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Assessing Genotype-By-Environment Interactions in *Aspergillus* Ear Rot and Pre-Harvest Aflatoxin Accumulation in Maize Inbred Lines

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Received: 14 November 2017; Accepted: 18 December 2017; Published: 20 December 2017

Abstract: *Aspergillus flavus*, causal agent of the Aspergillus ear rot (AER) of maize, also produces aflatoxins that cause aflatoxicosis in humans and livestock. Ten maize inbred lines were evaluated in replicated trials in two aflatoxicosis outbreak hot spots in Kenya and in three maize-growing areas in South Africa for resistance to AER, *A. flavus* colonization, and pre-harvest aflatoxin accumulation during the 2012/2013 growing season. AER severity was measured by visual assessment, while *A. flavus* colonization and aflatoxin content were quantified by real-time polymerase chain reaction (PCR) and liquid chromatography tandem mass spectrometry, respectively. Genotype by environment interaction (GEI) was determined using analysis of variance (ANOVA), additive main effects and multiplicative models (AMMI), and genotype plus by environment (GGE) biplot analyses. Stability of genotypes was evaluated using AMMI analysis. AER severity and fungal colonization significantly ($p < 0.001$) varied between genotypes. GEI influenced the severity of AER symptoms and aflatoxin accumulation significantly ($p < 0.001$), while fungal colonization was not affected. The inbred lines response was consistent for this trait in the test environments and was thus considered a desirable measure to indicate maize lines with a high risk of aflatoxin accumulation. CML495, CKL05019, LaPosta, and MIRTC5 were the least diseased lines, with the lowest aflatoxin contamination and a stable phenotypic response across the environments. Kiboko was determined as the ideal representative test environment, with discriminative ability of the genotypes for selection of the desired stable responses of the three traits.

Keywords: GGE biplot; environment; GGI; AMMI stability index; aflatoxins; *Aspergillus flavus* colonization; *Aspergillus* ear rot

1. Introduction

Aflatoxin is a toxic and carcinogenic compound produced mostly by the fungus *Aspergillus flavus*. Aflatoxins can be found at detrimentally high concentrations in maize grain and other cereals in the tropics due to the prevailing conducive environment for fungal growth and toxin production. Human exposure in Africa is more serious than in other continents because the crops that are highly susceptible to infection by the fungus are the primary staple. Furthermore, the region lacks the capacity to monitor maize grown and consumed at a local level [1]. As the levels that cause regulatory concerns are continually being lowered, it is increasingly difficult for this continent to produce maize that can

meet the accepted levels of aflatoxins [2]. Thus, it is necessary to develop methods that will prevent aflatoxin production in susceptible crops. Several approaches ranging from good agricultural practices through biocontrol to decontamination and use of binders are being exploited to manage exposure levels [1,3,4]. However, breeding of maize varieties for *A. flavus* resistance is considered an effective and environmentally safe method for controlling contamination, and successful maize cultivars need to be adapted to a range of environments. Genotype × environment interaction (GEI) is commonly observed as the differential ranking of genotypes across environments, enabling precise prediction on genotype potentiality and environmental influences on them [5]. The objective of this study was to evaluate maize inbred lines for resistance to Aspergillus ear rot (AER), *A. flavus* colonization, and pre-harvest aflatoxin accumulation under different environmental conditions.

2. Results

2.1. Aspergillus Ear Rot Severity

Maize inbred lines varied significantly in their expression of AER symptoms in Kiboko (Kenya; $p < 0.01$), Makhatini (South Africa; $p < 0.05$), and across the five test environments ($p < 0.01$), with highly significant line by environment interaction ($p < 0.01$). Lines with the lowest AER symptoms in Kiboko were CKLO5022 (nil), CML 247 (nil), P502c2-185-3-4-2-3-B-2-B*5 (1.8%), LaPosta (3.4%), and MIRTC (4.6). In Makhatini, CML247 (nil), CKLO5015 (0.1%), CML264 (0.2%), and CML495 (0.3%) were least diseased (Table 1). The most diseased lines were CML264 (35.6%), CKLO5003 (18.9%), and CKL05019 (16.7%). Additive main effects and multiplicative interaction (AMMI) analysis of variance (ANOVA) showed that AER symptoms were significantly ($p < 0.001$) influenced by the environment (E), genotype (G), and their interaction (GEI). The environment accounted for the largest variation and explained 32% of the variation observed, followed by G (3.98%) and GEI (3.83%). The first and the second interaction principle component axis (IPCA) were significant, with IPCA1 accounting for 8.2% of the total G × E variation and IPCA2 for 3.2% of the variation observed. Furthermore, IPCA1 sum of squares (57.11) was greater than the second (18.41), indicating the presence of differences in ARE severity of the genotypes as a result of GEI. The stability response of inbred lines varied across the five environments, with ASV ranging from 0.39 to 3.95 with a mean of 1.76. Lines CML495 (0.39), MIRTC (0.41), LaPosta (0.66), CKL05003 (1.14), CKL05015 (1.25), and P502c2 (1.86) were the most stable in their response to AER across the environments. On the other hand, CML264 (3.95), CKL05022 (3.32), and CML247 (2.44) were least stable.

Table 1. Aspergillus ear rot severity for maize inbred lines grown in Kenya and South Africa during 2012/2013 maize growing seasons.

Inbred Line Name	*Aspergillus* Ear Rot Severity (%) [1,2]					
	Katumani	Kiboko	Makhatini	Potchefstroom	Vaalharts	Across Environment
CKL05003	4.9 a	18.9 a–c	1.5 ab	0.1 a	1.4 a	0.2 a
CKL05015	6.2 ab	8.5 a–d	0.1 c	0.1 a	0.6 a–c	0.1 a–c
CKL05019	0.0 c	16.7 ab	1.6 ab	0.0 a	0.1 bc	0.0 bc
CKL05022	0.3 bc	0.0 e	4.7 a	0.0 a	0.2 bc	0.1 a–c
CML247	1.9 a–c	0.0 e	0.0 c	0.0 a	0.0 c	0.0 c
CML264	0.0 c	35.6 a	0.2 bc	0.1 a	0.2 bc	0.1 a–c
CML495	0.2 bc	6.0 b–d	0.3 bc	0.0 a	0.4 bc	0.0 bc
La Posta	0.2 bc	3.4 c–e	2.2 ab	0.0 a	0.0 c	0.0 c
MIRTC5	1.3 bc	4.6 b–e	1.1 a–c	0.0 a	0.7 ab	0.1 a–c
P502c2	1.0 bc	1.8 de	2.3 ab	0.3 a	0.0 c	0.3 ab
Mean	1.6 bc	9.6 a	1.4 b	0.1 d	0.4 cd	-

[1] Percentage of maize ears covered with visual symptoms of Aspergillus ear rot; [2] means followed by the same alphabetical letter in each column are not significantly different according to the Student's *t*-test of least significant differences ($p \leq 0.05$).

