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The role of ear environment in postharvest susceptibility of maize to toxigenic *Aspergillus flavus*

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Abstract

A kernel screening assay (KSA) was used to assess the genetic and environmental effects on the vulnerability of maize to aflatoxin accumulation. Kernels of 26 inbred lines that had been grown in seven environments, and 190 lines of the Intermated B73xMo17 (IBM) population grown in one location in the United States, were inoculated with a toxigenic strain of *A. flavus* and incubated in the dark at 30°C for 6 days. Percent kernel colonization (PKC), sporulation and aflatoxin were influenced by the maize genotypes (G), the location ("ear environment" or E) and the GxE interactions. Overall, low broad-sense heritabilities were observed for PKC, sporulation and aflatoxin. PKC was significantly correlated with sporulation in all environments. Aflatoxin was positively correlated with colonization for two and with sporulation for all ear environments. Higher grain sulphur or magnesium in IBM was associated with less colonization or aflatoxin. Postharvest susceptibility of maize to aflatoxin is thus influenced by factors that are modulated by the ear environment. In a KSA, sporulation could be a proxy test for aflatoxin accumulation.

KEYWORDS
food safety, kernel screening assay, maize breeding, mycotoxins

1 | INTRODUCTION

Colonization of maize by toxigenic *Aspergillus* species reduces grain quality and threatens the health of maize consumers in the tropical and sub-tropical world (Shephard, 2008). *Aspergillus flavus* and *A. parasiticus* are the major fungi that contaminate cereal crops and nuts with aflatoxins, including aflatoxin B1, the most potent naturally occurring carcinogen known (Diener et al., 1987; Payne, 1992). Maize grain provides an excellent substrate for the growth of *A. flavus* (Diener et al., 1987; Payne, 1992). The extent of toxin accumulation depends on fungal prevalence and toxigenicity, the vulnerability of the maize variety, environmental conditions, and the interaction between the environment and the maize genotype (genotype-by-environment interaction or GxE; Atehrikeng et al., 2008; Fountain et al., 2014).

Maize grain can be colonized by aflatoxin-producing fungi before, during or after harvest (Diener et al., 1987; Sétamou, Cardwell, Schulthess, & Hell, 1997). Maize varieties vary in their susceptibility to colonization and toxin accumulation. Aflatoxin resistance is a complex trait with low heritability (Mideros et al., 2014; Warburton, Brooks, Windham, & Williams, 2011), which is influenced by multiple genes of small effect (Mayfield, Murray, Rooney, Isakeit, & Odvody, 2011; Mideros et al., 2014; Warburton et al., 2009). While genes conferring resistance are unknown, a range of plant and kernel traits are known or suspected to influence varietal susceptibility to *A. flavus* and aflatoxin accumulation (Balconi, Motto, Mazzinelli, & Berardo, 2010; Betrán & Isakeit, 2004; Mutiga et al., 2017). It is not known whether the mechanisms controlling pre- and postharvest resistance to aflatoxin accumulation are shared, but it is reasonable

to speculate that distinct mechanisms would operate at different stages of ear development.

Most aflatoxin resistance breeding and trait dissection efforts have focused on pre-harvest aflatoxin accumulation. However, peri- and postharvest aflatoxin accumulation is also important when whole grain is stored under conditions that permit colonization and growth of *A. flavus* (Mutiga et al., 2014; Sharma & Geeta, 2007; Tefera, 2012). For example, smallholder farmers in East Africa store maize for up to eight months, often under conditions conducive to fungal growth as they have limited capacity to manage temperature, humidity and pests (Hell & Mutegi, 2011). Postharvest infection of mature kernels occurs during shelling, when maize is dried on the ground, or when storage conditions favour colonization by *A. flavus* (Azziz-Baumgartner et al., 2005; Hell, Fandohan, Kiewnick, Sikora, & Cotty, 2008).

Aflatoxin accumulation occurs upon successful colonization of maize kernels by a toxigenic *A. flavus* strain (Payne, 1992). Postharvest colonization of maize by *A. flavus* involves direct invasion of kernels or continued growth of fungi that initiated the process during crop growth or harvest. Resistance mechanisms would involve structural barriers to entry into the kernel and/or inhibition of fungal growth once the fungus has entered the kernel. Both structural characteristics and chemical composition of the kernel have been associated with resistance to colonization of mature kernels by *A. flavus* (Brown, Cotty, Cleveland, & Widstrom, 1993; Gembeh, Brown, Grimm, & Cleveland, 2001). Environmental factors (e.g., water and nutrient stress) which influence kernel development have also been reported to influence susceptibility of maize to *A. flavus* and aflatoxin accumulation (Fountain et al., 2014). The extent of adaptation of a maize variety to the environment in which it is grown has been correlated with resistance to colonization by fungal pathogens (Betrán & Isakeit, 2004; Magan, Medina, & Aldred, 2011). GxE interactions could therefore influence postharvest vulnerability to fungal contamination via effects on grain structure and density (Mutiga et al., 2017).

In vitro inoculations have been used to assess resistance of various maize tissues to colonization by *A. flavus*. An in vitro inoculation of silks and developing maize kernels was not able to demonstrate good association between field aflatoxin accumulation and the response of the tissues to *A. flavus* (Mideros, Windham, Williams, & Nelson, 2012). Mature kernel inoculations have been used to investigate aflatoxin resistance in different maize varieties, for identification of aflatoxin resistance-associated proteins, and for mapping aflatoxin resistance quantitative trait loci (QTL; Brown et al., 2011, Brown et al., 2001). While genotypic responses to postharvest aflatoxin accumulation have been analyzed using a mature kernel assay, the effect of the environment in which the grain was produced has not been investigated for postharvest aflatoxin accumulation in maize.

The relationships between maize kernel structure, chemical composition and susceptibility of the kernels to infection by *A. flavus* have been associated with aflatoxin accumulation, but are not well understood. Previous studies conducted in our laboratory had shown negative correlations between pre-harvest aflatoxin and kernel fibre and ash content, and a positive correlation between aflatoxin and carbohydrate content (Mideros et al., 2012). Zein proteins regulate kernel texture by binding starch granules and enhancing compactness within the endosperm (Betrán, Bhatnagar, Isakeit, Odvody, & Mayfield, 2006; Ngonyamo-Majee, Shaver, Coors, Sapienza, & Lauer, 2008). Flint maize has more compact endosperm and was previously reported to have lower aflatoxin contamination compared to dent genotypes (Betrán et al., 2006; Mutiga et al., 2017). Certain antifungal proteins have been reported to play a role in resistance of maize to *A. flavus* colonization, but these lessons have been difficult to apply because of the strong GxE effects on the accumulation of these proteins (Baker, Brown, Chen, Cleveland, & Fakhoury, 2009). Recent studies have shown an association between kernel integrity and aflatoxin accumulation, with a higher toxin level in maize with low integrity than intact kernels (Betrán et al., 2006; Mutiga et al., 2014).

