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Dissection of mechanisms of resistance to *Aspergillus flavus* and aflatoxin using tropical maize germplasm

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RESEARCH ARTICLE

Abstract

Aspergillus flavus induced ear rots and subsequent contamination of maize (*Zea mays* L.) by aflatoxin is a serious food safety issue, especially in developing countries where the crop is mostly cultivated by smallholder famers for own consumption and income generation. A better understanding of the mechanisms of resistance could help breeders to develop resistant maize varieties. In this study, a set of six tropical maize inbred lines previously identified as resistant or susceptible under natural field conditions were evaluated for response to *A. flavus* colonisation and aflatoxin contamination. Fungal biomass was significantly higher ($P<0.05$) in susceptible than resistant maize inbred lines, and this was highly correlated ($P=0.001$) to aflatoxin levels. Maize inbred lines MRI, MR2 and MR3 had low fungal biomass and low aflatoxin levels, suggesting that resistance in these lines was mediated through restricted fungal colonisation and establishment. Among the three putatively resistant inbred lines mentioned above, MR2 had a relatively high colonisation compared to the other two lines, revealing that *A. flavus* could establish and colonise kernels that were injured during inoculation, but did not contain high levels of aflatoxin. This could signify the presence of host genes that interfere with the aflatoxin biosynthetic pathway.

Keywords: maize, *Aspergillus flavus,* aflatoxins, resistance

1. Introduction

Infection of maize (*Zea mays* L.) by toxigenic strains of *Aspergillus flavus* and *Aspergillus parasiticus,* and subsequent contamination with aflatoxin is a major health hazard, especially in developing countries where maize is a major staple. The adverse impact of aflatoxins on human and livestock health is well documented (Williams *et al.*, 2004; Wu, 2015). The ingestion of high levels of aflatoxins can be fatal (Lewis *et al.*, 2005), while chronic exposures may result in cancers, liver diseases, abortion, immune system suppression, interference with micronutrient metabolism, and retarded growth of children (Khlangwiset *et al.*, 2011; Williams *et al.*, 2004; Wu, 2015). Over five billion people in developing countries are estimated to be chronically exposed to aflatoxin, through their diets (Stronsnider *et al.*, 2006; Williams *et al.*, 2004). The economic costs associated with aflatoxin contamination include disposal of contaminated food and feed, and losses caused by lower productivity of both humans and livestock (Wu, 2015). In addition, losses in income are incurred through inspection, sampling and analysis before and after shipments, and rejection of lots due to high aflatoxin concentrations (Coulibaly *et al.*, 2008; Wu, 2015).

In developed countries, such as the USA, aflatoxin levels in maize are generally lower due to good production and post-harvest practices and a more temperate environment in the major maize-growing areas. The low aflatoxin levels are also reinforced through stringent food safety standards, monitoring and destruction of contaminated maize (Robens and Cardwell, 2005). The application of such strategies in developing countries is difficult because of differences in food production systems, in particular,

the prominence of subsistence farming, the decrease in available farm land that makes rotation difficult, a tropical environment that is conducive for growth of the fungus, and the lack of resources, technology and infrastructure for optimal drying, storage and certification. Most rural households in developing countries eat what they produce (De Groote *et al.*, 2013; Lewis *et al.*, 2005; Strosnider *et al.*, 2006; Suleiman and Rosentrater, 2015). As a result, it is difficult to put effective aflatoxin monitoring and regulation measures in place. Consequently, many small-scale farmers in many developing countries may be chronically exposed to aflatoxin.

Several pre- and post-harvest interventions are recommended to minimise aflatoxin contamination, but most have proved insufficient in eliminating the threat of the harmful health and economic effects due to contamination (Hell *et al.*, 2008). Biological control, using non-toxin producing *A. flavus* strains to prevent further infection by toxin-producing strains has proved very efficient, consistently reducing aflatoxin contamination by >80% (Bandyopadhyay *et al.*, 2016). The major drawback to the wide dissemination of the biological control strategy has been the need to develop and register a product for each country, as strains cannot be used across borders; thus, making the process costly and cumbersome (Bandyopadhyay *et al.*, 2016). The development and use of host resistance offers a possible and cost-effective solution. This technology is easy to transfer and disseminate to farmers, as it is packaged in seed, and thus requires no extra effort to implement by smallholder farmers. However, this requires identification and use of suitable maize inbred lines that restrict infection by aflatoxin-producing fungi and the subsequent contamination by aflatoxins to develop resistant maize varieties.