The genotype main effect and GEI on AER was visualized by GGE biplot analyses (Figure 1). The first two IPCs accounted for 85.51% (IPC1 = 66.29% and IPC2 = 19.22%) of the total variability of AER caused by G and GEI. MIRTC5 (#15), LaPosta (#14), CML495 (#12), and P502c2 (#16) were the most resistant to AER and the most stable. Line CKL05022 (#6) was resistant to AER but unstable in its expression of AER symptom across the environments (Figure 1A). Lines CKL05003 (#3), CKL05015 (#4), CML264 (#9), and CKL05019 (#5) were the most affected by AER and least stable. In Figure 1B, the center of the concentric circles of the genotype-comparison biplot is where an ideal genotype (resistant to AER) is located. Line CKL05022 (#6) is the most preferred across all environments followed by P502c2 (#16), MIRTC5 (#15), LaPosta (#14), and CML495 (#12). The results of the genotype-comparison biplot (Figure 1B) agree with those of genotype-ranking biplot in Figure 1A.

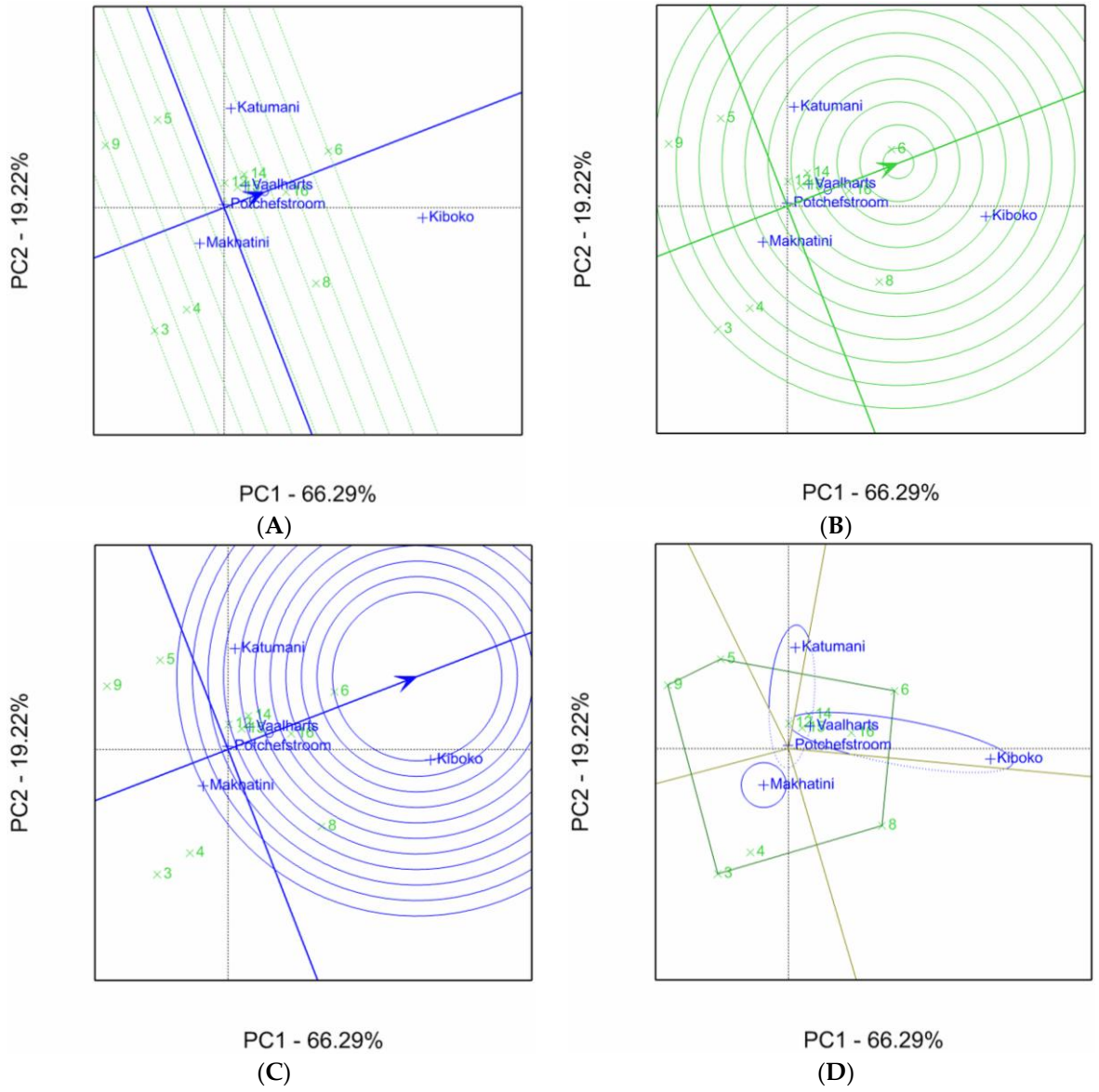

Figure 1. Genotype plus genotype by environment interaction biplot for Aspergillus ear rot severity in 10 maize inbred lines tested, based on environment-focused scaling for (**A**) genotype ranking, (**B**) genotype comparison, (**C**) environment comparison, and (**D**) the polygon view-exhibiting mega-environments.