The chemical composition of a maize kernel reflects both genetic factors and environmental conditions under which it develops (Baxter et al., 2014; Flint-Garcia, Bodnar, & Scott, 2009). Furthermore, availability of soil nutrients varies across environments. A lower aflatoxin accumulation was reported in maize that was grown in soil under high nitrogen (N) compared to low N (Blandino, Reyneri, & Vanara, 2008; Mutiga et al., 2017). Improved crop vigour, as a result of soil N amendment, was hypothesized to reduce pre-harvest susceptibility to fungal pathogens, including *A. flavus* (Blandino et al., 2008; Mutiga et al., 2017). In the present study, we hypothesize that maize grain mineral elemental content (ionomic content) might influence postharvest colonization by *A. flavus*.

Mapping populations, such as the Intermated B73xMo17 (IBM) and the nested association mapping (NAM) populations, can allow inference about trait genetic architecture (Lee et al., 2002; Yu, Holland, McMullen, & Buckler, 2008). We conducted mature kernel screening assays to investigate sources of variance for susceptibility of mature maize grain to aflatoxin accumulation in the NAM founder lines. In addition, a similar assay was conducted using kernels of the IBM recombinant inbred population, for which the mineral elemental content had been determined, to investigate the associations between susceptibility to *A. flavus* colonization, aflatoxin accumulation and ionomic content (Baxter et al., 2014).

The objectives of this study were to investigate whether ear environments influence postharvest susceptibility of maize to *A. flavus* and aflatoxin accumulation and to investigate whether grain ionomic profile is correlated with susceptibility of the maize to *A. flavus* and aflatoxin accumulation. We used a mature kernel screening assay that is intended to reveal genetic and physiological differences in vulnerability to fungal colonization and toxin accumulation. We compared kernel colonization, sporulation and aflatoxin accumulation in diverse inbred lines that had been grown in multiple environments in the US.

2 | MATERIALS AND METHODS

2.1 | Germplasm

Mature, dry kernels of 26 founders of the nested association mapping (NAM) population and Intermated B73xMO17 (IBM)

recombinant inbred lines (RILs), that had been grown in different ear environments (location/year combinations) in the USA, were used in this study. NAM founder seed was sourced from field trials conducted in Homestead, Florida (FL_2007), Ponce, Puerto Rico (PR_2007 and PR_2008), Columbia, MO (MO_2007 and MO_2009), Aurora, New York (NY_2009), Blacksburg, Virginia (VA_2009). Kernels of the IBM RI (n = 190), for which ionomic content (calcium, copper, iron, phosphorus, sulphur, magnesium, manganese, potassium and zinc) had been analyzed were sourced from a trial conducted in Clayton, NC in 2005 (Baxter, Gustin, Settles, & Hoekenga, 2013; Lee et al., 2002). All kernels used in the two studies were from selfed maize ears.

2.2 | Fungal inoculum and pilot mature kernel assay

The aflatoxin-producing NRRL 3357, a strain of *A. flavus* which is publicly available for research in the US, was used for in vitro inoculation of the kernels. Fungal culture for inocula was grown for 10 days on maize kernels that had been moistened and autoclaved. A conidial suspension was prepared for inoculation as previously described (Mideros et al., 2012).

A previously established developing kernel screening assay (DvKA) had revealed differences in susceptibility when diverse maize lines were inoculated with a 10-day-old *A. flavus* conidial suspension at 10^7 conidia/ml (Mideros et al., 2012). In the protocol of the DvKA, a visual score of the percentage of the surface of individual kernels colonized by *A. flavus* (percent kernel colonization or PKC) and conidia count (CC) from the sporulation on colonized kernels was collected after a 7-day incubation of kernels in the dark at 30°C.

A mature kernel assay was developed with a slight modification of the DvKA protocol. The effect of inoculum concentration and the timing of data collection were investigated with respect to percent kernel colonization. Mature, dry kernels of 10 maize inbred lines (a subset of the NAM founders, Figure 1) grown in Aurora, NY in 2008, were inoculated with *A. flavus* at three inoculum concentrations: 10^5, 10^6 and 10^7 conidia/ml. The control treatment included kernels of each genotype immersed in water instead of the inoculum. Kernels were surface-sterilized using 10% of commercial bleach solution (3.8% of sodium hypochlorite) and rinsed twice in deionized distilled water (DD H_2O) before they were dipped in *A. flavus* conidial suspension or in deionized H_2O for 30 s. There were three replicates per inoculum concentration treatment or control, and each replicate contained four kernels of each inbred line.

Kernels were transferred to uncapped 9-cm Petri plates that were placed side by side on filter paper that had been moistened with 30 ml of DD H_2O, avoiding direct kernel-to-kernel contact in the process. The plates were then placed in a capped and parafilm-wrapped clear tray (24.3 × 24.3 × 0.18 cm, Corning, NY). Trays were incubated in a dark chamber at 30°C for 7 days. The percent kernel colonization and the hours to appearance of *A. flavus* mycelia (latent period) were visually estimated at 24- and 12-hr intervals, respectively.

2.3 | Mature kernel assay for diverse inbred lines from multiple ear environments

Based on the findings of the pilot experiment above, kernels of the test genotypes were inoculated at 10^7 conidia/ml and incubated in uncapped 4-cm diameter Petri plates, each of which was placed on a moistened Whatman #1 filter paper (7 ml of DD H_2O) in a 9-cm diameter Petri dish, which was capped and wrapped with parafilm. Three kernels were used per genotype for each of the three laboratory replicates, due to limited seed stocks. The inbred lines CML322 and CML52 were used as susceptible and resistant checks, respectively. Data were collected on PKC, CC and aflatoxin after 6 days of incubation in the dark at 30°C. Kernels were kept in 15 ml falcon tubes at −20°C prior to conidia count. Conidia were washed from the surface of kernels by adding 2 ml of a 0.2% Tween-20 solution into a 15 ml tube and vortexing at the maximum speed for 1 min prior to pouring the suspension into a well in a 12-well multi-dish (Waltham, MA). Conidia were counted using a hemocytometer. Kernels were kept at −20°C until the time of aflatoxin quantification.