Use of host resistance could be facilitated by a clear understanding of *A. flavus* and aflatoxin genetics, tools to help identify effective resistance genes and availability of low-cost aflatoxin assays robust enough to correctly verify reduced aflatoxin accumulation in large numbers of breeding materials (Mahuku *et al.*, 2013). Resistance to *A. flavus* and aflatoxin accumulation is a highly quantitative trait (Warburton and Williams, 2014; Widstrom *et al.*, 2003), and probably different mechanisms contribute to resistance in different maize germplasm sources, and possibly under different environmental conditions (Guo *et al.*, 2008). Resistance may result from: (1) prevention of fungal infection of maize; (2) prevention of subsequent growth of the fungus once infection has occurred; (3) inhibition of aflatoxin formation following infection; and (4) degradation of aflatoxin by products or enzymes produced by the plant or by the fungus itself (Brown *et al.*, 1999; Williams *et al.*, 2015).

This study was designed to confirm previous classification of six tropical maize inbred lines, as resistant or susceptible following evaluation under natural field conditions and define mechanisms of resistance to *A. flavus* in these lines. To differentiate between mechanisms limiting infection and growth of the fungus vs production of aflatoxin, techniques to accurately quantify the amount of fungus and amount of aflatoxin are needed. Quantitative real-time polymerase chain reaction (qPCR) is a method that estimates the amount of fungal biomass based on the number of copies of fungal genes (Mideros *et al.*, 2009). Enzyme linked immunosorbant assay (ELISA) and fluorescence based assay (VICAM) can be used to directly quantify aflatoxin. The objective of this study was to use these techniques to quantify fungal biomass and aflatoxin levels in maize grain following artificial inoculation of tropical maize inbred lines with a virulent toxigenic isolate of *A. flavus*, and to use these measurements to distinguish resistance mechanisms acting in these lines. In the process, we confirmed the response of resistant maize inbred lines to infection by *A. flavus* and subsequent aflatoxin accumulation, and validated the utility and practicality of a low-cost in-house ELISA assay (Waliyar and Reddy, 2009) as a tool for quantifying aflatoxin and its application in a maize breeding program.

2. Materials and methods

Maize germplasm

A set of six maize inbred lines were selected from previous field studies that included 12 lines (Table 1). The 12 maize inbred lines had previously been classified as resistant or susceptible to *A. flavus* using visual rating under natural field conditions (Table 1). The lines were screened for response to aflatoxin accumulation under laboratory conditions using the kernel screening assay (KSA) (Brown *et al.*, 1993). A highly virulent isolate of *A. flavus*, PR96B-40, previously isolated from CIMMYT's experimental station at Agua Fria located in the state of Puebla, Mexico was used for the kernel assay screening and subsequent field studies. This isolate was selected from a group of 40 isolates that were screened for ability to colonise maize kernels and produce aflatoxin under laboratory conditions (data not shown). Based on results from KSA, six maize inbred lines were selected for further studies to elucidate the mechanism of *A. flavus* and aflatoxin resistance under field conditions. The inbred line designated MS1 was used as a susceptible check.

Experiential site, layout and management

Field experiments were conducted at CIMMYT's experimental station at Agua Fria in Mexico. Agua Fria (20º45' N, 97º63' W, 110 m.a.s.l.) is a humid site situated near the Gulf of México with a minimum average temperature of 16 °C and maximum of 33 °C. Trials were laid out in an alpha lattice design (Petterson and Williams,

Table 1. Characteristics of tropical maize inbred lines used to elucidate the mechanism of *A. flavus* and aflatoxin resistance.

Entry	Pedigree	Visual classification	Kernel assay (µg/kg)	Classification[1]
1	CML 52	Resistant	167.10	MS3
2	(P36STE-28*36STE-38)-BBBB-###-B*8-B	Susceptible	113.20	NS
3	CL-02510	Resistant	188.41	NS
4	CML 495. CL-RCW01	Resistant	3.52	MR1
5	CML 247	Resistant	2.06	MR2
6	CML-155	Resistant	204.18	NS
7	DERRC2 15-3-1-#2-#1-1-#-#-B	Susceptible	2.10	MR3
8	DTPWC9-F115-1-2-1-2-B-B-B	Susceptible	205.60	NS
9	90[SPMATC4/P500(SELY)]#-B-4-2-B-B	Susceptible	10.90	NS
10	P502c1F9-2-2-1-B-B-1-3-1-1-B-1-B-B-1-B-B-B-B	Susceptible	208.10	MS1
11	DTPWC9-F67-2-2-1-B-B-B	Susceptible	189.20	MS2
12	S.AM.TSR-76-1-1-B-1-BBBB-5-##-B*9-B-B-B	Susceptible	77.10	NS

[1] Classification: MR = resistant; MS = susceptible; NS = not selected for this study.