Similar to the ideal genotype, it is possible to rank test locations according to their discriminating ability and suitability of representation. Centers of the concentric circles in Figure 1C are where

an ideal location should be. Thus, Kiboko was the best test environment that imposed the most separation among genotypes. Environments closer to the origin of the biplot suggest that the variation is relatively low; therefore, the separation among genotypes will be low as well. This is the case for Vaalharts, Potchefstroom, and Makhatini (Figure 1C). The which-won-where polygon view of GGE biplot is shown in Figure 1D. Genotypes at the polygon vertices are the highest interactive and most affected positively or negatively. In our study, the genotype markers were CML264 (#9), CKL05019 (#5), CKL05022 (#6), CML247 (#8), and CKL05003 (#3). Line CKL05022 (#6) was the most resistant in Kiboko and Vaalharts forming a mega-E. Line CKL05003 (#3) was the most resistant in Makhatini, forming a second mega-E. Katumani and Potchefstroom formed a third mega-E but with no winning marker. Three other vertex genotypes, CKL05019 (#5), CML264 (#9), and CML247 (#8), were located away from all test environments and within sectors lacking an environmental marker, meaning that the genotypes were responsive in most test environments and their response to AER was not specific to a particular environment. The genotypes within the polygon were less responsive, compared to the vertex genotypes, within the sectors that they were confined. MIRTC5 (#15), LaPosta (#14), CML495 (#12), and P502c2 (#16) were less responsive compared to CKL05022 (#6) in Kiboko (Figure 1D).

2.2. Aspergillus Flavus Colonization

Grain colonization by *A. flavus*, as determined by the quantity of fungal target DNA, varied significantly within Kiboko ($p < 0.01$) and Makhatini ($p < 0.05$). Inbred line by environment interactions were significant ($p < 0.01$), with inbred lines CKL05019 (4.67), CML247 (4.27), CML495 (5.77), and MIRTC5 (6.03) being the most resistant to colonization in Kiboko in addition to CML264 (nil) and LaPosta (nil) in Makhatini (Table 2). Inbred lines CKL05003, CKL05015, CKL05022, and CML264 were the most colonized by *A. flavus*.

Table 2. *Aspergillus flavus* maize grain colonization of maize inbred lines grown in Kenya and South Africa during 2012/2013 maize growing seasons.

Inbred Line Name	*A. flavus* Target DNA (ng/μL) [1,2]					
	Katumani	Kiboko	Makhatini	Potchefstroom	Vaalharts	Across Localities
CKL05003	0.26 ab	53.4 a	0.01 b	0.44 ab	2.36 a	0.26 ab
CKL05015	0.06 b	14.87 ab	0.02 b	0.03 c	5.70 a	0.03 bc
CKL05019	0.11 b	4.67 b	0.00 b	0.08 bc	16.20 a	0.06 bc
CKL05022	0.08 b	12.07 ab	0.78 a	0.37 a–c	10.08 a	0.46 a
CML247	0.97 a	4.27 b	0.02 b	0.07 bc	6.21 a	0.21 a–c
CML264	0.07 b	11.10 ab	0.00 b	0.08 bc	7.60 a	0.05 bc
CML495	0.06 b	5.77 b	0.00 b	0.03 c	26.35 a	0.03 c
La Posta	0.15 b	10.53 b	0.00 b	0.05 c	2.23 a	0.05 bc
MIRTC5	0.08 b	6.03 b	0.01 b	0.46 a	10.41 a	0.23 a–c
P502c2	0.07 b	43.5 a	0.09 b	0.12 a–c	4.81 a	0.10 bc
Mean	0.19 c	16.62 a	0.09 c	0.17 c	9.19 b	-

[1] Absolute concentration of *A. flavus* target DNA; [2] means followed by the same alphabetical letter in each column are not significantly different according to the Student's *t*-test of least significant differences ($p \leq 0.05$).

GEI did not influence fungal DNA content among genotypes across the environments ($p = 0.40$). The ranking of genotypes according to their fungal DNA content did not vary between environments. This is, therefore, a desirable trait for use in selection of germplasm for breeding for resistance to fungal infestation, since the phenotypic performance of genotypes across environments is consistent. Significance of GEI makes it difficult in breeding, since phenotype is dependent on environment, and the mean quantification of this trait is not a sufficient and meaningful indicator. Only IPCA 1 was significant and accounted for 3% of the variation. The stability of the inbred lines to *A. flavus* colonization as determined by the ASV and ranged between 0.35 and 7.36, with a mean of 3.35. Lines CML264 (0.35), CKL05022 (0.45), LaPosta (1.46), and CKL05015 (1.80) were the most stable in their

response to fungal colonization across localities, while CKL05003 (7.36), P5o2c2 (6.91), CML247 (5.50), and CKL05019 (4.13) were the least stable.

Figure 2 shows G and GEI on *A. flavus* colonization, as visualized by GGE biplot analysis (Figure 2). The first two principal components accounted for 93.4% (IPC1: 88.6% and IPC2: 4.8%) of the total variation of fungal colonization. Lines CML495 (#12), CKL05019 (#5), and CML247 (#8) were the most resistant to fungal colonization across localities and also most stable (Figure 2A). Line LaPosta (#14) was also favorable being close to CML495 (#12). Lines P502c2 (#16) and CKL05003 (#3) were least resistant. Genotype comparison biplot identified CML247 (#8) as the most desirable genotype against fungal colonization followed by CKL05019 (#5), CML495 (#12), LaPosta (#14), and MIRTC5 (#15) (Figure 2B).

