Grain Chemical Composition as Affected by Genetic Backgrounds and Toxigenic Aspergillus flavus Inoculation in Corn Hybrids

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Abstract

Mycotoxins are toxic secondary metabolites commonly found in corn and known to cause health issues to human and animals. Information on the relationship between corn grain inoculated with mycotoxins and grain nutrients (protein, oil, fatty acids, sugars, and amino acids) in corn hybrids, especially stacked-gene hybrids is very limited. The objective of this study was to investigate the nutritional advantage of stacked-gene hybrids (Stgene) over non-genetically modified organisms (non-GMO) corn or glyphosate-resistant (RR). The experiment was conducted in two locations (clay and sandy soils) in 2011, irrigated, and inoculated with toxigenic Aspergillus flavus using four hybrids of stacked-gene, four of RR, and two non-GMO (conventional). Non-inoculated plots were used as control. The results showed that stacked-gene hybrids had no observable nutritional advantage over RR or non-GMO as all hybrid classes accumulated adequate nutrients in their grains; this effect could be due to adequate concentrations of nutrients in the soil. Higher levels of grain protein, carbohydrates (glucose, reducing sugars, and starch), oleic acid, total amino acids, and some minerals were observed under inoculated conditions in clay and sandy soils indicated a possible osmotic adjustment role of these compounds as stress indicators and osmoprotectants under inoculated conditions. Grain nutrients in clay soil were higher than those in sandy soils; nutrient differences could be due to sandy soil possibly suffering drier conditions, especially inoculated soil, reducing nutrient uptake and nutrients mobility to the grain. This study provides advanced knowledge on the relationship between grain nutrients and mycotoxins in corn hybrids. It is also useful to the corn breeders to understand the responses of grain nutrients to fungal diseases in corn hybrids.

Keywords: Grain Nutrition, Grain Chemical Composition, Stacked-Gene Corn, Hybrids, Mycotoxins, Aflatoxin, Fumonisin.

Introduction

Aflatoxins are toxic secondary metabolites commonly found in corn produced by Aspergillus spp., predominantly Aspergillus flavus. The amount of aflatoxins in crops is regulated by the government of each country; the United States has set the maximum aflatoxin level for direct human consumption at 20 ppb. Aflatoxin is known to cause liver cancer in humans as well as acute toxicity and death in poultry (Abbas, 2005; CAST, 2003). Cyclopiazonic acid (CPA) is also a mycotoxin produced by Aspergillus and Penicillium spp., including A. flavus fungi growing on feedstuff, and it is often found along with aflatoxins in animal feeds. CPA is also produced by Penicillium cyclopium (Abbas et al., 2011; King et al., 2011; Miller et al., 2011). CPA causes a disturbance of calcium metabolism in muscles, often causing death in animals. Fusarium spp. are vectors for mycotoxin contamination, including zearalenone, which causes vulvaginitis (hyperestrogenism) in swine, and deoxynivalenol (DON), which causes feed refusal, nausea, and vomiting (CAST, 2003). Fumonisins are mycotoxins...
produced by Fusarium spp. such as Fusarium verticillioides and Fusarium proliferatum, and are known to cause leukoencephalomalacia in horses and pulmonary edema in pig. The allowable level of fumonisins in maize and maize products has been set by FDA at 2–4 ppm (CAST, 2003; NTP, 1999).

Corn grown under heat and drought stress is susceptible to infection by A. flavus and Fusarium spp., resulting in kernels contaminated with aflatoxins and fumonisins. Insect infestation promotes fungal inoculation and mycotoxin contamination through insects-burrowing through the husks or down the silk channels, opening infection routes for air- or dust-borne fungal pathogens, causing further contamination and kernel damage. It has been reported that insects managed by Bt toxins can act as vectors of fungal pathogens (Abbas et al., 2009), and if this vector is controlled, Bt corn is thought to be less susceptible to mycotoxin-producing plant pathogens. It is well known that European corn borer (O. nubilalis) is one of most economically important insect pests in corn producing areas of the United States. In the southern United States, larvae enter diapause, overwinter primarily in the stalks, and in spring adults emerge as moths, travel before mating and laying eggs on the upper surface of maize leaves, feeding on corn ear (Cook et al., 2016). It has been reported that in spite of that, not all types of Bt toxins would give the same level of protection (NTP, 2001), and transgenic corn may be protected against European corn borer (Abbas et al., 2016). The possible effects of transgenic corn hybrids on mycotoxin control and the advantage of transgenic corn hybrids, including stacked gene hybrids, was studied, and its influence on reduced mycotoxin contamination, resulted from insect infection, was also evaluated (Abbas et al., 2016). They used what is called “stacked-gene” hybrids (hybrids released with multiple insect resistance genes and herbicide resistance genes). They compared ten commercially available corn hybrids: two non-transgenic, four stacked-gene transgenic, and four transgenic with glyphosate tolerance only to determine if any hybrid class had the advantage of reduced mycotoxin contamination. They found that mycotoxin production was affected by soil type, with lower levels of mycotoxins in the hybrids planted in clay soil vs. sandy soil. Also, they found that there were no significant differences in mycotoxin production amongst the hybrid classes. They concluded that further research is needed to identify conditions where transgenic hybrids might produce higher yields and lower mycotoxin levels. Contaminated corn with mycotoxins, including aflatoxins and fumonisins leads to major food safety issues, production losses, and grain waste (Robens and Cardwell, 2003). Aflatoxins and fumonisins can be a major problem in the southern U.S., although fumonisins are spread more northerly areas of the U.S. (Abbas, 2005; Abbas et al., 2015; CAST, 2003). Mycotoxin production is affected by environmental conditions such as heat, drought, insect infestation and various plant diseases (Dienster et al., 1987; Payne, 1992; Abbas et al., 2002; Bruns and Abbas, 2005; De la Campa et al., 2005; Bruns and Abbas, 2006; Abbas et al., 2007). It was reported that stacked-gene hybrids can reduce mycotoxins, as they are formulated to affect mycotoxin production.

