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Maize (*Zea mays* L.) yield and aflatoxin accumulation responses to exogenous glycinebetaine application

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Abstract

Exogenously applied glycinebetaine (GB) accumulates at high levels in maize (*Zea mays* L.). Under water deficit and high temperature conditions GB application produces yield benefits. These sub-optimum conditions often result in high levels of aflatoxin accumulation which reduces grain quality. A 3-year (2008, 2009 and 2010) field experiment was conducted to determine the effects of GB on maize yield and aflatoxin accumulation. Weekly and alternate weekly GB application increased plant biomass by 10 and 13%, respectively. Net photosynthesis increased by 6% with GB application; however, stomatal conductance, transpiration rate and electron transport rate were not significantly affected. Grain yield increased by 6 and 13% with GB applied alternate weekly and weekly, respectively, over control plots averaged over years. GB application resulted in a trend of reduced aflatoxin accumulation in inoculated ears compared with non-inoculated controls in 2009 and 2010; however, inherent field and sampling variation did not allow us to conclude statistically any advantage attributable to GB application. We can conclude that GB did not significantly reduce aflatoxin production in the inoculated treatments.

Keywords: Maize; Glycinebetaine; Photosynthesis; Growth; Aflatoxin accumulation.

Running title: Glycinebetaine effect on maize yield and aflatoxin levels.

Introduction

Drought and high temperatures limit profitable maize production in the USA ′and′ particularly in the Southern USA. Drought stress occurs in high

ambient temperature and low relative humidity conditions which can reduce grain yields by >50% in brief periods of wilting during anthesis (Çakir, 2004; Nielsen et al., 2010). Furthermore, moisture stress may increase the crop's vulnerability to other biotic and abiotic stresses. For example, drought and high temperatures during flowering and grain-filling period can lead to increased aflatoxin accumulation that result in extensive economic losses in Mississippi and other maize growing states (Hawkins et al., 2008). Maize yield is highly sensitive to changes in temperature with a 17% yield reduction for every 1 °C rise in temperature in the US corn producing states (Lobell and Asner, 2003). Wheat, maize and barley yields have also been negatively correlated with recent warming trends (Lobell and Field, 2007). Moreover, increases in greenhouse gases, are projected to be between 1.5 and 11 °C by 2100 (Stainforth et al., 2005). These changes in temperature and precipitation will influence maize yield and quality.

To abate the effects of abiotic stresses on crop growth, plants accumulate or synthesize compatible solutes in response to these stresses. Glycinebetaine (GB), an amphoteric amine, plays an important role as a compatible solute in plants under various environmental stresses such as moisture, temperature (high and low) and salinity. Plants species vary in their capacity to produce GB. Spinach (*Spinacia oleracea*) and barley (*Hordeum vulgare*) produce high levels of GB, which allows the crops to sustain growth despite stressful conditions (Sakamoto and Murata, 2002). Maize, however, is not a significant producer of GB. The action of GB is not confined to osmoregulation but also acts as an osmoprotectant by stabilizing the proteins, enzymes and membrane functions against the adverse effects of high and low temperatures and salt stress (Holmström et al., 2000). For example, GB protects various components of photosynthetic machinery under stress conditions (Incharoensakdi et al., 1986; Yang and Lu, 2005). Therefore, GB has become a tool to enhance the abiotic stress tolerance of crops via genetic engineering (McCue and Hanson, 1990; Sakamoto and Murata, 2002). Glycinebetaine enhanced lines for maize (Rhodes et al., 1989), wheat (*Triticum aestivum*) (Naidu et al., 1990) and sorghum (*Sorghum bicolor*) (Grote et al., 1994) have been identified. Transgenic lines of maize with enhanced GB synthesis have reduced cell membrane damage by 13% relative to wild type maize (Quan et al., 2004). Breeding for enhanced GB accumulation has been impeded by the inability to isolate the dominant allele determining stress-induced GB production in sufficient quantities (Rhodes et al., 1989). Therefore, exogenous application of GB has been