2.4 | Mature kernel assay for the IBM RILs

Seed of the IBM population that had been grown at Clayton, NC in 2005 was subjected to the established mature kernel assay described above. Prior to inoculation with the toxigenic *A. flavus* strain, seeds of each of the 190 lines/field replicate were sorted based on kernel size into large (>1 cm in length; presumed to have originated from the mid part of the ear) and small (<1 cm in length; presumed to have originated from the tip and base of the ear) categories. To compare the susceptibility of kernels obtained from different points of the maize ear, the two kernel size categories were inoculated separately. Sample inoculation and incubation were based on a completely randomized design. Data were collected for PKC and aflatoxin level after 6 days of kernel incubation in the dark at 30°C. Kernels were kept at −20°C until the time of aflatoxin quantification.

2.5 | Aflatoxin quantification

Aflatoxin was quantified from a 0.5-g (NAM founder lines) and 1-g (IBM population) samples using Vicam AflaTest® method (Waters, Watertown, MA, USA). Briefly, kernels were removed from −20°C and thawed at room temperature for 15 min prior to grinding using a mortar and pestle. A finely ground sub-sample of 0.5 g (NAM founder lines) or 1-g (IBM population) was weighed into a sterile falcon tube prior to addition of NaCl and 80% methanol (the slurry consisted of the sample, salt and methanol in the ratios of 1:10:10), respectively. The slurry was vortexed at maximum speed for 3 min and allowed to settle for 10 min. The supernatant (extract) was decanted in a sterile 14 ml tube, diluted at 1 ml of extract: 4 ml of DD H_2O and mixed well prior to passing through a microfibre filter (Waters). Two millilitres of the filtered diluted extract was passed through an AflaTest® column (Waters) at a flow rate of 1–2 drops/s.

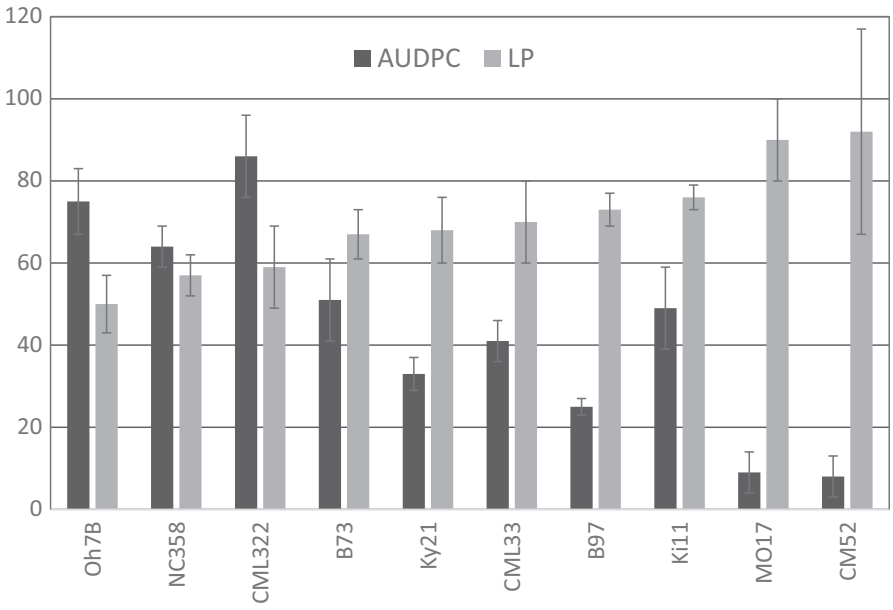

FIGURE 1 Area under disease progress curve (AUDPC) and latent period (LP) for colonization by *Aspergillus flavus* in 10 maize inbred lines that were grown at Cornell's Robert Musgrave Research Farm, Aurora, NY in 2008. Data were collected during the pilot experiment for the establishment of a mature kernel assay

The column was rinsed twice using 5 ml of DD H_2O water and eluted with 1 ml of HPLC grade methanol into a disposable cuvette. One millilitre of AflaTest developer was added to the 1 ml eluate prior to reading in a Vicam fluorometer calibrated at 110/−2.0 (ng/g) per AflaTest protocol. The fluorometer readings were multiplied by the appropriate dilution factors. Samples with aflatoxin above the fluorometer calibration limit of 300 ng/g were diluted and re-tested.

2.6 | Statistical analysis

Data were analyzed using JMP software version 13 (SAS Institute Inc., 2016, Cary, NC, USA). To attain normal distribution, data were transformed using the most appropriate methods. For the pilot experiment, PKC data were arcsine transformed prior to computing area under disease progress curve (AUPDC; Shaner & Finney, 1977) for each replicate of a treatment. For the NAM founders experiment, PKC and conidia count were cube root transformed prior to statistical analysis. Aflatoxin data from the NAM founders experiments were transformed to log (ng/g + 1). For the IBM population, aflatoxin and percent kernel colonization were cube root transformed prior to input in statistical models. Back-transformed means and least square means are reported in summary tables. Data arising from the pilot studies were used in streamlining of the assay and to identify some genotypes to serve as checks, based on trends of latent period and PKC (Figures 1 and 2). Statistical assessment of the response of inbred lines across the ear environments was conducted based on a model with all-random factors (genotype, environment and replicates) and variance component prediction using Restricted Maximum Likelihood (REML) method. Genotypic mean responses were compared across the ear environments using Tukey's Honest Significant Difference (HSD, $\alpha = 0.05$). Broad-sense heritability (H^2) was computed using variance components as follows. $H^2 = \frac{\sigma^2 G}{\sigma^2 P}$, where $\sigma^2 G$ is to variance due to genotype, $\sigma^2 P$ is the whole phenotypic variance (a sum of genotype, ear environment, interaction between ear environment and genotype, replication and the residual variances). To determine the relationship between the grain ionomic content, PKC and aflatoxin, a regression model was established with the concentrations of all elements as predictor variables for either PKC or aflatoxin responses.

3 | RESULTS

3.1 | Assay development

A pilot kernel assay experiment showed that inoculum concentration of 10^5 conidia/ml had significantly less kernel colonization (26%) than the other two concentrations of 10^6 conidia/ml (51%) and 10^7 conidia/ml (51%), which did not differ significantly ($p \leq 0.05$). Analysis of the AUDPC at different days after inoculation revealed that most kernels were visibly colonized and germinated by the 7th day. Because kernels were germinated by the 7th day of incubation, the assays were terminated at that time. Significant ($p < 0.05$) differences in AUDPC among inbred lines were observed at the 5th and 6th days post-inoculation, but not later. The 6th day after inoculation was chosen as the sampling date for subsequent assessment of percent kernel colonization. Based on latent period (the time between inoculation and the visual manifestation of symptoms) and AUDPC, the 10 inbred lines differed significantly ($p < 0.0001$) in kernel colonization when inoculated with conidial suspensions at 10^6 conidia/ml (Figure 1). The latent period among the inbred lines ranged from 38 hr (for OH7B, NC358 and CML322) to 96 hr (for CML52), with a mean of 55 hr at an inoculum concentration of 10^7 conidia/ml and 60 hr at an inoculum concentration of 10^6 conidia/ml. The latent period and the AUDPC did not differ between the inoculum concentrations of 10^6 and 10^7 conidia/ml ($p > 0.05$). As expected, the latent period was negatively correlated with AUDPC ($r = -0.53$, $p = 0.002$; Figure 1).