Effects of transgenics on aflatoxin contamination in corn have been studied, but no conclusion or recommendations have been made. For example, Williams et al. (2010) and Wiatrak et al. (2005) reported lower levels of aflatoxin in Bt lines and hybrids, respectively. Other studies found no significant effects of Bt hybrids on aflatoxin or inconsistent effects of Bt hybrids on fumonisins. While studying the western bean cutworm and European corn borer infestation, it was found that there was no significant effect of Bt on aflatoxin and variable effect of Bt on fumonisin content (Catangui and Berg, 2006). Abbas et al. (2009) studied the effect of 65 hybrids on the aflatoxin level over three years, but the effect of transgene on aflatoxin levels could not be demonstrated. Abbas et al (2013) reported that Bt corn lacks protection against aflatoxin contamination or no protection against the insects (e.g., corn earworm or fall army-worm) that limit the spreading of Aspergillus infection. Bt corn hybrids showed clearer effects on reducing fusarium mycotoxins. For example, the first-generation Bt corn hybrids demonstrated a resistance to fusarium ear rot along with lower levels of fumonisin contamination in harvested kernels (Munkvold et al., 1999). Meta-analysis concluded that Bt corn showed lower level in one or more fusarium mycotoxins in 19 of 23 studies (Ostry et al., 2010).

Relationship between corn grain mycotoxins and grain nutrients (protein, oil, fatty acids, sugars, and amino acids) has been studied in glyphosate-resistant hybrids. For example, Viquez et al. (1994) evaluated the correlation between aflatoxin produced by Aspergillus flavus and nutrients (Mg$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$) and the polynomial and sugars (xylose, fructose, glucose, and mannose) content in corn grain. They found that the levels of aflatoxin were highly correlated ($P \leq 0.01$) with minerals (Mg$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$) and their polynomial effect. No effects of xylose, fructose, and glucose/mannose content in corn grain on aflatoxin level were seen, but a multicollinearity relationship between glucose/mannose and aflatoxin level was observed. They concluded that aflatoxin production by A. flavus was correlated with zinc, magnesium, and calcium, and temperature, and environmental factor, and played a stimulatory role for aflatoxin production by A. flavus (Viquez et al., 1994).

Aflatoxin production is influenced by catabolic activity, reduced coenzyme level, energy charge, and metal ions (Dutton, 1988; Llewellyn et al., 1988). Other studies identified that biological, chemical, and environmental factors are associated with the biosynthesis of aflatoxin production and had a direct relationship with them (Ellis et al., 1991), and among these chemical factors were high level of carbohydrates and a low level of proteins (Thalmann, 1990; Ellis et al., 1991). Carbohydrates supply the two carbon precursors for toxin synthesis, and in this regard, corn is considered a good substrate for aflatoxin production because it has high a level of carbohydrates (approximately 63%) and low nitrogen (10%) content (Wiseman and Buchanan, 1987). Also, the biosynthesis of aflatoxin is influenced by some metal ions such as cadmium (Cd$^{2+}$), magnesium (Mg$^{2+}$), calcium (Ca$^{2+}$), and zinc (Zn$^{2+}$) (Failla et al., 1986; Stossel, 1986; Ellis et al., 1991; Viquez et al., 1994). Specific attention was given to Zn and Ca as Zn is required for aflatoxin biosynthesis (Failla et al., 1986; Stossel, 1986), and Ca2+ content may reduce aflatoxin contamination (Mixon et al., 1984). Other researchers Abdollahi and Buchan (1981a) investigated the ability of compounds in-
ducing aflatoxin biosynthesis by Aspergillus parasiticus by transferring the cultured organism from peptone basal medium that does not support aflatoxin production to a medium containing a test compound. They found that ribose, xylene, glucose, fructose, sorbose, mannose, galactose, maltose, sucrose, raffinose, and glycerol induced aflatoxin production. Also, they found that aflatoxin biosynthesis was not induced by lactose, lactic acid, sodium pyruvate, oleic acid, citric acid, sodium acetate, \( \alpha \)-methyl-D-glucoside, 3-O-methyl-D-glucoside, cAMP, or cGMP. They concluded that the induction of aflatoxin was due to the specific available metabolizable carbon sources that regulate aflatoxin formation. It was Abdollahi and Buchanan (1981b) shown that aflatoxins were not produced by A. parasiticus when the organism cultured medium containing peptone plus mineral salts. However, when the microorganism was transferred to a glucose-containing medium, aflatoxin production occurred. They also showed that aflatoxin synthesis could be blocked by cycloheximide or actinomycin D (Abdollahi and Buchanan, 1981b). They concluded that glucose or its metabolites act at the transcriptional level, inducing enzymes involved for aflatoxin synthesis.

The relationship between nitrogen assimilation, metabolism and aflatoxin formation has been investigated in a toxigenic and a non-toxigenic strain of Aspergillus parasiticus. Ammonia from the medium is mainly assimilated via NADP-requiring glutamate dehydrogenase. During growth NAD-requiring glutamate dehydrogenase followed an inverse pattern of activity with respect to NADP glutamate dehydrogenase. Alpha-ketoglutarate, the product of NAD glutamate dehydrogenase, stimulated acetate incorporation into aflatoxins. Glutamine synthetase, ornithine transcarbamylase, both utilizing glutamate as substrate were assayed under different growth conditions. An important regulatory role for glutamine synthetase is suggested. The metabolic route of asparagine utilization was also investigated. Both the known pathways, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase are operative simultaneously. Aflatoxin production is influenced by chemical compounds, including a) carbon source, and glucose, ribose, xylose, and glyceral were considered excellent substrates for both growth and aflatoxin production by A. parasiticus (Davis and Diener, 1968), whereas oleic and fumaric acids enhanced growth, but not aflatoxin production (Davis and Diener, 1968). This is due to the fact that carbohydrates induce aflatoxin synthesis, and the use of a readily metabolizable carbohydrate results in an elevated energy, which in turn induce the aflatoxin biosynthesis pathway (Abdollahi and Buchanan, 1981a; Abdollahi and Buchanan, 1981b); b) amino acids were found not to induce aflatoxin production, except aspartic acid; c) nitrogen has been reported to stimulate aflatoxin synthesis, for example, organic nitrogen sources especially casamino acids and NH\(_4\)NO\(_3\), although NH\(_4\)Cl and NaNO\(_3\) resulted in lower aflatoxin production. Proline and asparagine stimulated aflatoxin production than the other nitrogen sources (Payne and Hagler, 1983; Reddy et al., 1971).

