekalb (DK668, CK687), Asgrow (RX889), and Garst (8300GLSIT, 82857Y35). The materials tested were hybrids of temperate by subtropical experimental crosses.

Inoculation

Two non-wounding inoculation methods were used, dependent upon the location and environmental conditions of the trial. At all locations in

2000 and 2004, at CS and W in 2001, and at CS in 2003, the silk channel technique was used (Zummo and Scott, 1989). Plants were inoculated with a conidial suspension containing 3 x 10^7 conidia of *A. flavus* in 3 mL distilled water injected 6 to 10 days after midsilk. The alternative method, which was used for all other trials, involves inoculation by placing *A. flavus* colonized autoclaved corn kernels on the soil surface between treatment rows when the first hybrids reached mid-silk stage. Both methods have shown effectiveness in discriminating genotypes and in detecting significant differences in aflatoxin accumulation (Odvody *et al.*, 1996, Zummo and Scott, 1989). The *A. flavus* isolate used was NRRL3357. At harvest, infected ears were husked, dried, shelled, and bulked, then analyzed for aflatoxin content.

Environments

All hybrid trials were grown in three locations located in south and central Texas where aflatoxin contamination is a frequent problem: Weslaco (latitude 26°09, elevation 22.5 m), Corpus Christi (latitude 27°46, elevation 12.9 m), and College Station (latitude 30°37, elevation 96 m). Parental inbred lines were grown in Weslaco only. Repeatabilities were measured for each location individually and across locations. Historically, conditions in Corpus Christi and Weslaco have been more optimal for aflatoxin production than those in College

Station. Six years of trials (1999, 2000, 2001, 2002, 2003, and 2004) were analyzed to determine repeatability of relevant traits. Climatic conditions differed drastically over these years, including both dry and rainy seasons.

Analysis of Phenotypic Traits

Several phenotypic traits were measured for these trials, including aflatoxin concentration, maturity, endosperm texture, husk cover, and ear rot infection. Not all traits were measured for every trial. Maturity was measured in days from planting to 50% silking in each plot. Inoculated ears were harvested by hand. Lines were rated for the tightness of husk cover (1= loose, open husk, 5 = long, tight husk covering).

At harvest, infected ears were husked, dried, shelled, and bulked. Grain was visually rated in the field for texture (1 indicating flinty endosperm, 5 indicating floury endosperm). Visual ratings of *Aspergillus flavus* colonization (1 indicating no ear rot, 5 indicating completely colonized ears) were also recorded. The whole kernel sample was ground using a Romer mill (Union, MO). Aflatoxin quantification was conducted using 50-g subsamples of ground material from each plot with monoclonal

antibody affinity columns and flouresence determination using the Aflatest by Vicam (Watertown, MA).

All data was analyzed using SAS procedures. Means were obtained using REMLtool^{TM} software, which utilizes restricted maximum likelihood (REML) methods to estimate variance components in mixed linear models. Repeatability was measured using the variance components for each trial to determine the ratio of genetic variance to total phenotypic variance. For individual locations, repeatability (*R*) was measured using the formula:

$$R = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{r}}$$

where σ_g^2 is the genotypic variation, σ_e^2 is the variance due to error, and *r* is the number of replications. The formula for across location estimates was:

$$R = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{ge}^2}{e} + \frac{\sigma_e^2}{r'e}}$$

where σ_g^2 is the genotypic variation, σ_{ge}^2 is the estimate of genotype by environment interaction variance, σ_e^2 is the variance due to error, and *r'*

is the harmonic mean of the number of replications, and *e* is the number of environments.

Logarithmic transformation of aflatoxin measurements was used in analysis to equalize variance and normalize the data (Fig. 2.1). Repeatabilites were also measured for silking dates, kernel texture, husk cover, and *A. flavus* infection. Pearson's correlations were estimated using SPSS software (SPSS, Inc., 1999). The formula for the Pearson product moment correlation coefficient, *r*, is:

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

where x and y are the sample means of trait x to trait y.

RESULTS

White Hybrids

In all trials, aflatoxin concentration measured in parts per billion (ng g^{-1}) was not normally distributed (Figure 2.1). Logarithmic transformation of aflatoxin contamination was used to equalize variance and normalize the data. Mean aflatoxin concentration ranged from 27 ng g^{-1} to 577 ng g^{-1} (Table 2.1). Corpus Christi had the highest mean aflatoxin concentration in 2001 and 2002. Weslaco had the highest mean aflatoxin

concentration in all other years, as well as the highest overall mean ($\bar{x}$ = 134 ng g^{-1}) across all years. College Station had the lowest overall mean ($\bar{x}$ = 39 ng g^{-1}). Year 1999 had the highest overall mean ($\bar{x}$ = 269 ng g^{-1}) and maximum aflatoxin concentration (6194 ng g^{-1}).

Aflatoxin is a highly variable trait across environments. The variation due to environment and genotype by environment interactions were estimated for each year. The environment was the largest variation component in 2001 and 2002. Error was the largest variance component in all other years. In 1999 and 2000, the GxE interaction and the environmental variance were both higher than the genotypic variation. Genotypic variation was not the largest contributor to phenotypic variance for any of the years studied.

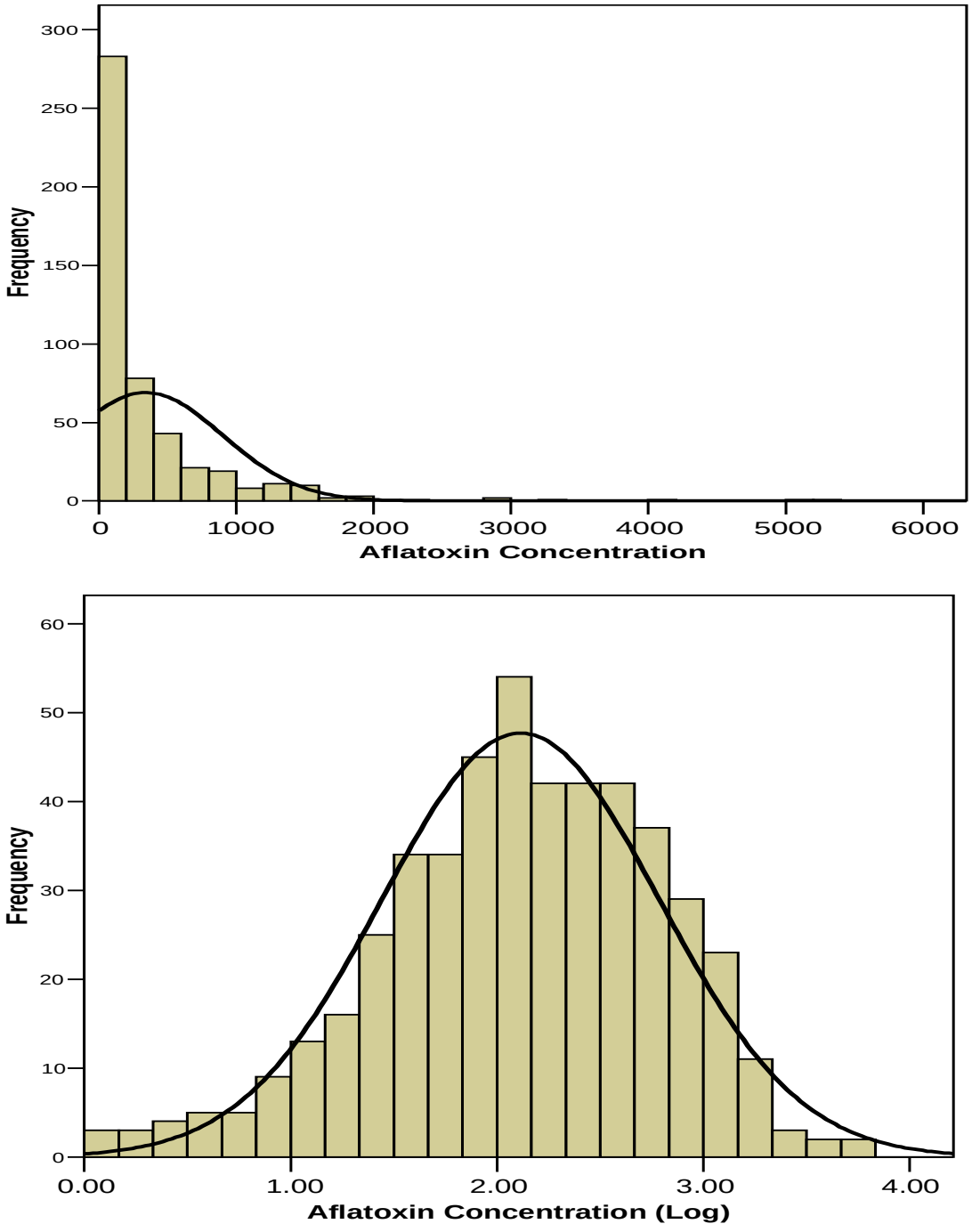

Figure 2.1. Distribution of aflatoxin concentration for white hybrids in Corpus Christi trials (1999-2004) in (a) parts per billion (ng g^{-1}) and (b) logarithmic transformation of parts per billion.

Table 2.1. Statistics and variance components of aflatoxin concentration in white maize hybrids in inoculated trials per and across locations.

						Variance Components†					
Year	Location	IM*	$\bar{x}$ (Log)**	$\bar{x}$ §	Maximum §§	Rep	Block	σ_g^2	σ_e^2	GxE	Env
1999	College	CK	2.300 ± 0.048	200	1300	0.015	0.034	0.059	0.090		
	Corpus	CK	2.247 ± 0.052	177	6194	0.018	0.000	0.164	0.028		
	Weslaco	CK	2.594 ± 0.054	393	4699	0.006	0.012	0.115	0.083		
	Across		2.430 ± 0.040	269		0.014	0.030	0.009	0.082	0.072	0.044
2000	College	SC	1.557 ± 0.073	36	729	0.052	0.000	0.080	0.314		
	Corpus	SC	2.087 ± 0.065	122	640	0.020	0.000	0.136	0.013		
	Weslaco	SC	2.132 ± 0.063	135	2301	0.011	0.000	0.131	0.182		
	Across		1.979 ± 0.050	95		0.027	0.000	0.054	0.220	0.065	0.157
2001	College	SC	1.843 ± 0.052	70	750	Variance component estimates did not converge.					
	Corpus	CK	2.761 ± 0.042	577	5297						
	Weslaco	SC	2.530 ± 0.035	339	2799						
	Across		2.343 ± 0.033	220		0.000	0.000	0.027	0.142	0.049	0.225
2002	College	CK	0.237 ± 0.053	2	300	0.016	0.000	0.101	0.225		
	Corpus	CK	2.295 ± 0.047	197	3199	0.006	0.001	0.189	0.080		
	Weslaco	CK	1.732 ± 0.078	54	2301	0.000	0.000	0.284	0.454		
	Across		1.425 ± 0.058	27		0.006	0.000	0.116	0.255	0.074	1.123

Table 2.1 continued

Year	Location	IM*	$\bar{x}$ (Log)**	$\bar{x}$ §	Maximum §§	Variance Components†					
						Rep	Block	σ_g^2	σ_e^2	GxE	Env
2003	College	SC	1.787 ± 0.042	61	1099	0.100	0.002	0.019	0.365		
	Corpus	CK	NA								
	Weslaco	CK	1.996 ± 0.037	99	1698	0.005	0.003	0.103	0.262		
	Across		1.894 ± 0.028	78		0.052	0.000	0.047	0.314	0.015	0.004
2004	College	SC	1.916 ± 0.068	82	1413	0.038	0.012	0.053	0.331		
	Corpus	CK	1.446 ± 0.064	28	575	0.000	0.000	0.055	0.302		
	Weslaco	CK	1.954 ± 0.050	90	1400	0.014	0.021	0.074	0.275		
	Across		1.776 ± 0.040	60		0.019	0.007	0.066	0.303	0.000	0.069

* Inoculation Method (CK = Colonized Kernels, SC = Silk Channel).
** Mean ± Standard Error (Log transformation of aflatoxin contamination in ng g^{-1}).
§ Antilog of means.
§§ Antilog of maximum aflatoxin concentration,
† Variance components: replications, blocks, genotype, error, genotype by environment interaction, environment.

Significant repeatability estimates for the white hybrids ranged from R = 0.204 to R = 0.877 (Table 2.2). Trials at Corpus Christi showed the highest repeatability per location ($\bar{x}$ = 0.730), followed by Weslaco ($\bar{x}$ = 0.662), and College Station ($\bar{x}$ = 0.415). Overall, 2002 had the highest repeatabilities, with lowest repeatabilities in 2003 and 2004.

Pearson's Correlations to Aflatoxin Concentration

Pearson's correlations were taken between all traits measured across years for each location (Table 2.3). Significance was determined using the degrees of freedom for each pairwise comparison. Aflatoxin concentration was significantly correlated to endosperm texture at all three locations (College Station r = 0.207**, Weslaco r = 0.197**, and Corpus Christi r = 0.227**). There was a significant positive correlation of aflatoxin concentration to *A. flavus* infection at College Station (r = 0.557**) and Corpus Christi (r = 0.420**). Maturity was significantly positively correlated to aflatoxin concentration at Corpus Christi (r = 0.543**), but was significantly negatively correlated at Weslaco (r = -0.213**). Husk cover was not recorded for enough trials at Corpus Christi to establish any correlation measurements.

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Table 2.2. Repeatabilities with standard errors of aflatoxin concentration in white maize hybrids in inoculated trials per and across locations.

Year	Entries	College Station	Weslaco	Corpus Christi	Across Locations	Yearly Mean*
1999	25	0.396 ± 0.152	0.848 ± 0.063	0.854 ± 0.047	0.161 ± 0.418	0.699
2000	20	0.204 ± 0.120	0.743 ± 0.097	0.835 ± 0.052	0.563 ± 0.185	0.594
2001	30	0.579 ± 0.134	0.743 ± 0.096	NA	0.477 ± 0.169	0.661
2002	30	0.574 ± 0.116	0.653 ± 0.096	0.877 ± 0.038	0.716 ± 0.092	0.701
2003	90	0.132 ± 0.168	0.540 ± 0.087	NA	0.542 ± 0.125	0.336
2004	30	0.324 ± 0.221	0.447 ± 0.190	0.354 ± 0.211	0.663 ± 0.095	0.375
Location Mean*		0.415	0.662	0.730	0.592	

* Mean does not include repeatabilities that are zero or non-significant.

Table 2.3. Pearson's correlations of phenotypic traits§ by location for the white hybrid maize trials for 1999-2004 in (a) College Station, (b) Corpus Christi and (c) Weslaco.

(a)

	Aflatoxin Concentration	Maturity	Endosperm Texture	Husk Cover
Maturity	0.054			
Endosperm Texture	0.207**	-0.134**		
Husk Cover	-0.074	-0.114	0.147	
A. flavus infection	0.557**	-0.104	0.283**	0.038

(b)

	Aflatoxin Concentration	Maturity	Endosperm Texture
Maturity	0.543**		
Endosperm Texture	0.227**	-0.227**	
A. flavus infection	0.42**	NA	0.561**

Table 2.3 continued.

(c)

	Aflatoxin Concentration	Maturity	Endosperm Texture	Husk Cover
Maturity	-0.213**			
Endosperm Texture	0.197**	-0.198**		
Husk Cover	0.011	0.025	0.140**	
A. flavus infection	0.027	-0.243**	0.365**	0.220**

§ Units for traits: Aflatoxin Concentration (logarithmic transformation of ng g^{-1}), Maturity (Days to 50% Silking), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Husk Cover (1 = loose open husk, 5 = tight husk cover), *Aspergillus flavus* infection (1 = no visible ear rot infection, 5 = ear/cob completely visibly infected).

** Significant at 0.01.

Yellow Hybrids

Mean aflatoxin concentration ranged from under 10 ng g^{-1} to 1091 ng g^{-1} (Table 2.4). Corpus Christi had the highest mean aflatoxin concentration in 2000, 2001, and 2002. Weslaco had the highest mean aflatoxin concentration in all other years, as well as the highest overall mean per location across all years ($\bar{x}$ = 165 ng g^{-1}). College Station had the lowest overall mean ($\bar{x}$ = 45 ng g^{-1}). In 2001, aflatoxin concentration reached its highest level (7194 ng g^{-1}).

The environment was the largest variance component in 1999, 2001, 2002, and 2004 for across location analysis. In 2000 and 2003, variance due to error was the largest component. Across locations, error was always a higher proportion of the total variance than genotype. At individual locations, genotypic variation was the largest variance component for only three trials, Corpus Christi in 2000 and 2001, and Weslaco in 2002.

Repeatabilities were highest at Corpus Christi ($\bar{x}$ = 0.744), followed by Weslaco ($\bar{x}$ = 0.710), and College Station ($\bar{x}$ = 0.587) (Table 2.5). Across location repeatabilities were significant for all trials except 2000. In 1999, across location repeatability estimates included only College

Table 2.4. Statistics and variance components of aflatoxin concentration in yellow maize hybrids in inoculated trials per and across locations.

						Variance Components†					
Year	Location	IM*	$\bar{x}$ (Log)**	$\bar{x}$§	Maximum§§	Rep	Block	σ_g^2	σ_e^2	GxE	Env
1999	College	CK	1.921 ± 0.064	83	1099	0.010	0.008	0.124	0.127		
	Corpus	CK	NA								
	Weslaco	CK	2.518 ± 0.041	330	2301	0.001	0.000	0.043	0.080		
	Across					0.005	0.005	0.048	0.102	0.035	0.168
2000	College	SC	1.708 ± 0.068	51	933	0.000	0.000	0.134	0.232		
	Corpus	SC	2.628 ± 0.040	425	3698	0.005	0.000	0.089	0.028		
	Weslaco	SC	2.292 ± 0.066	196	3499	0.019	0.000	0.158	0.184		
	Across					0.000	0.329	0.296	60.95	30.11	0.000
2001	College	SC	1.763 ± 0.052	58	1413	0.000	0.000	0.070	0.358		
	Corpus	CK	3.038 ± 0.047	1091	7194	0.004	0.003	0.155	0.041		
	Weslaco	SC	2.558 ± 0.032	361	2399	0.007	0.004	0.053	0.098		
	Across					0.001	0.004	0.015	0.192	0.063	0.414

Table 2.4 Continued.

Year	Location	IM*	$\bar{x}$ (Log)**	$\bar{x}$ §	Maximum§§	Variance Components†					
						Rep	Block	σ_g^2	σ_e^2	GxE	Env
2002	College	CK	0.500 ± 0.069	3	320	0.000	0.016	0.000	0.540		
	Corpus	CK	2.787 ± 0.053	612	4498	0.000	0.010	0.087	0.073		
	Weslaco	CK	1.875 ± 0.066	75	1698	0.000	0.064	0.253	0.218		
	Across					0.000	0.031	0.027	0.333	0.076	1.320
2003	College	SC	1.941 ± 0.045	87	1698	0.020	0.000	0.114	0.234		
	Corpus	CK	1.240 ± 0.046	17	330	0.000	0.022	0.112	0.241		
	Weslaco	CK	1.950 ± 0.047	89	2999	0.006	0.000	0.166	0.233		
	Across					0.008	0.001	0.057	0.243	0.072	0.116
2004	College	SC	2.201 ± 0.057	159	2999	0.019	0.010	0.038	0.216		
	Corpus	CK	1.389 ± 0.050	24	260	0.008	0.000	0.062	0.158		
	Weslaco	CK	2.294 ± 0.069	197	4603	0.006	0.005	0.031	0.227	0.041	0.242
	Across										

* Inoculation Method (CK = Colonized Kernels, SC = Silk Channel).
** Mean ± Standard Error (Log transformation of aflatoxin contamination in ng g^{-1}).
§ Antilog of means.
§§ Antilog of maximum aflatoxin concentration,
† Variance components: replications, blocks, genotype, error, genotype by environment interaction, environment.

Table 2.5. Repeatabilities with standard errors of aflatoxin concentration in yellow maize hybrids in inoculated trials per and across locations.

Year	Entries	College Station	Weslaco	Corpus Christi	Across Locations	Yearly Mean*
1999	20	0.796 ± 0.087	0.679 ± 0.127	NA	0.614 ± 0.207	0.738
2000	20	0.698 ± 0.113	0.775 ± 0.085	0.906 ± 0.033	0.014 ± 0.492	0.793
2001	30	0.437 ± 0.147	0.683 ± 0.085	0.920 ± 0.027	0.278 ± 0.234	0.680
2002	30		0.823 ± 0.057	0.703 ± 0.114	0.318 ± 0.232	0.763
2003	90	0.661 ± 0.083	0.741 ± 0.064	0.650 ± 0.091	0.564 ± 0.114	0.684
2004	30	0.345 ± 0.224	0.560 ± 0.152	0.541 ± 0.148	0.445 ± 0.183	0.482
Location Mean*		0.587	0.710	0.744	0.372	

Station and Weslaco. Repeatabilities for yellow hybrids were highest in 2000 ($\bar{x}$ = 0.793), the same year with the highest across location repeatability (R = 0.614). Repeatability was lowest in 2004 ($\bar{x}$ = 0.482).

Pearson's Correlations to Aflatoxin Concentration

Pearson's correlations were measured for yellow hybrid trials (Table 2.6). Aflatoxin concentration was significantly and positively correlated at all locations to maturity (r = 0.122** in College Station, r = 0.389** in Corpus Christi, and r = 0.095* in Weslaco) and to *A. flavus* infection (r = 0.403**, 0.462**, and 0.276** respectively). Husk cover was positively correlated to aflatoxin concentration at College Station (r = 0.201**) and Weslaco (r = 0.241**), but was not recorded for enough trials for any correlations to be determined in Corpus Christi. Endosperm texture was correlated significantly to aflatoxin concentration at Corpus Christi (0.221**) and Weslaco (0.276**) but not at College Station.