1976) with four replications. Each entry was established in plots 4.5 m long with three rows each, with 0.75 m between rows and 0.2 m between plants in a row to give a total of 20 plants per row. Standard agronomic practices for the location were followed. In the field, appropriate weed, control measures were undertaken as needed. The study was conducted for two seasons (years).

Aspergillus flavus inoculum preparation

A highly virulent and toxigenic isolate of *A. flavus* (PR96B-40), isolated from Agua Fria was used for inoculum preparation. The fungus, stored as spores immobilised on silica gel at 4 °C, was recovered by sprinkling a few silica gel grains onto potato dextrose agar (PDA) medium and incubated at 28 °C for 7 days. After 7 days, *A. flavus* spores were harvested by flooding PDA plates with sterile distilled water containing Tween®-20 surfactant at the rate of 0.2 ml/l. The spore suspension was passed through two layers of cheese cloth to remove mycelial and agar fragments and the spore concentration was estimated using a haemocytometer and adjusted to 1×10^6 spores/ml. 25 ml of the diluted spore suspension was used to inoculate a jar containing 250 g of maize kernels, previously sterilised by autoclaving overnight soaked kernels. The jars were shaken vigorously to thoroughly and completely mix the spores and maize kernels before incubation at 28 °C in the dark for 2 weeks. During this period, the jars were shaken every other day, to avoid clumping of colonised kernels and to make sure that *A. flavus* growth was evenly distributed within the jar. After two weeks, *A. flavus* colonised maize kernels were emptied into a container with sterile distilled water containing Tween-20 surfactant, and vigorously shaken to dislodge spores before filtering through two layers of cheese cloth to remove maize kernels and mycelium fragments. The spore concentration was estimated using a haemocytometer, and adjusted to 1×10^6 spores/ml and used for field inoculations.

Artificial inoculation of plants

Plants were inoculated 12 to 14 days after female flowering (silking) when kernels were in the soft dough stage, using a pinbar dipped in to an *A. flavus* spore suspension (1×10^6 spores /ml) and pressed through the husk of the mid-section of each primary ear (Windham *et al.*, 2003). To reduce within plot (i.e. genotype) variation due to age, inoculations were only done on primary ears that silked within 48 h of each other. Two of the rows were inoculated while one row was mock-inoculated using sterile distilled water to serve as the control. Irrigation was withheld starting two weeks before flowering in the trials to simulate drought conditions that are known to predispose maize plants to *A. flavus* infection and aflatoxin accumulation.

Sample and data collection

Samples of developing maize ears were collected at different time intervals, starting just before inoculation (day 0), then at 7, 14, 21, 28 and 49 days post-inoculation. The final samples were collected at harvest, 49 days after inoculation. For each sampling time, two cobs were collected from each row to give a total of 4 cobs from inoculated and 2 cobs from non-inoculated plots for each entry per replication. Sampled cobs were immediately put on dry ice and taken to the laboratory and stored in a deep freezer at -80 °C until the time of analysis. Climatic data including relative humidity, maximum and minimum temperatures, and sunlight intensity were recorded every 30 min for the duration of the experiment (i.e. from planting to harvesting).

Agronomic data for flowering time, plant and ear height and incidence of rotten cobs at harvest were recorded.

Aflatoxin analysis

For aflatoxin and fungal biomass analysis, collected cobs were removed from -80 °C and immediately lyophilised. After lyophilisation, cobs from the same entry for each collection point and replication were combined, the grain shelled by hand and the whole sample ground to a fine powder using a kitchen blender, and an aliquot of 50 g was drawn from a thoroughly mixed sample and used for aflatoxin extraction and analysis using the enzyme-linked immunosorbent assay (ELISA) and/or VICAM Aflatest (Watertown, MA, USA). The blender was thoroughly cleaned and disinfected between samples to prevent cross contamination. For the ELISA assay, aflatoxins were extracted following the method described by Waliyar and Reddy (2009). Briefly, the maize flour was homogenised in 100 ml of methanol extraction buffer (70:30, methanol:sterile distilled water), containing 0.5% KCl and homogenised for 30 min at 300 rpm. The homogenate was filtered through Whatman filter No 1 (Maidstone, UK), the filtrate collected and used immediately for ELISA analysis. Indirect ELISA was used to estimate content of AFB_1 in kernels from each entry and treatment. Briefly, antigens were immobilised on the surface of an ELISA plate, followed by competition for antibody binding sites between AFB_1 present on the surface of the plate and AFB_1 molecules present in the sample or standard. Aflatoxin specific antibodies were detected using enzyme labelled secondary antibodies that were obtained from ICRISAT (Reddy *et al.*, 2001; Waliyar and Reddy, 2009). To validate ELISA results, a subset of the samples collected at harvest were analysed using the VICAM Aflatest, as per the manufacturer's instructions.