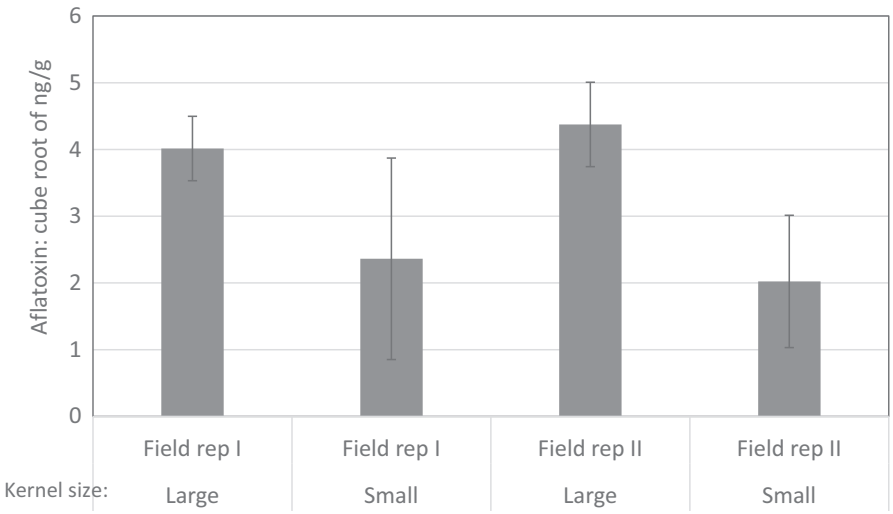

FIGURE 2 Aflatoxin concentration (ng/g) in dry mature kernels of the Intermated B73xMo17 (IBM) Recombinant Inbred (RI) maize population that was grown at Clayton, NC, 2005. Error bars denote *SD*

3.2 | Responses of seed of NAM founders grown in diverse ear environments to postharvest inoculation with *A. flavus*

The variance components and heritabilities derived from the responses of the kernels of the NAM founders across the seven ear environments are shown in Table 1. Based on all-random model involving all seven environments, genotypes accounted for significant ($p < 0.05$) variation in sporulation (11%) and aflatoxin (14%), but not in PKC. As expected, GxE significantly influenced the three phenotypes, accounting for huge percentages of variances (PKC 26%; sporulation 28% and aflatoxin 18%). Ear environment did not influence aflatoxin accumulation, but significantly influenced PKC and sporulation (Table 1). Analysis of variances for data from individual ear environments showed that genotypes influenced PKC and sporulation in all, but FL_07 and MO_07. Similarly, genotypes significantly ($p < 0.05$) influenced aflatoxin in four ear environments (MO_09, NY_09, PR_08 and VA_09).

A pairwise comparison of kernel colonization, sporulation and aflatoxin for the 10 inbred lines across the seven ear environments showed a strong positive correlation between aflatoxin and sporulation, and a marginal positive correlation between aflatoxin and percent kernel colonization (Table 2). Aflatoxin was significantly correlated with colonization in seed from NY_09, VA 2009 and MO_09, and PR_08 (four of the seven ear environments), but the overall correlation was insignificant ($r = 0.37$, $p > 0.05$). Aflatoxin was positively correlated with sporulation for seed from all ear environments except NY in 2009, and the two traits had a significant positive grand correlation ($r = 0.52$, $p = 0.0055$) (Table 2). Kernel colonization and sporulation were highly correlated ($r = 0.85$, $p < 0.0001$), and the correlations ranged from 0.42 (FL_07) to 0.91 (VA_09) (Table 2).

Summaries of the response of the 26 inbred lines to the toxigenic *A. flavus* in the kernel screening assay are presented in Supporting Information Tables S1–S3. The evidence suggested that the properties of the seed were different based on the ear environment. For example, kernels of the inbred lines grown in VA_09 and in FL_07 were less susceptible to colonization and aflatoxin accumulation compared to other ear environments (Supporting Information Tables S1–S3). The most susceptible were kernels that were grown in NY_09 and in PR_08 (Supporting Information Tables S1–S3). Genotypes did not differ in PKC for kernels from FL_07 and MO_07, but in the rest of the ear environments. Sporulation differed significantly ($p < 0.05$) among the inbred lines for kernels from six ear environments, but not for kernels from FL_07. Furthermore, aflatoxin differed significantly ($p < 0.05$) among the kernels of inbred lines from different ear environments, but for FL_07, MO_09 and PR_07. The responses of certain lines (CML52, Oh7B, CML322 and NC358) were inconsistent when the seeds grown in multiple ear environments were subjected to the assay. B73 was the most consistent line and was generally ranked (based best linear unbiased predictors, BLUPs of individual traits) in the most resistant category across majority of ear environments (Supporting Information Tables S1–S3). Although Mo17 had lower PKC (between 4% and 12%) and ranked among the consistent lines together with B73, kernels of the line had more aflatoxin than half of the rest of the inbred lines from across the ear environments.

By using data for kernels from ear environments which had the largest number of seed stocks of the inbred lines ($n = 21$), rank correlation analysis was conducted to test the consistency of the performances of the lines (Table 3). Although the GxE interaction influenced each of the three traits, the ranks of mean aflatoxin levels in maize inbred lines were consistent between the two years (2007 and 2009; $\rho = 0.46$, $p = 0.037$) when maize was grown at Missouri (Table 3). However, notable GxE was seen between kernels from MO_07 and from PR_08. PKC observed in kernels from MO_07 was negatively correlated with that observed in kernels from PR_08 ($\rho = -0.46$, $p = 0.04$). Similarly, sporulation in kernels from MO_07 was negatively correlated with PKC in kernels from PR_08 ($\rho = -0.53$, $p = 0.013$; Table 3). The interaction was also reflected in the inverse response of the kernels of the genotypes that were grown at the two ear environments. For example, the lines with the

TABLE 1 Variance components and broad-sense heritability (H^2) of resistance to kernel colonization, sporulation and aflatoxin production by toxigenic *Aspergillus flavus* on dry mature kernels of maize inbred lines that were grown in seven environments in the US