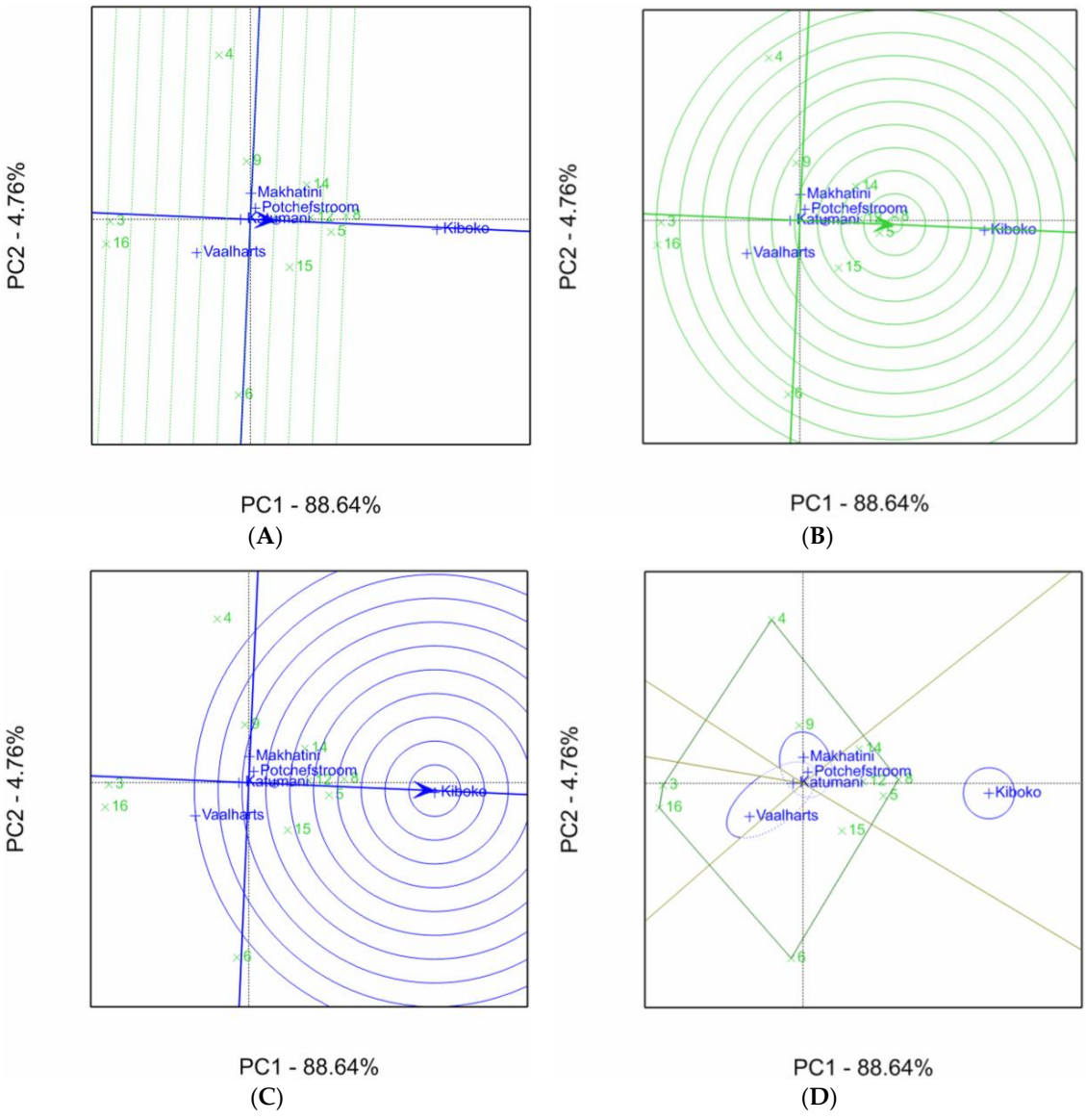

Figure 2. Genotype plus genotype by environment interaction biplot for *Aspergillus flavus* colonization in 10 maize inbred lines that were tested based on environment-focused scaling for (**A**) the genotype ranking, (**B**) genotype comparison, (**C**) environment comparison, and (**D**) the polygon view exhibiting mega-environments.

Environment-comparison biplot revealed Kiboko as the best test environment for this trait (Figure 2C). The which-won-where polygon in Figure 2D view identifies the genotype markers as

CKL05015 (#4), CML247 (#8), CKL05022 (#6), CKL05003 (#3), and P502c2 (#16). Three mega-E are demarcated. Line CML247 (#8) is the most resistant in Kiboko mega-environment. Lines CKL05019 (#5) and CML495 (#12) are also favorable in this environment, as they are close to CML247 (#8). Line LaPosta (#14) is within the polygon and also within the Kiboko sector, but it is far from the vertex, and so it is considered less responsive in this environment. The second mega-E consists of Makhatini, Potchefstroom, and Katumani with CKL05015 (#4) as the winning genotype. The third mega-E consists of Potchefstroom, Katumani, and Vaalharts with CKL05003 (#3) and P502c2 (#16) as the winning genotypes (Figure 2D).

2.3. Aflatoxin Accumulation in Maize Inbred Lines

Maize inbred lines accumulated significantly different levels of aflatoxins within Kiboko ($p < 0.05$) alone and across the five test environments ($p < 0.01$). The highly affected genotypes were P502c2 (7.1 µg/kg), CKL05003 (4.7 µg/kg), and CKL05015 (3.9 µg/kg), while the most resistant were CML247 (0.3 µg/kg), MIRTC5 (1.1 µg/kg), CML 264 (0.67 µg/kg), LaPosta (0.55 µg/kg), CML 495 (0.50 µg/kg), and CKL05019 (1.2 µg/kg) (Table 3).

Table 3. Aflatoxin accumulation in maize inbred lines grown in Kenya and South Africa during 2012/2013 maize growing seasons.

Inbred Line Name	Total Aflatoxins (µg/kg) [1,2]					
	Katumani	Kiboko	Makhatini	Potchefstroom	Vaalharts	Across localities
CKL05003	0.00 b	4.72 ab	0.00 a	0.00 ab	0.00 a	0.001 a
CKL05015	0.00 b	3.86 a–c	0.00 a	0.00 ab	0.06 a	0.000 ab
CKL05019	0.00 b	1.15 cd	0.00 a	0.00 b	0.03 a	0.000 ab
CKL05022	0.00 b	2.12bd	0.00 a	0.00 b	0.07 a	0.001 ab
CML247	0.19 a	0.30 d	0.00 a	0.00 b	0.07 a	0.000 ab
CML264	0.00 b	0.67 d	0.00 a	0.00 b	0.02 a	0.000 ab
CML495	0.00 b	0.50 d	0.00 a	0.00 b	0.00 a	0.000 b
La Posta	0.00 b	0.55 d	0.00 a	0.00 b	0.00 a	0.001 ab
MIRTC5	0.00 b	1.10 cd	0.00 a	0.01 a	0.05 a	0.000 ab
P502c2	0.00 b	7.09 a	0.00 a	0.00 b	0.02 a	0.000 ab
Mean	0.02 b	2.20 a	0.00 b	0.00 b	0.03 b	-

[1] Total aflatoxins content; [2] means followed by the same alphabetical letter in each column are not significantly different according to the Student's *t*-test of least significant differences ($p \leq 0.05$).