Quantification of *Aspergillus flavus* biomass using qPCR

To detect *A. flavus* and quantify fungal biomass, we used the method developed by Mideros *et al.* (2009), using qPCR-TaqMAN. Fungal DNA from infected grain from field samples and pure fungal isolate used for inoculations (isolate PR96B-40) was extracted using the CTAB method (Doyle and Dickson, 1987). PCR was conducted in 25 µl volumes, containing 4 pmol of each forward (Af2F) and reverse (Af2R) primers, 5 pmol of Af2Taq Man probe, 3 µl DNA, 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 7.12 µl of sterile distilled water. PCR cycling conditions included an initial cycle at 50 °C for 2 min, then 1 cycle denaturing at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 30 s, annealing at 59 °C for 30 s and extension at 72 °C for 30 s. The extent of fungal colonisation (infection coefficient (IC)), estimated as amount of fungal biomass, was calculated per Mideros *et al.* (2009) by dividing the amount of fungal DNA estimated by qPCR using CFX96 Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and total DNA determined by Nanodrop ND-1000 (Thermo Fisher, Waltham, MA, USA).

Statistical analysis

Aflatoxin (AFB_1) and IC data was log transformed [Log_{10} (x+1)] before analysis. Analysis of variance was performed using Proc MIXED option in SAS (2013). Differences between inbred lines within years were determined using Fisher's protected least significant difference (LSD) test at 5% significance level. Pearson correlation coefficient was used to establish the correlations between different parameters and was performed using PROC CORR in SAS. All statistical analysis were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

Response of maize lines to aflatoxin contamination

Aflatoxin quantification allowed us to classify maize lines as resistant or susceptible based on standards set by regulatory authorities in Mexico (<20 µg/kg). Significant differences in the levels of aflatoxin ($P<0.0001$) were observed between susceptible and resistant inbred lines. The response of the lines was the same in the two years of evaluation, revealing that the classification of these lines as resistant or susceptible was correct and the performance of each line across years was stable. Therefore, subsequent analysis used combined data for the two seasons.

Validation of the ELISA assay for aflatoxin quantification

To validate the utility of ELISA for aflatoxin quantification in a breeding program, a subset of the samples collected at harvest, 49 days after inoculation were assayed using VICAM. Although aflatoxin results obtained using ELISA were lower than those obtained using VICAM, these were highly correlated ($r^2=0.98$) (Table 2). This is because VICAM measures total aflatoxins, whereas the ELISA assay used in this study was specific for AFB_1, with cross-reactivities of 10, 8, 0.2 and 0.1% with aflatoxin B_2, G_1, G_2 and M_1, respectively (Reddy *et al.*, 2001).

Response of maize inbred lines to *Aspergillus flavus* and aflatoxin

To test whether natural infection is reliable for differentiating resistant from susceptible maize germplasm, aflatoxins were estimated in the same maize inbred lines from inoculated and non-inoculated plots. In all cases, large variation in aflatoxin levels were observed in maize from control compared to inoculated plots (Figure 1). Maize from non-inoculated plots consistently gave lower aflatoxin levels than those from inoculated plots and these varied between

Table 2. Validation of the suitability of ELISA as a tool to reliably quantify aflatoxins in a maize breeding program.

Code	Maize inbred line	Aflatoxin levels (µg/kg)[1]	
		VICAM aflatoxins	ELISA AFB_1
MR1	CML495	4.0±0.95	3.5±3.39
MR2	CML247	2.9±1.50	2.1±1.18
MR3	DERRC2	15.0±5.59	2.1±0.57
MS1	P502c1F9	280.0±38.84	208.1±37.99
MS2	DTPWC9-F67	220.0±18.90	189.2±7.40
MS3	CML52	260.0±40.60	167.1±55.5

[1] Aflatoxin values are means of 4 replications of results from 2010 ± the standard deviation of the mean. For each replication and sample, two independent aflatoxin analyses were conducted and where huge variations were observed, an additional analysis was conducted.

replications and years. However, for maize from inoculated plots, no statistically significant differences were observed between replications and years. These results reveal that establishment of adequate disease pressure is necessary for identifying resistant inbred lines, and therefore, artificial inoculation is essential in a breeding program.