Inbred Lines

Inbred lines that served as parental material for the hybrid trials were grown in Weslaco only (Table 2.7). White inbreds had lower mean repeatability (R = 0.648) than yellow inbreds (R = 0.876). Genotypic variation was higher than error for all yellow inbred trials.

Table 2.6. Pearson's correlations of phenotypic traits§ by location for the yellow hybrid maize trials for 1999-2004 in (a) College Station, (b) Corpus Christi and (c) Weslaco.

(a)

	Aflatoxin Concentration	Maturity	Endosperm Texture	Husk Cover
Maturity	0.122**			
Endosperm Texture	0.051	-0.214**		
Husk Cover	0.204**	0.022	0.051	
A. flavus infection	0.403**	0.013	0.245**	0.009

(b)

	Aflatoxin Concentration	Maturity	Endosperm Texture
Maturity	0.389**		
Endosperm Texture	0.221**	0.142	
A. flavus infection	0.462**	0.332**	0.195**

Table 2.6 Continued.

(c)

	Aflatoxin Concentration	Maturity	Endosperm Texture	Husk Cover
Maturity	0.095*			
Endosperm Texture	0.208**	0.051		
Husk Cover	0.241**	0.224**	0.068	
A. flavus infection	0.276**	0.012	0.224**	0.124

§ Units for traits: Aflatoxin Concentration (logarithmic transformation of ng g^{-1}), Maturity (Days to 50% Silking), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Husk Cover (1 = loose open husk, 5 = tight husk cover), Aspergillus flavus infection (1 = no visible ear rot infection, 5 = ear/cob completely visibly infected).

* Significant at 0.05.
** Significant at 0.01.

Table 2.7. Repeatabilities with standard errors of aflatoxin concentration in inbred trials in inoculated trials in 2000-2003.

	Year	$\bar{x}$ (Log)*	$\bar{x}$*	Maximum*	σ_g^2	σ_e^2	Repeatability ± Standard Error
White Inbreds	2000	2.482	303	1.546	0.03	0.12	0.493 ± 0.293
	2001	2.967	927	2.185	0.103	0.095	0.813 ± 0.081
	2002	1.279	19	3.322	0.346	0.686	0.668 ± 0.158
	2003	1.728	53	3.23	0.18	0.445	0.618 ± 0.152
Mean		2.114	326				0.648

Table 2.7 Continued.

	Year	$\bar{x}$ (Log)*	$\bar{x}$*	Maximum*	σ_g^2	σ_e^2	Repeatability ± Standard Error
Yellow Inbreds	2000	2.518	330	2.713	0.286	0.107	0.914 ± 0.052
	2001	3.011	1026	1.23	0.063	0.059	0.811 ± 0.082
	2002	2.04	110	3.732	0.6039	0.444	0.845 ± 0.071
	2003	1.951	89	3.62	0.6884	0.192	0.935 ± 0.025
Mean		2.380	389				0.876

* Mean (Log transformation of aflatoxin Concentration in ng g^{-1}), Antilog of means, variance components: genotype, error, repeatabilities ± standard error.

Repeatabilities across years were more for yellow inbreds than white inbreds (Figure 2.2).

Mean aflatoxin concentration was significantly correlated to repeatability across locations (r = 0.457) for all trials. The antilog of the maximum aflatoxin concentration for a given trial was highly correlated to repeatability (r = 0.517). Overall, yellow inbreds had higher repeatabilities than hybrids or white inbreds (Figure 2.2).

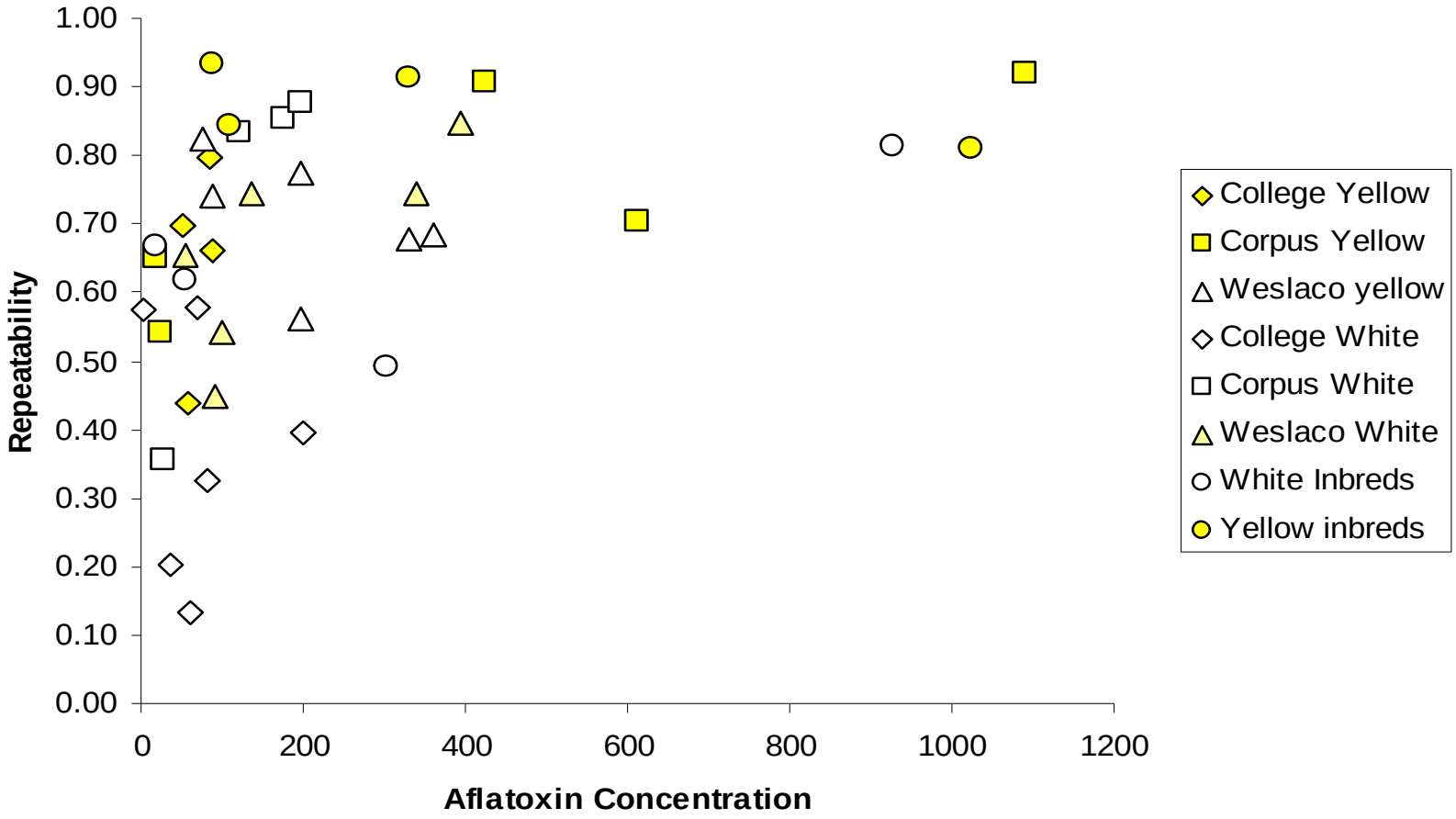

Figure 2.2. Aflatoxin concentration (ng g^{-1}) by repeatability for white and yellow hybrid and inbred trials in three Texas location (College Station, Corpus Christi, and Weslaco) from 1999-2004.

Quality Protein Maize

Quality protein maize (QPM) trials were grown in College Station and Weslaco in 2000 and 2001, and in all three locations in 2002 and 2003 (Table 2.8). Mean repeatability was highest in Corpus Christi ($\bar{x}$ = 0.844), followed by Weslaco ($\bar{x}$ = 0.762) and College Station ($\bar{x}$ = 0.540). QPM trials in 2003, which included more entries than all other years, had a higher yearly mean ($\bar{x}$ = 0.786) than all other years.

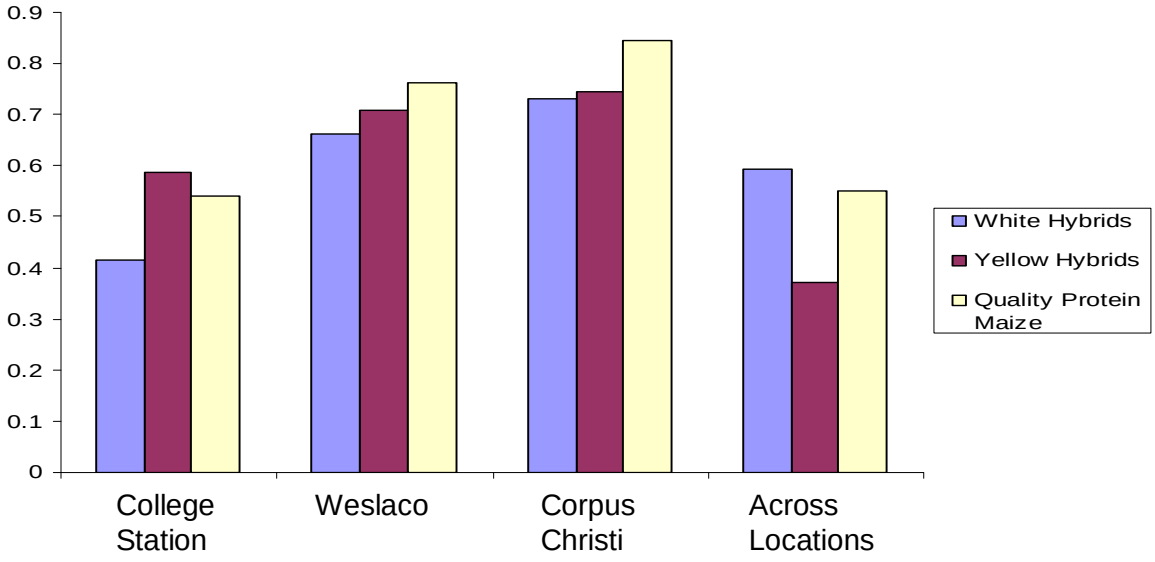

Figure 2.3. Mean repeatabilities of aflatoxin concentration in hybrid trials for 1999-2004 and quality protein maize trials in 2000-2003 under inoculation.

Table 2.8. Repeatabilities with standard errors of aflatoxin concentration in quality protein maize in inoculated trials per and across locations.

Year	Entries	College Station	Weslaco	Corpus Christi	Across Locations	Yearly Mean*
2000	20	0.563 ± 0.193	0.743 ± 0.097	NA	0.734 ± 0.143	0.653
2001	30	0.242 ± 0.350	0.628 ± 0.166	NA	0.469 ± 0.317	0.628
2002	30	0.449 ± 0.123	0.806 ± 0.043	0.810 ± 0.046	0.161 ± 0.418	0.688
2003	90	0.608 ± 0.122	0.872 ± 0.039	0.877 ± 0.038	0.838 ± 0.052	0.786
Location Mean*		0.540	0.762	0.844	0.551	

* Mean does not include repeatabilities that are zero or non-significant.

QPM trials had higher mean repeatability at all locations than white hybrids (Figure 2.3), and were more repeatable at Corpus Christi and Weslaco than yellow hybrids. At College Station, however, yellow hybrids had higher repeatabilities than QPM trials.

Secondary Traits

Several of the secondary traits measured were phenotypically correlated to aflatoxin concentration in hybrid trials (Table 2.6); therefore repeatability was measured for these traits. Not all traits were measured at every location each year. Mean repeatabilities were higher for maturity, endosperm texture and husk cover than mean repeatabilites for aflatoxin concentration across locations and years. There was also less variation for repeatability for these traits across years and locations than for aflatoxin concentration.

Repeatabilities for maturity, endosperm texture, and husk cover differed significantly for white and yellow hybrid trials. White hybrids had higher repeatability for maturity ($\bar{x}$ = 0.976) than yellow hybrids ($\bar{x}$ = 0.871). Yellow hybrids had higher repeatability for endosperm texture ($\bar{x}$ = 0.973) and husk cover ($\bar{x}$ = 0.887) than white hybrids ($\bar{x}$ = 0.898, and $\bar{x}$ = 0.664 respectively).

At all locations, texture was significantly and positively correlated to *A. flavus* for both white hybrids (r = 0.283** in College Station, r = 0.561** in Corpus Christi, r = 0.365** in Weslaco) and yellow hybrids (r = 0.245**, 0.195**, 0.224** respectively).

For white hybrids, endosperm texture was negatively and significantly correlated to maturity at all locations (r = -0.134**, -0.227**, -0.198** respectively). Endosperm texture and maturity were only significantly correlated at College Station (r = -0.214**) for yellow hybrids.

DISCUSSION

Both inoculation methods, the colonized kernel and the silk channel technique, yielded concentrations of aflatoxin which were high enough to offer variability for selection at the locations where used. The colonized kernel technique (Odvody *et al.*, 1996; Olanya *et al.*, 1997) was less effective in years with heavy rainfall during inoculation or flowering (2003, 2004). Heavy rains after colonized kernels have been placed between rows can cover the kernels with soil, providing a physical barrier that prevents inoculation. Should these environmental conditions occur, either colonized kernels should be reintroduced after rains have passed, or an alternative method of inoculation employed. Because drought stress combined with high temperatures during

flowering is more conducive to aflatoxin production (Payne, 1998), increased moisture during flowering reduces aflatoxin concentration. In this situation, the inoculation method will be less influential on aflatoxin concentration.

Repeatabilities for aflatoxin concentration were higher than expected. Corpus Christi generally has environmental conditions most favorable to aflatoxin concentration, and at that location the highest levels of aflatoxin were recorded. Weslaco, however, had the highest mean aflatoxin concentration for both white and yellow hybrids. Highest repeatabilites for aflatoxin concentration were reported in Corpus Christi also, indicating that range of aflatoxin concentration is more important for repeatability than mean aflatoxin concentration, as evidenced also by the higher correlation of maximum aflatoxin concentration to repeatability.

Previous studies have shown large GxE effects for aflatoxin concentration (Hamblin and White, 2000; Widstrom *et al.*, 1984; Zuber *et al.*, 1983). Trials examined in this study exhibited large GxE interactions and/or environmental variance. Across location repeatabilities for aflatoxin concentration were consistently lower than individual location repeatabilities. Corpus Christi had the highest mean

repeatability for aflatoxin concentration for white and yellow hybrids and QPM lines, followed by Weslaco and College Station. The high GxE interaction and environmental variance indicates that any future breeding efforts for aflatoxin resistance must include testing over several environments, although higher genotypic variance at Corpus Christi indicates that this environment provides a more optimal environment for selection for aflatoxin resistance.

While husk cover has been previously indicated as a morphological barrier to aflatoxin, the use of silk channel inoculation bypassed that barrier. Husk cover was not measured consistently enough in the trials when colonized kernel inoculation was used. Thus, despite this study finding no correlation between husk cover and aflatoxin concentration, the relationship of these traits remains unclear.

Endosperm texture was positively and significantly correlated to aflatoxin concentration for both white and yellow hybrids at Corpus Christi and at Weslaco. More floury endosperm yielded higher aflatoxin concentration. Because endosperm texture is highly heritable per and across locations, it is a potential characteristic for indirect selection for lower aflatoxin accumulation.

CHAPTER III

PHENOTYPIC EVALUATION OF RECOMBINANT INBRED LINE POPULATION CML176 x Tx811

INTRODUCTION

Within any crop-breeding program, several traits must be considered for selection, including complex ones. Simple or qualitative traits are controlled by few genes, are easy to score in early generations, and are highly heritable with very low response to environmental effects. With these traits, one can generally accomplish improved germplasm relatively quickly. Quantitative traits, on the other hand, are generally controlled by several genes with variable effects, are laborious to measure, and have a high level of environmental response. These types of traits are often the ones most crucial to breeders, such as grain yield and response to disease or pests. In order to have effective selection, a breeder must consider the amount of variance within the breeding populations that is attributable to genetics, that is, the proportion of variance that is heritable (Bernardo, 2002). This may be ascertained using field experiments that are designed such that the components contributing to overall phenotypic variance can be estimated.

One method of studying genetic contributions to phenotypic variance of complex traits is to develop and characterize a mapping population. A mapping population is developed by crossing phenotypically divergent parental inbreds, and exploring the variance in the offspring at a given level of inbreeding. The type of mapping population chosen depends on the needs and available resources of the program. Recombinant inbred line (RIL) populations, which are developed by selfing for several generations after crossing two divergent parents, are most commonly used, despite the longer development time, because they may be propagated indefinitely, provide higher resolution in maps, and may be used to study more than one trait. They allow for estimation of heritability of a trait and may be used for mapping quantitative trait loci (QTL) for complex traits.

A complex trait of particular interest to corn breeders in the southeastern United States is the response to preharvest aflatoxin concentration. Aflatoxin is a mycotoxin produced by the ear-rot fungus *Aspergillus flavus*. It creates serious economic losses as well as profound health problems in both animals and humans. Aflatoxin accumulation has been associated with high temperatures and drought at time of flowering (Payne *et al.*, 1998). These conditions are common in the southeastern USA, and thus aflatoxin is a persistent problem in

this region, with exceptionally large losses in the early 1990's. The World Health Organization has set the maximum residue level for aflatoxin in human foods at 20 parts per billion (ng g^{-1}), and in the USA, grain with levels of aflatoxin B1 higher than that is banned from interstate commerce. Grain with more than 300 ng g^{-1} may not be used as livestock feed. While there are some genetic sources for reduced levels of aflatoxin accumulation, there are currently no commercial corn hybrids that are resistant to aflatoxin production or *A. flavus* infection. Although some germplasm has less susceptibility to aflatoxin accumulation, this germplasm also has less desirable agronomic characteristics. Selection for aflatoxin resistance is difficult due to environmental variation and expense and difficulty in quantifying it.

Objectives of the Study

(i) Obtain statistical measurements (means for parental inbred lines, overall mean, and minimum and maximum values) for several phenotypic characteristics of the CML176 x Tx811 RIL mapping population: days to silking, root lodging, grain texture, aflatoxin concentration, grain yield', test weight, and thousand-kernel weight.

(ii) Estimate heritability with standard error and variance components for all characteristics per individual location and across locations.

(iii) Determine correlations between all phenotypic characteristics per location, and across locations for aflatoxin concentration and root lodging.

REVIEW OF LITERATURE

History of Aflatoxin Research

A. flavus has been recognized as a pathogen on corn since 1920 (Taubenhaus, 1920), but aflatoxin concentration was not considered to be problematic until the 1960s, when it was linked to poultry disease. While some studies showed preharvest infection with *A. flavus* (Barnsetetter, 1927; Butler, 1947; Eddins, 1930), it was considered predominantly to be a storage problem. It was first established in field trials as a preharvest problem in corn in the 1970's (Anderson *et al.*, 1975; Rambo *et al.*, 1974). Subsequent studies were implemented for field evaluation of corn genotypes in order to identify resistance sources to *A. flavus* and/or aflatoxin accumulation (Lillehoj *et al.*, 1976; Widstrom *et al.*, 1981). There are currently no commercial corn hybrids resistant to aflatoxin. Some of the limiting factors in developing aflatoxin

resistant corn are: the variation in aflatoxin accumulation that requires inoculation, more replications, and increased number of locations; the lack of a reliable, rapid, high throughput, and inexpensive screening methodology; and the low metabolic activity of corn plants after physiological maturity (Payne, 1992).

Environmental Conditions favoring Aflatoxin Production

Aflatoxin accumulation has been shown to be highly dependent on environmental conditions as well as inconsistent in expression, even under optimal conditions when subject to natural inoculum. *A. flavus* was originally classified as a storage fungus on corn, based on studies done in temperate climates. However, in temperate climates with hot, dry growing seasons, such as the southern United States, aflatoxin infection of corn is more likely to be a preharvest concern (Wilson and Abramson, 1992).

Temperature and moisture have been shown to be the factors that most influence the level of concentration with aflatoxin (Payne *et al.*, 1998; Widstrom, 1996). Specifically, high temperatures and drought stress resulted in high levels of aflatoxin concentration (Payne, 1998). Jones *et al.* (1981) noted the role of moisture, and McGee *et al.* (1996) found that high soil temperatures increased aflatoxin accumulation. Cole *et al.*

(1995) found that neither high temperatures nor drought stress alone were sufficient for the higher levels of concentration by using field trials where soil moisture and temperature were controlled. Some have suggested that higher night temperatures are important to concentration, both in corn (Ashworth *et al.*, 1969b) and also in almonds (Doster and Michailides, 1995).

Aspergillus flavus is one of the mycotoxin-producing fungi that is well adapted to grow on substrates with low moisture. Significant infection and aflatoxin concentration do not occur until the kernel moisture is below 32% (Payne, 1998). Aflatoxin can continue to be produced in kernels until the moisture reaches 15% (Payne *et al.*, 1988). When high temperatures and drought conditions are combined particularly during kernel filling, aflatoxin levels are highest (Lisker and Lillehoj, 1991; Vincelli *et al.*, 1995).

These conditions are prevalent in the southern United States, and therefore aflatoxin is a persistent problem in that region. They were also associated with a high incidence when the U.S. Corn Belt experienced higher than usual temperatures combined with low rainfall in the 1980's (Hurburgh, 1991). Other regions with these conditions that have

reported preharvest aflatoxin concentration include southern China, southeast Asia, and Africa (Hall and Wild, 1994).