suggested as an alternative approach to genetic engineering to improve crop productivity under stressful environments (Mäkelä et al., 1996). Foliar GB application improved yield in various crops under water stressed conditions, for example, wheat by 18% (Díaz-Zorita et al., 2001), cotton (*Gossypium hirsutum*) by 18-22%, maize by 18-34% (Agboma et al., 1997) at a cost of less than 1US $ ha^{-1} (Naidu et al., 1998). This was linked with increased stomatal conductance and net photosynthesis (Mäkelä et al., 1998) and enhanced water status in the GB-treated plants (Saneoka et al., 1995).

Aflatoxins are fungal metabolites produced by fungi in the *Aspergillus* genus. Some species like *A. flavus* produces highly potent naturally occurring carcinogens that cause developmental and immune system disorders (Groopman et al., 2008). Infection of maize kernels with *Aspergillus* and subsequent aflatoxin accumulation is a major economic concern in the southeastern United States grain industry (Windham and Williams, 1998). Aflatoxin production depends on interacting factors that stress the maize plant. Some of these factors include water deficit and high temperatures (Betran and Isakeit, 2004; Abbas et al., 2006; Hawkins et al., 2008; Windham et al., 2009) which are common in the maize growing states of the southern USA, especially during critical stages of reproductive development. Controlling aflatoxin severity is possible with cultural practices that mitigate plant stress (Abbas et al., 2002), therefore, GB application may indirectly reduce aflatoxin accumulation by alleviating high temperature and water deficit stress in maize. Previous attempts at reducing aflatoxin levels in maize using foliarly-applied glufosinate-ammonium and urea were unsuccessful (Bruns and Abbas, 2006). With increasing maize acreage in the southern US and erratic weather patterns particularly drought and heat, there is an increased risk of aflatoxin impacting the US maize industry (Betran and Isakeit, 2004; Magan et al., 2011; Wu et al., 2011). The objectives of this study were to investigate the effects of foliar application of GB on maize under rainfed conditions and to determine whether GB application can indirectly reduce aflatoxin accumulation.

Materials and Methods

Field experiments

Experiments were conducted in 2008, 2009 and 2010 at the R. R. Foil Plant Science Research Center, Mississippi State University (33° 28’ N,

88° 47' W), Mississippi, USA on a 65% Marietta fine sandy loam and 35% Leeper silty clay loam soil. A Terral maize hybrid, TV25R31, was grown in 2008 and 2009 experiments and a similar hybrid, TV25R19, was grown in 2010. The planting and harvesting dates are presented in Table 1. Fertilizer and herbicides were applied in accordance with standard production practices and neither fertility nor weed pressure was limiting to crop growth at any time. The experimental design was a randomized complete block with split plot arrangement of treatments with four replications. Treatments were frequency of GB application (control, alternate weekly and weekly) as whole plots and aflatoxin inoculation (with and without) as subplots in a split-plot arrangement. Plots in 2008 and 2009 were 12 m wide by 152 m long for total area of 0.16 ha. Plots were half as long in 2010 for 0.07 ha. For all years, yield and aflatoxin data were recorded from the middle 8 rows with two border rows on either side of the plots. Rows were seeded 0.96 m apart in all years with a plant density of 74,000 plants ha^{-1}.

Table 1. Planting, glycinebetaine (GB) application, aflatoxin inoculation and harvesting dates for each year of the study.

Field Activity	2008	2009	2010
Planting	May 6 (127)[a]	April 24 (115)	April 7 (98)
GB application (W, AW)[b]	June 3 (155)	May 22 (143)	May 26 (147)
GB application (W)	June 9 (161)	May 29 (150)	June 2 (154)
GB application (W, AW)	June 17 (169)	June 8 (160)	June 9 (161)
GB application (W)	June 20 (172)	June 17 (169)	
GB application (W, AW)	June 24 (176)	June 19 (171)	
GB application (W)		June 22 (174)	
GB application (W, AW)		June 25 (177)	
Aflatoxin inoculation	July 17 (199)	July 10 (192)	June 22 (174)
Harvesting	September 25 (269)	October 1 (275)	August 27 (161)

[a] Numbers in parentheses are the Julian calendar day.
[b] W and AW is the weekly and alternate weekly glycinebetaine (GB) application.