Maize inbred lines previously identified as resistant did not contain high levels of aflatoxin following inoculation with a highly virulent and toxigenic isolate of *A. flavus* (Figure 1B). Although not statistically significant (P<0.05), differences in aflatoxin levels were observed between years for some resistant lines for inoculated plots. In both years, susceptible lines had high levels of aflatoxin compared to resistant lines, and for some of them, statistically significant differences were observed between inoculated and non-inoculated lines (Figure 1). One line, MS1 was consistently susceptible in both years and under both natural and artificial inoculation, revealing that this line was highly susceptible. The resistant line, MR3 had low levels of aflatoxins in both inoculated and non-inoculated plots in both years, and this line can be used as a resistant check.

Aspergillus flavus colonisation and establishment

To establish the extent of maize colonisation by *A. flavus*, fungal biomass was estimated using qPCR. Susceptible lines had significantly higher fungal biomass (IC) compared to resistant lines (Table 3), revealing rapid colonisation and establishment of *A. flavus* in susceptible genotypes both under natural and artificially created conditions. The infection coefficient (IC) values for susceptible lines were significantly higher (P<0.05) for inoculated plots compared to non-inoculated plots. For example, the highly susceptible line, MS1, fungal biomass was higher in inoculated plots

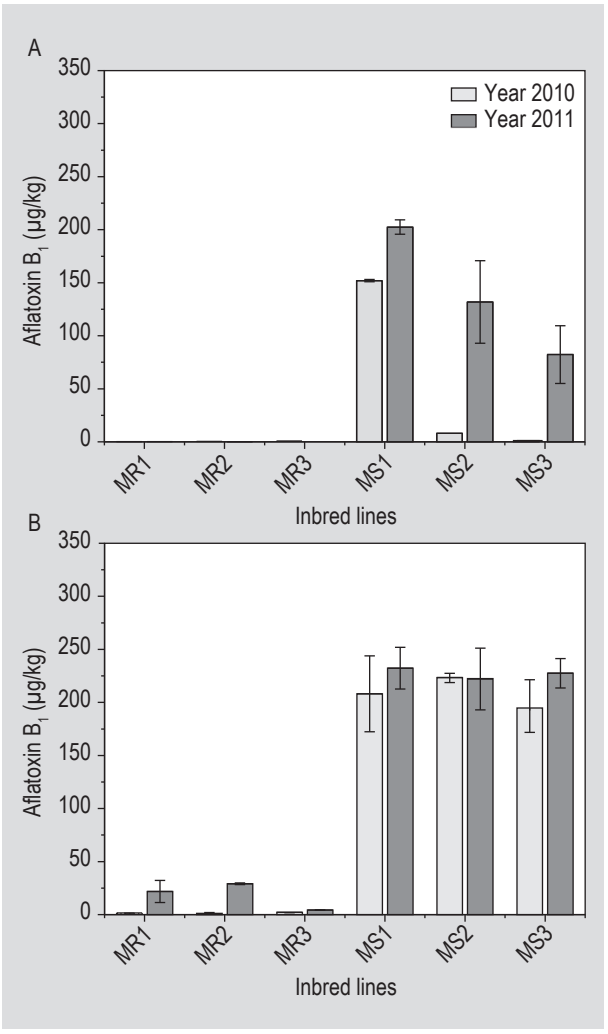

Figure 1. Aflatoxin levels in tropical maize inbred lines not inoculated (A) or inoculated (B) with a toxigenic isolate of *Aspergillus flavus*, and evaluated over two seasons (2010 and 2011). For susceptible lines, large variations were observed for the non-inoculated trails compared to inoculated trials.

(1.89×10^{-1}) compared to non-inoculated plots (5.21×10^{-3}). For resistant lines, no statistically significant differences were observed for inoculated and non-inoculated plots, except for line MR2 (CML247) that had significantly higher fungal biomass (2.01×10^{-3}) following inoculation with *A. flavus* (Table 3) compared to 9.94×10^{-7} under natural epidemics (Table 3). Very low fungal biomass was observed in the resistant line MR3 across years, and between inoculated (6.59×10^{-5}) and non-inoculated (3.38×10^{-5}) plots (Table 3; Figure 2).

Relationship between fungal biomass and aflatoxin content

Although fungal biomass and aflatoxin levels increased slightly in 2011, no statistically significant differences (P<0.05) were observed for each maize inbred line and

Table 3. Mean aflatoxin levels and fungal biomass estimated by ELISA and qPCR, respectively, in maize inoculated with *Aspergillus flavus* and non-inoculated controls.