The AMMI analysis of variance for total aflatoxins in grains was significantly ($p < 0.001$) influenced by G, E, and GEI, accounting for 3.91%, 83.61%, and 4.23% of the total variation observed, respectively (Table 4). Further analysis of GEI revealed that only IPCA 1 was significant and accounted for 12.6% of the variation influencing fungal contamination. This implied that the interaction of the genotypes with five environments was predicted by the first component of genotypes and environments. The stability of inbred line response to aflatoxin accumulation, as determined by the ASV, ranged between 1.18 and 169.12 with a mean of 78.80. The high ASV values observed with this trait indicated the wide variability in aflatoxin accumulation in grains. Inbred line CKL05022 (1.18) was the most stable genotype, with ASV value closer to zero, followed by MIRTC5 (28.47) and CKL05019 (32.66). Inbred lines P502c2 (169.12) and CKL05003 (133.2) were the least stable.

Table 4. Correlations between *Aspergillus* ear rot severity (%), *A. flavus* colonization, and total aflatoxins content at field localities in Kenya and South Africa and across localities.

Locality	Country	AER Severity (%) versus Total Aflatoxin (µg/kg)	AER Severity (%) versus *A. flavus* DNA Content (ng/µL)	*A. flavus* DNA Content (ng/µL) versus Total Aflatoxin (µg/kg)
Makhatini	South Africa	0.200 ^{NS} (p = 0.289)	0.149 ^{NS} (p = 0.433)	0.321 ^{NS} (p = 0.084)
Potchefstroom	South Africa	0.055 ^{NS} (p = 0.774)	−0.33 ^{NS} (p = 0.864)	0.328 ^{NS} (p = 0.077)
Vaalharts	South Africa	0.094 ^{NS} (p = 0.623)	0.290 ^{NS} (p = 0.120)	0.237 ^{NS} (p = 0.207)
Katumani	Kenya	0.027 ^{NS} (p = 0.887)	0.066 ^{NS} (p = 0.073)	0.951 ** (p = 0.000)
Kiboko	Kenya	0.010 ^{NS} (p = 0.957)	0.243 ^{NS} (p = 0.196)	0.772 ** (p = 0.000)
Combined	-	0.257 ** (p = 0.002)	0.176 * (p = 0.031)	0.761 ** (p = 0.000)

Significance: NS means not significant; * means p-value less than 0.05; ** means p-value less than 0.01.

The effect of G and GEI on aflatoxin accumulation in maize inbred lines was visualized by GGE biplot analysis (Figure 3). IPC1 (99.31%) and IPC2 (0.56%) accounted for a total of 99.87% of the variation observed. In this study, ideal genotypes were CKL05019 (#5), MIRTC5 (#15), and LaPosta (#14). Lines CML495 (#12) and CML264 (#9) are also desirable given that they are close to LaPosta (#14). Similar results were obtained from Genotype Comparison biplot, which grouped LaPosta (#14), CML495 (#12), and CML264 (#9) as the best selection for resistance to aflatoxin accumulation and stability (Figure 3B). Lines CKL05019 (#5) and MIRTC5 (#15) were also desirable, as they were close to the center of concentric circles (Figure 3B). Figure 3C shows the graphical evaluation of the test environments for their power to discriminate between the genotypes. Kiboko was the ideal test environment, since it was at the center of the concentric circle and also close to the average environment axis (AEA) (Figure 3C).

Which-won-where' biplot for aflatoxin accumulation in grains is represented in Figure 3D. The equality lines divided the biplot into five sectors, of which two retained all three locations. One mega-E was Kiboko with CML247 (#8) as the ideal genotype, while the other mega-E was made up of the other four locations with P502c2 (#16) as the ideal genotype. However, the latter mega-E was at the center of the origin of the biplot, meaning that it had low discriminative ability. There was no environmental marker within the sector that had CML495 (#12) and CML264 (#9), as the vertices genotype markers, meaning that these genotypes were highly resistant to aflatoxin accumulation across all the environments tested. Lines CKL05019 (#5) and MIRTC5 (#15) were within the polygon of this sector and close to the vertex genotypes, and thus were desirable genotypes that were also not environmentally specific.

Significant positive correlations were obtained between AER severity, fungal colonization, and total aflatoxins content across the test environments (Table 4). Positive correlations were observed within Katumani and Kiboko between total aflatoxins and total fungal DNA content in the maize. This result further supports fungal colonization as a useful indicator for prediction of aflatoxin accumulation, since Kiboko emerged as the best test environment.