Glycinebetaine application

Plots were sprayed with 4 kg ha^{-1} of GB (Nutristim, 97% betaine anhydrous, Finnfeeds Finland Ltd., Finland) mixed in water and with a 1% non-ionic surfactant (Tween 20) with a tractor-driven sprayer. The dates of the two GB treatments include weekly (W) and alternating weekly (AW) application as shown in Table 1. Depending upon environmental conditions and soil moisture in the given years, GB applications were initiated 4 weeks

after emergence. Control plots were sprayed with equal amounts of water plus a 1% surfactant. There was no rainfall on the day of or after each GB application event in all three years of this study. Glycinebeatine is readily absorbed and translocated throughout the plant almost immediately after application (Mäkelä et al., 1996), therefore there is a very low chance of reduction in absorption and translocation potential with precipitation.

Inoculum production and application

Apergillus flavus isolate NRRL 3357 has produced high levels of aflatoxin in maize grain in prior studies (Windham and Williams, 1998; Windham and Williams, 1999; Windham and Williams, 2002; Windham et al., 2009) and was used as the inoculum in all tests. Inoculum was increased according to Windham et al. (2009). Briefly, *A. flavus* was grown on sterile maize cob grits (size 2040, Grit-O-Cobs®, The Andersons, Maumee, OH, USA) in 500-ml flasks. Each contained 50 g of grits and 100 ml of sterile, distilled water and was incubated at 28 °C for 3 weeks. Conidia in each flask were washed from the grits using 500 ml sterile distilled water containing 0.1% Tween 20 per liter and filtered through four layers of sterile cheesecloth. The concentrations of conidia were determined with a hemacytometer and adjusted to 10^7 conidia per ml.

The top ear of each plant was inoculated with *A. flavus* with a side-needle technique using an Idico tree-marking gun fitted with a 14-guage hypodermic needle (Zummon and Scott, 1989; Windham et al., 2005). Ears were inoculated 7 to 14 days after midsilk by inserting the needle through the husk midway up the ear injecting 3.4 ml of the *A. flavus* conidia inoculum. Plants inoculated and harvested for aflatoxin analysis were selected from a single-row within a given GB treatment. Approximately 40 plants were inoculated per plot and 20 ears from those inoculated were selected for toxin analyses.

Growth and physiological measurements

Plant height, node numbers, leaf, stem and plant biomass were measured between 60 and 65 days after seeding in all years. Gas-exchange parameters such as leaf photosynthesis, stomatal conductance and transpiration rate were measured using Li-COR 6400 Photosynthesis system (LI-COR Inc., Lincoln, NE, USA) with an integrated fluorescence chamber set at 1,500 µmol photon m^{-2} s^{-1} photosynthetically active radiation, 30 °C cuvette temperature and 360-ppm CO_2 concentration. Measurements were made in

2009 and 2010 only using the topmost fully expanded leaf, from three plants per replicate, between 10:30 to 13:00 h between 60 and 65 days after planting in both years. Total chlorophyll and carotenoids concentrations and leaf water potential were measured from the topmost fully expanded leaves 65 days after planting. The pigments were extracted by placing 5, 0.38 cm^2, leaf disks in a vial containing 5 ml of dimethyl sulfoxide and incubating in darkness for 24 h. Thereafter, the absorbance of the supernatant was measured at 648, 662 and 470 nm with a Bio-Rad UV/VIS spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The total chlorophyll and carotenoids were estimated and expressed on a leaf area basis (μg cm^{-2}) using an equation developed by Lichtenthaler (1987).