Code	Maize line	AFB_1 (µg/kg)		Infection coefficient	
		Non-inoculated[1]	Inoculated	Non-inoculated	Inoculated
MR1	CML495	0.19^{aC}	11.65^{aD}	2.30×10^{-4aC}	5.58×10^{-5aC}
MR2	CML247	0.27^{aC}	15.08^{aD}	9.94×10^{-7aC}	2.01×10^{-3aD}
MR3	DERRC2	0.85^{aC}	3.15^{aD}	3.38×10^{-5aC}	6.59×10^{-5aC}
MS1	P502c1F9	177.23^{bD}	220.22^{bE}	5.21×10^{-3bC}	1.89×10^{-1bD}
MS2	DTPWC9-F67	69.02^{bD}	205.65^{bF}	8.49×10^{-4aC}	1.36×10^{-2bD}
MS3	CML52	41.68^{bD}	197.30^{bF}	5.81×10^{-3bC}	1.52×10^{-2bD}

[1] Values are means of combined data from 2010 and 2011 evaluations. Means followed by the same letter are statistically not significant at $P<0.05$. The letters a and b within a column designate differences between lines, while letters C, D, E and F stand for differences between inoculated and non-inoculated, for each maize line.

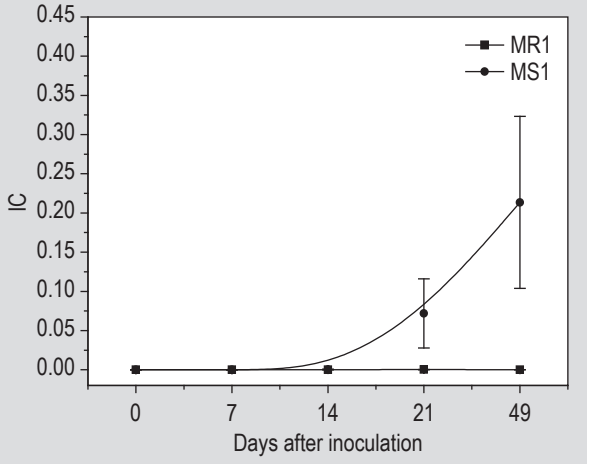

Figure 2. *Aspergillus flavus* biomass estimated using qPCR in a resistant (MR1) and susceptible (MS1) tropical maize inbred lines over time. Fungal biomass (measured as infection coefficient (IC)) increased dramatically in the susceptible compared to the resistant maize line.

between years, revealing that line performance was consistent across years (Figure 1B). As a result, data from the two years were combined for analysis. Low and non-significant correlations were observed in non-inoculated lines (r=0.1) while significant correlations were observed (r=0.7 and 0.9 respectively) between fungal biomass and aflatoxin levels for resistant and susceptible lines following inoculations with *A. flavus* (Figure 3). Among the resistant lines, MR2 (CML247) had a relatively high fungal biomass (2.01×10^{-3}) following inoculation with a virulent *A. flavus* isolates, but aflatoxin levels (15.08 µg/kg) were low and non-significantly different from those obtained for other (MR1 and MR3) resistant lines (Table 3).

4. Discussion

Several management strategies may reduce aflatoxin contamination of maize, and of these, host resistance may prove the most effective strategy. Host resistance is a preventative approach that is economical, easy to disseminate, requires no additional production resources from the farmer, leaves no harmful residues, and is compatible with other control measures, including biological control (Mahuku *et al.*, 2013). Improving host resistance via plant breeding is more efficient when the genetics and mechanisms of aflatoxin resistance is understood, so that suitable maize germplasm is used in breeding programs and the useful diversity maintained during selection.

In this study, we tested three hypotheses that were meant to elucidate the mechanism of resistance in a selected set of tropical maize germplasm. We hypothesised that resistance to aflatoxin might result from scenarios where: (1) colonisation and establishment of the *A. flavus* is restricted, manifested as low fungal biomass which translates into reduced aflatoxin production and accumulation; (2) fungal infection occurs but is restricted to infected kernels, while within infected kernels, *A. flavus* continues to grow and produce aflatoxin; (3) *A. flavus* infection and colonisation occurs, but aflatoxin production by the fungus is restricted, possibly as a result of host genes that interfere or block the aflatoxin biosynthetic pathway. Alternatively, (4) lack of resistance occurs due to a susceptible interaction, where fungal growth is not restricted leading to higher fungal biomass in infected kernels and higher aflatoxin levels. These four outcomes were tested in field experiments conducted over two years under artificial inoculation with a highly virulent and toxigenic strain of *A. flavus*, and fungal biomass was estimated using real time quantitative PCR