Traits in Maize Affecting Aflatoxin Accumulation

Chemical methods of resistance that are under genetic control have been identified. The enzyme b-1-3-Glucanase may have a role in the inhibition of *A. flavus* growth on the grain when present in maize kernels (Lozovaya, 1998). Huang *et al.* (1997) has identified two other kernel proteins that appear to confer resistance. One inhibits aflatoxin production with no effect on fungal growth, while the other protein inhibits the growth of the fungus. Chen *et al.* (1998) also suggested a trypsin inhibitor in kernels that may confer resistance when present in high concentrations. Tubajika and Damann (2001) also implicated a trypsin inhibitor for increased resistance to aflatoxin concentration.

Additionally, physical methods of resistance are known to exist, such as kernel pericarp wax and husk covering over the ear. Wax and cutin layers on the surface of maize kernels have been indicated in conferring resistance to aflatoxin accumulation (Guo *et al.*, 1995; Russin *et al.*, 1997). Thicker pericarp layers may prevent initial infection by *A. flavus* conidia in undamaged kernels (Tubajika and Damann, 2001). Other research has focused on indirect protection of kernels by

breeding for better husk coverage (McMillian *et al.*, 1985; Lisker and Lillehoj, 1991). Tighter husk coverage may reduce insect susceptibility, and therefore fewer damaged kernels for *A. flavus* infection.

Efforts to accurately identify tolerant genotypes are hindered by environmental effects on phenotype. When environmental conditions are optimal, genotypic differences are displayed and selection is possible. When conditions do not favor aflatoxin production, however, phenotypic variance is lower and selection power is diminished. If more consistently expressed characteristics were associated with reduced aflatoxin accumulation, these traits might be used for indirect selection.

Heritability

In order to make progress through breeding, there must be genetic variation within breeding material that is identifiable and selectable by breeders. In complex traits, identification of genetic variation can be complicated by genotype x environment interaction (GxE). Additionally, the number of genes as well as their mode of action needs to be charcacterized. Genetic variation may be due to additive, dominant, or epistatic effects. The portion of genetic variation that is available to accumulate genetic gain through selection is termed additive variance. The ratio between the additive genetic variance and the total phenotypic

variance is known as narrow-sense heritability. With lower heritability traits, breeders have less selection power, and environmental effects affect genetic gains. Classic measurements of heritability are estimated by evaluating random genotypes in several environments. Cooper *et al.* (1993) utilized a measurement of repeatability that estimates the genetic variation available for breeding when genotypes are considered fixed. Response to aflatoxin concentration has long been considered to be lowly heritable due to the large environmental effect and the GxE interactions (Hamblin and White, 2000; Widstrom *et al.*, 1984; Zuber *et al.*, 1983).

Mapping Populations

Mapping is accomplished using populations that are constructed with some degree of genetic recombination. The mapping populations most commonly used are backcross populations, doubled haploids (DH), F_2 populations, recombinant inbred lines (RILs), and near isogenic lines (NILs). Selection of the type of population is dependent upon resource ability and research needs, such as economic feasibility, project time requirements, available labor force, lab/field space, and institutional infrastructure.

F_2 populations, backcross populations, and doubled haploids all undergo a single cycle of meiosis. Backcross populations are quickly developed and are recombinant only for one of the chromosomes of each homologous pair. They provide the least amount of information of possible mapping populations. Double haploid (DH) populations have the advantage of complete homozygosity, as well as being able to be propagated indefinitely, and contain the same amount of recombinant information as backcross populations. Development of a DH population is labor intensive, may result in some variation or aberrant segregation rations due to the tissue culture process, and is not feasible for some crops.

F_2 populations contain all possible combinations of parental alleles and can be rapidly and relatively easily developed. Both homologous chromosomes are recombinant in F_2 populations, which provides twice the information of a backcross population. However, these populations have a finite supply of seed, which limits the amount of testing that can be done on them.

RIL populations, which are developed by selfing for several generations after crossing two divergent parents, have many advantages for QTL mapping. A major advantage provided by RIL populations is that they

may be propagated indefinitely. This allows for multilocation and multiyear testing, which allows the partition of phenotypic differences in genotypic, environmental and GxE. Since RILs undergo several meiotic events in the process of development, fewer individuals are needed to detect linkage of the same magnitude as an F_2 population, which results in higher resolution in the maps. RILs take longer to develop than F_2 populations, backcross populations, or DH. Due to the lack of heterozygosity, DH and F_2 populations do not provide any estimate of dominant gene effects, which is important in hybrid crops that exploit heterosis. This is less of a concern for the study of aflatoxin production since genetic response to aflatoxin has been found primarily of additive in nature (Campbell and White, 1995b, Campbell *et al.*, 1997, Hamblin and White, 2000).

The final type of mapping population currently in use is near isogenic lines (NIL). This population consists of individuals that differ in the locus of interest but share the same genetic background elsewhere in the genome. Production of these populations is time consuming, and each population can only be used to map one trait.

MATERIALS AND METHODS

Germplasm and Population Development

Two inbred lines were used to create a mapping population of RILs to study response to aflatoxin concentration. The parents used were two quality protein maize (QPM) inbreds that differed for many agronomic characteristics, CML176 and Tx811. Tx811 is a temperate line released in 2003 with intermediate maturity that is susceptible to aflatoxin accumulation. CML176 is an subtropical line with late maturity that has been shown to be less susceptible to aflatoxin accumulation, but has susceptibility to root lodging. These lines were crossed and then selfed for at least 6 generations to produce a RIL mapping population with 160 S_6 RILs.

Environments

The entire population was grown in two Texas locations, College Station (latitude 30°37, elevation 96 m) and Weslaco (latitude 26°09, elevation 22.5 m). An alpha lattice design was used with 3 reps at each location, with additional reps of each of the parental inbreds included as checks. Experimental units consisted of single rows plots of 4.047 m^2 in College Station and 5.079 m^2 in Weslaco . Plant populations was 50604 plants/ha in Weslaco and 66220 plants/ha in College Station. Standard

cultural practices in both locations were applied. Limited irrigation was applied around flowering to induce some level of drought stress.

Inoculation

All trials were inoculated with *A. flavus* isolate NRRL3357. Aflatoxin production exhibits high spatial and environmental variation, therefore both trials were inoculated to eliminate possibility of environmental variation of natural inoculum. Inoculation was accomplished using the nonwounding silk channel inoculation technique (Zummo and Scott, 1989). Plants were inoculated with a conidial suspension containing 3 x 10^7 conidia of *A. flavus* in 3 mL distilled water injected by syringe 6 to 10 days after midsilk.

Phenotypic Traits Measured

Silking date, defined as the days from planting to the date at which 50% of the plants in a plot exhibited emerged silks, was taken for all plots at both locations. Prior to harvest, each plot was rated for percentage of root lodging, defined as the percent of plants with stalks leaning greater than 30° from vertical.

Inoculated ears were harvested by hand. In the field, grain was rated for texture (1 indicating flinty endosperm with round crown kernel and

vitreous appearance, 5 indicating floury endosperm with pronounced dentation) and kernel integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage). At harvest, infected ears were husked, dried, shelled, and bulked. Grain yield' was measured in grams per ear and test weights (g/L) and thousand kernel weights (g) were taken for each bulked sample. The whole kernel sample was ground using a Romer mill (Union, MO) and quantified for aflatoxin concentration. Quantification of aflatoxin was conducted using 50-g subsamples of corn meal from each plot with monoclonal antibody affinity columns and flouresence determination using Vicam Aflatest (Watertown, MA). Aflatoxin concentration was measured in parts per billion (ng g^{-1}).

Analysis

Data was analyzed using SAS procedures and REMLtool™ software. Genotypic means were obtained using REMLtool™ software, which utilizes restricted maximum likelihood (REML) methods in mixed linear models. All effects (lines, environments, replications, block within replications) were considered random. Variance components, heritability estimates and standard errors were computed using SAS codes developed by Holland *et al.* (2003). Heritability was estimated for all

measured traits both per location and across locations. For individual locations, heritability (h^2) was measured using the formula:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{r}}$$

where σ_g^2 is the genotypic variance, σ_e^2 is the variance due to error, and r is the number of replications. The formula for across location estimates was:

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{ge}^2}{e} + \frac{\sigma_e^2}{r'e}}$$

where σ_g^2 is the genotypic variance, σ_{ge}^2 is the estimate of genotype by environment interaction, σ_e^2 is the variance due to error, and r' is the harmonic mean of the number of replications, and e is the number of environments.

Correlations were estimated using Pearson's correlation function, with significance determined for 150 degrees of freedom. The formula for the Pearson product moment correlation coefficient, r, is:

$$r = \frac{\sum (x - \bar{x})(y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

where x and y are the sample means of trait x to trait y. Biplots from lines x traits two way table were develop after standardized the traits to illustrate the relationship among traits at single environments using the Biplot v1.1 in Excel (Smith, E.P., Virginia Tech; http://www.stat.vt.edu/facstaff/epsmith.html). Genotypic correlations for each trait were obtained for each location and across locations with SAS codes developed by Holland (2003) using estimated variance components. Genotypic correlation was estimated as follows:

$$r = \frac{Cov_{xy}}{\sigma^2_{gx}\sigma^2_{gy}}$$

Where Cov_{xy} is the genetic covariance between trait x and trait y and σ^2_{gx} and σ^2_{gy} are the genetic variance for traits x and y respectively. Phenotypic correlation was estimated from variance component using the formula:

$$r = \frac{Cov_{xy}}{\sigma^2_{px}\sigma^2_{py}}$$

Where Cov_{xy} is the phenotypic covariance between trait x and trait y and σ^2_{px} and σ^2_{py} are the phenotypic variance for traits x and y respectively. Expected genetic gain for direct selection for each trait was estimated using following Falconer and Mackay (1996):

$$\text{Genetic gain} = 1.75 * \sqrt{\sigma^2_g} * \sqrt{h^2}$$

Where 1.75 is the selection differential for 10% selection, σ^2_g is the genetic variance and h^2 is the heritability. Expected indirect genetic gain or correlated response (CR_y) for aflatoxin concentration was measured as:

$$CR_y = 1.75 * r_{gxy} * \sqrt{h^2_x} * \sqrt{\sigma^2_{gy}}$$

Where 1.75 is the selection differential for 10% selection, r_{gxy} is the genotypic correlation between trait x and y, h^2_x is the heritability of the secondary trait x, and σ^2_{gy} is the genetic variance of the trait of interest.

RESULTS

For all traits measured, the population had significant phenotypic variation (Figures 3.1-3.10). The means for parental inbred lines CML176 and Tx811 incorporate a total of 12 reps per location.

Phenotypic Variation and Heritabilities

Aflatoxin Accumulation

Aflatoxin concentration was measured in parts per billion (ng g^{-1}). Distribution of aflatoxin concentration was skewed at both College Station (Figure 3.1a) and Weslaco (Figure 3.2a). Logarithmic transformation of aflatoxin measurements was used in analysis to equalize variance and normalize the data, for both locations (Figures 3.1b and 3.2b).

The RIL population had significant variation for aflatoxin accumulation in both locations. Aflatoxin concentration reached a maximum of almost 3900 ng g^{-1} and had a mean of 292 ng g^{-1} in College Station, and was over 4400 ng g^{-1} with a mean of 214 ng g^{-1} in Weslaco (Table 3.1). Across locations, the overall mean was 250 ng g^{-1} with a heritability estimate of 0.508, which is intermediate to the values of heritability at College Station (h^2 = 0.315) and Weslaco (h^2 = 0.663).

In both trials, Tx811 had higher mean aflatoxin concentration than CML176, although the differences were not significant. CML176 was among the top 10 lines with least aflatoxin concentration in both locations, although only one of the offspring also exhibited such low

Table 3.1. Statistical measurements and estimates for variance components and heritabilities of aflatoxin concentration in ng g $^{-1}$ for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	195	163	154
Mean CML176	154	32	50
Overall Mean	292	214	250
Range	3864	4416	4198
	Variance Components		
Rep	0.012	0.000	0.008
Block	0.040	0.023	0.000
Genotype	0.047	0.217	0.085
Residual	0.308	0.331	0.349
Environment	NA	NA	4.321
Genotype by Environment Interaction	NA	NA	0.048
Heritability¥	0.315 ± 0.097	0.663 ± 0.050	0.508 ± 0.076

¥ ± Standard Error.

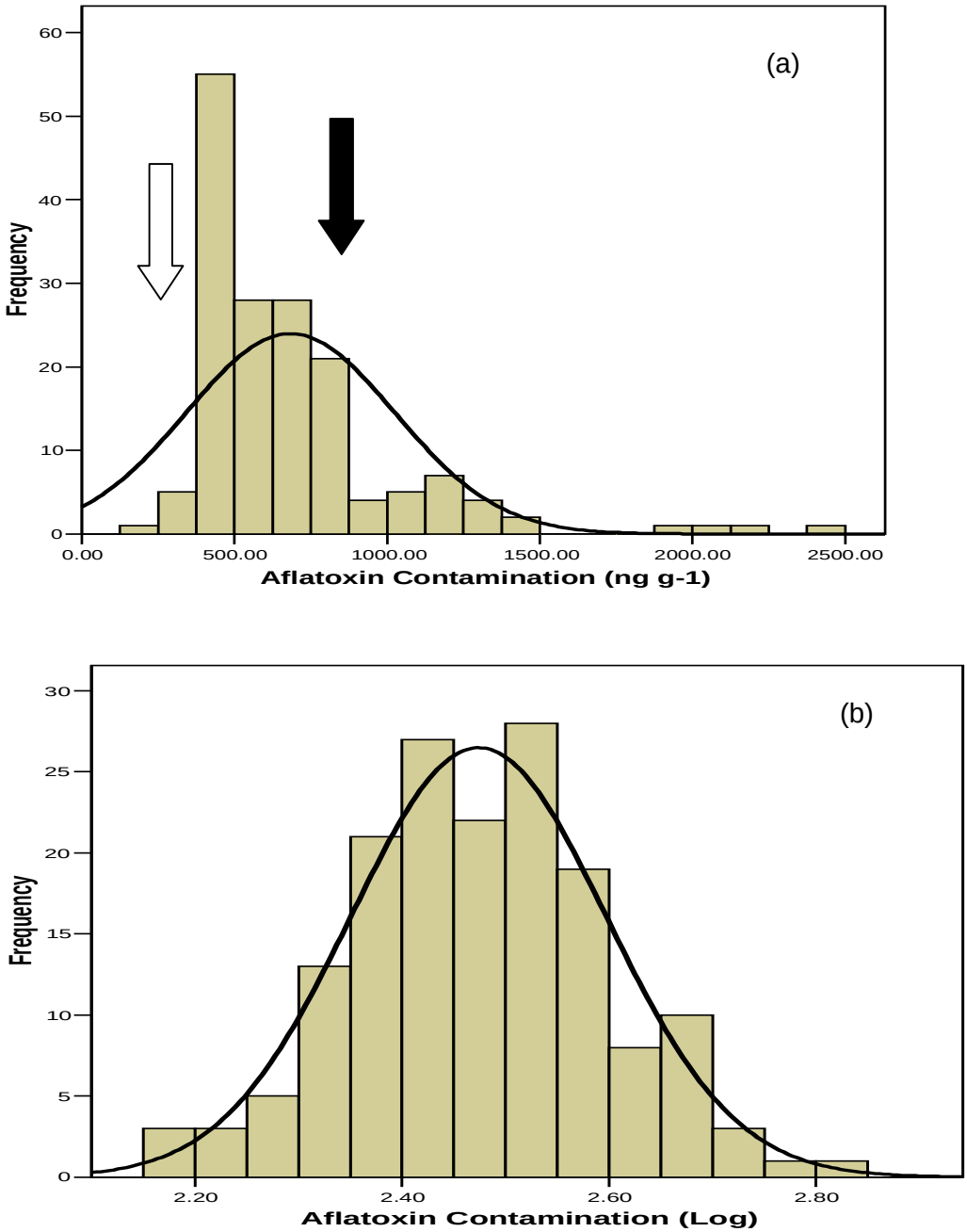

Figure 3.1. Distribution of RIL population for aflatoxin concentration in College Station. Concentration is measured in parts per billion (ng g^{-1}) (a) and the logarithmic transformation of ng g^{-1} measurement (b). Arrows indicate mean concentration for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

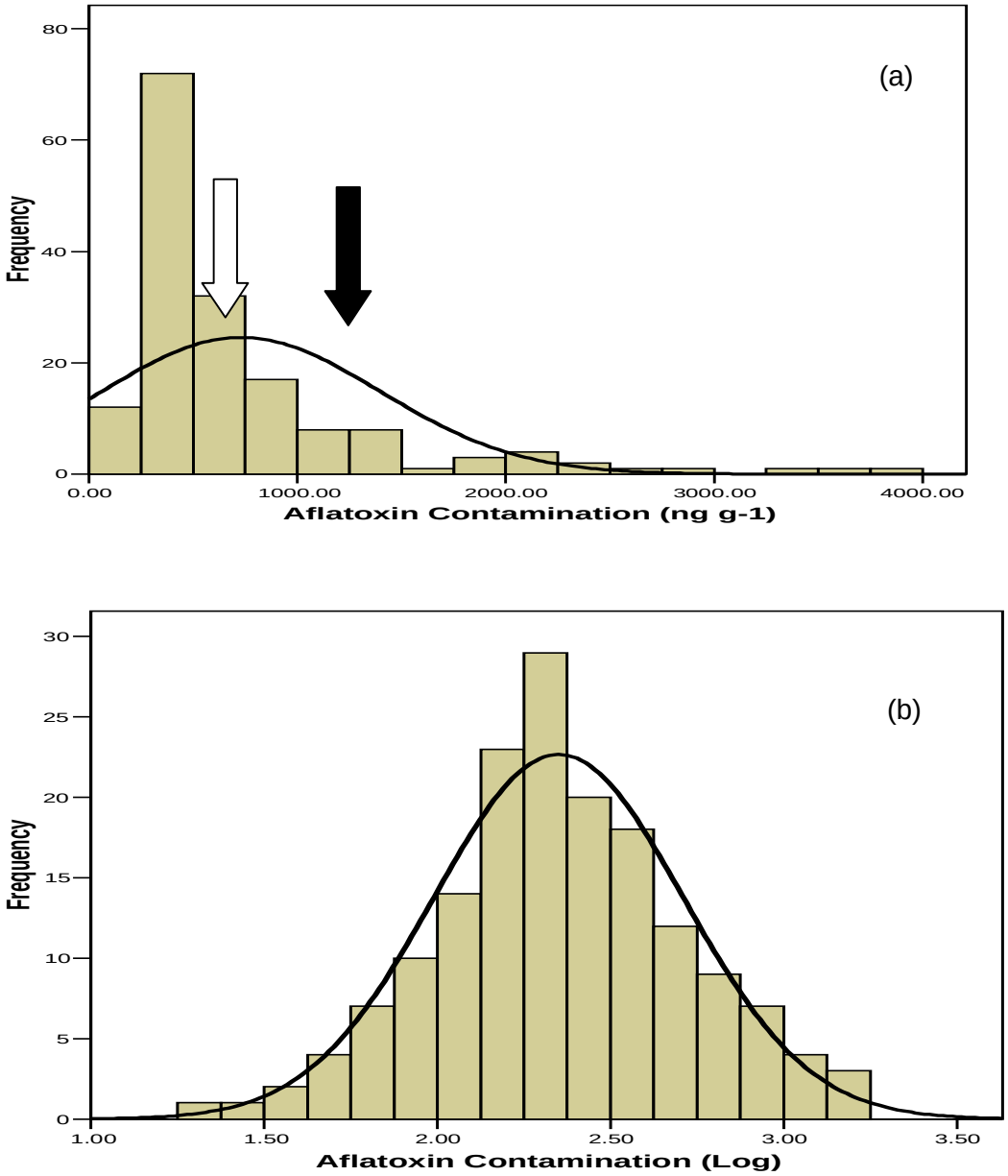

Figure 3.2. Distribution of RIL population for aflatoxin concentration in Weslaco. Concentration is measured in parts per billion (ng g^{-1}) (a) and the logarithmic transformation of ng g^{-1} measurement (b). Arrows indicate mean concentration for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

accumulation in both locations (data not shown). Rankings between locations were not consistent, due to a low correlation of aflatoxin concentration between locations (Figure 3.3). At each location, the component contributing the most to variance was error (Table 3.1). The genotypic variance component was a minimal contribution relative to error in College Station, resulting in a heritability estimate there that was less than half the heritability at Weslaco. While genotypic variance was higher than GxE interaction across locations, variance between environments contributed to the majority of overall phenotypic variance for aflatoxin concentration.

Because of this environmental variance, mean aflatoxin concentration for College Station was compared to Weslaco (Figure 3.3). While there was some correlation between the two locations (R^2 = 0.200), it was not strong enough to predict the performance of a line in one location by data in the other environment.