Harvest and aflatoxin analyses

Inoculated ears were hand-harvested at kernel maturity, 63 days after midsilk and dried at 38 °C for 7 days. Ears were machine shelled and grain samples from each row were poured into a sample splitter twice to mix the grain. Grain samples for aflatoxin analyses were ground with a Romer mill (Romer Laboratories Inc., Union, MO, USA). The VICAM AflaTest® (VICAM, Watertown, MA, USA) was used to determine aflatoxin contamination in a 50-g subsample from each plot. This procedure can detect aflatoxins (B_1, B_2, G_1, G_2) at concentrations as low as 2 ng g^{-1}.

Data analysis

Growth, biomass, gas exchange and pigments were subjected to analysis of variance using PROC GLM procedure in SAS (SAS Institute Inc, 2004). Yield and aflatoxin accumulation were analyzed using repeated measure mixed model analysis of variance, PROC MIXED procedure in SAS (SAS Institute Inc, 2004) for treatment interaction effects. Aflatoxin data were log transformed (ln(aflatoxin value+1)) before analysis and back transformed to the original scale for presentation. Means were compared using the PDIFF option in PROC MIXED ($P>0.05$).

Results and Discussion

This is the first field study to document the benefits of repeated foliarly applied GB application on maize growth and yield and the indirect

alleviation of grain aflatoxin accumulation. A previous study indicates that GB enhances yield and growth characteristics during periods of water deficit and high temperatures in maize (Agboma et al., 1997), but this experiment did not study the seasonal effects on GB efficacy to protect grain yield and quality under field conditions. In addition, the seasonal impacts of GB on aflatoxin accumulation under field conditions have not been quantified previously. Previous reports on the enhanced effects of GB application on maize stress tolerance were conducted in controlled greenhouse environments (Yang and Lu, 2006). This study attempted to determine whether these findings are stable under variable field conditions and provide definitive answers on the utility of GB as a stress ameliorant under field conditions. Management practices employed in this 3-year field study are similar to farmer's production practices for rainfed maize from planting to harvesting with GB application being the additional management activity to alleviate drought-and high-temperature conditions. In addition, alflatoxin accumulation is enhanced under stress conditions; therefore we determined whether GB application can indirectly influence aflatoxin accumulation.

Growing season precipitation (April to August) totaled 362, 772 and 350 mm in 2008, 2009 and 2010, respectively and was 25 (2008) and 29% (2010) lower than the long-term average precipitation (452 mm). In 2009, precipitation was 41% higher than the long-term average precipitation and was an extremely wet year. Within the same period, mean air temperatures were 22.4, 21.6 and 24.9 °C in 2008, 2009 and 2010, respectively, differing slightly from the long-term average temperature (23.5 °C). The 2010 production year was the driest year with a 1.4 °C increase in seasonal temperature which was considered hot and dry for this region (Table 2).

Table 2. Total monthly precipitation and mean monthly air temperature for 2008, 2009 and 2010 growing season and the 15-yr average (1995-2010) at the Plant Science Research Farm at Mississippi State University, MS.

Month	Precipitation (mm)				Average temperature (°C)			
	2008	2009	2010	15 yr-avg	2008	2009	2010	15 yr-avg
April	149.6	105.2	108.0	102.1	14.7	15.6	18.1	17.3
May	100.1	266.7	71.1	87.8	20.6	19.7	22.8	22.1
June	46.7	103.1	70.4	89.2	24.4	24.2	26.7	25.6
July	33.5	128.0	57.4	83.0	25.8	24.7	27.8	27.1
August	32.3	168.7	42.7	90.3	26.4	23.9	28.9	26.8

Growth characteristics

To quantify the effects of GB on growth processes, we measured plant height, node numbers, leaf area and biomass components. Total above ground dry matter (DM) was affected by treatment (P=0.0465) and year (P<0.0001); however, there was no interaction. A 10% (800.5 g plant^{-1}) to 13% (821.4 g plant^{-1}) increase in DM was found across years for the weekly and alternate weekly treatments, respectively (Table 3). Significant year effects were observed for plant height, node numbers, leaf area index (LAI) and leaf and stem dry weights. Across all years, there was a trend of increased plant height, LAI, leaf and stem dry weight with weekly and alternate weekly GB application; however, these increases were not significant relative to the control. Plant height increased from 4 to 5% with GB application while LAI increased by 6% for both weekly and alternate weekly GB application across all years (Table 3).