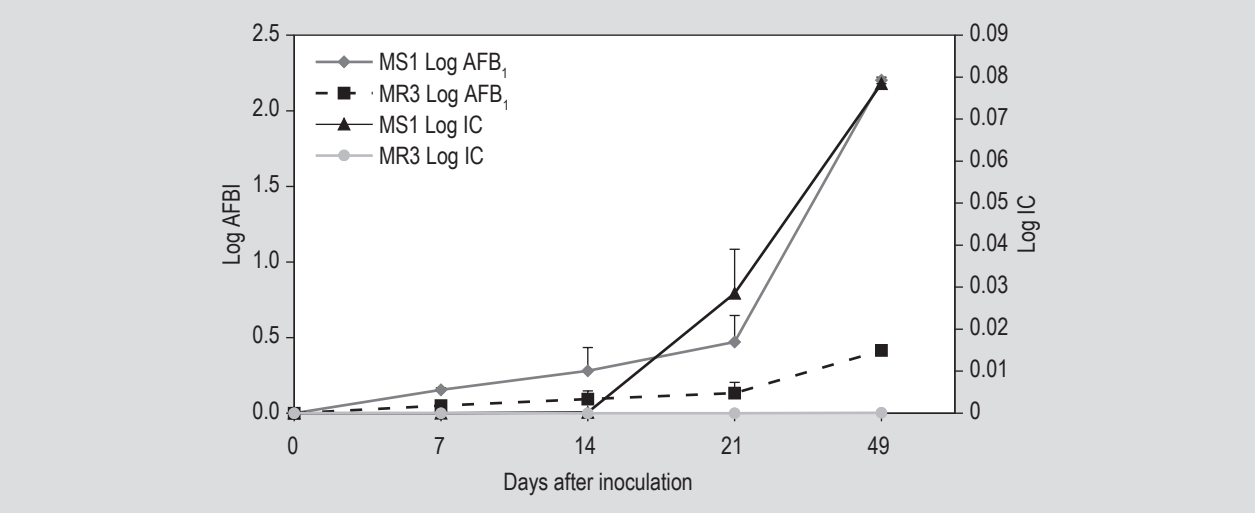

Figure 3. Relationship between aflatoxin B_1 concentration (Log AFB_1) measured using ELISA and fungal biomass (Log IC) estimated using qPCR for a highly resistant MR3 and a highly susceptible MS1 tropical maize inbred lines.

using the method described by Mideros *et al.* (2009) and theAFB_1 quantified using VICAM and/or ELISA.

The presence of low fungal biomass and low aflatoxin levels would signify restricted fungal colonisation and establishment, and possibly death of the fungus within infected cells, resulting in very little or no aflatoxin being produced, consistent with hypothesis 1. This is a resistance response ideal for breeding programs to mitigate the negative effects of aflatoxin in the maize food chain. The maize inbred lines MR1, and MR3, fit this scenario and these lines may now be used as donors of resistance to *A. flavus* and aflatoxin resistance in breeding programs. These inbred lines may also serve as resistant checks in field experimentation. Aflatoxin resistance might result from the inability of *A. flavus* to colonise maize kernels, resulting in absence or low levels of fungal biomass and subsequent low levels of aflatoxins (Fountain *et al.*, 2015). Resistance could be a result of preformed host barriers, such as pericarp wax, cutin and polyphenolic compounds impeding the fungus from passing through the pericarp to colonise the seed (pericarp resistance) of resistant genotypes; or cell wall fortification to restrict further colonisation and proliferation of *A. flavus* (Brown *et al.*, 1995; Guo *et al.*, 1995). The presence of preformed host barriers impeding *A. flavus* colonisation in resistant maize germplasm was demonstrated by Brown *et al.* (2001) following inoculation with an *A. flavus* mutant expressing GUS gene. Low fungal growth was observed in resistant maize lines, and this was correlated to low aflatoxin production.

Alternatively, resistance to colonisation by *A. flavus* and subsequent aflatoxin production could result from diverse factors that include biochemical, physiological, molecular and differential timing or level of expression of resistance genes (Bowles, 1993; Skriver and Mundy, 1990). Moore *et al.* (2003), reported elevated expression of two antifungal chitinases, pCh2 and pCh11 in damaged maize grains that were colonised by *A. flavus*. These enzymes were detected in the aleurone of maize grains and their activity peaked 36 days after pollination. The recognition of *A. flavus* by maize cells in contact with the pathogen and the subsequent transcriptional activation of defence signalling system constitute the first line of defence and response to infection (Skriver and Mundy, 1990). The WRKY transcription factors involved in regulating defence responses in developing maize kernels have been reported to be significantly upregulated by *A. flavus* inoculation in a resistant maize line TZAR101 (Fountain *et al.*, 2015). An ortholog of one WRKY gene, ZmWRKY53 in *Arabidopsis*, AtWRKY33, was demonstrated to function in necrotrophic pathogen defence responses and regulating chitinase and peroxidase gene expression. Magbanua *et al.* (2013) reported less colonisation of maize cob tissue of the resistant inbred Mp313e inoculated with a GFP expressing strain of *A. flavus,* compared to cobs of the more susceptible genotype SC212. Restricted *A. flavus* growth in Mp313e was attributed to the presence of highly cross-linked lignin found in in this line Mp313e and not SC212. In this study, we observed restricted fungal colonisation and establishment on some resistant maize lines, however, more studies are needed to identify metabolites in these lines and establish the link to resistance.