		Ear environment[a]						
	Overall	FL_07	MO_07	MO_09	NY_09	PR_07	PR_08	VA_09
Cube root of percent Kernel colonization								
Model								
R^2	0.75	0.04	0.02	0.81	0.67	0.66	0.58	0.86
Percentage of variance								
G	7.3	20.7	0.9	66.4**	51.1*	48.8*	43.6*	81.4*
E	35.0***	–	–	–	–	–	–	–
GxE	25.5***	–	–	–	–	–	–	–
Rep	2.0	0	0	6.7	5.4	6.4	1.8	0
H^2	0.07	0.21	0.09	0.66	0.51	0.49	0.43	0.81
Cube roof of conidia count								
Model								
R^2	0.72	0.27	0.34	0.75	0.75	0.67	0.65	0.87
Percentage of variance								
G	10.5*	20.4	23.4	60.7**	63.1*	50.4*	53.5*	83.6*
E	26.0**	–	–	–	–	–	–	–
GxE	27.7***	–	–	–	–	–	–	–
Rep	0.0	0	0	4.3	2.3	5.0	0	0
H^2	0.11	0.20	0.23	0.61	0.63	0.50	0.54	0.84
Aflatoxin, log (ppb+1)								
Model								
R^2	0.50	0.44	0.42	0.46	0.72	0.17	0.67	0.69
Percentage of variance								
G	14.3*	21.2	24.7	31.6*	48.4*	10.1	59.2*	58.3*
E	3.3	–	–	–	–	–	–	–
GxE	18.2***	–	–	–	–	–	–	–
Rep	4.2	13.6	5.9	1.5	14.1	0	0	0.9
H^2	0.14	0.21	0.25	0.32	0.48	0.10	0.59	0.58

[a]Ear environments: FL_07, Florida, 2007; MO_07/09, Missouri, 2007/2009; NY_09, New York, 2009; PR_07/08, Puerto Rico, 2007/2008 and VA_09, Virginia, 2009 (location and year combination where the inbred lines were grown). R^2, Coefficient of determination. G, genotype; E, ear environment. *, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.

least colonization and sporulation in kernels produced in MO_07 were CML322, NC358 and Ky21, but these genotypes were ranked among the five with most colonization and sporulation in kernels that were produced in PR_08 (Supporting Information Table S1). Kernels of B73 were marginally influenced by the interaction, and the line was ranked as moderately resistant to colonization across the two contrasting ear environments (Supporting Information Table S1).

3.3 | Responses of the IBM population to the KSA, and an association between kernel infection, aflatoxin and grain ionomic content

We investigated the possible ways through which grain physical and chemical composition could influence aflatoxin contamination by utilizing additional seed stocks of genotypes whose elemental profiles had been analyzed. Thus, we took advantage of an ongoing study in which the IBM RILS were being evaluated for ionomic content and had been grown in NC 2005 (Baxter et al., 2013). Kernels of the IBM RILs were thus utilized for a KSA with a premise that the population, which was developed by intermating the cross between B73 and Mo17, would not only reveal the aflatoxin vs. ionomic content relationship but also enhance QTL mapping for aflatoxin resistance. Following the KSA, PKC and aflatoxin were analyzed. Due to logistical issues, conidia were not counted. Ionomic content for the seed lots of the respective lines was used in a regression analysis with PKC and aflatoxin.

Kernels of 188 IBM RIs significantly differed in the extent of kernel colonization by *A. flavus* (Table 4). While the aflatoxin content in the RILs ranged widely (from 0 to 3,150 ng/g), the genotype effect

was not significant because of inconsistent values. As expected, heritabilities for the two traits were low, aflatoxin (H^2 = 0) and kernel colonization (H^2 = 0.26) (Table 4). The aflatoxin ranges of the parental lines, grown in the same ear environment, were 5.5–575 ng/g for B73, and 2–1,000 ng/g for Mo17. The aflatoxin mean of the IBM RI population (260 ± 306 ng/g) was between those of the parental lines (185 ± 170 ng/g, B73; 306 ± 297 ng/g, Mo17), but the standard deviations were very large. The PKC of the IBM RIs ranged from 4% to 42%. The ranges of colonization of the IBM parental lines in the same ear environment were 6% to 25% for B73, and 6% to 38% for Mo17. The mean PKC (17% ± 7%) of the IBM RIs was similar to that of the parental lines but closer to that of Mo17 (17% ± 9%) than to that of B73 (13% ± 6%).

Aflatoxin accumulation differed significantly ($p < 0.0001$) between the two kernel size categories (large and small; large kernels were presumed to be those from the middle of the ear kernels, and small kernels were presumed to be those from the tip and base of the ear) within the field replicates of the IBM population (Table 4, Figure 2). Larger kernels had significantly higher toxin levels than the small kernels (73 ng/g vs. 10 ng/g; t-ratio = −27.4, $p < 0.0001$, df = 750; Figure 2). No significant differences were observed in percent kernel colonization between the kernel size categories (Table 4). The field replicates differed in kernel colonization (replicate 2, 18%; replicate 1, 13%), but did not differ in aflatoxin level (Table 4).

A linear regression with all mineral elements as predictors of either PKC or aflatoxin showed that an increase in the concentrations of sulphur and magnesium was associated with a significant reduction percent kernel colonization. An increase in the concentrations of either magnesium or sulphur by 200 μg/g was associated with a unit decrease in PKC (Table 5). Similarly, more sulphur in the grain was significantly (p = 0.02) associated with less aflatoxin accumulation (Table 5). An increase in the concentration of sulphur by 4 μg/g was associated with a decrease in aflatoxin contamination by 1 ng/g. No associations were observed between either aflatoxin or PKC and the concentrations of rest of the minerals in the grain (Table 5).

TABLE 2 Correlation between kernel infection factors and aflatoxin (ng/g) in a mature kernel screening assay for maize inbred lines that were grown in seven environments in the US. Inbred lines of the kernels were grown at the following environment year combinations: Homestead, Florida, 2009 (FL_07), Columbia, Missouri, 2007, 2009 (MO_07, MO_09), Aurora, NY 2009 (NY_09), Ponce, Puerto Rico 2007, 2008 (PR_07, PR_08) and Blacksburg, Virginia 2009 (VA_2009)

		Ear environment[a]						
Correlation between	Overall correlation	FL_07	MO_07	MO_09	NY_09	PR_07	PR_08	VA_09
Kernel colonization & Aflatoxin	0.37	0.24	0.01	0.25*	0.30*	0.23	0.29*	0.43**
Kernel colonization & Sporulation	0.85***	0.42**	0.57**	0.63***	0.69***	0.57***	0.51***	0.90***
Sporulation & Aflatoxin	0.52**	0.58***	0.35**	0.28*	0.28	0.45***	0.68***	0.50**
Latent period & Sporulation		ND	−0.36	−0.60***	ND	−0.49**	−0.26	ND
Latent period & Aflatoxin		ND	−0.16	−0.23	ND	−0.21	−0.25	ND
Kernel colonization & latent period		ND	−0.45**	−0.58***	ND	−0.36	−0.17	ND

[a]Based on data from 10 inbred lines for which seed stocks were available across the seven environments. ND means that data for latent period was not collected, and hence the correlations could not be computed. *, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.