Node numbers averaged 15 per plant and was unaffected by GB application. Alternate weekly GB application produced plants with leaf and stem dry weights that were 12 and 14% numerically but not statistically greater than control plants, respectively, while weekly GB application produced plants with leaf and stem dry weights that were 9 and 11% numerically but not statistically greater than the control plants, respectively (Table 3). Glycinebetaine is more likely to produce an effect on plant growth characteristics if water availability is sub-optimum. In a pot experiment using a similar hybrid (TV25R19), Reddy et al. (2012) found that plant height, leaf area and plant biomass increased with GB application under mild water stress (0.5 L d^{-1}) conditions. In addition, GB application increased both total plant dry weight and ear dry weight by 35% under the mild water-stressed conditions. This effect was also demonstrated by Yang and Lu (2006) with plant height and dry weight increasing with GB concentration up to 10 m*M* while > 10 m*M* resulted in inhibitory effect on these growth characteristics. In this study, all management practices reflected farmer's current production practices and therefore the heterogeneity in seasonal water availability may have overwhelmed the beneficial effect of GB on the growth characteristics.

Table 3. Effect of glycinebetaine on maize growth and development and aflatoxin levels: plant height, node numbers, leaf area index, leaf, stem and total dry weight per plant, grain yield, 100-seed weight and aflatoxin levels for maize grown at the Plant Science Research Farm at Mississippi State University, MS during 2008, 2009 and 2010 growing seasons.

Treatment	Plant height (cm)	Node number (no.)	Leaf area index	Leaf dry weight (g)	Stem dry weight (g)	Total biomass (g)	Yield (Mg ha^{-1})	100-seed weight (g)	Aflatoxin concentration (ppb)[b]
Control	194.3	14.9	3.8	218.0	508.5	726.5[a]	8.0[b]	27.8	270.4
Alternate weekly	202.1	14.9	4.1	243.9	577.5	821.4[b]	8.5[ab]	29.5	181.3
Weekly	204.2	14.9	4.1	238.2	562.3	800.5[b]	9.0[a]	28.9	164.0
ANOVA									
Year	***[a]	***	***	***	***	***	***	*	***
Treatment	NS	NS	NS	NS	NS	**	***	NS	NS
Year × Treatment	*	NS	NS	NS	NS	NS	NS	*	NS

[a] *,**,*** significant at the P=0.05, 0.01, 0.001 probability level, NS=not significant (P>0.05).

[b] Aflatoxin concentrations are presented as geometric means. Data were log transformed (ln (aflatoxin value+1)) before analysis and back transformed to the original scale for presentation.

[c] Within columns, means followed by the same lowercase letters are not different using PDIFF option in PROC MIXED (P>0.05).

Physiology

We measured various physiological processes to understand the differences in growth parameters. Glycinebetaine did not influence cell membrane thermostability, pigment concentration and leaf water potential which were measured in 2009. Both weekly and alternate weekly GB application demonstrated a trend of increased membrane stability (9 and 13%), carotenoids (15 and 13%), total chlorophyll (15 and 19%) and leaf water potential (6 and 5%) (Table 4). Glycinebetaine application irrespective of weekly or alternate weekly applications increased net photosynthesis by 6.5%, however stomatal conductance, transpiration rate and electron transport rate were not affected by GB application (Table 4). Stomatal conductance in the weekly and alternate weekly GB treated plants was 9 and 11% numerically but not statistically greater than the control plants, respectively (Table 4). These findings support prior greenhouse and protected pot experiments (Yang and Lu, 2006). For example, GB increased CO_2 assimilation rate within a 2-20 m*M* concentration range through greater electron transport in PSII photochemistry and increased stomatal conductance augmented by increased turgor pressure in guard cells (Yang and Lu, 2006). Hussain et al. (2008) also found marginal but not significant increases in leaf water potential in GB treated sunflower under water-stressed conditions. Also, water regulation in common bean was enhanced under stressed conditions following GB applications at concentrations over 10 m*M* (Xing and Rajashekar, 1999). In these studies, the benefits to plant growth were attributed to the enhanced plant water status.