Maize germplasm accumulating low fungal biomass, but high aflatoxin levels would signify fungal infection, colonisation and establishment in infected kernels only; consistent with hypothesis 2. In this scenario, the maize seed would produce barriers that restrict further infection of neighbouring kernels, thus confining the fungus to infected kernels. However, within the infected kernel, the fungus would be able to establish and produce aflatoxins.

This scenario necessitates host genes inhibiting subsequent colonisation of adjacent kernels and has been reported before (Brown *et al.*, 1999; Ortega-Beltran *et al.*, 2014). In this study, none of the inbred lines tested conformed to this scenario. This is probably because of the small sample size used or possibly because this kind of scenario is uncommon. However, it has been reported that resistance to *A. flavus* and aflatoxin accumulation are not one and the same, and are probably mediated by different genetic factors (Hamblin and White, 2000), giving credence to the existence of such germplasm. Nevertheless, breeding programs to improve aflatoxin resistance in maize should incorporate aflatoxin quantification as a tool when selecting resistant germplasm. This type of resistance would probably not be ideal as many factors, e.g. damage from insects, birds, rodents, etc., can provide infection avenues for *A. flavus* and subsequent high aflatoxin levels. Therefore, aflatoxin quantification should be mandatory in breeding for aflatoxin and resistance based on kernel rot should not be considered as an option.

Resistance to aflatoxin could result from expression of host genes that interfere with the aflatoxin biosynthetic pathway. Under this scenario, *A. flavus* infection, colonisation and establishment resembles that of a susceptible interaction, but aflatoxin production and accumulation is severely reduced. The presence of high fungal biomass and low aflatoxin levels would represent this scenario and suggests the presence of host genes that block some key steps in the aflatoxin biosynthetic pathway. Of the three resistant maize inbred lines, only MR2 had significantly more fungal biomass in inoculated than in uninoculated ears, but aflatoxin levels were comparable to other resistant lines, and may express such genes. Using *A. flavus* mutant expressing GUS gene, Brown *et al.* (2001) observed that fungal growth in kernels of the resistant inbred line 1368 were as high as those observed in susceptible inbreds 603 and KU, but this line accumulated low levels of aflatoxins. In another study, Brown *et al.* (1995) observed greater fungal colonisation when kernels of some resistant lines were wounded before inoculation; however, and irrespective of wounding, aflatoxin levels remained the same. This could possible signify the presence of resistance mechanism(s) directly inhibitory to aflatoxin biosynthesis rather than to fungal infection. However, such a scenario would not be ideal for a breeding program, as ear rot could still be a problem in these lines, thus affecting the quality of kernels produced, resulting in poor quality kernels and yield loss.

A susceptible or compatible interaction is manifested by the presence of high fungal biomass and high aflatoxin levels. This scenario reveals the absence of host genes that impede fungal infection, colonisation and proliferation; and absence of host genes that interfere with aflatoxin biosynthetic pathway. This is the most common scenario observed in maize (Brown *et al.*, 1995; Guo *et al.*, 1995), and has also been observed in *A. flavus*/peanut interactions. Wang *et al.* (2016) showed that genes associated with mycelial growth, conidial development and aflatoxin biosynthesis were up-regulated in aflatoxin susceptible peanut lines compared with resistant lines, and showed that aflatoxin production was correlated to fungal establishment and proliferation. All susceptible lines used in this study conform to this scenario, and the most susceptible maize inbred line in this study, MS1, could be used as a check in future studies to identify lines with resistance to aflatoxin accumulation. Furthermore, it could be used to develop biparental populations that can be used to study the genetics of aflatoxin and *A. flavus* resistance in maize.

In this study, we identified maize inbred lines that appear to possess genes for restricting *A. flavus* colonisation and for impeding aflatoxin production; these lines are useful candidates for use in breeding programs to improve aflatoxin resistance, and to combine different types of resistance. The two mechanisms of resistance identified in this study should be the target of further research to elucidate the genetics of resistance in this set of inbred lines and develop molecular markers and protocols for marker assisted selection. We further confirmed the utility and suitability of an in-house ELISA assay as a low-cost tool amenable to large scale application in a breeding program for improving aflatoxin resistance.

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