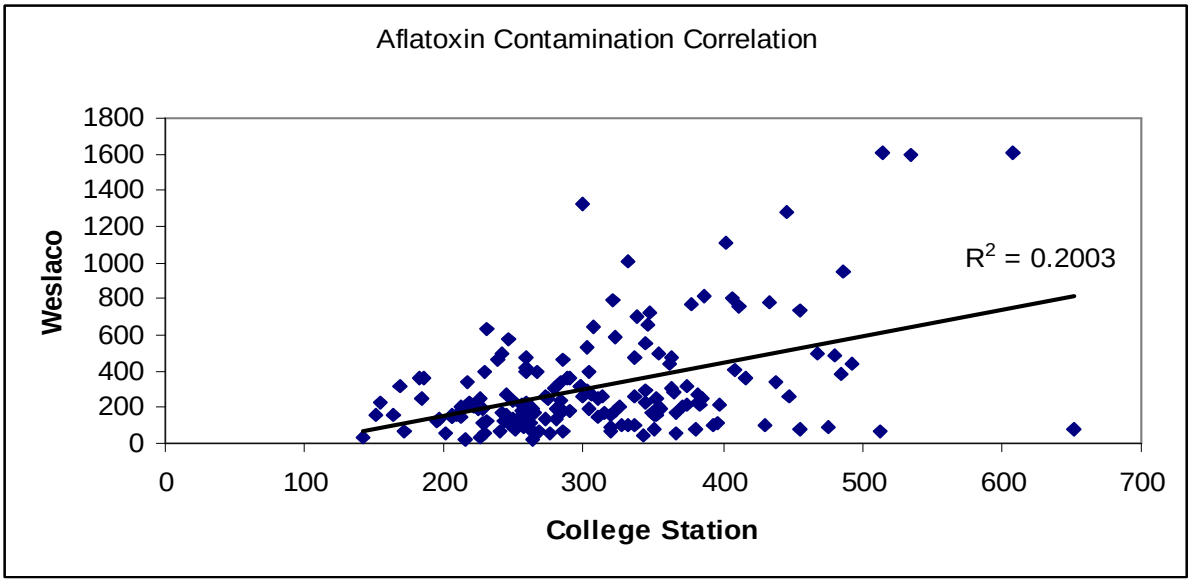

Figure 3.3. Correlation of mean aflatoxin concentration (anti-log) for recombinant inbred lines between College Station and Weslaco.

Female Flowering

The population had significant variation for female flowering at both locations (Figure 3.4), with parental lines differing significantly at both trials (Table 3.1). Tx811 has earlier maturity (83 days in College Station, 89 days in Weslaco) than CML176 (89 days in College Station, 93 days in Weslaco). Heritabilities were high for both College Station (h^2 = 0.888) and Weslaco (h^2 = 0.853) and across locations (h^2 = 0.831).

While there was significant variation for maturity between environments, maturity had little genotype by environment interaction (Table 3.2), and at each location, the most significant component contributing to

variation was genotype. Thus, maturity was a highly heritable trait, both at individual locations and across locations.

Table 3.2. Statistical measurements and estimates for variance components and heritabilities of maturity (days to 50% silking) for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	83	89	86
Mean CML176	89	93	91
Overall Mean	86	91	88
	Variance Components		
Rep	0.0891	0	0.0415
Block	0.2881	0.0579	0.2021
Genotype	6.8544	2.9033	3.9328
Residual	2.5886	1.5015	2.0303
Environment	NA	NA	12.522
Genotype by Environment Interaction	NA	NA	1.004
Heritability[¥]	0.888 ± 0.015	0.853 ± 0.020	0.831 ± 0.027

[¥] ± Standard Error.

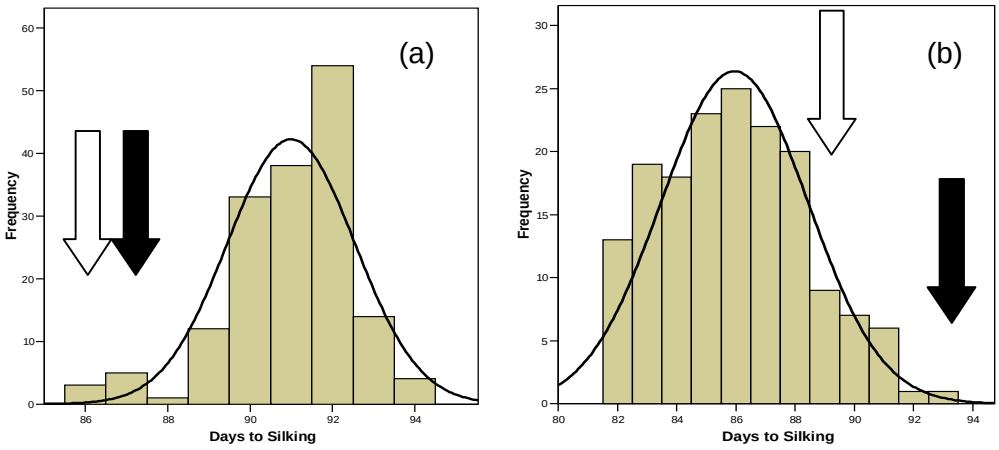

Figure 3.4. Distribution of RIL population for maturity, measured in days to 50 % silking at College Station (a) and Weslaco (b). Arrows indicate mean flowering time for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

Endosperm Texture

There were significant differences within the population for endosperm texture ratings (Figure 3.5). Tx811 was significantly more floury across locations (2.60) than CML176 (1.14). The parental inbreds were not significantly different in College Station, although in Weslaco the results were similar to results from across location analysis (Table 3.3). The mean for the population was 2.2 across locations. Texture was highly heritable at College Station (h^2 = 0.881) and Weslaco (h^2 = 0.809) as well as across locations (h^2 = 0.801).

Table 3.3. Statistical measurements and estimates for variance components and heritabilities of endosperm texture* for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	2.4	2.7	2.6
Mean CML176	1.6	1.6	1.4
Overall Mean	2.024	2.349	2.176
	Variance Components		
Rep	0	0.0044	0.0018
Block	0.0071	0.0171	0.0121
Genotype	0.4301	0.3581	0.3111
Residual	0.1741	0.2537	0.2137
Environment	NA	NA	0.0424
Genotype by Environment Interaction	NA	NA	0.0833
Heritability¥	0.881 ± 0.016	0.809 ± 0.026	0.801 ± 0.031

* 1 indicates flinty endosperm with round crown kernel and vitreous appearance, 5 indicates floury endosperm with pronounced dentation.
¥ ± Standard Error.

The most significant source of variation at each location and across locations was genotype. The error component of variation was higher at

Weslaco than at College station, resulting in a lower heritability estimate for Weslaco. Heritability of endosperm texture was high at both locations as well as across locations due lower environmental variation or GxE interaction.

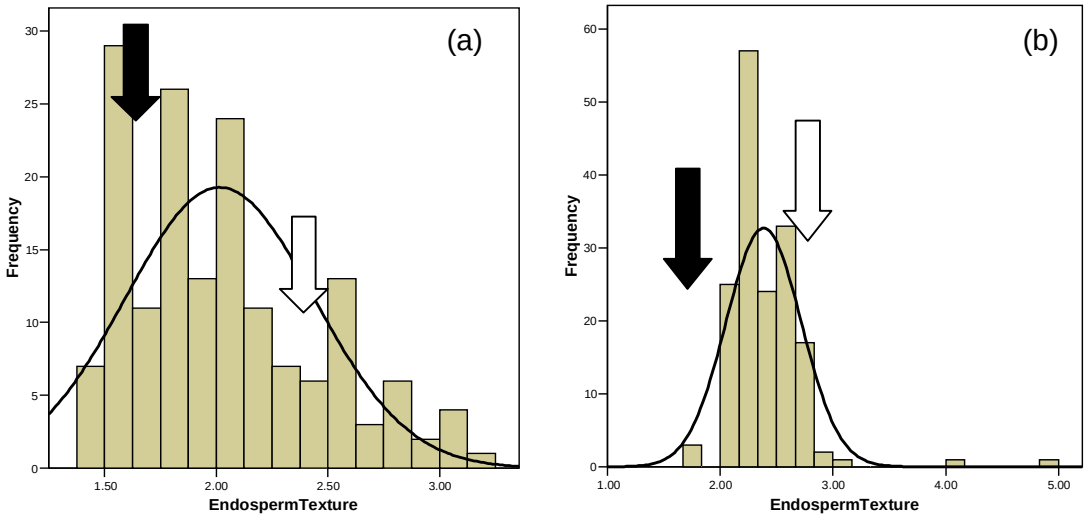

Figure 3.5. Distribution of RIL population for endosperm texture, scored from 1 (flinty endosperm with round crown kernel and vitreous appearance) to 5 (floury endosperm with pronounced dentation) at College Station (a) and Weslaco (b). Arrows indicate mean endosperm texture for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

Kernel Integrity

Parental inbreds were not significantly different for kernel integrity at either location (Table 3.4). The population, however, did show significant variation (Figure 3.6). Kernel integrity was more heritable at

College Station (h^2 = 0.829) than at Weslaco (h^2 = 0.716), while the across location heritability was lower than both (h^2 = 0.463).

Genotypic variance was the largest variance component for kernel integrity in College Station. In Weslaco and across locations, residual variance was higher than genotypic variance, which lowered the heritability. Across locations, however, environmental variance and GxE interaction were minimal and heritability overall remained high.

Root Lodging

The RIL population exhibited a large amount of variation for root lodging (Figure 3.7). CML176 has exhibited poor root characteristics in the past, and as such is susceptible to root lodging. Tx811 is less susceptible to root lodging, but in cases of extreme environmental stresses also exhibits lodging (Table 3.5). At College Station, the differences in susceptibility to root lodging were less visible due to the higher overall level of lodging (mean of 45.8 %). At Weslaco, where lodging was lower (mean of 16.2 %), CML176 had significantly higher levels of root lodging (40.6 %) than Tx811 (4.6 %). Across locations, the differences in the parents were still significant. At both individual locations, heritability for root lodging was over 0.70, while across locations heritability was 0.522.

Table 3.4. Statistical measurements and estimates for variance components and heritabilities of kernel integrity* for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	2.3	2.8	2.24
Mean CML176	1.8	1.7	1.5
Overall Mean	2.5	2.7	2.6
	Variance Components		
Rep	0.0040	0.0000	0.0019
Block	0.0000	0.0068	0.0000
Genotype	0.5696	0.4336	0.4070
Residual	0.3531	0.5161	0.4374
Environment	NA	NA	0.0124
Genotype by Environment Interaction	NA	NA	0.0965
Heritability¥	0.829 ± 0.023	0.716 ± 0.039	0.771 ±0.035

* 1 indicates all ears without split kernels or insect damage, 5 indicates most of the ears with splits and/or insect damage.
¥ ± Standard Error.

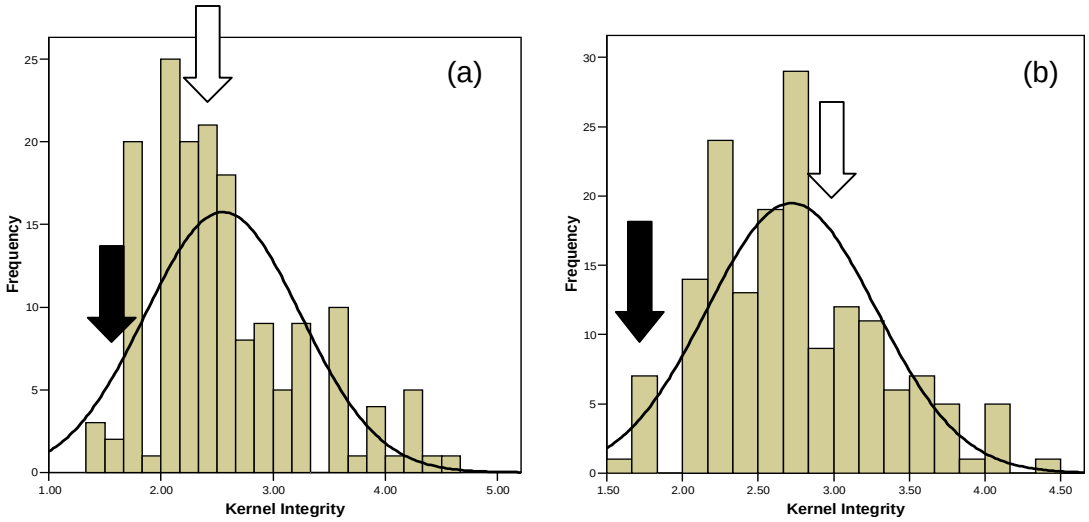

Figure 3.6. Distribution of RIL population for kernel integrity, scored from 1 (all ears without split kernels or insect damage) to 5 (most of the ears with splits and/or insect damage) at College Station (a) and Weslaco (b). Arrows indicate mean kernel integrity for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

While conditions at College Station were conducive to root lodging (heavy wind and rain at flowering), they were less optimal at Weslaco. At College Station, the largest component of phenotypic variance for root lodging was residual variance, which was close to the amount of genotypic variance. At Weslaco, genotypic variance was the greatest contributor to phenotypic variance. Across locations, variation due to environment was the greatest variance component, and variance due to GxE interaction was higher than genotypic variance. This high environmental influence on phenotype reduces the heritability of root lodging across environments.

Table 3.5. Statistical measurements and estimates for variance components and heritabilities of root lodging* for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	39.02	4.57	19.95
Mean CML176	58.13	40.64	50.75
Overall Mean	45.84	16.20	31.09
	Variance Components		
Rep	41.676	4.075	22.966
Block	98.622	42.673	69.023
Genotype	313.170	211.920	130.640
Residual	392.570	206.510	300.180
Environment	NA	NA	429.430
Genotype by Environment Interaction	NA	NA	132.680
Heritability¥	0.705 ± .041	0.755 ± 0.034	0.529 ± 0.075

* Measured as percent of plants with stalks leaning greater than 30° from vertical.

¥ ± Standard Error.

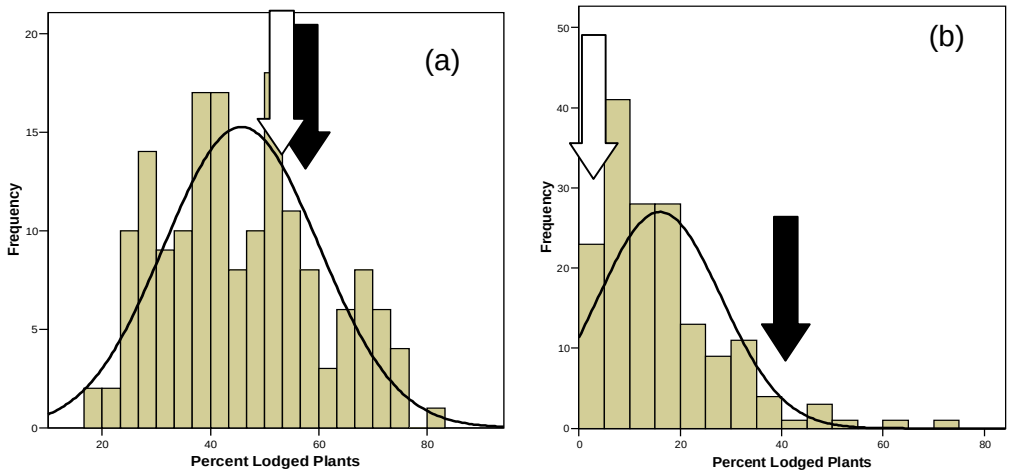

Figure 3.7. Distribution of RIL population for root lodging, measured as percent of plants with stalks leaning greater than 30° from vertical, at College Station (a) and Weslaco (b). Arrows indicate mean root lodging for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

Grain Yield' and Yield Components

Parental inbred lines were not significantly different for grain yield' (Table 3.6), thousand kernel weight (Table 3.7) or test weight (Table 3.8) at either location. Grain yield' was normally distributed with significant differences for the population (Figure 3.8). There were no significant differences in mean grain yield' across locations.

Grain yield' was less heritable at College Station (h^2 = 0.321) than at Weslaco (h^2 = 0.702). Residual variance was much higher than genotypic variance at College Station. They were not significantly different at Weslaco. Residual variance was high across locations, but

GxE interaction was also a significant component of overall variance, resulting in a lower across location heritability (h^2 = 0.296).

Thousand kernel weight was normally distributed at Weslaco, but distribution was skewed at College Station (Figure 3.9) due to a few genotypes with higher thousand kernel weight. Grain from College Station was heavier (212.2 grams) than that at Weslaco (169.9 grams). It was not significantly heritable at College Station or across locations, and was very lowly heritable (h^2 = 0.297) at Weslaco.

Both locations had a high level of residual or error variance. Thousand kernel weight showed a high level of genotype by environment interaction, but error remained the largest contributor to phenotypic variance.

Test weight was normally distributed at College Station, but distribution was slightly skewed at Weslaco. While mean test weight was not significantly different between locations, the range for test weight was significantly higher at College Station (658.7) than it was at Weslaco (372.7).

Test weight was not heritable at College Station due to large residual

Table 3.6. Statistical measurements and estimates for variance components and heritabilities of grain yield', measured in grams per ear, for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	57.247	48.749	51.845
Mean CML176	58.340	59.693	58.574
Overall Mean	58.867	50.529	54.650
Range	75.830	74.350	101.860
	Variance Components		
Rep	0.000	13.325	3.570
Block	0.000	20.318	8.761
Genotype	134.910	61.311	44.309
Residual	855.210	78.164	479.020
Environment	NA	NA	52.780
Genotype by Environment Interaction	NA	NA	202.040
Heritability [¥]	0.321 ± 0.091	0.702 ± 0.042	0.296 ± 0.109

[¥] ± Standard Error.

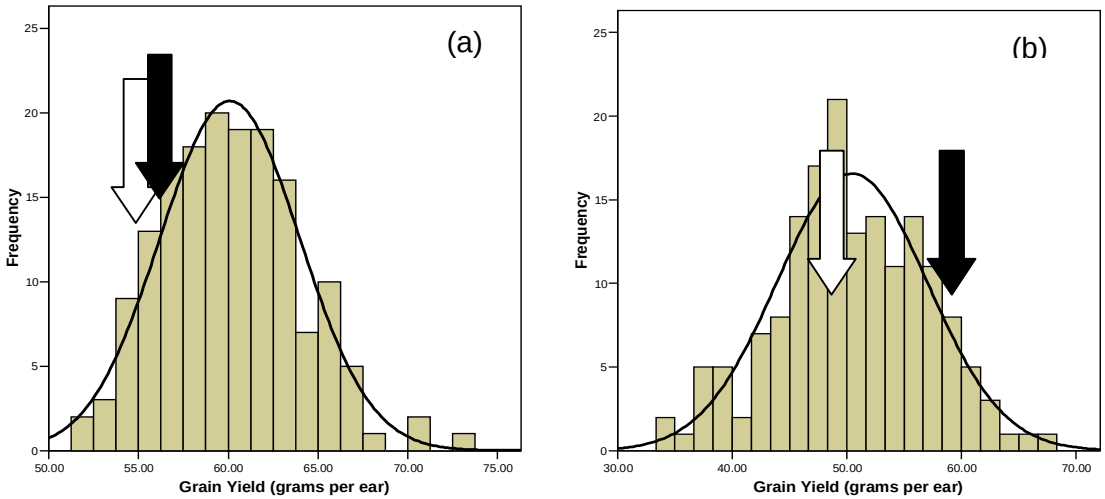

Figure 3.8. Distribution of RIL population for grain yield' in grams per ear at College Station (a) and Weslaco (b). Arrows indicate mean grain yield' for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

Table 3.7. Statistical measurements and estimates for variance components and heritabilities of thousand kernel weight in grams, for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	204.8	162.4	177.1
Mean CML176	216.6	187.8	213.2
Overall Mean	212.2	169.9	183.8
	Variance Components		
Rep	75.49	132.45	104.86
Block	797.38	306.13	556.31
Genotype	3.87	548.5	83.64
Residual	13146	3897.28	8505
Environment	NA	NA	831.89
Genotype by Environment Interaction	NA	NA	202.04
Heritability ¥	0.001 ± 0.142	0.297 ± 0.098	0.052 ± 0.156

¥ ± Standard Error.

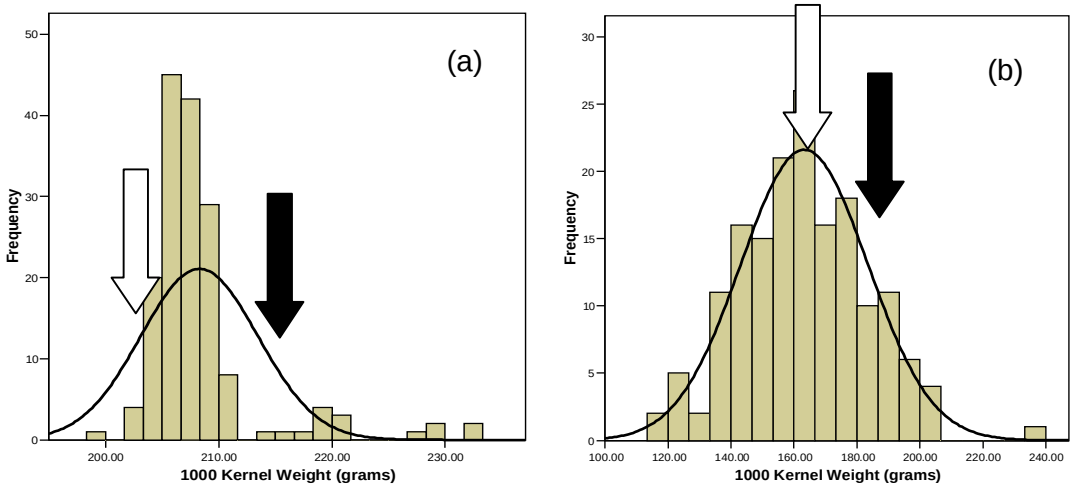

Figure 3.9. Distribution of RIL population for thousand kernel weight in grams at College Station (a) and Weslaco (b). Arrows indicate mean thousand kernel weight for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

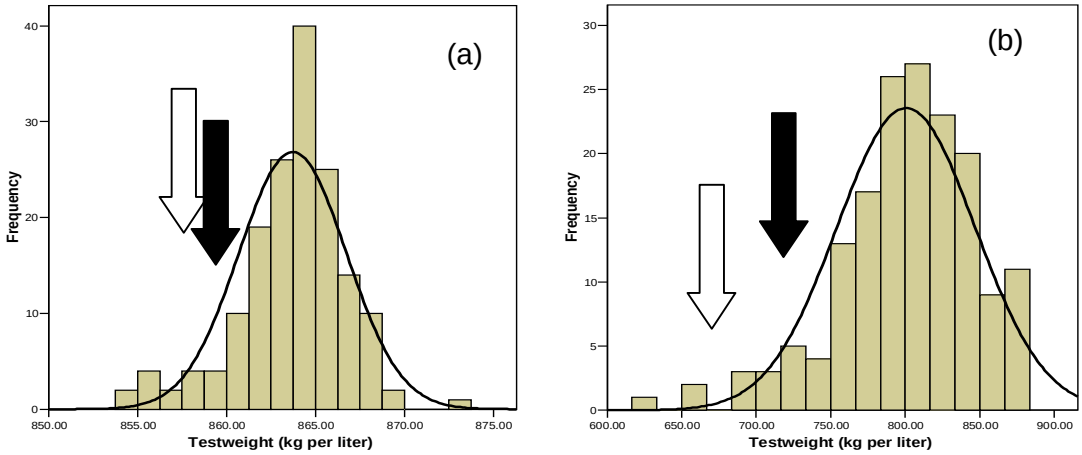

Figure 3.10. Distribution of RIL population for test weight in g L^{-1} at College Station (a) and Weslaco (b). Arrows indicate mean test weight for parental inbred lines Tx811 (white) and CML176 (black) in 2004.