Maize has the capacity to absorb and accumulate high levels of foliar applied GB (Yang and Lu, 2006) which is translocated throughout the plant almost immediately when applied and can remain unmetabolized for up to three weeks after application (Mäkelä et al., 1996). Glycinebetaine accumulation leads to a decrease in osmotic potential facilitating cell turgor pressure and cell membrane integrity under water deficit conditions. The leaf-level physiological benefits to GB application are difficult to replicate in the field where continuous variable diurnal and seasonal environmental conditions exist.

Table 4. Effect of glycinebetaine on maize physiology: cell membrane thermostability (CMTS), carotenoids, total chlorophyll, leaf water potential (LWP), photosynthesis, stomatal conductance, transpiration rate and electron transport rate (ETR) for maize grown at the Plant Science Research Farm at Mississippi State University, MS.

Treatments	CMTS	Carotenoids	Chlorophyll	LWP	Photosynthesis	Conductance	Transpiration	ETR
		μg cm^2		MPa	μmol m^{-2} s^{-1}	mol m^{-2} s^{-1}	mmol m^{-2} s^{-1}	μmol electrons m^{-2} s^{-1}
Control	56	8.2	47.7	-1.78	32.6[b]	0.39	9.7	199.3
Alternate weekly	63	9.3	56.6	-1.70	34.7[a]	0.35	10.0	203.9
Weekly	61	9.4	54.8	-1.68	34.8[a]	0.38	10.2	206.1
ANOVA								
Year					NS[a]	***	***	***
Treatment	NS	NS	NS	NS	*	NS	NS	NS
Year × Treatment					NS	NS	NS	NS

[a]*,**,*** significant at the 0.05, 0.01, 0.001 probability level, NS=not significant (P>0.05).

Yield and aflatoxin accumulation

Grain yield was affected by a year ($P<0.001$) and GB ($P=0.0248$) effect but there was no interaction. Yield differed in 2008 (10.8 Mg ha^{-1}), 2009 (8.9 Mg ha^{-1}) and 2010 (5.3 Mg ha^{-1}) when averaged across GB application treatments (Table 3). The drastic decline in 2010 was related to sustained seasonal water deficit in that year relative to the other years in the study. Grain yield increased by 6% (8.5 Mg ha^{-1}) and 13% (9.0 Mg ha^{-1}) following alternate weekly or weekly GB application compared with control plots (8.0 Mg ha^{-1}) when averaged over the 3 years in this study (Table 3). Exogenous GB and its role in drought and heat tolerance has been studied extensively in maize (Agboma et al., 1997), sorghum (Agboma et al., 1997), wheat and common beans (*Phaseolus vulgaris*) (Xing and Rajashekar, 1999), soybean (*Glycine max*) (Koti and Reddy, 2005; Brand et al., 2007), cotton (Meek and Oosterhuis, 2000), sunflower (*Helianthus annuus*) (Hussain et al., 2009); however, most of these studies were limited to small plots, greenhouse or other controlled environments which did not subject these crops to the variation present in field scale research plots and natural weather patterns. Variability in precipitation and temperature influences the responses of a crop to these foliarly-applied chemicals. Glycinebetaine is most effective in periods of water deficit (Hussain et al., 2009; LiXin et al., 2009; Reddy et al., 2012); therefore conducting multiyear experiments are important in establishing the efficacy of GB. In a 1-yr study in Australia, Agboma et al. (1997) found that maize was highly responsive to GB application and the response varied with the intensity of water stress. Yield was found to increase by 34 and 18% at suboptimum and optimum water levels when 6 kg GB was applied once. Glycinebetaine rates similar to rates applied in the current study (4 kg ha^{-1}) increased yield by 30 and 10% at suboptimum and optimum water levels in Agboma et al. (1997) study. In the current study, total biomass and photosynthesis varied with GB application while grain yields increased following GB application. Maize accumulates 1 μmol of GB per gram fresh weight; however, the crop responds well to foliarly-applied GB. Endogenous accumulation in maize depends on the concentration of exogenously applied GB and increases to 21 μmol of GB per gram fresh weight 3 weeks after application if GB is applied at 50 m*M* concentrations (Yang and Lu, 2006). Transgenic lines with enhanced endogenous GB synthesis compared to wild types were reported to be more tolerant to drought stress (Quan et al., 2004). These transgenic lines are not adapted to