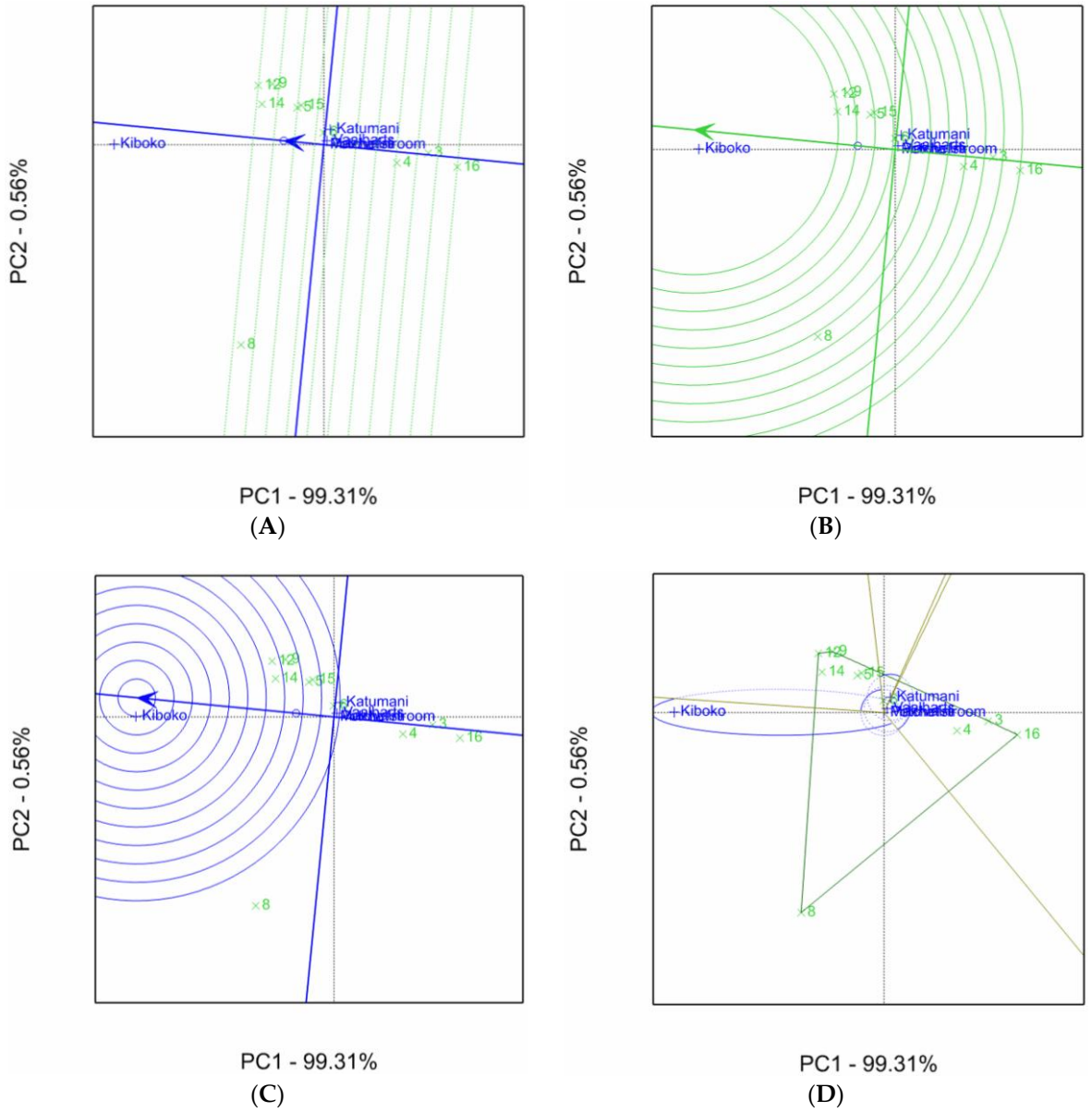

Figure 3. Genotype plus genotype by environment interaction biplot for aflatoxin accumulation in 10 maize inbred lines tested, based on environment-focused scaling for (**A**) the genotype ranking, (**B**) genotype comparison, (**C**) environment comparison, and (**D**) the polygon view exhibiting mega-environments.

3. Discussion

This study investigated maize inbred lines for resistance to AER and aflatoxin accumulation under diverse environments. The data collected was treated to ANOVA, AMMI, and GGE biplot analyses, which grouped the genotypes into three categories. The first category represented by lines CKL05003 and CKL05015 showed consistent stable high AER severity, *A. flavus* colonization, and aflatoxin accumulation across the environments. These lines are thus good sources of genetic material (a) for breeding where inclusion of heterozygotes are required [6]; (b) that can help in identifying differences between resistant and susceptible lines in development of gene and gene based markers associated with resistance of the three traits [7]; and (c) for inclusion as positive controls in breeding trials. The second category consists of inbred lines that showed low AER but were highly colonized with *A. flavus* and contained comparatively high aflatoxin levels. Lines P502c2 and

CKL05022 belonged to this category. This observation suggests the effect of environment in triggering aflatoxin accumulation in maize and thus the subjectivity of using AER symptoms alone for predicting aflatoxin contamination in maize. The third category consists of genotypes, which showed relatively low rating of all the three traits across the environments and were stable in their phenotypic responses. These desirable lines are CML495, LaPosta, CKL05019, and MIRTC5. Moreover, line CML495 was also shown to be resistant to *Fusarium verticillioides*, which causes Fusarium ear rot of maize, and the mycotoxins (fumonisins) it produces [8]. This line, therefore, represents a valuable source of resistance to multiple ear rot fungi and their mycotoxins at a time when the co-occurrence of mycotoxins is becoming more prevalent.

Genotype and GEI are considered relevant to meaningful cultivar evaluation and should be considered simultaneously when making selection decisions [9]. The stability of phenotypic responses of genotypes is also important, as traits are affected by environmental fluctuations [10]. The presence of GEI has been reported to complicate breeding, testing, and selection of superior genotypes, since it reduces the association between genotype and phenotype, and this leads to change in relative ranking and stability differences of genotypes across environments, diminishing the genetic progress expected from plant breeding [11–13]. In this study, AMMI analysis revealed high GEI for AER and aflatoxin accumulation. Fungal colonization measured by target DNA content, however, was not affected by GEI. Analysis of variance on the other hand revealed GEI for all the three traits. The significant correlations observed between *A. flavus* target DNA and total aflatoxin content of maize suggests that this trait is reliable for quantifying susceptibility of genotypes to fungal infection and possible eventual risk to aflatoxin accumulation compared with AER severity. Significant, though low, correlations were found between AER/fungal content and AER/aflatoxin content, demonstrating the inconsistency of the relationship between AER/fungal target DNA content and AER/aflatoxins. Mideros [14] reported the unreliability of using severity of AER as a measure of aflatoxin accumulation.

Environmental effect was significant for all the three traits measured and explained the predominant source of variation observed for each trait. Yan [15], however, pointed out that E was irrelevant for cultivar evaluation. Yan [15] and Yan and Kang [9] recommended the use of G and GEI, whose responses are more stable though relatively small. Which-won-where analysis involved G and GEI producing a pattern, which identified specific genotypes to their suitable mega-environment. Lines whose responses are unstable across environments may win for specific environments and also provide germplams, which can be included in breeding programs for specific traits. Lines CML264, CKL05022, and CML247, for example, showed low disease symptoms across the environments but were unstable. Line CML247 was identified as an ideal genotype for Kiboko environment with regard to resistance to fungal colonization and aflatoxin accumulation. Similarly, line CKL05022 was an ideal genotype resistant to AER in Kiboko and Vaalharts. Several studies have identified such useful germplasm and recommended further testing of indifferent locations and environments to evaluate potential usefulness in breeding for resistance to aflatoxin accumulation [16–18].