Minerals, such as K, Fe, Cu, Mn, Mg, Zn, Mo and Ca, are essential for fungal growth, are involved as prosthetic groups of enzymes (Fe, Cu, Zn), act as enzyme activators (Mn, Zn, Co, Fe, Ca), and are important in secondary metabolite biosynthesis such as manganese, iron and zinc. The function of minerals in secondary metabolites is due to the possibility that they function as metalloenzymes for enzymatic activities (Detroy and Ciegler, 1971) and as catalysts of lipid peroxidation (Maggon et al., 1973). Zn has been shown to play a major role in fumarate synthesis and carbohydrate metabolism because of its effects on the utilization of carbohydrates (Berry, 1975). For example, Zn was reported to affect glycolytic and tricarboxylic acid cycle enzymes, glycolysis by the EMP pathway in A. parasiticus, leading to pyruvate accumulation (Gupta et al., 1976) and phosphogluconate cycle (Grigorov et al., 1979).

Since information about the effects of genetic background of corn hybrids and mycotoxin on corn grain chemical composition is limited, especially for stacked-gene hybrids, the current research was conducted. The objective of this study was to investigate the possible influence of grain chemical composition (protein, oil, fatty acids, sugars and minerals) as affected by genetic backgrounds classes (Non-GMO, RR, and stacked-gene) and toxigenic Aspergillus flavus inoculation in corn hybrids under irrigated field conditions in the Mississippi Delta. The current study was a part of a bigger study, previously conducted by Bruns (2014) and Abbas et al. (2016).

**Materials and Methods**

**Experimental Site and Conditions**

The experiment was conducted at two sites (a Bosket fine sandy loam: fine-loamy, mixed, active, thermic Mollic Hapludolls, located at the Mississippi State University Delta Branch Research and Extension Center, Stoneville, MS; and a Tunica clay: clayey over loamy, smectitic, nonacid, thermic Vertic Haplapod at a private property 1.5 km north of Elizabeth, MS, USA). Corn hybrids, their growth, and field management were reported previously (Bruns, 2014; Abbas et al., 2016). Ten corn hybrids, four stacked-gene (Stgene), four glyphosate-tolerant (RR), and two non-GMO, were used as plant materials. The experiment was planted on April 7, 2011 in conventional tillage plots, furrow irrigated, whereas field management was conducted as described previously (Bruns, 2014). Rows within each plot were inoculated at the V10 stage of growth with toxigenic Aspergillus flavus K54 (NRRL 58987), isolated from corn kernels in Mississippi, grown on wheat, and applied at a rate of 22.42 kg ha\(^{-1}\) (Accinelli et al., 2014). The remaining six rows that were naturally infected with disease-producing fungi, including Fusarium and Aspergillus spp., were used as control. The cost, yield, net return per acre, and yield components for the ten hybrids as well as the evaluation of transgenic corn hybrids for mycotoxin control were described previously (Bruns, 2014; Abbas et al., 2016).

**Grain Mineral, N, S, and C Analysis**

Mature, dry corn grains were ground to pass through a 1-mm sieve using a Laboratory Mill 3600 (Perten, Springfield, IL, USA), and the ground dried samples were used for analysis. Briefly, a 0.6 g ground sample was digested in HNO\(_3\) with a microwave digestion system, and the mineral content was determined using inductively coupled plasma spectrometry (Thermo Jarrell-Ash...
Grain Analysis for Protein, Oil, and Fatty Acids

Dried corn grains were processed as described above. Ground corn grain samples from each replicate were analyzed for protein, oil, and starch content using a near-infrared reflectance diode array feed analyzer (Perten, Spring Field, IL). Calibrations were carried out using PertenThermoGalactic Grams PLS IQ (Perten, Spring Field, IL). The reference samples were analyzed based on the AOAC Official Methods for protein, oil, and starch as described previously (Reddy et al., 2010). The analysis was determined based on dry matter (Reddy et al., 2010). Fatty acids were determined using the AOAC Official Method 996.06 using methyl esters analysis by capillary GC. Total amino acids were calculated from the different individual amino acid according to the AOAC Official Method 982.30 E (a, b, c). Reducing sugars were determined colorimetrically as described by DuBois et al. (1956).

Glucose Determination in Grains

The concentration of glucose in corn grains was determined by an enzymatic reaction using a Glucose (HK) Assay Kit, Product Code GAHK-20 (Sigma-Aldrich Co, St Louis, MO USA) as described previously (Bellaloui et al., 20014). The concentration of glucose was measured at 340 nm using the Beckman Coulter DU 800 spectrophotometer. The concentration was expressed as mg g⁻¹ dry weight.