Table 3.8. Statistical measurements and estimates for variance components and heritabilities of test weight in kilograms per liter for CML176 x Tx811 recombinant inbred line population per and across locations in 2004.

	College Station	Weslaco	Across Locations
Mean Tx811	857.5	797.6	814.7
Mean CML176	869.8	878.4	883.7
Overall Mean	849.1	802.1	825.5
	Variance Components		
Rep	0	36.79	0
Block	1030.69	116.09	588.87
Genotype	2329.24	2392.29	1130.69
Residual	127243	987.54	63467
Environment	NA	NA	1772.78
Genotype by Environment Interaction	NA	NA	1156.31
Heritability ¥	0.052 ± 0.127	0.879 ± 0.017	0.092 ± 0.141

¥ ± Standard Error.

variance. Error was much lower at Weslaco, and test weight was highly heritable there (h^2 = 0.879). Environmental variation and GxE

interactions were both high in the across location analysis, and test weight was not heritable across locations.

Pearson's Correlations among Traits

Correlation estimates are given for all traits at College Station (Table 3.9) and Weslaco (Table 3.10). The relative relationship of each trait to all others is shown for College Station (Figure 3.11) and Weslaco (Figure 3.12).

In College Station, kernel integrity (r = 0.535**) and texture (0.249**) were positive and significantly correlated to aflatoxin accumulation (Table 3.11). At Weslaco, aflatoxin accumulation was correlated to days to silking (r = -0.229**) in addition to kernel integrity and endosperm texture (r = 0.605** and r = 0.187*, respectively). Correlations between aflatoxin concentration and other traits were similar at College Station (Figure 3.11) and Weslaco (Figure 3.12).

Maturity was significantly and negatively correlated to grain yield (r = -0.166*) and thousand kernel weight (r = -0.299**) in College Station (Table 3.9). In Weslaco, it was significantly correlated to both traits, and it was also significantly and negatively correlated (r = -0.229**) to aflatoxin accumulation.

Table 3.9. Pearson's correlations of phenotypic traits[§] in College Station for the CML176 x Tx811 recombinant inbred line population in 2004.

	Maturity	Kernel Integrity	Endosperm Texture	Grain yield'	Root Lodging	Aflatoxin Concentration	1000 Kernel Weight	Test weight
Maturity	1.000							
Kernel integrity	0.077	1.000						
Endosperm texture	0.028	0.466**	1.000					
Grain yield'	-0.166*	-0.268*	-0.127	1.000				
Root Lodging	0.121	0.018	0.024	-0.135	1.000			
Aflatoxin concentration	0.133	0.535**	0.249**	-0.106	-0.101	1.000		
1000 Kernel weight	-0.299**	-0.083	-0.112	0.105	-0.009	-0.003	1.000	
Test weight	-0.010	-0.155	-0.063	0.172*	-0.101	0.001	0.078	1.000

§ Units for traits: Maturity (Days to 50% Silking), Kernel Integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Grain Yield (grams per ear), Root Lodging (% of plants with stalks leaning greater than 30° from vertical), Aflatoxin Concentration (logarithmic transformation of ng g^{-1}), 1000 Kernel Weight (grams), Test weight (g L^{-1}).

* Significant at 0.05 (150 df).

** Significant at 0.01 (150 df).

Table 3.10. Pearson's correlations of phenotypic traits§ in Weslaco for the CML176 x Tx811 recombinant inbred line population in 2004.

	Maturity	Kernel Integrity	Endosperm Texture	Grain Yield	Root Lodging	Aflatoxin Concentration	1000 Kernel Weight	Test weight
Maturity	1.000							
Kernel integrity	-0.209**	1.000						
Endosperm texture	0.035	0.306**	1.000					
Grain Yield	-0.421**	-0.092	0.029	1.000				
Root Lodging	0.470**	-0.188*	-0.094	-0.094	1.000			
Aflatoxin concentration	-0.229**	0.605**	0.187*	0.018	-0.133	1.000		
1000 Kernel weight	-0.149	-0.041	-0.059	0.509**	-0.023	0.022	1.000	
Test weight	-0.039	-0.561**	-0.371**	0.200*	0.199*	-0.374**	0.438**	1.000

§ Units for traits: Maturity (Days to 50% Silking), Kernel Integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Grain Yield (grams per ear), Root Lodging (% of plants with stalks leaning greater than 30° from vertical), Aflatoxin Concentration (logarithmic transformation of ng g^{-1}), 1000 Kernel Weight (grams), Test weight (g L^{-1}).

* Significant at 0.05 (150 df).

** Significant at 0.01 (150 df).

Kernel integrity, in addition to being significantly correlated to aflatoxin accumulation, is significantly and positively correlated to endosperm texture at both College Station (r = 0.466**) and Weslaco (r = 0.306**). There was inconsistent correlation with grain yield characteristics. At College Station, kernel integrity was negatively but significantly correlated with grams per ear (r = -0.268*) while at Weslaco, it was correlated to test weight (r = -0.561**).

Root lodging was not significantly correlated to any other traits in College Station, where lodging was extensive. At Weslaco, root lodging was significantly correlated to maturity (r = 0.470**), kernel integrity (r = -0.188*), and test weight (r = 0.199*).

At College Station, yield components were not consistently correlated. Grain yield and test weight were significantly correlated (r = 0.172*), but neither were correlated to thousand kernel weight. At Weslaco, grain yield was significantly correlated to test weight (r = 0.200*) and thousand kernel weight (r = 0.509**), and test weight and thousand kernel weight were correlated (r = 0.438**).

Table 3.11. Genotypic and Phenotypic Correlations between aflatoxin concentration and secondary traits[§] at College Station in 2004.

	Genotypic Correlation	Phenotypic Correlation
Maturity	0.250 ± 0.159	0.099 ± 0.050
Kernel Integrity	0.847 ± 0.416	0.412 ± 0.041
Endosperm Texture	0.456 ± 0.204	0.157 ± 0.047
Root Lodging	-0.335 ± 3.211	-0.067 ± 0.081
Grain Yield	-0.321 ± 0.285	-0.043 ± 0.048
1000 Kernel Weight	0.084 ± 0.070	-0.001 ± 0.001
Test weight	0.726 ± 1.760	0.021 ± 0.047

§ Units for traits: Maturity (Days to 50% Silking), Kernel Integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Grain Yield (grams per ear), Root Lodging (% of plants with stalks leaning greater than 30° from vertical), 1000 Kernel Weight (grams), Test weight (g l^{-1}).

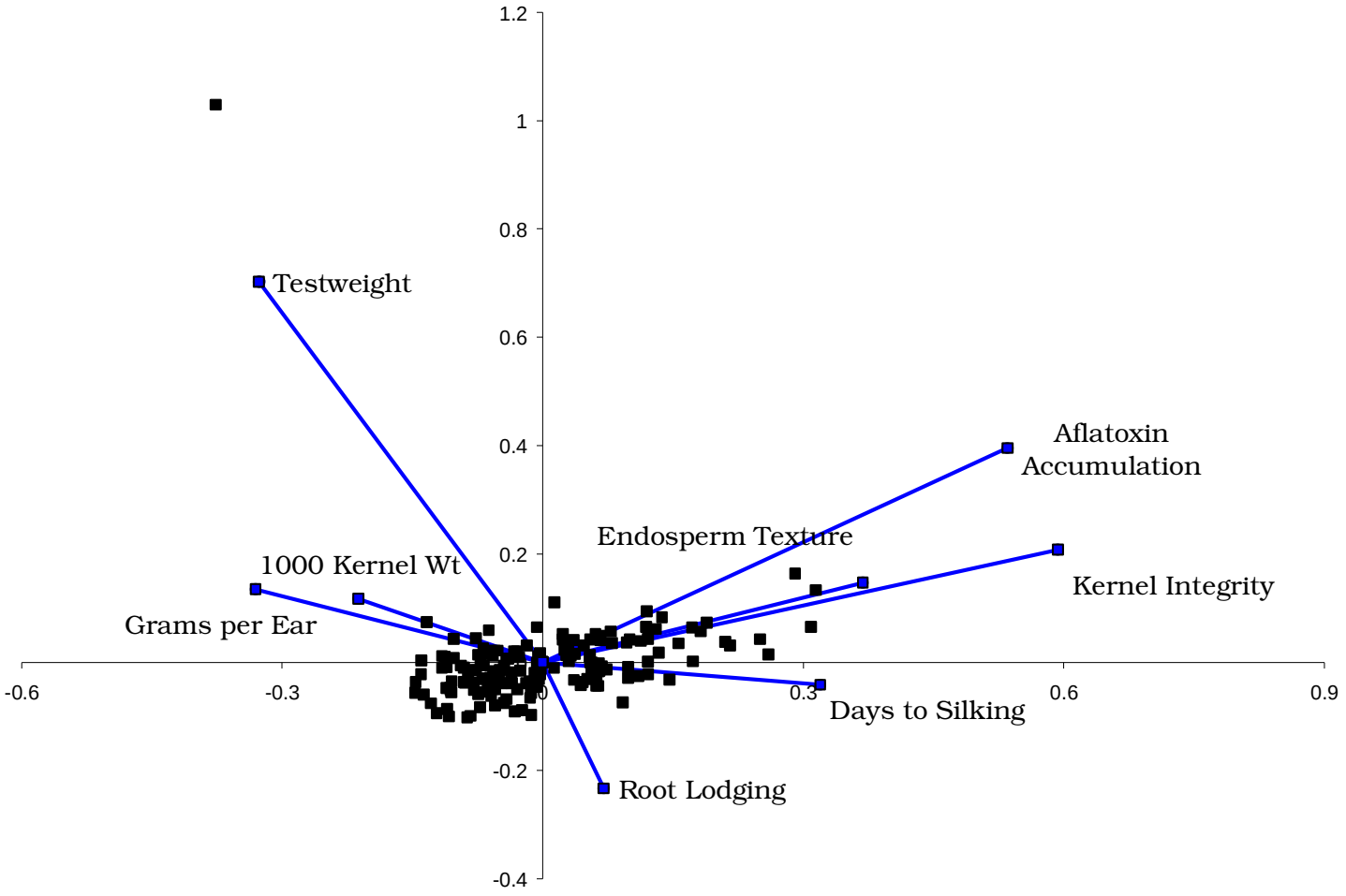

Figure 3.11 Single value decomposition biplot showing correlations among traits at College Station in 2004.

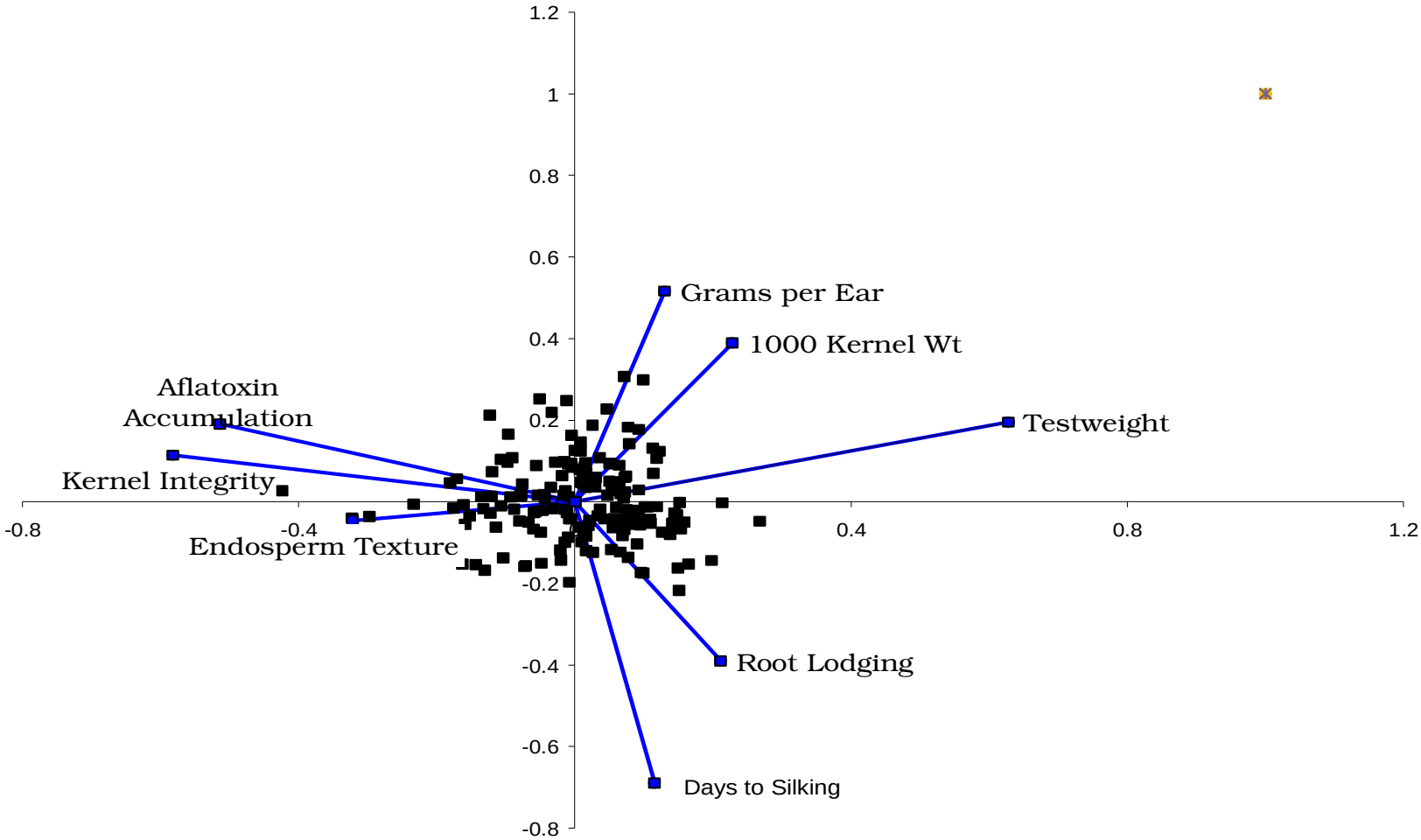

Figure 3.12. Single value decomposition biplot showing correlations among traits at Weslaco in 2004.

At Weslaco (Table 3.12), aflatoxin concentration was significantly genotypically correlated with maturity (r = -0.276**), endosperm texture (r = 0.492**), and kernel integrity (r = 0.705**), as well as to test weight (r = -0.454**). Phenotypic correlations using variance components were slightly lower than Pearson's correlations for kernel integrity (r = 0.544**) but higher for endosperm texture (0.272**).

Table 3.12. Genotypic and phenotypic correlations between aflatoxin concentration and secondary traits[§] at Weslaco in 2004.

	Genotypic Correlation	Phenotypic Correlation
Maturity	-0.276 ± 0.101	-0.147 ± 0.055
Kernel Integrity	0.705 ± 0.071	0.544 ± 0.037
Endosperm Texture	0.492 ± 0.095	0.272 ± 0.051
Root Lodging	-0.291 ± 0.112	-0.075 ± 0.054
Grain Yield	0.059 ± 0.122	-0.042 ± 0.054
1000 Kernel Weight	-0.028 ± 0.120	-0.015 ± 0.056
Test weight	-0.454 ± 0.088	-0.340 ± 0.050

§ Units for traits: Maturity (Days to 50% Silking), Kernel Integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Grain Yield (grams per ear), Root Lodging (% of plants with stalks leaning greater than 30° from vertical), 1000 Kernel Weight (grams), Test weight (g l^{-1}).

Table 3.13. Genotypic and phenotypic correlations between aflatoxin concentration and secondary traits[§] across locations in 2004.

	Genotypic Correlation	Phenotypic Correlation
Maturity	-0.330 ± 0.185	0.007 ± 0.035
Kernel Integrity	0.923 ± 0.075	0.491 ± 0.029
Endosperm Texture	0.630 ± 0.103	0.266 ± 0.037
Root Lodging	-0.131 ± 0.817	-0.060 ± 0.085
Grain Yield	-0.023 ± 0.232	-0.036 ± 0.034
1000 Kernel Weight	0.155 ± 0.050	-0.024 ± 0.002
Test weight	-0.702 ± 0.247	-0.334 ± 0.022

§ Units for traits: Maturity (Days to 50% Silking), Kernel Integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Grain Yield (grams per ear), Root Lodging (% of plants with stalks leaning greater than 30° from vertical), 1000 Kernel Weight (grams), Test weight (g L^{-1}).

Across locations (Table 3.13), aflatoxin concentration was significantly genotypically correlated with maturity (r = -0.330*), endosperm texture (r = 0.630**), and kernel integrity (r = 0.923**), as well as to test weight (r = -0.702**).

Expected Genetic Gain through Selection

Heritability measurements allow estimation of expected genetic gain through selection. Higher heritability of a trait indicates that more genetic gain can be accomplished with fewer breeding cycles. Traits with the highest heritabilites, like kernel integrity, endosperm texture, root lodging, and maturity had high expected genetic gains (Table 14). Traits like grain yield and yield components and aflatoxin concentration with lower heritabilities had lower relative expected genetic gain through direct selection.

Expected genetic gain through indirect selection was tabulated for the traits that were significantly genotypically correlated to aflatoxin concentration (Table 3.15). Theoretically, rapid advancement could be made to reduce aflatoxin concentration using the characteristics of endosperm texture and kernel integrity as selection criteria. Maturity would be less feasible due to the differences in sign at locations.

DISCUSSION

High temperatures and drought stress are more conducive to high levels of aflatoxin concentration (Payne 1998), although neither condition alone is sufficient (Cole *et al.* 1995). Weslaco traditionally has

Table 3.14. Expected direct genetic gain to selection for recombinant inbred line population per and across locations for each phenotypic trait[§].

	College Station	Weslaco	Across Locations
Aflatoxin Concentration	0.214	0.663	0.364
Maturity	4.318	2.754	3.163
Kernel Integrity	1.203	0.975	0.980
Endosperm Texture	1.077	0.942	0.874
Root Lodging	26.008	22.133	14.552
Grain Yield	11.522	11.479	6.338
1000 Kernel Weight	0.109	22.336	3.650
Test weight	19.260	80.249	17.858

§ Units for traits: Aflatoxin Concentration (logarithmic transformation of ng g^{-1}), Maturity (Days to 50% Silking), Kernel Integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation), Grain Yield (grams per ear), Root Lodging (% of plants with stalks leaning greater than 30° from vertical), 1000 Kernel Weight (grams), Test weight (kg l^{-1}).

Table 3.15. Expected indirect genetic gain or correlated response of aflatoxin concentration to secondary traits.

	College Station	Weslaco	Across Locations
Maturity	189.423	-356.526	-899.693
Kernel Integrity	333.832	834.359	1878.332
Endosperm Texture	639.230	618.937	1686.308

§ Units for traits: Aflatoxin Concentration (ng g^{-1}), Maturity (Days to 50% Silking), Kernel Integrity (1 = all ears without split kernels or insect damage, 5 = most of the ears with splits and/or insect damage), Endosperm Texture (1 = flinty endosperm with round crown kernel and vitreous appearance, 5 = floury endosperm with pronounced dentation).

environmental conditions that are more conducive to aflatoxin concentration, and the colonized kernel technique (Odvody *et al.*, 1996; Olanya *et al.*, 1997) has been adequate. For Weslaco trials in 2004, however, rainy conditions during flowering may have compromised the inoculation, so the silk channel technique was employed at both locations rather than only at College Station. The environmental conditions during 2004 were less optimal at both locations for aflatoxin concentration, as evidenced by mean aflatoxin concentrations lower than previously recorded. Natural conditions and inoculum would not have produced concentrations necessary to detect significant differences. Inoculation with the silk channel technique, however, did

prove effective in producing levels of aflatoxin that were high enough to offer variability for selection/analysis at both locations.

Heritability is a measurement of the proportion of phenotypic variation that is genetic rather than environmental or due to error/chance. Higher heritability values indicate that phenotypic variance is more likely to be due to genotypic variance than to other sources. Traits that are highly heritable are more amenable to direct selection and increased genetic gain. High heritability across environments indicates that phenotypic expression of a trait is due to genetics rather than to environmental variance or genotype by environment interaction. Traits that are highly heritable in individual location but are lowly heritable across locations require multiple environments to make genetic gain that will be stable across locations.