TABLE 3 Rank correlations of genotypic means of aflatoxin, kernel colonization and sporulation based on laboratory kernel screening assay of 21 maize inbred lines (a subset of NAM founders) among the ear environments, Missouri in 2007 (MO_07) and Missouri 2009 (MO_09), and at Puerto Rico in 2008 (MO_2008)

		Aflatoxin			Colonization			Sporulation	
Trait	Ear environment[a]	MO_07	MO_09	PR_08	MO_07	MO_09	PR_08	MO_07	MO_09
Aflatoxin	MO_07								
	MO_09	0.46*			−0.18			−0.14	
	PR_08	0.36	0.3		−0.23	0.32		0.04	0.21
Colonization	MO_07	−0.12	−0.18						
	MO_09	0.29	0.63**		0			0.18	
	PR_08	0	0.18	0.48*	−0.46*	0.32		−0.31	0.21
Sporulation	MO_07	0.09	−0.14		0.74***				
	MO_09	0.21	0.68**		−0.21	0.83***		0.05	
	PR_08	−0.02	0.13	0.58**	−0.53*	0.02	0.81***	−0.32	0.08

[a]Ear environments refer to locations where maize was grown in different years. *, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.

4 | DISCUSSION

Postharvest storage of mature maize grain under conditions that could favour growth of moulds over several months necessitates research on postharvest resistance to colonization and aflatoxin accumulation. While standard procedures for screening for pre-harvest resistance have been established, very few assays have been developed for screening of maize for resistance to postharvest resistance to colonization by *A. flavus* and aflatoxin accumulation (Brown et al., 2001). In the current study, we used mature kernels of important maize germplasm that was available to us through the Cornell University–USDA-ARS research network to develop and to test the utility of a laboratory screening assay for resistance to colonization and aflatoxin accumulation in mature maize grain. Mature kernels represent a maize developmental stage that is biochemically stable at room temperature (Baxter et al., 2014). This study allows us to gain new insights into the *A. flavus*/maize pathosystem, particularly into how the ear environment influences susceptibility of grain to colonization and contamination by toxigenic moulds. The assay was not only effective in differentiating the response of diverse inbred lines but has also established that sporulation of *A. flavus* is a proxy for aflatoxin accumulation in mature kernels. In addition, we have shown that certain grain mineral elemental profiles (sulphur and magnesium) are associated with reduced colonization and aflatoxin accumulation in the germplasm tested.

TABLE 4 Variance components and broad-sense heritability (H^2) for kernel colonization and aflatoxin, based on a mature kernel screening assay of 188 Intermated B73xMo17 (IBM) recombinant inbred lines which were grown at Clayton, NC in 2005

	Variance component	Percentage of variance	Model	Heritability
Kernel colonization (%)				
Genotype	14.8***	26		
Field replicate	9.6***	17		
Kernel Size	0	0		
Residual	32.4	57		
Total	56.8	100		
r^2			0.53	–
H^2			–	0.26
Aflatoxin (ng/g)				
Genotype	0	0		
Field replicate	0	0		
Kernel Size	2.5***	70		
Residual	1.1	30		
Total	3.5	100		
r^2			0.65	–
H^2			–	0

Note. All factors were considered to have a random effect in the model. Kernel size was based on sorting into large or small grain of each RIL. r^2, coefficient of determination. *, **, ***; significant at 0.05, 0.001 and <0.0001 respectively.

The current study made use of a few mature kernels of individual maize genotypes grown across different ear environments to assess the potential variability in colonization, sporulation and aflatoxin accumulation, with a projection that the assay and the findings could be useful in maize breeding efforts for regions where the associated postharvest losses and food safety concerns are important. Previous studies have used mature kernel screening assay to identify aflatoxin resistance and to develop molecular markers for breeding for resistance in West and Central African maize (Brown et al., 2010, 2011). Additionally, a combination of mature kernel screening assay with proteomics and expression analysis has previously been used to identify aflatoxin resistance-associated proteins that were proposed as potential molecular markers for aflatoxin resistance breeding (Zhi-Yuan, Brown, Menkir, & Cleveland, 2012). However, the utility of the markers has not been effective in breeding because of the strong GxE, which has been well documented at pre-harvest stages in maize development. It had not been known whether the mechanisms of maize resistance to colonization and aflatoxin accumulation are distinct in developing and in mature kernels. Through the current study, three important components of the postharvest *A. flavus*–maize pathosystem (surface colonization, sporulation and aflatoxin accumulation) were investigated as responses for the diverse maize germplasm. Additionally, the responses (traits) were correlated in order to establish whether inexpensive approaches could be used as proxy for aflatoxin analysis in maize breeding programs.

A higher correlation was observed between sporulation and aflatoxin than between percent kernel colonization and aflatoxin. A lack of correlation between the apparent mouldiness and aflatoxin contamination has been reported in previous studies (Mutiga et al., 2014; Smart, Shotwell, & Caldwell, 1990). The score for colonization was a superficial assessment of the severity of infection on the grain and may not reflect cryptic (internal) kernel damage. Cryptic infection could occur when the fungus enters the kernel via the peduncle or through a damaged pericarp and could lead to aflatoxin accumulation, even with minimal or no apparent mouldiness symptoms (Smart et al., 1990). Based on the current findings, the sporulation could be a proxy measure for aflatoxin content. Maize endophytes (and possibly inoculum from the resident *A. flavus*) could be another cause of the observed differences in correlations across ear environments. In this study, however, we only observed random, minor colonization of kernels by *A. niger* (<1% of the samples).