Boron Determination

The concentration of B in corn grains was determined using the azomethine-H method (Lohse, 1982; Dordas et al., 2007). Briefly, dry grains were ground to pass through a 1-mm sieve using a Laboratory Mill 3600 (Perten, Springfield, IL, USA) as described above. A 1.0-g sample was ashed at 500°C and extracted with 20 ml of 2 M HCl at 90°C for 10 min. Then, a 2-ml sample of the filtered mixture was added to 4 ml of buffer solution. The buffer solution contained 25% ammonium acetate, 1.5% EDTA, and 12.5% acetic acid. A 4-ml sample of fresh azomethine-H solution (0.45% azomethine-H and 1% of ascorbic acid) (John et al., 1975) was then added. The concentration of B was measured by a Beckman Coulter DU 800 spectrophotometer (Beckman Coulter, Inc., Brea, CA, USA) at 420 nm (Bellaloui et al., 2014).

Iron Determination

The concentration of Fe in corn grains was determined as described by Bandemer and Schaible (1944) and Loeppert and Inskeep (1996). The dried grain was ground using a Laboratory Mill 3600 (Perten, Springfield, IL, USA) as described above. The samples were acid digested and extracted, and the resulting reduced ferrous Fe reacted with 1,10-phenanthroline as described by Bellaloui et al. (2011, 2014). Briefly, a 2-g ground sample was acid digested, and the soluble constituents were dissolved in 2 M of HCl. An aliquot of 4 ml, containing 1 to 20 µg of iron of the sample solution, was transferred into a 25 ml volumetric flask and diluted to 5 ml using 0.4 M HCl. The quinol solution of 1 ml was added to the 5 ml diluted sample solution and mixed. Then, the phenanthroline solution of 3 ml and 5 ml of the tri-sodium citrate solution (8% w/v) were added. After adding distilled water to 25 ml dilution, the samples were incubated at room temperature for 4 h. Phenanthroline reagent solution of 0.25% (w/v) in 25% (v/v) ethanol and quinol solution (1% w/v) was prepared. The standard curve for Fe was conducted by preparing concentrations, ranging from 0.0 to 4 µg ml⁻¹ of Fe in 0.4 M HCl. The concentration of Fe was performed by the Beckman Coulter DU 800 spectrophotometer at an absorbance of 510 nm.

Phosphorus Determination

The concentration of P in corn grain was determined by the yellow phosphor-vanado-molybdate complex method according to Cavell (1955). The method was previously described and reported by others Bellaloui et al. (2009, 2014). Briefly, dry corn grains were ground by a Laboratory Mill 3600 (Perten, Springfield, IL, USA) as described above. A ground sample of 2 g was ashed at 500°C, and then 10 ml of 6 M HCl were added. Then, the samples were placed in a water bath at 100°C to dryness. Extraction of P was conducted with 2 ml of 36% v/v HCl under heat and filtration, then 5 ml of 5M HCl and 5 ml of ammonium molybdate–ammonium metavanadate reagent were added to 5 ml of the filtrate. Ammonium molybdate–ammonium metavanadate was prepared by dissolving 25 g of ammonium molybdate and 1.25 g of ammonium metavanadate in 500 ml of distilled water. The P standard curve was performed by preparing a range of P concentrations from 0 to 50 µg ml⁻¹ using dihydrogen orthophosphates. The concentration of P was determined at 400 nm using the Beckman Coulter DU 800 spectrophotometer.

Experimental Design and Statistical Analysis

The experimental was a randomized complete block design with four replicates. Analysis of variance (ANOVA) was performed using PROC MIXED in SAS 6.1 (Cary, NC, USA). Genotype and soil were modeled as fixed effects. Repetition was considered as a random effect. Residuals of the random effect factor were shown as covariance parameters. Residuals refer to Restricted Maximum Residual Likelihood (REML) values, reflecting the total variance of the random parameters in the model. Means in each hybrid class (Non-GMO, RR, and Stgene) separated by Fisher’s protected LSD (0.05).
Results

ANOVA

The results showed that soil (clay or sandy), genetic background, treatments, and their interactions had significant (P ≤ 0.05) effects on seed composition (protein, starch, glucose, stearic, oleic, and linoleic acids), macro-nutrients (Ca, Mg, C, and N) (Table 1-4). However, K and P were affected by soil and genetic background, but not their interactions (Table 2). Treatments had significant effects on all seed composition constituents and macro- and micro-nutrients, except for S (Table 1-4). Soil did not have significant effects on glucose and reducing sugars (Table 1).

Table 1. Analysis of variance (F values and P values) for the effects of soil, genetic background (Genback: Non-GMO, RR, or stacked gene) of hybrids classes, treatments (Trt: inoculated or non-inoculated with Aspergillus flavus K54), and their interactions on chemical composition compounds (%) in corn grown in sandy and clay soils under Mississippi delta conditions.

| Effect        | DF | Protein | Oil   | Starch | Glucose | Reducing Sugars |
|---------------|----|---------|-------|--------|---------|-----------------|
| Soil          | 1  | 980     | <.0001| <.0001 | 242     | 1.3            |
| Genback       | 2  | 8.2     | 0.000 | 1.1    | 0.345   | 4.7            |
| Soil×Genback  | 2  | 10.4    | <.0001| 2.1    | 0.132   | 7.2            |
| Trt           | 1  | 429     | <.0001| 88.1   | <.0001  | 335            |
| Soil×Trt     | 1  | 1.3     | 0.259 | 0.0    | 0.909   | 13.4           |
| Genback×Trt  | 2  | 0.0     | 1.000 | 0.7    | 0.511   | 0.1            |
| Soil×Genback×Trt | 2 | 0.0 | 1.000 | 0.7  | 0.511 | 0.1 |
| Residuals     |    | 0.138   | 0.407 | 0.774  | 6.124   | 0.226          |

Table 2. Analysis of variance (F values and P values) for the effects of soil, genetic background (Genback: Non-GMO, RR, or stacked gene) of hybrids classes, treatments (Trt: inoculated or non-inoculated with Aspergillus flavus K54), and their interactions on macro-nutrients (%) in corn grown in sandy and clay soils under Mississippi delta conditions.