Kiboko emerged as the best test environment. Kiboko formed mega-E with Vaalharts for AER evaluation and with Vaalharts, Makhatini, and Potchefstroom for aflatoxin accumulation. However, in both cases, all the other environments scored low in discriminative ability and are thus not recommended for evaluation of the three traits. These findings support Ayahlneh [12], who reported that the knowledge of GEI could help to minimize the cost of wider genotype evaluation by eliminating unwanted testing sites.

4. Materials and Methods

4.1. Germplasm and Field Experimental Design

Ten maize inbred lines from the International Maize and Wheat Improvement Centre (CIMMYT) in Kenya were evaluated for AER and grain colonization by *A. flavus*, as determined by quantity of fungal target DNA and aflatoxin accumulation (Table 5). The inbred lines included four of mid-altitude

and six of lowland tropical adaptation. The experimental design for all trials was conducted in a randomized complete block design with three replications. Each entry was planted in 10-m long plots, spaced 0.90 m apart. Each plot consisted of 33 plants, spaced 0.30 m apart. The trials were established in 2012/2013 planting seasons. Four border rows of a commercial hybrid were planted around the experimental plot. Standard agronomic practices including application of irrigation water and fertilizer were followed at each location.

Table 5. Maize inbred lines, their origin, and their characteristics.

Name/Pedigree	Line Code	Characteristics
CKL05003	3	MA adaptation, turcicum leaf blight (TLB) and gray leaf spot (GLS) tolerant, semi-dent, white grain
CKL05015	4	MA adaptation, TLB and maize streak virus (MSV) tolerant, semi-flint
CKL05019	5	MA adaptation, intermediate maturity, TLB and GLS tolerant, flint, white
CKL05022	6	MA adaptation, TLB and GLS tolerant, flint, white
CML247	8	Lowland tropical (LT) adaptation, GLS tolerant, semi-dent, white
CML264	9	LT adaptation, flint, white
CML495	12	LT adaptation, flint, white
La Posta Seq C7-F103-2-1-1-1xMIRTC5 Bco F80-4-2-1-1-1-3-1-B-B-B	14	LT adaptation, drought tolerant background
MIRTC5 Bco F78-2-2-1-1-1xDERRc2 15-3-7-1-1-B-B-B-B	15	LT adaptation, semi-flint, white
P502c2-185-3-4-2-3-B-2-B-B-B-B-B-B	16	LT adaptation, semi-dent, white

Designated line code for the inbred lines used in this study.

4.2. Field Locations

The evaluation trials in Kenya were done at Kenya Agricultural and Livestock Research Organisation (KALRO) stations at Katumani (1°35′ S, 37°14′ E, 1600 m above sea level) and Kiboko (37°75′ E, 2°15′ S; 975 m above sea level). These stations are situated in Machakos County in the semi-arid eastern Kenya, which is considered an aflatoxin hot spot (18). The region has bimodal rainfall with long rains falling in March to May and short rains from October to December/January. Field trials in South Africa were conducted at Potchefstroom (grid reference 26°73′ S, 27°07′ E; altitude, 1349 m) in the North West province, Vaalharts (grid reference 27°95′ S, 24°83′ E; altitude, 1180 m) in the Northern Cape province, and in Makhatini (grid reference 22°39′ S, 32°17′ E; altitude, 77 m) in the KwaZulu-Natal province.

4.3. Inoculum Production and Field Inoculation

Three *A. flavus* isolates (V201365, V100130, and V100095) from Machakos County were used as inoculum for the field trials conducted in Kenya. These isolates are known to produce high levels of aflatoxins in maize grain [19,20]. For inoculation of the field trials in South Africa, three toxigenic strains of *A. flavus* (MRC 3951, MRC 3952, and MRC 3954) were obtained from the Medical Research Council's Programme on Mycotoxins and Experimental Carcinogenesis unit (MRC-PROMEC, Tygerberg, South Africa). The inoculum for field trials was prepared according to Okoth et al. [21].

The primary maize ear on each plant was inoculated using the silk channel method [22]. Inoculation was done after at least 50% of the individual plants in the plot had emerged silks, and when the silk length was at least 2.5 cm long. The ears were inoculated once. Two mililiter of the well-mixed

conidial suspension was slowly injected into each maize ear through the silk channel using a 10-mL syringe and sterile needle (gauge 18). Due to the diversity between inbred lines in terms of maturity, inoculation was done at different times for the lines. All inoculated ears were labelled for identification at harvest.

4.4. Assessment of Aspergillus Ear Rot (AER) Rating

Inoculated ears in each plot were harvested by hand at 12–18% moisture. The percentage of AER symptoms per ear was visually estimated using a method adapted from Henry et al. [23]. The percentage ear rot assigned per ear estimated the area of a rotten ear whereby 0% = no ear rot symptoms, 25% = rot covering a quarter of the ear, 50% = rot covering half of the ear, 75% = rot covering three quarters of the ear, and 100% = rot covering the entire ear. The average AER per plot was then calculated. The ears from each plot were bulked and hand shelled. The grains were then thoroughly mixed and a 250-g sample collected, milled, and stored at 4 °C until aflatoxin extraction was performed.

4.5. Quantification of Aspergillus Flavus in Maize Grain

Freeze dried mycelia of the *A. flavus* isolates (V201365, V100130, and V100095), as well as the maize samples, were preserved at −20 °C until genomic DNA was extracted. DNA was extracted as described by Boutigny et al. [24] using DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA), including an initial DNA isolation step consisting of CTAB/PVP and an additional phenol extraction steps for fungal cultures performed prior to the use of the commercial kit. These modifications ensured maximum DNA recovery of superior quality. DNA from water-inoculated maize grain was also isolated and served as target-free host DNA for the preparation of matrix (maize)-matched standards.