The genetic proportion of phenotypic variation of aflatoxin response was higher at Weslaco than at College Station, which is consistent with previous data. For trials in which the genotypes are fixed rather than random, a measurement called repeatability (*R*) is used (Cooper *et al.* 1993). Weslaco traditionally has environmental conditions more conducive to aflatoxin accumulation, and results in higher aflatoxin levels with greater variability. The heritability had a high variance

between locations, due to high environmental component in variance, reiterating that multilocation testing is necessary in order to make progress in selection for lower aflatoxin accumulation. Previous studies of hybrid trials across six years have shown similar results, with Weslaco having higher repeatability of aflatoxin response than College Station (Chapter II).

Maturity has been indicated in previous studies as being negatively but significantly correlated (r = -0.59**) to aflatoxin concentration (Betran and Isakeit., 2004). Higher r values indicate stronger correlations. A negative correlation indicates that as the value of one trait increases, the value of the other trait decreases. In this case, later maturity is associated with lower aflatoxin accumulation. Weslaco, early maturation was also significantly correlated to higher aflatoxin accumulation both genotypically and phenotypically. Tx811 matures earlier than CML176, and is also more susceptible to aflatoxin concentration. At College Station in 2004, however, later maturity was correlated to higher aflatoxin concentration. The most critical time for aflatoxin concentration is during kernel filling, when drought and high stress are most likely to promote high aflatoxin levels (Lisker and Lillehoj, 1991; Vincelli *et al.*, 1995). In this instance, wetter weather during kernel fill for the earlier material may have resulted in lower aflatoxin

concentration than previously indicated. The earlier material, despite higher susceptibility, therefore exhibited lower levels of aflatoxin than the later material due to early wetter and cooler conditions during that crucial time.

Maturity has a high heritability, both per and across locations. The lack of variation across environments indicates that maturity is a more simply inherited trait, and that selection for maturity is possible early in the breeding process, without multiple year or location trials. It is also easy to select for in the field. A strong and highly significant correlation with aflatoxin response indicates that maturity may be a viable option for indirect selection, but the reversal of signs based on environment preclude maturity as the optimal trait for indirect selection. If maturity were considered in selecting for response to aflatoxin, environmental conditions at flowering time would have to be considered and somehow incorporated into selection criteria and decisions.

The consistent and highly significant correlation of aflatoxin with kernel integrity and endosperm texture indicates that lines with softer endosperm and a higher percentage of damaged kernels have increased aflatoxin concentration, which are also attributes of Tx811. Kernel integrity and endosperm texture have high heritabilities both per and

across locations. These are, therefore, also more simply inherited traits, which may be selected for early in the breeding process, without multiple year or location trials.

Kernel integrity had the highest overall correlation with aflatoxin response. While kernel integrity is not directly a measurement of wax and cutin layers on the surface of maize kernels or thicker pericarp layers, all of these characteristics could contribute to higher levels of kernel integrity. These individual characteristics have been previously indicated in conferring resistance to aflatoxin accumulation (Guo *et al.*, 1995; Russin *et al.*, 1997, Tubajika and Damann, 2001). Kernel integrity ratings may provide a way to select for these morphological characteristics that promote resistance to infection and aflatoxin accumulation.

Susceptibility to root lodging is an attribute that has been associated with parental inbred CML176. Under extreme situations, as in College Station in 2004, high levels of root lodging throughout the population reduced phenotypic variability, and genotypic variability was more difficult to ascertain. This lowered the overall heritability in that environment. In conditions that were less conducive to root lodging, CML176 does have significantly higher levels of root lodging than Tx811,

and the proportion of genotypic variance (heritability) was increased. Material with higher thousand kernel weight and that with later maturity had significantly higher root lodging.

For the secondary traits with high heritability and high genotypic correlation to aflatoxin concentration, potential for genetic gain through indirect selection is possible. However, caution must be exhibited in using these traits due to the environmental and spatial variation exhibited for aflatoxin concentration. For initial decrease in breeding stock mean aflatoxin concentration, therefore, these traits provide an excellent preliminary screening method. Once the overall mean had been reduced, however, genotypic variance would need to be reassessed to determine the usefulness of these secondary traits for indirect selection.

This population showed high levels of phenotypic variation for both locations for both aflatoxin accumulation and root lodging. This data, when combined with genotypic information, may provide information about quantitative trait loci for these traits.

CHAPTER IV

MOLECULAR MARKER CHARACTERIZATION OF THE RECOMBINANT INBRED LINE POPULATION CML176 x Tx811 AND QTL MAPPING

INTRODUCTION

Genetic resistance to disease is an important goal for many plant breeders. In some cases, genetic resistance is the only feasible disease management possibility. One problem of particular concern to corn breeders in the southern United States is that of pre-harvest aflatoxin infection. Aflatoxin is a mycotoxin that is produced by the fungus *Aspergillus flavus* when environmental conditions are favorable. Drought and high temperature stress, particularly at flowering time are conducive to aflatoxin production. Aflatoxin is particularly problematic since it causes serious health problems in both humans and livestock.

There are some management techniques that can help to minimize aflatoxin contamination. These are similar to those for increasing overall plant health; plant adapted material, irrigate to prevent drought stress, particularly during flowering, proper nutrient management and weed control. For many regions, however, these management techniques are not an option. Additionally, while these techniques are helpful overall in

reduction of aflatoxin, aflatoxin production is still possible despite optimal management. For this reason, stable genetic resistance to aflatoxin contamination is desirable.

Some sources of resistance to aflatoxin production that are under genetic control have been identified. Despite this, aflatoxin production is a complex trait that is affected by both genetic and environmental influences. Integration of the traits increasing resistance has been difficult through traditional breeding methods. Although breeding for aflatoxin resistance has been underway for over 30 years, there are still no resistant commercial hybrids.

Another agronomic trait of interest is root lodging, defined as the failure of plants to maintain upright stature, which can greatly impact yield. While many factors may contribute to root lodging, such as height of plant, environmental conditions, and overall plant health, resistance to root lodging is due to a combination of many morphological traits. Root lodging is often measured in percent of lodged plants per plot. This measurement is difficult to reproduce, however, due to the necessity of particular environmental conditions. The number of morphological traits as well as the environmental component for screening makes root lodging

another complex trait with limitations on genetic advancement possible through traditional breeding.

Molecular breeding offers a tool for identification and integration of the genetic components of resistance to either aflatoxin production or root lodging that may be more difficult to ascertain or isolate through traditional breeding methods. Development of a mapping population allows genetic correlations between marker data and phenotypic expression. While several types of mapping populations exist, recombinant inbred lines (RILs) are the most commonly used. Parental lines that are phenotypically different are crossed and the offspring are self-pollinated for several generations. The resulting population will consist of lines that are predominantly homozygous with random combinations of parental DNA.

Markers are selected that are polymorphic between parents. Although there are many types of molecular markers available, simple sequence repeat (SSR) markers are most commonly used for extensively researched crops like maize. Markers that are polymorphic for the parents are then characterized on the entire population.

A RIL population for studying response to aflatoxin was developed using the inbred lines CML176 and Tx811 as parents. These lines differ for many agronomic traits, including response to aflatoxin as well as root lodging. Phenotypic data from different locations combined with genotypic data may be used to map genes or quantitative trait loci (QTL) that affect traits of interest.

Objectives of the Study

(i) Screen markers to determine polymorphism for parental lines.

(ii) Characterize polymorphic markers in the parents on entire RIL population.

(iii) Explore associations between markers and aflatoxin concentration and root lodging.

(iv) Identify regions of the genome or QTLs associated with these traits.

REVIEW OF LITERATURE

Limitations of Traditional Breeding for Aflatoxin Resistance

There are two major limiting factors in producing aflatoxin resistant corn through traditional breeding: the variation in aflatoxin accumulation that requires inoculation, several replications, and multiple locations, and the

lack of a reliable, rapid, high throughput, and inexpensive screening methodology (Payne, 1992). Experimental design has lowered the amount of variation due to factors other than genetics that potentially biases selection decisions. Optimal designs often require too many trials in different environments (years or locations) or too many genotypes for screening to be viable based on the resources available. Additionally, even under inoculation, variations in aflatoxin accumulation due to genetic differences may be difficult to identify due to sporadic expression in the field. Molecular breeding could help to minimize the necessity for extensive field trials with high replications in numerous locations, as well as reduce the number of plots to be screened for aflatoxin.

Economic Costs of Aflatoxin

In addition to the hazards presented to human and animal health by aflatoxin, the economic losses incurred must be considered. Loss of profitability is the largest economic consideration. Contaminated corn is worth less and farmers either cannot sell corn that tests positive for aflatoxin or receive reduced remuneration for it. Additionally, livestock is less profitable when aflatoxin contaminated corn is used as feed due to lower productivity, health problems, or death. Profitability is reduced when farmers must increase inputs due to risk of aflatoxin contamination, such as irrigation, crop rotation, proper fertilization, or

pest control. Another, often overlooked economic expense is the cost of research that is necessitated for monitoring aflatoxin exposure and contamination.

Research Approaches to Aflatoxin

Research regarding aflatoxin includes empirical studies of *A. flavus*, biochemical studies of the aflatoxin production pathway, physiological studies of aflatoxin production within corn, and genetic studies of both *A. flavus* and corn. Traditional breeding efforts to reduce pre-harvest aflatoxin concentration have been underway since the mid-1970's. Through these efforts, including germplasm screening studies, some corn genotypes have been found in public sources with lower levels of aflatoxin production (Campbell and White, 1995a, Darrah *et al.*, 1987; Scott and Zummo, 1988, 1990; Thompson *et al.*, 1984; Widstrom *et al.*, 1987; Windham and Williams, 1998). Additionally, some inbred lines of corn (Brown *et al.*, 1998; Campbell and White, 1995a; Huang *et al.*, 1997; Widstrom, 1996) with low levels of resistance to aflatoxin accumulation *per se* have been identified. Despite these efforts, there are currently no commercial hybrids resistant to aflatoxin. With the promise of marker assisted selection (MAS), research increasingly includes molecular studies.

Research Approaches to Root Lodging

Root lodging is defined as the failure of plants to maintain upright stature, and many factors contribute to lodging. It is often measured in percent of lodged plants per plot, however, this measurement is difficult to reproduce due to the necessity of particular environmental conditions. Several root or aerial morphological traits have been shown to contribute to root lodging, including: root mass, volume, and number, diameter of roots, angle of root growth from stem, stalk diameter, ratios of ear height to plant height, and length of base internodes (Bruce *et al.*, 2003). While there are some studies on mapping quantitative trait loci (QTL) for particular morphological characteristics that affect root lodging, few for maize root lodging *per se* exist. The number of morphological traits contributing to this trait, as well as the environmental component for screening, make root lodging another complex trait with limitations on genetic advancement possible through traditional breeding.

Genotype by Environment Interaction

While the expression of some traits is completely under genetic control, response to aflatoxin and root lodging both are influenced by the environment. In breeding, environmental effects must be accounted for and removed in order to accurately assess genetic differences and select superior genotypes. These influences are easily determined and do not

affect selection decisions when environmental influence affects all genotypes similarly. When the environment affects some genotypes differently than others, genotype by environment (GxE) interaction is significant (Fehr, 1987). This interaction complicates breeding efforts, and requires more extensive evaluation over multiple years and environments in replicated trials. For root lodging in particular, trials in multiple years and environments may not produce the necessary phenotypic variance due to a lack of sufficiently adverse conditions (e.g., high winds) necessary to register variation. Some trials have attempted to compensate for this lack with mechanical perturbations, but these too are limiting (Beck *et al.*, 1987; Guingo and Hebert, 1997; Kato and Koinuma, 1999). No measurements for GxE of root lodging per se were found. Genotype by environment interaction was noted for both root biomass and root number in shading situations, which are both traits correlated to root lodging (Hebert *et al.*, 2001).

GxE interactions have been significant in several studies on the genetics of aflatoxin production in corn (Payne, 1992; Brown *et al.*, 1999). Efforts to accurately identify genotypes that accumulate lower levels of aflatoxin are hindered by these interactions, since part of the phenotypic variation is not due to genotypic variation. In general, when environmental conditions are optimal, genotypic differences are displayed and selection

is possible. When conditions do not favor aflatoxin production, however, not only is selection power diminished because phenotypic variance lower, but what minimal phenotypic variance exhibited may be due to genotype by environment interactions rather than evidence of a superior genotype.

Molecular Breeding

Despite some indications of physical and chemical sources under genetic control that prevent or reduce *A. flavus* infection or aflatoxin accumulation, which individually may have high heritabilities, aflatoxin resistance remains a complex trait under both environmental and genetic influence. Genotypic response to aflatoxin is variable and, due to the environmental components as well as the nature of the fungal interaction with genotypes, is considered to be lowly heritable. Similarly, root lodging is a complex trait under genetic control but with environmental components that is difficult to score consistently. Molecular markers provide a potential for improving genetic gain for such complex traits (Lee, 1995). They not only offer direct genotypic information, but can also improve efficiency in selection. When combined with phenotypic evaluation, molecular marker genotyping can be a valuable tool for breeding. Genotypic and phenotypic information can be combined to locate QTL. In order to make progress with

molecular breeding, more than identification of QTL is necessary. The location and total number of QTLs affecting the phenotype, as well as a measurement of their relative importance, must be ascertained (Stuber, 1992). Identification of candidate QTL, elucidation of epistatic and pleiotropic relationships, as well as the genetic basis of heterosis, may provide the necessary tools to allow significant advances in plant improvement and elite germplasm identification (Stuber, 1992).

QTL mapping, with the eventual goal of marker assisted selection (MAS), is possible using specific mapping populations with a known number of meioses. From these, molecular linkage maps can be constructed which allow estimation of the number and location of QTL in the genome affecting genetic variation. Tanksley *et al.* (1989) maintain that the construction of a saturated marker linkage map is the most fundamental step required for a detailed genetic study and MAS approach in any crop. With a sound linkage map, associations between the marker alleles and the QTL might be found and utilized to develop improved lines or populations (Dudley, 1993). Those markers that are tightly linked to implicated genes are preferred, with linked markers flanking important QTL being optimal. The ultimate goal in QTL-MAS is to be able to accurately predict which progeny will exhibit the desired phenotype (Klein *et al.*, 2001; Paterson *et al.*, 1991a).

Quantitative traits with low heritability traditionally are difficult to improve efficiently using only phenotypic selection. Paterson *et al.* (1991a) found that genotypic selection was more effective than phenotypic selection in predicting the phenotype of F_3 progeny based on molecular marker data of the F_2 parents. Since QTL-MAS is costly, it is most indicated when the trait in question is controlled by a few major genes which have a large environmental variance, or a large number of genes with small effects. The large environmental variance of aflatoxin accumulation, as well as the expense of screening for aflatoxin contamination, makes it a suitable candidate for QTL-MAS.

Mapping Populations

Mapping is accomplished using populations that are constructed with a particular level of genetic recombination between the parental inbred lines. The most commonly used ones are backcross populations, doubled haploids (DH), F_2 populations, recombinant inbred lines (RILs), and near isogenic lines (NILs). Selection of the type of population is dependent upon the mating system of the species, resource ability, and research needs, such as economic feasibility, project time requirements, available labor force, lab/field space, and institutional infrastructure.

RIL populations, which are developed by selfing for several generations after crossing two divergent parents, are the most commonly used mapping populations. A major advantage provided by RIL populations is that they may be propagated indefinitely. This allows for multilocation/multiyear testing, possibly decreasing error variance while increasing phenotypic variance. Since RILs undergo several meiotic events in the process of development, fewer individuals are needed to detect linkage of the same magnitude as an F_2 population, which results in higher resolution in the maps. RILs take longer to develop than F_2 populations, backcross populations, or DH. Due to the lack of heterozygosity, DH and RIL populations do not provide any estimate of dominant gene effects, which is important in hybrid crops that exploit heterosis. Unlike NILs, RIL populations can be used to study any traits for which there is significant variation within the population.

Molecular Markers

There are several DNA-based markers available for mapping, including restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) or microsatellites, single nucleotide polymorphisms (SNPs), as well as

others and RNA-based markers. There are thousands of mapped, publicly available SSRs for maize which cover the entire genome. These markers are PCR-based, are highly repeatable, tend to be highly polymorphic, and lend themselves to high throughput. As such, they are often used in genotyping mapping populations.

Statistical Analysis of QTL

Genotypic and phenotypic data are statistically combined in order to identify associations between marker alleles and QTL. There are three major approaches to this process. Single marker analysis considers the association between the trait and one marker locus at a time. Interval mapping (Lander and Botstein, 1989) considers pairs of adjacent markers as a unit and tests for the presence of a QTL within each unit by comparing flanking marker information. Composite interval mapping (Zeng, 1994) combines interval mapping and multiple regression analysis in order to control for the presence of multiple QTL which may be linked to the interval under consideration. This increases the power and precision of mapping by reducing the error caused by nearby QTL that may be affecting the interval.

QTL Stability

Environmental factors that cause differential performance in cultivars is known as the genotype x environment (GxE) interaction. As previously indicated, breeding programs must identify factors influencing the GxE interaction and minimize it when possible in order to more effectively select superior genotypes. Experimental design is used to control for the effect of GxE interactions, particularly by increasing the number of locations and optimizing conditions to select the trait of interest. QTL-MAS may be utilized to select more optimal genotypes while bypassing the masking effects of GxE interaction (Paterson *et al.*, 1991b).

While identification of QTL that show consistent expression across diverse environments is ideal for MAS (Velboom and Lee, 1996), Bubeck *et al.* (1993) found no consistency between environments in markers associated with QTL in corn. Many factors have been found to influence the ability to detect significant associations between environment, QTL, and marker loci. These include the characteristics of the trait under study, gene actions affecting the trait, type and size of the population used to study the QTL, geographical adaptation of the material studied, generation of evaluation, number of environments, and experimental design (Beavis, 1994; Dudley, 1993).

There are two alternatives for the identification of QTL that are stable across environment. Phenotypic data from different environments may be combined and only QTL that are statistically significant on average across environments are selected. Alternatively, QTL analyses may be performed for each environment separately to determine QTL specific to that environment. Environment-specific QTL that are present in a determined number of environments are declared significant. While some traits may be analyzed using data averaged across environments, others must be analyzed through the latter (environment specific) analyses. The methodology chosen will be dependent upon the trait to be studied. For a complex trait such as response to aflatoxin, the environment specific analysis is more likely to be employed.

Research has shown that QTL stability across environments is trait dependent (Dudley, 1993; Zhuang *et al.,* 1997). QTL that explain a large amount of phenotypic variance were more likely to be evidenced across environments (Xu, 2002). Increasing the number of environments used in the QTL analysis decreased the stability of the detected QTL, however gathering phenotypic information across environments is important to accurately identify genomic blocks affecting important traits of interest that can be introgressed into elite genotypes. Identification of environment-specific QTL may offer alternative alleles that may be

pyramided with more stable QTL to provide genotypic buffering (Paterson *et al.* 1991b).

Congruency of QTL in corn was found for kernel weight, protein concentration, and plant height and was mainly attributable to one or few QTL of moderate to large effect. If more cost-effective than phenotypic selection, MAS is promising for these traits. (Mihaljevic *et al.,* 2004). Aflatoxin quantification can be quite expensive and time consuming, and environmental effects can complicate phenotyping enough that any potential genetic gain is unidentifiable. For these reasons, QTL-MAS may provide a cost-effective alternative for breeding to increase resistance to aflatoxin accumulation, even though identification of QTL (particularly environmentally stable QTL) is an expensive and complicated process.

QTL for Aflatoxin Resistance

Because of the desirability of incorporating QTL-MAS into breeding programs for commercial corn breeding programs, several mapping studies have been done. Two studies used the resistant maize inbred Mp313E as one of the parents (Brooks *et al.*, 2005; Davis *et al.*, 2000). Another study involved the resistant inbred Tex6 (Paul *et al.*, 2003). All studies identified a significant QTL on chromosome 4. Brooks *et al.* also

identified a region on chromosome 2 that was consistent across locations. Other chromosomes were implicated in different years/environments for each study. Paul *et al.* (2003) also identified QTL significantly associated with aflatoxin on all chromosomes except 1 and 8.

MATERIALS AND METHODS

Germplasm and Population Development

Two inbred lines were used to create a mapping population of RILs to study response to aflatoxin concentration. The parents used were CML176 and Tx811, which are quality protein maize (QPM) inbreds that differ for many agronomic characteristics. Tx811 is a temperate line released in 2003 with intermediate maturity that is susceptible to aflatoxin accumulation and has lower root lodging and less grain hardness than other QPM lines (Betran *et al.*, 2003). CML176 is a subtropical line with late maturity and flinty grain texture that has been shown to be less susceptible to aflatoxin accumulation, but has susceptibility to root lodging. These lines were crossed and then selfed for at least 6 generations to produce a RIL mapping population with 160 S_6 RILs.

Environments

The parents and the entire population were grown in two Texas locations, College Station (latitude 30°37, elevation 96 m) and Weslaco (latitude 26°09, elevation 22.5 m). An alpha lattice design was used with 3 reps at each location, with additional reps of each of the parental inbreds included as checks for a total of 12 reps of each parental inbred per location. Experimental units consisted of single rows plots of 4.047 m^2 in College Station and 5.079 m^2 in Weslaco. Plant populations was 50604 plants/ha in Weslaco and 66220 plants/ha in College Station. Standard cultural practices in both locations were applied. Limited irrigation was applied around flowering to induce some level of drought stress.

Inoculation

All trials were inoculated with *A. flavus* isolate NRRL3357. Aflatoxin production exhibits high spatial and environmental variation; therefore both trials were inoculated to eliminate possibility of environmental variation of natural inoculum. Inoculation was conducted using the nonwounding silk channel inoculation technique (Zummo and Scott, 1989). Plants were inoculated with a conidial suspension containing 3 x 10^7 conidia of *A. flavus* in 3 mL distilled water injected by syringe 6 to 10 days after midsilk.