| Ca     | K   | Mg   | P    | C    | N    |
|--------|-----|------|------|------|------|
| Soil   | 1   | 82.0 | <.0001| 122  | <.0001| 63.3 |
| Genback| 2   | 59.7 | <.0001| 22.2 | <.0001| 6.4  |
| Soil×Genback| 2 | 59.7 | <.0001| 0.7  | 0.509 | 10.2 |
| Trt    | 1   | 179  | <.0001| 37.0 | <.0001| 69.4 |
| Soil×Trt| 1 | 179  | <.0001| 112.5| <.0001| 38.6 |
| Genback×Trt| 2 | 51.5 | <.0001| 6.9  | 0.001 | 0.9  |
| Soil×Genback×Trt| 2 | 51.5 | <.0001| 4.4  | 0.014 | 9.3  |
| Residuals|    | 0.0004| 0.002 | 0.0002| 0.0003| 0.029 |

Table 3. Analysis of variance (F values and P values) for the effects of soil, genetic background (Genback: Non-GMO, RR, or stacked-gene) of hybrids classes, treatments (Trt: inoculated or non-inoculated with Aspergillus flavus K54), and their interactions on micro-nutrients (%) in corn grown in sandy and clay soils under Mississippi delta conditions.

| B    | Cu  | Fe   | Mn    | Zn    |
|------|-----|------|-------|-------|
| Soil | 1   | 0.8  | 21.4  | <.0001| 64.5  |
| Genback| 2 | 5.6  | 0.005 | 1.8   | 0.436 | 1.8  |
| Soil×Genback| 2 | 4.7  | 0.011 | 1.9   | 0.159 | 1.5  |
| Trt  | 1   | 156  | <.0001| 17.7  | <.0001| 45.2 |
| Soil×Trt| 1 | 0.0  | 9.75  | <.0001| 0.5   | 0.477|
| Genback×Trt| 2 | 0.0  | 0.992 | 0.1   | 0.954 | 0.2  |
| Soil×Genback×Trt| 2 | 0.3  | 0.738 | 0.1   | 0.954 | 0.1  |
| Residuals|    | 0.294| 0.230 | 27.605| 0.292 | 4.190 |
Table 4. Analysis of variance (F values and P values) for the effects of soil, genetic background (Genback: Non-GMO, RR, or stacked-gene) of hybrids classes, treatments (Trt: inoculated or non-inoculated with Aspergillus flavus K54), and their interactions on total amino acids (%) and fatty acids (% of total oil) in corn grown in sandy and clay soils under Mississippi delta conditions.

| Effect | DF | Total | Palmitic | Stearic | Oil | Linoleic | Linolenic |
|--------|----|-------|---------|---------|-----|----------|-----------|
|        |    | F-value | P-value | F-value | P-value | F-value | P-value | F-value | P-value |
| Soil   | 1  | 96.3   | <.0001  | 229.3   | <.0001 | 34.8    | <.0001  | 41.7    | <.0001  | 26.4    | <.0001  | 1.0     | 0.328 |
| Genback| 2  | 8.4    | 0.000   | 1.0     | 0.368  | 4.5     | 0.012   | 24.0    | <.0001  | 13.3    | <.0001  | 11.6    | <.0001 |
| Soil×Genback | 2   | 3.4    | 0.037   | 0.5     | 0.629  | 6.0     | 0.003   | 6.7     | 0.002   | 3.1     | 0.050   | 8.2     | 0.000 |
| Trt    | 1  | 470    | <.0001  | 1.4     | 0.000  | 61.3    | <.0001  | 631     | <.0001  | 235     | <.0001  | 32.0    | <.0001 |
| Soil×Trt | 1   | 20.3   | <.0001  | 1.8     | 0.185  | 48.3    | <.0001  | 11.8    | 0.001   | 14.1    | 0.000   | 12.5    | 0.001 |
| Genback×Trt | 2   | 5.7    | 0.004   | 1.8     | 0.175  | 1.1     | 0.350   | 0.1     | 0.888   | 0.2     | 0.814   | 3.0     | 0.054 |
| Soil×Genback×Trt | 2   | 10.3   | <.0001  | 1.0     | 0.383  | 1.1     | 0.350   | 0.0     | 0.983   | 1.3     | 0.283   | 3.0     | 0.054 |
| Residuals | 3.0  | 0.202  | 0.189   | 0.020   | 1.222  | 2.202   | 0.010   |          |          |          |          |          |          |

Effect of Genetic Backgrounds on Grain Chemical Composition and Mineral Nutrition in Sandy Inoculated and Non-Inoculated Soil

In sandy and inoculated soil, grains in Non-GMO and stacked gene hybrids had higher starch, glucose, N, but reducing sugars, total amino acids, B, and Cu were higher in Non-GMO and RR (Table 9 and 10). Stacked gene hybrids had higher oil, P, and C than Non-GMO and RR (Table 9 and 10). In sandy soil and non-inoculated soil, Non-GMO and stacked gene hybrid showed higher starch, glucose, and Mg than RR, but total amino acids

Table 5. Corn grain chemical composition components (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked-gene) in inoculated clay soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid Classes | Protein | Oil | Fiber | Starch | Glucose | Reducing sugars | Total amino acids | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
|----------------|---------|-----|-------|--------|---------|----------------|--------------------|----------|--------|-------|---------|-----------|
| Non-GMO        | 11.6463 | 4.06| 1.10  | 66.24  | 60.90   | 1.84          | 10.98             | 13.89    | 2.20   | 33.47 | 47.55   | 1.24      |
| RR             | 11.7063 | 3.94| 1.10  | 66.21  | 60.51   | 1.75          | 9.84              | 14.08    | 2.07   | 32.60 | 49.09   | 1.36      |
| Stacked genes  | 11.6481 | 3.78| 1.04  | 65.64  | 57.31   | 1.88          | 10.14             | 14.00    | 2.21   | 31.26 | 49.89   | 1.21      |
| LSD            | 0.0406  | 0.22| 0.05  | 0.25   | 0.56    | 0.13          | 0.09              | 0.03     | 0.25   | 0.31  | 0.31    | 0.03      |