the growing conditions of the southern US. Because there is no regionally adapted enhanced GB producing hybrids available, exogenous GB application is a viable option.

Drought stress and high temperatures decreased not only yield but also the maize quality by providing a favorable environment for pathogenesis, especially during flowering and grain-filling periods of maize development. High temperatures increase the production of *A. flavus* conidia, their dispersal and kernel infection rate, thereby contributing to high levels of aflatoxin accumulation under these conditions. A kernel infection rate of 2.4% at 24 °C increases drastically to 28% at 32 °C average daily temperature (Payne et al., 1988). A year × inoculation (P=0.0086) effect on aflatoxin concentration occurred from variation in aflatoxin concentration across years. Aflatoxin concentration was below the 20 ppb maximum level permitted by the USDA guidelines across all the non-inoculated plants. Inoculation significantly increased aflatoxin levels across all treatments (Table 5). Averaged across the 3 years, the level of aflatoxin accumulation in naturally infected plants did not differ with weekly or alternate weekly GB application, with one notable but abstruse exception in 2008 with aflatoxin levels increasing by 3.5 ppb with weekly GB application (Table 5). This may be an artifact of the sporadic nature of infection leading to aflatoxin accumulation. Small fluctuations of several ppb are common with alfatoxin data.

Aflatoxin concentration in the weekly GB treated plants was 81% lower in 2009 (99.5 ppb) and 2010 (99.5 ppb) when compared with 2008 (544.6 ppb). Relative to the inoculated plants, naturally infected plants accumulated the lowest aflatoxin concentrations in all years (Table 5). The weather during the three years of the study was highly variable with 2008 and 2010 experiencing 25 and 29% reduced precipitation relative to the 15-year average. In 2010, early season water deficit was sustained throughout the growing season and this significantly reduced yield across all treatments (Table 3). Aflatoxin contamination of inoculated maize was highest in 2008, which experienced the driest July and August conditions among the 3 years of the study. Glycinebetaine application, either weekly or alternate weekly did not influence the incidence of aflatoxin accumulation. It is possible that stress alleviating benefits provided by GB application were overcome by excessive drought and heat as also observed in Reddy et al. (2013).

Table 5. Aflatoxin concentration in maize hybrid treated with glycinebetaine and inoculated with *Aspergillus flavus.*

GB application	Inoculation	Aflatoxin concentration (ppb)[a]			
		2008	2009	2010	Mean
Control	Inoculated	544.6^{aAb}	270.4^{aB}	312.2^{aAB}	375.7
	Non inoculated	1.8^{cA}	4.1^{bA}	1.2^{bA}	2.4
Alternate weekly	Inoculated	365.0^{aA}	134.3^{aA}	109.9^{aA}	203.1
	Non inoculated	1.5^{cA}	5.5^{bA}	1.6^{bA}	2.9
Weekly	Inoculated	544.6^{aA}	99.5^{aB}	99.5^{aB}	247.8
	Non inoculated	6.7^{bA}	4.5^{bA}	1.0^{bA}	4.1

[a] Aflatoxin concentrations are presented as geometric means. Data were log transformed (ln(aflatoxin value+1)) before analysis and back transformed to the original scale for presentation.