DNA Extraction

DNA was extracted using a DNAZol protocol following manufacturer's recommendation (Invitrogen) with small modifications. DNA was extracted from a bulk of shoots from seedlings 5-10 days old, using 8-10 plants for each entry. A 50 mg sample of total bulked tissue was cut into 1 cm pieces and placed in a 1.5 mL epitube. To this was added 0.4 uL of sucrose buffer (0.35 M sucrose, 100 mM Tris pH 8.0, and 5 mM Na_2 EDTA pH 8.0) with 0.2% beta-mercaptoethanol, and 8 uL RNAaseA. The samples were ground by placing a tungsten carbide bar in each tube and using a mixer mill (SPEX Certiprep 2000 Geno/Grinder) according to manufacturer's directions for 30 sec at 1700 strokes min^{-1}. After the bar was removed with a magnet, 0.4 mL of Plant DNAZol (Invitrogen) was added. Tubes were inverted several times and allowed to incubate for 10 minutes. The supernatant (approximately 750 uL) was transferred to new labeled tubes. To this was added 750 uL of chloroform. Tubes were inverted several times and incubated for 10 minutes. Tubes were centrifuged for 10 minutes. All centrifuging was done at 13,000 rpm. The liquid upper layer was removed (approximately 500 uL) and put in a new epitube. To this was added 75 % volume (approximately 375 uL) of 100% ethanol. Tubes were gently inverted several times and allowed to incubate for 5 minutes. They were then

centrifuged for 4 minutes. Supernatant was poured off gently, leaving a pellet in the bottom of the tubes. Pellets were washed in a 0.4 mL of 3:2 mixture of plant DNAZol wash solution and 100% ethanol. They were incubated for 5 minutes in the wash mixture, then centrifuged for 2 minutes. The supernatant was removed, and the pellet was washed in 0.4 mL of 75% ethanol. Tubes were centrifuged for 2 minutes, and the supernatant was removed. Pellets were allowed to air dry completely. Dried pellets were dissolved in 15 mM Tris buffer, pH 7.9.

All DNA samples were quantified using a Turner Fluorometer. Original stock were diluted using 1x TE buffer to 50 ng uL^{-1}.

Molecular Markers

Simple sequence repeat markers were used due to their high polymorphism information content (PIC). They were screened on the parental inbreds, and those that were polymorphic were then run on the entire population (Table 4.1). Of 161 SSR marker primer pairs screened, 54 were considered polymorphic between Tx811 and CML176, or 34 percent. Paul *et al.* (2003) found that 47 percent of the SSR markers tried were polymorphic between parental inbreds used, higher than was found in this study. Polymerase chain reaction (PCR) conditions for the markers were slightly different based on the method of labeling the

marker: fluorescent labeling for Applied Biosystems Informatics system, infra-red fluorescent labeling for Li-cor system, M13 universal tail with M13 infra-red labeled primer for Li-cor system, or unlabeled for use with ethidium bromide-stained agarose gels. All PCRs were run using ABI GeneAmp 2700 or 9700 thermocyclers.

ABI Detection

The reverse primer for Initial markers was labeled fluorescently with FAM (6-carboxyflourescein) or HEX (4,7,2',4',5',7'-hexachloro-6-carboxyflourescein) for fluorescence-based detection on the ABI Prism 3700 DNA Analyzer (Applied Biosystems Informatics, 2003) . Reaction mixtures consisted of 6 ng of template DNA, 1.0 uL of 10x reaction buffer, 1.0 uL of 2.5 mM $MgCl_2$, 0.8 uL of dNTPs, 0.06 uL of Promega *Taq* polymerase, 1 uL of primer containing 2.5 pmol uL^{-1} of both forward and reverse primers, and 4.14 uL of water to make total reaction volume of 10ul.

PCR conditions for these markers were one cycle of 3 minutes at 94°C, forty cycles of 1 minute at 94°C, 1 minute at annealing temperature of primer, and 1 minute at 76°C, with a final single cycle of 30 minutes at 76°C. The final cycle is longer than absolutely necessary for reannealing

Table 4.1. Simple Sequence Repeat primers used on the CML176 x Tx811 recombinant inbred line mapping population.

Bin	Primer	Primer Sequence	Repeat	Label*
1.01	bnlg1124, A4616G06, bmc1124, A4616G05	TCTTCATCTCTCTATCAAACTGAC A//TGGCACATCCACAAGAACAT	AG(20)	IRDye™
1.05	umc2025	CGCCGTAGTATTTGGTAGCAGAA G//TCTACCGCTCCTTCGTCCAG TA	(AGCT)4	M13
1.08	umc1446	GCGCTGCTGCTTCTTAAATTATCT //GATGAGACCACCTACAAGTTC GCT	(TAA)7	IRDye™
2.01	phi96100	AGGAGGACCCCAACTCCTG// TTGCACGAGCCATCGTAT	ACCT	FAM
2.04	phi109642	CTCTCTTTCCTTCCGACTTTCC// GAGCGAGCGAGAGAGATCG	ACGG	HEX
2.07	umc1042	AAGGCACTGCTACTCCTATGGCT A//CTGACCTTTGAATTCTGTGCT CCT	GA17	M13
2.08	phi127	ATATGCATTGCCTGGAACTGGAA GGA//AATTCAAACACGCCTCCC GAGTGT	AGAC	FAM
3.01	umc1970	ACTGATGGTGTTCTTGGGTGTTTT //TTTTTACCCGAAGGTTCATCGT TT		M13
3.03	bnlg1447, A4651C03, bmc1447, A4651C04	GAGAGGAGAGGCTGAGCTGA// TCCTCCCACTGAATTTCCAC	AG(33)	IRDye™
3.05	phi073	GTGCGAGAGGCTTGACCAA// AAGGGTTGAGGGCGAGGAA	AGC	HEX
3.06	bnlg1047, A4637A08, A4637A07, bmc1047	ATGGAGATGGAGGAGAGAGAGA // GATGCGGCGATGGCTAA	AG(14)	M13

Table 4.1 continued.

Bin	Primer	Primer Sequence	Repeat	Label*
3.1	umc1136	CTCTCGTCTCATCACCTTTCCCT//CTGCATACAGACATCCAACCAAAG	(GCA)5	IRDye™
3.1	umc2048	GCTGAAGTCCCAACCACCAC//TTGACATGTTCTACCATCTCACCAA	(TC)6	Agarose
4.01	phi072	ACCGTGCATGATTAATTTCTCCAGCCTT//GACAGCGCGCAAATGGATTGAACT	AAAC	HEX
4.04	umc1117	AATTCTAGTCCTGGGTCGGAACTC//CGTGGCCGTGGAGTCTACTACT	(TCGCA)4	M13
4.05	bnlg1265, A4636B06, bmc1265, A4636B05	GGTTGTCCGTAAAGGCAAGA//TGTGAAGGCCAGACAGTCAG	AG(33)	IRDye™
4.11	phi006	AGGCGGCGTGCTGAACACCT//CGCTTCATCTCCCGTGACAATG	CCT	FAM
4.11	umc1058	AGCAAGCAGTTCGAAACAAGGAT// GACACCAGCACCACTTGAACG	(GC)7	IRDye™
5	bnlg1006, A4423A04, bmc1006, A4423A05	GACCAGCGTGTTGATCCC//GGAGACCCCGACTCTCTCTC	AG(20)	M13
5.03	umc1389	AAAACACAACGCTGGACATCAAC//GGTCGTTTTGCTTAGCCCATTTTA	(TGAC)4	M13
5.05	umc2111	CACGCAACCCACTCATCACTC//CTCACCGCTCTGCTCTGCTATC	(CTCA)4	M13
5.06	phi087	GAGAGGAGGTGTTGTTTGACACAC//ACAACCGGACAAGTCAGCAGATTG	ACC	FAM
5.07	phi085	AGCAGAACGGCAAGGGCTACT//TTTGGCACACCACGACGA	AACGC	FAM
5.09	umc1153	CAGCATCTATAGCTTGCTTGCATT//TGGGTTTTGTTTGTTTGTTTGTTG	(TCA)4	HEX

Table 4.1 continued.

Bin	Primer	Primer Sequence	Repeat	Label*
6.01	bnlg391, ZCAA391, bngl391	CAGATATCACAGCATCAGAAGATCA//AAAATGTAAGAACTTGTTTGGGATT		FAM
6.02	bnlg2191, A5151C05, A5151C06, bmc2191	CACACAATCCCCACAAAAAA//CGAAACATCCAGGAAACTGC	AG(33)	M13
6.04	phi031	GCAACAGGTTACATGAGCTGACGA//CCAGCGTGCTGTTCCAGTAGTT	GTAC	FAM
6.05	phi078	CAGCACCAGACTACATGACGTGTAA//GGGCCGCGAGTGATGTGAGT	AAAG	HEX
6.07	phi070	GCTGAGCGATCAGTTCATCCAG//CCATGGCAGGGTCTCTCAAG	AGCTG	HEX
7.0-7.02	umc1480	AATGAAGGTGGATGTGCTGCTACT//CTTCCCCATCTCCTCTTGAAGATT	(GAA)4	Agarose
7.02	phi034	TAGCGACAGGATGGCCTCTTCT//GGGGAGCACGCCTTCGTTCT	CCT	HEX
7.04	phi328175	GGGAAGTGCTCCTTGCAG//CGGTAGGTGAACGCGGTA	AGG	HEX
7.05	phi069	AGACACCGCCGTGGTCGTC//AGTCCGGCTCCACCTCCTTC		FAM
7.06	phi116	GCATACGGCCATGGATGGGA//TCCCTGCCGGGACTCCTG	ACTG/ ACG***	HEX
8.02	umc1304	CATGCAGCTCTCCAAATTAAATCC//GCCAACTAGAACTACTGCTGCTCC	(TCGA)4	FAM
8.03	phi2333376	CCGGCAGTCGATTACTCC//CGAGACCAAGAGAACCCTCA	CCG	HEX
8.04	phi014	AGATGACCAGGGCCGTCAACGAC//CCAGCTTCACCAGCTTGCTCTTCGTG	GGC	FAM

Table 4.1 continued.

Bin	Primer	Primer Sequence	Repeat	Label*
8.06	umc1161	GGTACCGCTACTGCTTGTTACTG C//GCTCGCTGTTGGTAGCAAGT TTTA	(GCTGGG)5	HEX
8.08	umc1663	GCTTGCACTAGCTTTAGCTCCAT C//CGGGATCAGTCGTTACAAAC ATAG	(ATG)8	IRDye™
8.09	phi015	GCAACGTACCGTACCTTTCCGA/ /ACGCTGCATTCAATTACCGGGA AG	AAAC	FAM
9.04	phi032	CTCCAGCAAGTGATGCGTGAC// GACACCCGGATCAATGATGGAAC	AAAG	FAM
9.08	umc1277	TTTGAGAACGGAAGCAAGTACTC C//ACCAACCAACCACTCCCTTTT TAG	(AATA)5	HEX
10.02	phi059	AAGCTAATTAAGGCCGGTCATCC C//TCCGTGTACTCGGCGGACTC	ACC	HEX
10.03	umc2180	ATCAGCATCGATAGCGAAGAAAG A//ATTGCTACTAGGGTTGTTGTT GCC	(GGCC)4	M13
10.03	bnlg1712, bmc1712, A4753H05, A4753H06	CTCAGGCTTCACGTGGGTTT// GTTACACTCCCCTGCCAAAA	AG(20)	M13
10.04	umc2163	AAGCGGGAATCTGAATCTTTGTT C//GAAATTGCTGGGGTTCTCATT TCT	(AG)28	M13
10.06	bnlg2190, bmc2190, A5151C03, A5151C04	TCCTCCTTCATCCCCTTCTT// CCCAGTATCATTGCCCAATC	AG(31)	IRDye™

* HEX and FAM are fluorescently labeled for use with ABI Prism 3700 DNA Analyzer, IRDye™ are labeled markers with infrared fluorescence for use with the LI-COR Long ReadIR™ DNA Sequencer, M13 are markers with the universal M13 tail which are combined with a labeled M13 primer for use on LI-COR, and Agarose are primer combinations that are unlabelled and are visualized on an agarose gel stained with ethidium bromide.

in order to minimize stuttering due to poly-A tails. After dilution, all plates were sent to the Laboratory for Plant Genome Technology for allele calling. Allele determination is done using ABI Prism Genotyper® software (Applied Biosystems Informatics, 2003).

LI-COR Detection

For some of the primers, allele detection was done using infrared fluorescence, with the LI-COR Long ReadIR™ DNA Sequencer (model 4200L-1 or 4200L-2). Gels were run with a 64 lane comb and loaded with a Hamilton 8-barrel syringe. PCR reaction products were multiplexed, and 1 uL of the mixtures was loaded into a well of a 7% polyacrylamide gel (25-cm in length and 0.25-mm in thickness). Also, 1 µl of a LI-COR broad range (700 or 800 bp) molecular weight standard (Cat. No. 4200-60) was loaded at the left and right lane of each gel. Key electrophoresis parameters include voltage set at 1500 V, current at 20 mA, power at 25 W, and temperature at 45 °C. SSR fragments were scored, analyzed, and converted into numerical data using Base ImagIR software, LI-COR.

Some primers had forward primers labeled with Li-cor IRDye™ 700 or 800 for use with the LI-COR 4200 system. The reaction mixtures for these primers consisted of 3 uL of 5 ng uL^{-1} of template DNA, 1.0 uL of

10x reaction buffer, 1.0 uL of 2.5 mM $MgCl_2$, 0.8 uL of dNTPs, 0.06 uL of Promega *Taq* polymerase, 1 uL of glycerol, 1 uL of primer containing 1 pmol uL^{-1} of both forward and reverse primers, and 3.14 uL of water to make total reaction volume of 10ul. The PCR conditions for these primers were one cycle of 3 minutes at 94°C, twenty-five cycles of 1 minute at 94°C, 30 seconds at annealing temperature of primer, and 30 seconds at 76°C, with a final single cycle of 4 minutes at 76°C.

Other primers were not specifically labeled with infrared fluorescence. Instead, these primers had an extended tail of the 5' of the forward primer corresponding to the universal M13 primer (Oetting *et al.* 1995), and were run with an additional primer of the M13 tail labeled with the Li-cor IRDye™ 700 or 800. The reaction mixtures for these primers consisted of 3 uL of 5 ng uL^{-1} of template DNA, 1.0 uL of 10x reaction buffer, 1.0 uL of 2.5 mM $MgCl_2$, 0.8 uL of dNTPs, 0.06 uL of Promega *Taq* polymerase, 1 uL of glycerol, 1 uL of reverse, unlabeled primer (1 pmol uL^{-1}) 0.64 uL of forward M13 tailed primer (1 pmol uL^{-1}) and 1.5 uL of M13 primer labeled with infra-red fluorescent dye to make total reaction volume of 10ul. The PCR conditions for these primers were one cycle of 3 minutes at 94°C, twenty-five cycles of 1 minute at 94°C, 30 seconds at annealing temperature of primer, and 30 seconds at 76°C, 10 cycles of 1 minute at 94°C, 30 seconds at 50°C (annealing temperature

of M13 primer), and 30 seconds at 76°C, with a final single cycle of 4 minutes at 76°C.

Agarose Detection

For a few of the markers, the difference in allele size was enough to be distinguished using unlabelled markers and then running the PCR product on gel electrophoresis in an ethidium bromide stained gel of 3% agarose. The gels were run at 250 volts for approximately 1.5 hrs. More product was necessary to visualize on agarose gels. For these primers, reaction mixtures were the same as ABI protocol, except that primer concentration was 5 pmol uL^{-1}, and the mixture was doubled for a total of 20 uL. The same PCR conditions as those for ABI were also used.

Data Analysis

Phenotypic data was analyzed using SAS procedures and REMLtool™ software. Genotypic means at each location were obtained using REMLtool™ software, which utilizes restricted maximum likelihood (REML) methods in mixed linear models. All effects (lines, environments, replications, block within replications) were considered random. These phenotypic means were then used in conjunction with individual marker data in order to conduct a single marker analysis. Single marker

analysis was done for each marker using SAS codes, which compare the means for each class. For most markers, three classes were compared: parental alleles from Tx811, parental alleles from CML176, and heterozygotes. The amount of the variation explained by each significant marker was also calculated (R^2).

Map distances were determined using MAPMaker© software (Whitehead Institute, 1997), with all heterozygotes scored as missing data so that the RIL function could be used. Because the distance between markers was too great to form known linkage groups, interval mapping was not employed.

QTL Stability

Temperature, rainfall and humidity after inoculation can greatly affect the development of fungal biomass and aflatoxin production. Evaluation of only two environments does not provide an adequate sampling of different environments to be able to confidently make more general statements about QTLs for resistance beyond these two environments (Paul *et al.*, 2003). There have been a number of specific inbreds and hybrids that were associated with relatively low levels of aflatoxin after evaluation in just two environments that were subsequently associated with high levels of aflatoxin production upon evaluation in multiple

environments (Campbell and White 1995a, b, Paul *et al.*, 2003). Although there are no current QTL studies of root lodging per se, the high variability by environment indicates that any QTLs for resistance to root lodging based solely on two environments are not adequate for generalizations outside of these environments. Thus, consistent with this observation, the QTLs identified as associated with lower levels of aflatoxin or root lodging in this study, which are strictly considered valid for just these two environments, require evaluation in other genetic studies and environments to further support their validity.

RESULTS

Heterozygosity and Segregation Distortion

Most markers used in this study had significant levels of heterozygosity. Average heterozygosity across markers was 16 percent. No lines were found to have levels of heterozygosity across markers that were high enough to eliminate them from the study.

A chi squared test was used to determine if segregation distortion was present. Significant segregation distortion (at the .05 level) was found for 51 percent of markers. All markers on chromosome 8 had segregation distortion. A total of 35 % of the markers had significantly higher levels

of CML176 alleles than Tx811 alleles. Tx811 alleles were favored at 16% of the markers.

Markers Associated with Aflatoxin Concentration

College Station

In College Station, there were two markers that were significantly associated with both aflatoxin concentration and the logarithmic transformation (Table 4.2). These markers were umc1042, located on chromosome 2, and umc1663, located on chromosome 8. The amount of variation explained by these two markers was almost 10%. Additional markers on chromosomes 6, 8, and 9 were significantly associated only with the logarithmic transformation of aflatoxin concentration.

The markers associated with log transformation accounted for 4.06-5.35% of the variation, altogether counting for almost 30%. Two of the markers, phi015 and umc1663, had no significant additive effects. For all markers except umc1304, the CML176 allele was associated with higher aflatoxin concentration than the Tx811 allele.

Weslaco

At Weslaco (Table 4.3), several markers were significantly associated with aflatoxin concentration (ng g^{-1}). Two each were found on

Table 4.2. Markers significantly associated with aflatoxin concentration (ng g^{-1} or logarithmic transformation) in CML176 x Tx811 recombinant inbred line population at College Station in 2004.

Location	Primer	R^2	Mean values of aflatoxin concentration by class			
			Tx811	CML176	Heterozygote	Additive Effect
Aflatoxin Concentration						
2.07	umc1042*	0.0506	602	753	780	76**
8.08	umc1663*	0.045	683	736	535	NS
Total		0.0956				
Logarithmic transformation						
2.07	umc1042*	0.0458	2.45	2.5	2.48	0.0265**
6.02	bnlg2191*	0.0465	2.44	2.5	2.47	0.0294**
8.02	umc1304*	0.0527	2.5	2.45	2.4	-0.0235*
8.02	phi015*	0.0503	2.45	2.49	2.42	NS
8.08	umc1663*	0.0406	2.47	2.5	2.43	NS
9.04	phi032*	0.0535	2.39	2.49	2.47	0.0506**
Total		0.2894				

* Significant at the 0.05 level.
** Significant at the 0.01 level.

chromosomes 4, 5, and 10, each explaining between 3.9 and 7.3 percent of the variation. The combined variation explained by these markers is over 30%. For the markers on chromosome 4, there was no significant additive effect. For the markers on chromosome 5, the Tx811 allele was more associated with lower aflatoxin concentration, while for the markers on chromosomes 4 and 10, the CML176 allele was more associated with lower aflatoxin.

Table 4.3. Markers significantly associated with aflatoxin concentration (ng g^{-1}) in CML176 x Tx811 recombinant inbred line population at Weslaco in 2004.

Location	Primer	R^2	Mean values of aflatoxin concentration by class			Additive Effect
			Tx811	CML176	Both	
4.01	phi072**	0.0734	953	577	551	NS
4.11	umc1058*	0.0447	774	510	825	NS
5.06	phi087**	0.0534	582	958	696	188**
5.07	phi085*	0.0441	549	860	745	155**
10.02	phi059*	0.0528	878	533	682	-173**
10.04	umc2163*	0.0393	849	625	459	-112*
Total		0.3077				

* Significant at the 0.05 level.
** Significant at the 0.01 level.

Only one marker was significantly associated with the logarithmic transformation of aflatoxin concentration- umc1480 located in the first bin of chromosome 7. This marker accounted for almost 5% of the variance for this trait, with additive effect of -0.0825*, indicating that the CML176 allele was associated with lower aflatoxin concentration.

No markers were associated with aflatoxin or its log transformation at both College Station and Weslaco. In fact, the markers associated with aflatoxin at each of these locations were not on the same chromosomes.

Markers Associated with Root Lodging

College Station

There were three markers that were found to have associations with root lodging in College Station (Table 4.4). These markers accounted for 4.5-6.2% of the variation seen. The marker at 2.04 (phi109642), had significant and positive additive effect (3.83**) while that at 2.08 (phi127) had significant but negative effect (-3.045*). For two of the markers, the CML176 allele was associated with lower root lodging, while at the other locus, the Tx811 allele promoted lower root lodging.