Table 6. Corn grain macro- and micro-nutrients (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked-gene) in inoculated clay soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid Classes | Ca | K | Mg | P | C | N | S | B | Cu | Fe | Mn | Zn |
|----------------|----|---|----|---|---|---|---|---|----|----|----|----|
| Non-GMO        | 0.026 | 0.521 | 0.149 | 0.265 | 42.70 | 1.40 | 0.11 | 2.76 | 2.09 | 28.99 | 3.73 | 27.45 |
| RR             | 0.017 | 0.536 | 0.142 | 0.262 | 42.44 | 1.45 | 0.12 | 3.43 | 1.98 | 30.27 | 3.78 | 28.57 |
| Stacked genes  | 0.011 | 0.431 | 0.130 | 0.276 | 42.60 | 1.36 | 0.11 | 3.10 | 2.14 | 28.58 | 3.81 | 28.23 |
| LSD            | 0.001 | 0.011 | 0.004 | 0.004 | 0.065 | 0.023 | 0.002 | 0.19 | 0.14 | 2.01  | 0.14 | 0.70  |

Table 7. Corn grain chemical composition components (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked-gene) in non-inoculated clay soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid Classes | Protein | Oil | Fiber | Starch | Glucose | Reducing sugars | Total amino acids | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
|----------------|---------|-----|-------|--------|---------|----------------|--------------------|----------|--------|-------|---------|-----------|
| Non-GMO        | 10.30   | 5.09| 1.02  | 63.97  | 60.27   | 3.24           | 8.19              | 14.11    | 2.55   | 28.08 | 51.98   | 1.24      |
| RR             | 10.36   | 4.64| 1.36  | 64.17  | 63.67   | 2.24           | 8.56              | 14.16    | 2.42   | 27.32 | 54.31   | 1.28      |
| Stacked genes  | 10.30   | 5.00| 1.01  | 63.51  | 64.09   | 2.48           | 8.33              | 14.22    | 2.56   | 26.15 | 54.42   | 1.39      |
| LSD            | 0.041   | 0.221| 0.058 | 0.681  | 0.563   | 0.131          | 0.140             | 0.103    | 0.035  | 0.241 | 0.364   | 0.026     |
Table 8. Corn grain macro- and micro-nutrients (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked-gene) in non-inoculated clay soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid class | Ca    | K     | Mg    | P     | C     | N     | S     | B     | Cu    | Fe    | Mn    | Zn    |
|--------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Non-GMO      | 0.06  | 0.369 | 0.100 | 0.321 | 42.78 | 1.71  | 0.117 | 3.97  | 2.87  | 36.03 | 4.57  | 29.98 |
| RR           | 0.014 | 0.383 | 0.125 | 0.327 | 42.49 | 1.72  | 0.147 | 4.56  | 2.76  | 37.22 | 3.79  | 32.52 |
| Stacked genes| 0.006 | 0.372 | 0.101 | 0.342 | 42.65 | 1.60  | 0.131 | 4.15  | 2.92  | 34.12 | 3.82  | 31.47 |
| LSD          | 0.0004| 0.011 | 0.003 | 0.004 | 0.1   | 0.681 | 0.002 | 0.191 | 0.144 | 2.007 | 0.132 | 0.713 |

Table 9. Corn grain chemical composition components (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked-gene) in inoculated sandy soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid classes | Protein | Oil | Fiber | Starch | Glucose | Reducing sugars | Total amino acids | PALmitic | Stearic | Oleic | Linoleic | Linolenic |
|----------------|---------|-----|-------|--------|---------|-----------------|-------------------|-----------|---------|-------|----------|-----------|
| Non-GMO        | 10.08   | 2.85| 1.11  | 64.88  | 59.35   | 1.71            | 9.32              | 14.77     | 2.43    | 33.94 | 47.66    | 1.19      |
| RR             | 9.43    | 2.99| 1.02  | 63.81  | 57.28   | 1.64            | 9.36              | 15.19     | 2.51    | 32.45 | 48.84    | 1.16      |
| Stacked genes  | 9.48    | 3.22| 1.05  | 64.17  | 60.42   | 1.58            | 9.06              | 15.01     | 2.45    | 32.61 | 49.00    | 1.23      |
| LSD            | 0.140   | 0.127| 0.047 | 0.260  | 0.784   | 0.122           | 0.681             | 0.143     | 0.051  | 0.366 | 0.436     | 0.026     |

Table 10. Corn grain macro- and micro-nutrients (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked-gene) in inoculated sandy soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid classes | Ca    | K     | Mg    | P     | C     | N     | S     | B     | Cu    | Fe    | Mn    | Zn    |
|----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Non-GMO        | 0.010 | 0.343 | 0.107 | 0.243 | 42.19 | 1.20  | 0.118 | 3.19  | 2.24  | 24.16 | 2.52  | 21.66 |
| RR             | 0.010 | 0.360 | 0.110 | 0.256 | 42.22 | 1.18  | 0.738 | 3.08  | 2.16  | 22.50 | 2.71  | 22.36 |
| Stacked genes  | 0.010 | 0.312 | 0.111 | 0.287 | 42.28 | 1.20  | 0.112 | 2.79  | 2.03  | 21.95 | 2.75  | 21.84 |
| LSD            | 0.000 | 0.015 | 0.003 | 0.004 | 0.032 | 0.018 | 0.443 | 0.109 | 0.116 | 0.487 | 0.146 | 0.339 |