[b] Within columns, means followed by the same lowercase letters and within rows, means followed by the same uppercase letters are not different using PDIFF option in PROC MIXED (P>0.05).

The effect of moisture and temperature stresses on preharvest aflatoxin contamination of maize is well documented (Cotty and Jaime-Garcia, 2007; Hawkins et al., 2008; Windham et al., 2009; Wu et al., 2011). These conditions highly favor growth, conidiation and dispersal of *A. flavus* and simultaneous infection in maize resulting in increased concentrations of aflatoxins. An 8 °C reduction in average temperature reduced kernel infection by 26% (Payne et al., 1988). Although the growing season precipitation and temperature among the three years were variable, the aflatoxin contamination levels of non inoculated maize did not vary. Although the level of aflatoxin accumulation generally increased during years of moisture stress, we did not identify an increase in contamination levels in 2010, the driest year during the study. In fact there was no difference in contamination levels across years despite the wide variation in soil moisture availability caused by differences in precipitation patterns across the three years. Despite yearly variability in aflatoxin accumulation in 3 maize hybrids, Hawkins et al. (2008), found similar accumulation rates in Pioneer 3223 averaging 665 ppb across 3 years. Further, Windham et al. (2009) reported that aflatoxin accumulation in susceptible maize hybrid TV2100 was similar in 4 out 6 years, giving credence to the sporadic nature of aflatoxin accumulation. Nevertheless, environmental variation is associated with *Aspergillus flavus* and aflatoxin accumulation. Aflatoxin levels generally increase with

temperature but decrease with rainfall 14 days prior to and following inoculation (Windham et al., 2009). Cultivar TV25R31 was evaluated for aflatoxin accumulation in a related genotype screening trial by Daves et al. (2010) which was planted at similar date and location using the same inoculation technique. Aflatoxin levels in their study were in the range of 735 and 601 ppb while in our study, aflatoxin levels were in the range of 545 and 270 ppb in 2008 and 2009, respectively.

In previous studies, aflatoxin accumulation varied significantly ′among′ years. This variation was attributed to environmental conditions (temperature and drought), genotype, soil fertility and insect pressure (Hawkins et al., 2008). In North Mississippi, the greatest aflatoxin accumulation occurs between 65 to 85 days after planting regardless of maize maturity group or physiological growth stage (Hawkins et al., 2008). In our study, ears were inoculated 7 to 14 days after mid-silk which coincides with the period of greatest accumulation. Perhaps concentrating or continuing the GB application during this period until harvest maturity to coincide *A. flavus* infection and late season heat and drought stress may elicit a different response for aflatoxin suppression. The utility of multiple GB application will have to compete with the cost of the product and its application. The final grain yield may not economically compensate for inputs and therefore multiple applications may not be justified.

There are many strategies to control the levels of aflatoxin in maize including avoidance by shifting planting dates to avoid periods of drought and high temperatures during critical stages of development, use of resistant genotypes, irrigation, weed control and keeping insect pressure minimal. Although we found a yield benefit with GB application, it seems that the product may not be effective at suppressing aflatoxin accumulation when tested over diverse environmental conditions.

Conclusions

Glycinebetaine can protect yields during periods of water deficit and high temperatures, even under field conditions with seasonal variability. Foliar application of GB applied weekly or alternate weekly increased maize grain yield by 13 and 6%, respectively, relative to the control. Increased yields are attributed to stress alleviation by GB which resulted in higher photosynthesis and total plant biomass. Each application of GB will incur additional costs; therefore producers have to determine whether the increased yields will

warrant GB application. Glycinebetaine application did not consistently or significantly lower aflatoxin accumulation; however, the trend in two of the three years of this study was a reduction of at least one half aflatoxin accumulation following GB application in inoculated treatments. In no years of the study did GB application increase aflatoxin accumulation in the inoculated treatments.

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