Weslaco

Six markers were found to be significantly associated with root lodging at Weslaco (Table 4.5). For half of these markers, higher root lodging was associated with the CML176 allele, while for the other half, the Tx811 allele contributed to greater root lodging. The amount of effect of each of these loci ranged from 5.17% to 10.42%. These six markers accounted for over 40% of the variation in the population. Two of the markers were in adjacent bins on chromosome 10. None of the markers that were found to have significant effects on root lodging at Weslaco were identical to those that affected root lodging at College Station.

Table 4.4. Markers significantly associated with root lodging (percent of plants with stalks at greater than 30% vertical) in CML176 x Tx811 recombinant inbred line population at College Station in 2004.

Location	Primer	R^2	Mean values of root lodging by class			
			Tx811	CML176	Both	Additive Effect
2.04	phi109642*	0.0584	40.97	48.63	46.55	3.83**
2.08	phi127*	0.045	49.31	43.22	42.92	-3.045*
3.06	umc1047*	0.0619	49.94	42.53	46.39	NS
Total		0.1653				

* Significant at the 0.05 level.
** Significant at the 0.01 level.

Table 4.5. Markers significantly associated with root lodging (percent of plants with stalks at greater than 30% vertical) in CML176 x Tx811 recombinant inbred line population at Weslaco in 2004.

			Mean values of root lodging by class			
Location	Primer	R^2	Tx811	CML176	Heterozygote	Additive Effect
1.01	u1124**	0.0596	12.38	18.887	Not applicable	
2.02	u1422*	0.0517	13.02	18.52	13.22	2.75**
8.09	p015*	0.0551	10.27	16.22	16.49	2.98**
9.08	u1277**	0.0758	18.57	10.97	16.47	-3.798**
10.03	u2180**	0.0602	18.63	12.61	17.64	-3.058**
10.04	u2163**	0.1042	19.21	11.15	16.87	-4.028**
Total		0.4066				

* Significant at the 0.05 level.
** Significant at the 0.01 level.

Epistatic Interactions

Complex traits are affected by several different genes or QTLs and often these loci have complex interactions called epistasis. All markers were compared pairwise to ascertain any epistatic interactions for each trait studied using the mean data per location. Many of the markers that were not significantly associated with the traits individually exhibited highly significant epistatic interactions. While an individual locus may be highly associated with desired genotypes, epistatic interactions can

mask these effects, limiting the effectiveness of MAS. Additionally, parental lines with less desirable attributes may actually have alleles that are beneficial. Analyses of epistatic effects can reveal beneficial alleles from less desirable parental inbreds for MAS.

Aflatoxin

Epistatic interactions were measured for logarithmic transformation of aflatoxin concentrations for both College Station and Weslaco (Table 4.6). None of the markers with significant epistatic interactions were individually associated with the traits. Only one epistatic interaction was found for aflatoxin concentration in Weslaco, however either parental type produced lower aflatoxin than a recombination of alleles for these two loci.

In College Station, several significant interactions were identified. As with the interaction for the Weslaco trials, each of these marker pairs had lower aflatoxin concentrations when the alleles at both loci were of only one parental type rather than a recombination of Tx811 alleles at one loci and CML176 alleles at the other.

Root Lodging

Significant epistatic interactions for root lodging are shown in Table 4.7.

Table 4.6. Significant epistatic interactions between loci for root lodging (percent plants with stalks at greater than 30 degrees vertical) in CML176 x Tx811 mapping population at College Station and Weslaco.

	Locus 1	Locus 2	Tx811 both	Tx811/ CML176	CML176/ Tx811	CML176 both
Weslaco						
	bnlg1447	phi072	2.22	2.33	2.61	2.22
College Station						
	bnlg2048	umc1277	2.439	2.51	2.52	2.43
	bnlg2191	phi078	2.409	2.51	2.53	2.46
	phi014	phi073	2.449	2.52	2.52	2.42

Only one interaction was found to be significant for the College Station trial. While having Tx811 alleles at both loci produced less lodging than CML176 at both, having the Tx811 allele at the bnlg1124 loci combined with the CML176 allele at phi014 produced much lower lodging rates.

At Weslaco, umc1663 had significant interactions with 3 other loci, and umc1048 had interactions with 2 other loci. As with aflatoxin concentration, none of the markers that had significant epistatic interactions were individually associated with root lodging.

Table 4.7. Significant epistatic interactions between loci for kernel integrity in CML176 x Tx811 mapping population at College Station and Weslaco.

	Locus 1	Locus 2	Tx811 both	Tx811/ CML176	CML176/ Tx811	CML176 both
College Station						
	bnlg1124	phi014	46.871	35.37	44.07	56.1
Weslaco						
	bnlg2048	phi233376	8.8879	21.05	20.96	13.88
	bnlg2048	umc1663	11.264	19.57	21.95	13.61
	bnlg391	umc1048	15.631	9.4	9.93	28.36
	phi96100	phi073	9.057	21.07	18.22	15.96
	phi078	umc1446	19.137	12.89	11.33	20.62
	umc1006	umc1663	6.743	17.86	27.85	12.55
	umc1048	umc1663	18.377	12.12	8.6	22.99

Kernel Integrity

Markers with significant epistatic interactions for kernel integrity by location are given in Table 4.8. Phi031 had significant interactions with two other loci at Weslaco. There were no common markers between the locations with epistatic effects for kernel integrity. At individual

locations, there were no markers that had epistatic effects for both aflatoxin concentration and kernel integrity.

Table 4.8. Significant epistatic interactions between loci for endosperm texture in CML176 x Tx811 mapping population at College Station and Weslaco.

	Locus 1	Locus 2	Tx811 both	Tx811/ CML176	CML176/ Tx811	CML176 both
College Station						
	bnlg2180	phi96100	2.967	2.31	2.41	2.72
Weslaco						
	phi014	phi078	2.822	2.38	2.67	3.11
	phi031	phi034	2.435	3.01	2.99	2.58
	phi031	umc1277	2.776	2.25	2.57	3.7

Endosperm Texture

Both locations had several significant epistatic interactions for endosperm texture (Table 3.9), with umc1048 significant at both but affecting different loci. Phi034 had a epistatic effects on both kernel integrity and endosperm texture at Weslaco, but with different loci for each trait. Phi 014 had epistiatic effects on both endosperm texture and aflatoxin concentration at College Station, interacting with different loci for each trait.

Table 3.9. Significant epistatic interactions between loci for endosperm texture in CML176 x Tx811 mapping population at College Station and Weslaco.

	Locus 1	Locus 2	Tx811 both	Tx811/ CML176	CML176/ Tx811	CML176 both
College Station						
	bnlg1124	umc1389	1.814	2.08	2.24	1.92
	bnlg1542	phi014	1.827	2.33	2.02	1.82
	phi059	phi087	1.911	2.21	2.09	1.84
	phi116	umc1048	1.852	2.5	2.08	1.97
Weslaco						
	bnlg1447	bnlg1970	2.26	2.39	2.62	2.35
	bnlg1970	phi078	2.261	2.54	2.38	2.31
	phi032	umc2163	2.263	4	2.45	2.34
	phi034	umc1389	2.486	2.39	2.13	2.4
	umc1048	umc1122	2.338	2.34	2.77	2.22

DISCUSSION

Lines in this study had a higher level of heterozygosity than expected from an S_6 RIL population. In the creation of the population, a high level of heterozygosity was inadvertently maintained. Plants with some level of heterozygosity tend to be more vigorous, and thus may have

been advanced due to higher levels of seed at earlier stages. Similarly, the high level of segregation distortion was unexpected. Parental inbred CML176 has less desirable agronomic characteristics, and therefore would be less likely to be unconsciously selected for in the field.

Aflatoxin levels at both locations in this study were lower than previous years due to increased moisture at flowering time. There were no markers that were consistently associated with aflatoxin concentration at both locations. None of the markers found to be significantly associated with aflatoxin in College Station were in the same bins as those found by Paul *et al.* (2003) or Brooks *et al.* (2005). At Weslaco, phi085, which is in bin 5.07, was significantly associated with aflatoxin levels. Paul *et al.* (2003) also identified markers in 5.07, bmc1346 and bng1118, as affecting aflatoxin in two different populations in different years. These markers were not stable across environments. Previous studies have found QTL for aflatoxin on chromosome 4. At Weslaco, chromosome 4 was also indicated as containing potential QTL for aflatoxin.

While the markers specifically used in previous trials may not be polymorphic for these parental lines, others located nearby might indicate QTLs in the same regions. Brooks *et al.* (2005) found QTL

associated with response to aflatoxin at 3.05, 5.05, and 6.05. This study did not include the same markers, but different markers in those same bins did not show any association with aflatoxin levels. Several markers that were not found to be directly associated with response to aflatoxin did have epistatic interactions that were significant in relation to aflatoxin concentration. None of these interactions, however, were consistent across locations. For the correlated traits of kernel integrity and texture, there were also several epistatic interactions. Only one marker, phi014 was implicated as having epistatic effects on both aflatoxin concentration and endosperm texture, and only at College Station.

Root lodging at College Station was unusually high in 2004 due to high winds and rain. As such, variation among lines was minimized, and parental lines were not significantly different from one another for this trait. Few associations were identified between root lodging and markers. The alleles from Tx811 were beneficial at one locus while the alleles from CML176, which has a record of poor root stock qualities, were beneficial at another locus. At Weslaco, root-lodging measurements were more variable, and the differences between the parental lines were significant. Several markers were found to be related to root lodging, accounting for approximately 40% of the variation. In

particular, two markers in adjacent bins on chromosome 10 were found to be significantly and highly associated with root lodging. Further study in this region might elucidate QTL affecting root lodging.

Several markers that were not associated with root lodging individually were found to have epistatic interactions that affected this trait. These interactions were not consistent across locations.

More extensive map coverage is necessary in order to precisely locate QTL affecting root lodging. This population was created from parental inbreds with significant differences in root lodging characteristics. The conditions conducive to root lodging are opposite those conducive to aflatoxin concentration. For the trials in 2004, conditions were more favorable to explore root lodging than response to aflatoxin.

CHAPTER V

CONCLUSIONS

STUDY 1: REPEATABILITIES OF AFLATOXIN ACCUMULATION IN FIELD TRIALS OF WHITE AND YELLOW MAIZE HYBRIDS AND INBRED LINES IN TEXAS

In an effort to better understand the variance components related to aflatoxin, maize trials from six years in three different Texas locations were analyzed. These trials included white and yellow hybrids as well as their inbred parental lines, and quality protein maize. While aflatoxin was the primary concern, several secondary traits were also analyzed in order to ascertain any correlations between traits. All aflatoxin trials were conducted under inoculation; however two different methods were used. Both inoculation methods, the colonized kernel and the silk channel technique, yielded concentrations of aflatoxin which were high enough to offer variability for selection at the locations where used. The colonized kernel technique (Odvody *et al.*, 1996; Olanya *et al.*, 1997) was less effective in years with heavy rainfall during inoculation or flowering (2003, 2004). Heavy rains after colonized kernels have been placed between rows can cover the kernels with soil, providing a physical barrier that prevents inoculation. Should these environmental conditions occur, either colonized kernels should be reintroduced after

rains have passed, or an alternative method of inoculation employed. Because drought stress combined with high temperatures during flowering is more conducive to aflatoxin production (Payne, 1998), increased moisture during flowering reduces aflatoxin concentration. In this situation, the inoculation method will be less influential on aflatoxin concentration.

From the variance components, repeatabilities, which are estimates of the proportion of additive variance of a trait, were measured for each trait. Repeatabilities for aflatoxin concentration were higher than expected. Corpus Christi generally has environmental conditions most favorable to aflatoxin concentration, and at that location the highest levels of aflatoxin were recorded. Weslaco, however, had the highest mean aflatoxin concentration for both white and yellow hybrids. Highest repeatabilites for aflatoxin concentration were reported in Corpus Christi also, indicating that range of aflatoxin concentration is more important for repeatability than mean aflatoxin concentration, as evidenced also by the higher correlation of maximum aflatoxin concentration to repeatability.

Previous studies have shown large genotype by environment (GxE) effects for aflatoxin concentration (Hamblin and White, 2000; Widstrom

et al., 1984; Zuber *et al.*, 1983). Trials examined in this study exhibited large GxE interactions and/or environmental variance. Across location repeatabilities for aflatoxin concentration were consistently lower than individual location repeatabilities. Corpus Christi had the highest mean repeatability for aflatoxin concentration for white and yellow hybrids and QPM lines, followed by Weslaco and College Station. The high GxE interaction and environmental variance indicates that any future breeding efforts for aflatoxin resistance must include testing over several environments, although higher genotypic variance at Corpus Christi indicates that this environment provides a more optimal environment for selection for aflatoxin resistance.

While husk cover has been previously indicated as a morphological barrier to aflatoxin, the use of silk channel inoculation bypassed that barrier. Husk cover was not measured consistently enough in the trials when colonized kernel inoculation was used. Thus, despite this study finding no correlation between husk cover and aflatoxin concentration, the relationship of these traits naturally remains unclear.

Endosperm texture was positively and significantly correlated to aflatoxin concentration for both white and yellow hybrids at Corpus Christi and at Weslaco. More floury endosperm yielded higher aflatoxin

concentration. Because endosperm texture is highly heritable per and across locations, it is a potential characteristic for indirect selection for lower aflatoxin accumulation.

STUDY 2: PHENOTYPIC EVALUATION OF RECOMBINANT INBRED LINE POPULATION CML176 x Tx811

A recombinant inbred line (RIL) was developed from divergent parental inbreds Tx811 and CML176 in order to study the genetic components of aflatoxin. This S_6 population was characterized phenotypically in the field in both College Station and Weslaco. Traits measured included aflatoxin concentration, percent root lodging, kernel integrity, endosperm texture, and maturity. Variance components were estimated, as well as heritability and genotypic and phenotypic correlations.

The genetic proportion of phenotypic variation of aflatoxin response was higher at Weslaco than at College Station, which is consistent with previous data. For trials in which the genotypes are fixed rather than random, a measurement called repeatability (*R*) is used (Cooper *et al.* 1993). Weslaco traditionally has environmental conditions more conducive to aflatoxin accumulation, and results in higher aflatoxin levels with greater variability. The heritability had a high variance between locations, due to high environmental component in variance,

reiterating that multilocation testing is necessary in order to make progress in selection for lower aflatoxin accumulation. Previous studies of hybrid trials across six years have shown similar results, with Weslaco having higher repeatability of aflatoxin response than College Station (Chapter II).

Maturity has been indicated in previous studies as being negatively but significantly correlated (r = -0.59**) to aflatoxin concentration (Betran and Isakeit., 2004). Higher r values indicate stronger correlations. A negative correlation indicates that as the value of one trait increases, the value of the other trait decreases. In this case, later maturity is associated with lower aflatoxin accumulation. Weslaco, early maturation was also significantly correlated to higher aflatoxin accumulation both genotypically and phenotypically. Tx811 matures earlier than CML176, and is also more susceptible to aflatoxin concentration. At College Station in 2004, however, later maturity was correlated to higher aflatoxin concentration. The most critical time for aflatoxin concentration is during kernel filling, when drought and high stress are most likely to promote high aflatoxin levels (Lisker and Lillehoj, 1991; Vincelli *et al.*, 1995). In this instance, wetter weather during kernel fill for the earlier material may have resulted in lower aflatoxin concentration than previously indicated. The earlier material, despite

higher susceptibility, therefore exhibited lower levels of aflatoxin than the later material due to early wetter and cooler conditions during that crucial time.

Maturity has a high heritability, both per and across locations. The lack of variation across environments indicates that maturity is a more simply inherited trait, and that selection for maturity is possible early in the breeding process, without multiple year or location trials. It is also easy to select for in the field. A strong and highly significant correlation with aflatoxin response indicates that maturity may be a viable option for indirect selection, but the reversal of signs based on environment preclude maturity as the optimal trait for indirect selection. If maturity were considered in selecting for response to aflatoxin, environmental conditions at flowering time would have to be considered and somehow incorporated into selection criteria and decisions.

The consistent and highly significant correlation of aflatoxin with kernel integrity and endosperm texture indicates that lines with softer endosperm and a higher percentage of damaged kernels have increased aflatoxin concentration, which are also attributes of Tx811. Kernel integrity and endosperm texture have high heritabilities both per and across locations. These are, therefore, also more simply inherited traits,

which may be selected for early in the breeding process, without multiple year or location trials.

Kernel integrity had the highest overall correlation with aflatoxin response. While kernel integrity is not directly a measurement of wax and cutin layers on the surface of maize kernels or thicker pericarp layers, all of these characteristics could contribute to higher levels of kernel integrity. These individual characteristics have been previously indicated in conferring resistance to aflatoxin accumulation (Guo *et al.*, 1995; Russin *et al.*, 1997, Tubajika and Damann, 2001). Kernel integrity ratings may provide a way to select for these morphological characteristics that promote resistance to aflatoxin infection/accumulation.

Susceptibility to root lodging is an attribute that has been associated with parental inbred CML176. Under extreme situations, as in College Station in 2004, high levels of root lodging throughout the population reduced phenotypic variability, and genotypic variability was more difficult to ascertain. This lowered the overall heritability in that environment. In conditions that were less conducive to root lodging, CML176 does have significantly higher levels of root lodging than Tx811, and the proportion of genotypic variance (heritability) was increased.

Material with higher thousand kernel weight and that with later maturity had significantly higher root lodging.

For the secondary traits with high heritability and high genotypic correlation to aflatoxin concentration, potential for genetic gain through indirect selection is possible. However, caution must be exhibited in using these traits due to the environmental and spatial variation exhibited for aflatoxin concentration. For initial decrease in breeding stock mean aflatoxin concentration, therefore, these traits provide an excellent preliminary screening method. Once the overall mean had been reduced, however, genotypic variance would need to be reassessed to determine the usefulness of these secondary traits for indirect selection.

This population showed high levels of phenotypic variation for both locations for both aflatoxin accumulation and root lodging. This data, when combined with genotypic information, may provide information about quantitative trait loci for these traits.

STUDY 3: MOLECULAR MARKER CHARACTERIZATION OF THE RECOMBINANT INBRED LINE POPULATION CML176 x Tx811 AND QTL MAPPING

The RIL population was characterized genotypically using simple sequence repeat markers throughout the genome. This genotypic data was then compared with phenotypic data in order to locate QTL affecting response to aflatoxin and root lodging.

Lines in this study had a higher level of heterozygosity than expected from an S_6 RIL population. In the creation of the population, a high level of heterozygosity was inadvertently maintained. Plants with some level of heterozygosity tend to be more vigorous, and thus may have been advanced due to higher levels of seed at earlier stages. Similarly, the high level of segregation distortion was unexpected. Parental inbred CML176 has less desirable agronomic characteristics, and therefore would be less likely to be unconsciously selected for in the field, and yet parental alleles from CML176 were maintained in a higher than expected ratio.

Aflatoxin levels at both locations in this study were lower than previous years due to increased moisture at flowering time. There were no markers that were consistently associated with aflatoxin concentration

at both locations. None of the markers found to be significantly associated with aflatoxin in College Station were in the same bins as those found by Paul *et al.* (2003) or Brooks *et al.* (2005). At Weslaco, phi085, which is in bin 5.07, was significantly associated with aflatoxin levels. Paul *et al.* (2003) also identified markers in 5.07, bmc1346 and bng1118, as affecting aflatoxin in two different populations in different years. These markers were not stable across environments. Previous studies have found QTL for aflatoxin on chromosome 4. At Weslaco, chromosome 4 was also indicated as containing potential QTL for aflatoxin.

While the markers specifically used in previous trials may not be polymorphic for these parental lines, others located nearby might indicate QTLs in the same regions. Brooks *et al.* (2005) found QTL associated with response to aflatoxin at 3.05, 5.05, and 6.05. This study did not include the same markers, but different markers in those same bins did not show any association with aflatoxin levels. Several markers that were not found to be directly associated with response to aflatoxin did have epistatic interactions that were significant in relation to aflatoxin concentration. None of these interactions, however, were consistent across locations. For the correlated traits of kernel integrity and texture, there were also several epistatic interactions. Only one

marker, phi014 was implicated as having epistatic effects on both aflatoxin concentration and endosperm texture, and only at College Station.

Root lodging at College Station was unusually high in 2004 due to high winds and rain. As such, variation among lines was minimized, and parental lines were not significantly different from one another for this trait. Few associations were identified between root lodging and markers. The alleles from Tx811 were beneficial at one locus while the alleles from CML176, which has a record of poor root stock qualities, were beneficial at another locus. At Weslaco, root-lodging measurements were more variable, and the differences between the parental lines were significant. Several markers were found to be related to root lodging, accounting for approximately 40% of the variation. In particular, two markers in adjacent bins on chromosome 10 were found to be significantly and highly associated with root lodging. Further study in this region might elucidate QTL affecting root lodging.

Several markers that were not associated with root lodging individually were found to have epistatic interactions that affected this trait. These interactions were not consistent across locations.

More extensive map coverage is necessary in order to precisely locate QTL affecting root lodging. This population was created from parental inbreds with significant differences in root lodging characteristics. The conditions conducive to root lodging are opposite those conducive to aflatoxin concentration. For the trials in 2004, conditions were more favorable to explore root lodging than response to aflatoxin.

Aflatoxin is more heritable at individual locations than expected. It is strongly correlated with kernel integrity ratings. While the correlations are not strong enough to accurately predict aflatoxin concentration, they do give indications of relative aflatoxin levels for selection purposes.

Heritability and repeatability estimates were similar for the traits measured. While repeatability is not a widely used estimate, these studies indicate that it gives a good indication of the heritability of the trait. While variance components are sometimes difficult to estimate due to lack of necessary populations, approximations can be achieved using repeatability measurements. This allows breeders the possibility of rough estimation of variance components for individual program needs.

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