Table 11. Corn grain chemical composition components (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked-gene) in non-inoculated sandy soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid classes | Protein | Oil | Fiber | Starch | Glucose | Reducing sugars | Total amino acids | PALmitic | Stearic | Oleic | Linoleic | Linolenic |
|----------------|---------|-----|-------|--------|---------|-----------------|-------------------|-----------|---------|-------|----------|-----------|
| Non-GMO        | 8.87    | 3.86| 1.08  | 65.58  | 62.95   | 2.83            | 8.24              | 15.50     | 2.42    | 29.84 | 51.24    | 1.36      |
| RR             | 8.22    | 4.00| 1.01  | 64.55  | 60.80   | 2.87            | 8.04              | 15.35     | 2.48    | 28.50 | 51.20    | 1.39      |
| Stacked genes  | 8.27    | 4.23| 1.04  | 65.08  | 63.11   | 2.58            | 7.61              | 15.23     | 2.56    | 28.68 | 51.66    | 1.29      |
| LSD            | 0.140   | 0.127| 0.048 | 0.215  | 0.805   | 0.144           | 0.099             | 0.136     | 0.036  | 0.352 | 0.511     | 0.032     |

Table 12. Corn grain chemical composition components (%) as affected by genetic backgrounds (Non-GMO, RR, or stacked gene) in non-inoculated sandy soil with Aspergillus flavus K54 to produce mycotoxins under Mississippi Delta.

| Hybrid classes | Ca    | K     | Mg    | P     | C     | N     | S     | B     | Cu    | Fe    | Mn    | Zn    |
|----------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Non-GMO        | 0.01  | 0.37  | 0.112 | 0.3038| 42.3   | 1.385 | 0.1125| 4.238 | 2.21  | 30.375| 3.100 | 28.2  |
| RR             | 0.01  | 0.39  | 0.0987| 0.3068| 42.4   | 1.371 | 0.1163| 4.179 | 2.01  | 27.0563| 3.6063 | 28.8  |
| Stacked genes  | 0.01  | 0.35  | 0.1029| 0.3062| 42.3   | 1.380 | 0.1125| 4.014 | 1.89  | 27.0000| 3.7500 | 27.4  |
| LSD            | 0.000 | 0.012 | 0.005 | 0.006 | 0.681  | 0.012 | 0.002 | 0.131 | 0.587 | 0.181  | 0.481  |
Observations were recorded for protein, oleic acid, Cu, starch, and total amino acids (data not shown). Although protein was higher in Inoc treatment in sandy soil than in Non-GMO and stacked gene, oil was higher in Non-inoc treatment than in Non-GMO and stacked gene. For example, in Non-GMO oil was 3.9% in Non-inoc vs. 2.8% in Inoc, and similar pattern was observed in stacked gene hybrid (data not shown).

**Discussion**

The effects of soil, genetic background, and inoculation treatment on seed nutrients show the significant contribution of abiotic factors, such as soil, and biotic factors, such as genotype and diseases, mycotoxins, and nutrients. It has been reported that mycotoxin production is affected by the soil type, since corn planted in clay soil has higher levels of mycotoxin than this grown in sandy soil. The level of nutrients in grains grown in clay soil were higher than that grown in sandy soil. However, this observation cannot be due necessary to the inoculation treatment only. For example, heat and drought stress can induce both inoculated and non-inoculated plots, reducing the uptake of nutrients by the crop and limiting the movement of nutrients from source (leaves) to sink (grains). It was reported that mycotoxin production is influenced by environmental conditions such as heat, drought, insect infestation level, and various plant diseases (Diener et al., 1987; Payne, 1992; Abbas et al., 2002; Bruns and Abbas, 2005; De la Campa et al., 2005; Bruns and Abbas, 2006; Abbas et al., 2007). In the Mississippi delta, drought and heat are major environmental factors that affect corn growth, limiting factor nutrient uptake and accumulation.

**Table 13.** Corn grain chemical composition, macro-, and micro-nutrients (%) as affected by inoculated (Inc) and non-inoculated (Non-inc) clay soil in Non-GMO hybrid class under Mississippi Delta.

| Inc | Oil | Starch | Oleic acid | Linolenic acid | Glucose | Reducing sugars | Total amino acids |
|-----|-----|--------|------------|---------------|---------|----------------|------------------|
| Non-GMO | 11.65 | 4.06 | 66.24 | 33.47 | 51.98 | 60.27 | 3.24 | 10.98 |
| Non-Inc | 10.30 | 5.09 | 63.97 | 28.08 | 47.55 | 57.31 | 1.84 | 8.19 |
| LSD | 0.052 | 0.252 | 0.217 | 0.156 | 0.169 | 0.541 | 0.169 | 0.131 |

| Macro- and Micro nutrients |
| Ca | K | P | N | S | B | Fe |
| Inc | 0.03 | 0.52 | 0.32 | 1.71 | 0.12 | 3.97 | 36.03 |
| Non-GMO | Non-Inc | 0.01 | 0.37 | 0.27 | 1.40 | 0.11 | 2.76 | 28.99 |
| LSD | 0.001 | 0.008 | 0.004 | 0.026 | 0.003 | 0.285 | 4.359 |

**Table 14.** Corn grain chemical composition, macro-, and micro-nutrients (%) as affected by inoculated (Inc) and non-inoculated (Non-inc) clay soil in glyphosate-resistant hybrid class (RR) under Mississippi Delta.

| Inc | Oil | Starch | Oleic acid | Linolenic acid | Glucose | Reducing sugars | Total amino acids |
|-----|-----|--------|------------|---------------|---------|----------------|------------------|
| RR | 11.71 | 4.64 | 66.21 | 32.60 | 54.31 | 63.67 | 2.24 | 9.84 |
| Non-Inc | 10.36 | 3.94 | 64.17 | 27.32 | 49.09 | 60.51 | 1.75 | 8.56 |
| LSD | 0.028 | 0.194 | 0.297 | 0.233 | 0.407 | 0.385 | 0.084 | 0.169 |

| Macro- and Micro nutrients |
| Ca | K | P | N | S | B | Fe |
| Inc | 0.02 | 0.54 | 0.33 | 1.72 | 0.15 | 4.56 | 37.22 |
| RR | Non-Inc | 0.01 | 0.38 | 0.26 | 1.45 | 0.12 | 3.43 | 30.27 |
| LSD | 0.001 | 0.013 | 0.005 | 0.029 | 0.002 | 0.174 | 0.933 |