Inconsistencies in correlations were observed for the three traits (colonization, sporulation and aflatoxin) across ear environments. Differences in the correlations between the three traits could be due to environmental factors on kernel integrity and other physical and chemical characteristics of the kernel, and their effect on fungal growth and toxigenicity. Previous in vitro studies have shown that fungal growth and formation of secondary metabolites are influenced by the nutrient status of the substrate (Morton, 1961). Furthermore, non-motile fungi form spores in order to escape harsh

TABLE 5 Relationship between kernel colonization, aflatoxin accumulation and the concentration of mineral elements in maize IBM RIs grown at Clayton, NC in 2005

	Regression coefficient				Confidence limits (95%)	
Factor/element	*β*	*SE*	*t* Ratio	*P*-value	Lower	Upper
Percent Kernel Colonization						
Intercept	21.61382	2.52784	8.55	<0.0001	16.6485	26.5791
Calcium	−0.01838	0.03021	−0.61	0.5431	−0.0777	0.0410
Copper	−0.28721	0.66888	−0.43	0.6678	−1.6011	1.0266
Iron	0.10636	0.12188	0.87	0.3832	−0.1330	0.3458
Potassium	0.00017	0.00080	0.21	0.8328	−0.0014	0.0017
Magnesium	−0.00520	0.00258	−2.02	0.0442	−0.0103	−0.0001
Manganese	0.12924	0.19145	0.68	0.4999	−0.2468	0.5053
Phosphorous	0.00003	0.00026	0.10	0.9202	−0.0005	0.0005
Sulphur	−0.00529	0.00236	−2.24	0.0255	−0.0099	−0.0006
Zinc	0.11349	0.11703	0.97	0.3326	−0.1164	0.3434
Aflatoxin (ppb)						
Intercept	409.97861	110.05417	3.73	0.0002	193.8050	626.1522
Calcium	1.99396	1.31511	1.52	0.1300	−0.5892	4.5772
Copper	14.48407	29.12109	0.50	0.6191	−42.7170	71.6851
Iron	1.28020	5.30633	0.24	0.8094	−9.1427	11.7032
Potassium	−0.01769	0.03464	−0.51	0.6098	−0.0857	0.0503
Magnesium	−0.12054	0.11218	−1.07	0.2830	−0.3409	0.0998
Manganese	11.98207	8.33502	1.44	0.1511	−4.3900	28.3541
Phosphorous	0.00323	0.01113	0.29	0.7716	−0.0186	0.0251
Sulphur	−0.24936	0.10286	−2.42	0.0157	−0.4514	−0.0473
Zinc	4.04592	5.09509	0.79	0.4275	−5.9621	14.0539

*, **, ***; significant at 0.05, 0.001 and <0.0001 respectively. NS; not significant.

conditions, and nutrient starvation may lead to reduced mycelial formation, increased sporulation and aflatoxin production in *A. flavus* (Adams, Wieser, & Yu, 1998; Mehl & Cotty, 2013; Morton, 1961; Son et al., 2014). Aflatoxin production by *A. flavus* has been correlated with increased oxidative stress, a condition that is influenced by the nutrient status of the media (Brown et al., 2014; Kim et al., 2008). Variations in biotic stress factors, such as insect pressure, could also play a role in distinct response of maize inbred lines across the environments (Wu, 2007).

Significant GxE and low heritabilities were observed in the current study. These findings are consistent with previous studies which mainly focused on pre-harvest resistance to aflatoxin accumulation in maize. Here, the low heritabilities and strong GxE are based on an assay which is relevant to aflatoxin accumulation in stored maize, a component for which many researchers have not dissected using such a set of diverse inbred lines grown in multiple environments (Yu et al., 2008). Although moderately high heritability values were occasionally observed for some traits for some ear environments, there is a caution in the interpretation because kernels were from a single season. The observed overall low heritabilities, for the NAM founders and IBM populations (aflatoxin, H^2 = 0–0.14; sporulation, H^2 = 0.11 and PKC, H^2 = 0.07–0.26), are within the ranges reported in pre-harvest aflatoxin resistance breeding efforts and imply the quantitative nature of the trait. To obtain accurate estimates of the response of individual maize genotypes, there is need to a more robust experimental design with multiple seasons and field replicates within the target environments.

The inbred lines differed in susceptibility to colonization and aflatoxin accumulation, but the magnitude of the response varied across the ear environments. NAM founders consist of inbred lines of different climatic adaptation, but no obvious trends in response to *A. flavus* could be associated with the weather conditions during seed production or adaptation to either tropical or temperate conditions (Flint-Garcia et al., 2009). The response of the tropical lines, developed by the International Maize and Wheat Improvement Center (CIMMYT), would have been expected to be consistent in kernels that were grown at PR and FL, while temperate lines would be consistent at MO and NY. Colonization was lower (2%) in kernels of CML322 that were grown at FL 2007 compared to those that had been produced at PR_07 (93%) and at PR_08 (88%). Similarly, a lower colonization (9%) was observed in kernels of CML52 that were produced in FL_07 compared to those that were grown at PR_08 (39%) and at MO_09 (38%). A significant infestation of the crop by fall armyworm had been observed in PR_07 and PR_08. However, kernels (from PR_08 and PR_08) used in the current KSA did not have obvious damage and the non-inoculated controls were not colonized by any other moulds. Pre-harvest damage of maize by insects could interfere with the nutrient availability to the developing

TABLE 6 Means and ranking of sporulation, infection coefficient and aflatoxin in field inoculated [data from Mideros et al., 2009] and in vitro inoculation of mature kernels at 10^6 conidia/ml (this paper) of diverse inbreed lines

	In vitro inoculation of mature kernels[a]			Field inoculation at 50% silking[b]		
Line	Sporulation	Aflatoxin (ng/g)	Aflatoxin (ng/g) rank	Infection coefficient	Aflatoxin	Rank for aflatoxin
B73	38.7 C	9 D	1	0.85 B,C,D,E	4,596 A,B	14
M37W	45.9 B,C	14 D	2	0.2 E	541 B,C,D,E,F,G	5
P39	42.9 B,C	33 C,D	3			
CML103	57.2 B,C	109 B,C,D	4	1.08 A,B,C	5,173 A,B	15
NC358	56.2 B,C	119 B,C,D	5	0.26 E	661 B,C,D,E,F	6
Tx303	62.6 A,B	501 A,B,C,D	6	0.38 C,D,E	874 A,B,C,D,E,F	8
CML247	59.1 A,B,C	661 A,B,C,D	7	0.37 C,D,E	233 E,F,G	2
CML333	53.3 B,C	714 A,B,C,D	8			
CML69	52.7 B,C	1,012 A,B,C,D	9			
CML52	55.1 B,C	1,040 A,B,C,D	10	0.31 C,D,E	136 F,G	1
CML277	60.3 A,B	1,139 A,B,C,D	11			
Mo17	46 B,C	1,514 A,B,C,D	12	1.63 A,B	6,596 A	16
B97	43.8 B,C	1,932 A,B,C,D	13	1.75 A	3,932 A,B,C	13
Ky21	56.4 B,C	2,138 A,B,C,D	14	0.91 A,B,C,D	1,046 A,B,C,D,E,F	10
Ki3	80.1 A	2,293 A,B,C,D	15	0.23 E	268 D,E,F,G	3
Oh7B	54.5 B,C	2,918 A,B,C	16		3,423 A,B,C,D	12
HP301	58.1 B,C	4,096 A,B	17			
Oh43	57.8 B,C	4,499 A,B	18	0.71 B,C,D,E	1,440 A,B,C,D,E	11
MS71	61.9 A,B	5,122 A,B	19	0.73 B,C,D,E	705 B,C,D,E,F	7
Ki11	61.3 A,B	5,547 A	20			
NC350	57.2 B,C	5,703 A	21		889 A,B,C,D,E,F	9
CML322	57.6 B,C	6,190 A	22	0.28 C,D,E	389 C,D,E,F,G	4

[a]Inbred lines were grown in Missouri in 2007 and 2009 and in Puerto Rico in 2008. Kernels of Mo17 were not available from Puerto Rico in 2008.
[b]Pre-harvest inoculation of the inbred lines with aflatoxigenic NRRL 3,357 *A. flavus* strain, in 2008, 2009, and 2010 at the R. R. Foil Plant Science Research Center at Mississippi State University. Means followed by the same letters within a column do not differ significantly (α = 0.05).

kernels, and hence affecting their chemical and physical composition. While differences in soil composition could have affected the kernel characteristics and their response in the KSA, soil analysis was not within the scope of the current research.

To assess whether resistance mechanisms were similar for developing (pre-harvest) vs. mature kernels (stored grain), we correlated data from three-year field inoculation trials at Mississippi (Mideros, Windham, Williams, & Nelson, 2009; Mideros et al., 2012) with mature kernel assays of inbred lines that were grown at Missouri (2007 and 2009) and in Puerto Rico (2008) in the current study. A pairwise comparison of the ranking of mean aflatoxin levels of inbred lines showed that the trend of the response of the lines was dissimilar between the two studies and the aflatoxin levels were not correlated between the two experiments (r = −0.12, p = 0.65; Table 6). Similarly, sporulation in mature kernel assays was not correlated with the kernel infection coefficient in field-inoculated trials (r=−0.35, p = 0.21). Furthermore, the trend of the genotypes was not similar to those reported in the earlier study (Mideros et al., 2012). Inconsistency in response of genotypes between the two studies is demonstrated as follows. Mo17 was moderately resistant to colonization in the current study, but was ranked among the most susceptible based on the field-inoculated trials (Mideros et al., 2012). Similarly, while B73 was found to be the resistant based on the current study, but it was listed among the most susceptible in the field experiments (Mideros et al., 2012). CML322 and CML247 were ranked among the resistant category in the field-inoculated trials, but tended to be among the most susceptible in the current study. Although these findings imply that the pre- and postharvest resistance mechanisms are distinct, and a need to develop complementary strategies to prevent aflatoxin contamination at the two stages in maize value chain, it is possible that the distinct trends in response of the genotypes between the two experiments were due to GxE.

The IBM RI lines included in the current study differed in kernel colonization but not in aflatoxin accumulation. The IBM RI population of 302 involved a B73×Mo17 bi-parental cross followed by five cycles of random intermating prior to development of the recombinant inbred lines, achieving a high resolution for mapping of genes associated with quantitative traits (Lee et al., 2002). In the current study, a subset of the IBM RI population and their parental lines was evaluated, albeit being grown at different ear environments. The

IBM RI parental lines were found to be resistant (B73) and moderately resistant (Mo17) to postharvest colonization by *A. flavus* and aflatoxin accumulation, and the RILs did not show consistent reactions. These findings imply that either the parental lines did not differ in QTL for resistance to aflatoxin-related traits or the error variance in the current studies was too large to allow us to detect differences. A cross with greater difference between parents, analyzed with many more replications (both in the field and in the postharvest assay), may have been needed to allow mapping of loci influencing colonization.

Significant differences in aflatoxin concentration were observed between kernels of different sizes (high in large vs. low in small kernels) of the same genotype. Different kernel sizes were assumed to have originated from different positions in the maize ear. Previous studies had shown a huge aflatoxin range (0–80,000 ng/g) in kernels from different positions of the same ear (Lee, Lillehoj, & Kwolek, 1980). The variations in susceptibility to colonization and toxin accumulation could be caused by intra-ear variation in either physical or chemical characteristics. However, no apparent physical damage was observed on the kernels. From the ionomic profiling study, a higher grain sulphur content at the tip compared to the middle or the base of the ear was reported (Baxter et al., 2014). In a related study, protein and carbohydrates were found to be uniform along deciles (arbitrary equal regions demarcated for research purposes) of the maize ear (Seebauer, Singletary, Krumpelman, Ruffo, & Below, 2010). Interestingly, significant negative correlations were found between sulphur and colonization or aflatoxin, and between magnesium and kernel colonization. These findings imply that the variations in colonization and aflatoxin accumulation in kernels of different sizes might be related to differences in elemental profiles. In the maize ionomic profiling project, QTL for high sulphur and magnesium content were identified, and these loci are not linked to those associated with resistance to kernel colonization and aflatoxin (Baxter et al., 2013; Mideros et al., 2014; Warburton et al., 2011). Plants are known to accumulate elemental sulphur or sulphur-rich compounds in order to prevent attack by pathogenic species (Chok-Fun & Carl, 1995; Choudhury & Goswami, 2013). Genes encoding high-sulphur zein proteins in maize endosperm have been reported and are thought to provide antimicrobial protection during seed storage (Bolchi, Petrucco, Luigi Tenca, Foroni, & Ottonello, 1999). While magnesium is an important compound in formation of chlorophyll, its antimicrobial role has not been demonstrated in plants (Walker & Weinstein, 1994). Breeding for high sulphur and magnesium could be a potential approach to reducing aflatoxin in maize.

The observed differences in responses of kernels between the two field replicates of the IBM RI population reflect the expected variability in response of maize to *A. flavus* across environments. The strong GxE requires multiple replications within and among environments to facilitate credible inferences about aflatoxin resistance in maize. There is need to use kernels from a more robust study in future screening of maize for resistance to aflatoxin. The observed strong association between fungal sporulation and aflatoxin means that researchers can use conidia count as a proxy for aflatoxin quantification in a kernel screening assay, hence reducing the cost. Additionally, segregating maize populations could be developed to further study the associations between sulphur, magnesium and aflatoxin accumulation in maize and to include the associated genomic regions in breeding programs. Further studies are needed to establish the mechanism through which the concentration of these elements would hinder maize colonization by *A. flavus* and the subsequent reduction in contamination. Because storage of mature maize grain is common in Africa, there is need to test the associations of aflatoxin accumulation and magnesium or sulphur in the adapted hybrid maize and other germplasm for breeding efforts in the region.

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CONFLICT OF INTEREST

All authors declare no conflict of interest in this manuscript.

AUTHOR CONTRIBUTIONS

SKM and RJN conceived and designed the study. SFG provided maize kernels. OAH provided kernels and grain mineral content data. SKM and NC conducted mature kernel screening assays in RNelson Lab, Cornell University. SKM and RJN wrote the manuscript with a review and input from the rest of the team.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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