**Table 15.** Corn grain chemical composition, macro-, and micro-nutrients (%) as affected by inoculated (Inc) and non-inoculated (Non-inc) clay soil in stacked-gene hybrid under Mississippi Delta.

| Inc | Oil | Starch | Oleic acid | Linolenic acid | Glucose | Reducing sugars | Total amino acids |
|-----|-----|--------|------------|---------------|---------|----------------|------------------|
| Stacked genes | 11.65 | 5.00 | 65.64 | 31.26 | 54.42 | 64.09 | 10.14 | 2.48 |
| Non-Inc | 10.30 | 3.78 | 63.51 | 26.15 | 49.89 | 60.90 | 8.33 | 1.88 |
| LSD | 0.041 | 0.192 | 0.135 | 0.228 | 0.204 | 0.611 | 0.074 | 0.135 |

| Macro- and Micro nutrients |
| Ca | K | P | N | S | B | Fe |
| Inc | 0.02 | 0.54 | 0.33 | 1.72 | 0.15 | 4.56 | 37.22 |
| Stacked genes | Non-Inc | 0.01 | 0.38 | 0.26 | 1.45 | 0.12 | 3.43 | 30.27 |
| LSD | 0.001 | 0.013 | 0.005 | 0.029 | 0.002 | 0.174 | 0.933 |
Although the current experiment was irrigated, the possibility that the crop suffered drought cannot be excluded. Different response of nutrients in hybrid classes grown in sandy soil compared with those grown in clay soil could be due to the fact that sandy soil creates additional stress due to warmer and drier season, especially during the kernel-filling stage and may contribute to higher aflatoxin contamination in sandy soils. In addition, the field water holding capacity of sandy soils is lower than that of clay soils, exposing the grain to higher drought and heat stress. It was recently found that the level of aflatoxin in sandy loam soil was higher compared with that in clay soil, possibly leading to lower yield of hybrids grown in sandy soil compared with that grown in clay soil even when irrigation takes place (Abbas et al., 2007; Bruns, 2014; Kebede et al., 2012). Under clay and sandy soil conditions with inoculation, the levels of protein, oleic acid, total amino acids, and starch, and some nutrients such as Ca, K, and Mg responded positively, indicating that these compounds are stress indicators, supporting the observation that a stress condition may occur for the grain grown under inoculated conditions. Meanwhile, the higher protein and oleic acid resulted in lower oil and linoleic acid in some cases, reflecting the inverse relationship between protein and oil (Kebede et al., 2012) and oleic and linoleic and linolenic acids. Previous studies reported that aflatoxin production is stimulated by high carbohydrates, low protein, (Thalmann, 1990; Ellis et al., 1991) and metal ion, such Cd²⁺, Mg²⁺, Ca²⁺, and Zn²⁺ (Failla et al., 1986; Stossel, 1986; Ellis et al., 1991; Viquez et al., 1994) levels. Zn is required for aflatoxin biosynthesis (Failla et al., 1986; Stossel, 1986) and Ca²⁺ content may reduce aflatoxin contamination (Mixon et al., 1984). Abdollahi and Buchman (1981a) found that ribose, xylose, glucose, fructose, sorbose, mannose, galactose, maltose, sucrose, raffinose, and glycerol induced aflatoxin production, but aflatoxin biosynthesis was not induced by lactose, lactic acid, sodium pyruvate, oleic acid, and citric acid. The involvement of these carbohydrates may be due to specific carbon source metabolites, inducing enzymes involved for aflatoxin synthesis and aflatoxin production. It was reported that glucose, ribose, xylose, and glycerol were considered excellent substrates for growth and aflatoxin production by A. parasiticus (Davis and Diener 1968), because they elevate energy level that is used for aflatoxin biosynthesis pathway (Abdollahi and Buchman, 1981a; Abdollahi and Buchman, 1981b). Minerals, such as K, Fe, Cu, Mn, Mg, Zn, Mo, and Ca, are essential for fungal growth, are involved as prosthetic groups of enzymes (Fe, Cu, and Zn), act as enzyme activators (Mn, Zn, Co, Fe, and Ca), and are important in secondary metabolite biosynthesis such as Mn, Fe, and Zn. In our study, protein, oleic acids, carbohydrates (glucose, reducing sugars, and starch), and total amino acids were higher under inoculated conditions in clay and sandy soils, indicating the stress indicators role of these compounds. The results showed that the level of these nutrients was higher under inoculated conditions. However, further research is needed in nutrient deficient soils with inoculation.

Conclusions

The current study showed that no nutritional advantage was observed using the stacked gene hybrids over glyphosate-resistant or non-GMO hybrids, and this could be due to the soils used had adequate nutrient concentrations. However, these results are not conclusive as the current experiment was irrigated and the level of inoculation may not be severe enough to impact the yield and nutrient uptake and nutrient mobility to the grains. Therefore, conducting a multi-year experiment with a bigger number of hybrids under different conditions of irrigated and non-irrigated and with a severe level of infection would contribute to further knowledge. Higher levels of protein, carbohydrates, and total amino acids, and some minerals were noticed under inoculated condition, but further investigation is needed. The higher levels of these compounds under inoculated conditions could be due to a possible osmotic adjustment role and osmoprotectants (Zhu, 2002; Serraj and Sinclair, 2002; Farooq et al., 2009; Marschner, 2012) under inoculation stress. Research on the effects of mycotoxin on the performance of Non-GMO, RR, and especially stacked-gene hybrids is limited and needs further investigation (Abbas et al., 2016), especially on the nutritional aspect.

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