After extraction, approximately 150 µg of each fungal and grain DNA sample was cleaned to remove impurities using OneStep PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA, USA). The quantity and purity of the DNA yield was determined with a NanoDrop ND-1000 spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa) and adjusted to 10 ng μL^{-1}. The same procedure was applied to DNA that was used to generate a standard curve.

The fungal target DNA in maize samples was determined by quantitative polymerase chain reaction (qPCR) according to Rose et al. [8]. To quantify *A. flavus* target DNA in inoculated maize samples, the qPCR assays contained each sample in duplicate, and *A. flavus* DNA (4^2–4^3 and 4^4-fold dilution of pathogen DNA in maize DNA free of *A. flavus* infection) of a known concentration was analyzed in triplicate. All the assays contained a no template control (NTC). Using the matrix specific standard curves, the Ct values were transformed into DNA concentrations using the Rotor-Gene™ 2.0.2.4 software (Qiagen, Valencia, CA, USA).

4.6. Toxin Analysis

Aflatoxin content in maize inbred lines was determined by the dilute-and-shoot method using liquid chromatographic tandem mass spectrometry (LC-MS/MS). Aflatoxins were extracted from sub-samples of 5 g each according to Rose et al. [25] and submitted to the Central Analytical Facility (CAF) at Stellenbosch University, South Africa for the quantification of AFB_1, AFB_2, AFG_1, and AFG_2. A mixture of aflatoxins B and G, guaranteed 95% pure, was purchased from Sigma Aldrich (St. Louis, MO, USA) to serve as standards. Ten percent of the total number of samples was evaluated in triplicate (3 samples per plot) to determine the sample variation for aflatoxin content, using LC-MS/MS. These were technical replicates to determine the within assay variation. A dilution series, ranging from 0.15×10^{-4} to 0.38 µg/kg for AFB_1 and AFG_1 and 0.44×10^{-5} to 0.11 µg/kg for AFB_2, and 0.83×10^{-5} and 0.08 µg/kg for AFG_2, was analysed with field trial samples. Each standard and sample (5 µL) was injected into the LC-MS/MS system, and samples with results above the calibration curve limit were diluted with 70% methanol and re-analysed.

4.7. Data Analysis

The data collected from the visual assessment of AER symptoms, *A. flavus* target DNA determination, and aflatoxin accumulation was subjected to univariate Analysis of Variance (ANOVA), employing the Generalized linear model (GLM) procedure of SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). The student's *t*-test, which determines least significant difference (LSD) between treatment means, was calculated at a 95% confidence interval. Non-parametric Pearson correlation coefficients were determined to assess the relationships between the traits using the CORR procedure in SAS, employing the log-transformed [ln(y + 1)] means of all variables.

The data was further subjected to additive main effects and multiplicative interaction (AMMI) analysis of variance [26] using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). The genotype by environment (G × E) interactions were partitioned amongst the first and second interaction principal components axes (IPCA) and the residual. The first principle component (IPC1), located on the *X*-axis, indicates the level of resistance where inbred lines with higher IPC1 values (positive or negative) are considered low risk to AER disease severity, *A. flavus* colonisation, or aflatoxin accumulation. The second principle component (IPC2), located on the *Y*-axis, represents performance stability of genotypes where IPC2 values near zero demonstrate greater adaptability of genotypes to different environments [8].

The stability of the genotypes across locations was determined by the AMMI stability values (ASV) [26], which are based on the first and second IPCA scores of the AMMI model for each genotype. The distance from a genotype's coordinate point to the origin in a two-dimensional scatter diagram determines the ASV value. Therefore, genotypes with the lowest ASV values exhibit the shortest projection from the biplot origin and are considered the most stable. Furthermore, the genotypic means relative to the principal components were graphically represented in GGE (genotype + genotype by environment) biplots. The GGE biplots were generated in GenStat 15th edition [27] by using the singular value decomposition (SVD) of IPC1 and IPC2, according to Yan [15]. The GGE biplot graphically represented the genotype main effect (G) and the genotype by environment (G × E) interaction [14,28]. The method is based on evaluating genotypes, firstly by considering only the effects of the genotype (G) and G × E as significant, while simultaneously taking these variables (G + GE) into consideration. Secondly, it enabled the evaluation and representation of genotypes in different environments using the biplot technique. Mega-environments (mega-E) were determined on the "ideal genotype for a particular environment" or the "which-won-where" approach. This approach determined the best performing genotypes shared in the same environments. An ideal genotype is a high performer with high stability across environments [29].

The estimation of resistance and stability of genotypes to AER, *A. flavus* colonisation, and aflatoxin accumulation was evaluated by an average environment coordination (AEC) method [15,16,28]. Stability of each genotype was explored by its projection onto the line drawn through the average environment and the biplot origin, the average environment axis (AEA; *X*-axis). The AEA arrow indicates the positive end of the axis ranking genotypes according to their performance. Resistance increases in the direction of the arrow. The smaller the absolute length of projection of a genotype on either side away from the biplot origin, the more stable it is. Longer projections are indicative of greater G × E interaction (GEI) and thus of reduced stability. Genotypes were projected on the AEA and ranked on resistance (low AER, *A. flavus* target DNA, aflatoxin) and stability. The average ordinate environment ((AEO), perpendicular to AEA) or stability axis (*Y*-axis) divides the genotypes into above-average resistance (to the right of the AEO) and below-average resistance (to the left of the AEO).

The IPC1 and IPC2 were also used to obtain the ideal test environment, characterized by large IPC1 scores (more power to discriminate genotypes in terms of the genotypic main effect) and small (absolute) IPC2 scores (more representative of the overall environments) [30]. GGE biplots were constructed with genotype-focus and symmetrical scaling.

5. Conclusions

The contamination of maize with aflatoxins remains a major concern for Africa's food security. Breeding for resistance is still considered to the best strategy currently available to lower aflatoxin accumulation in maize. Multi-environment analysis is useful in the evaluation of crop cultivars, as it enables selection of superior genotypes and eliminates unwanted test environments. In this study, Kiboko was the best test environment with high discriminative ability. Fungal DNA content in maize grain emerged as the stable trait to predict aflatoxin accumulation in maize. Furthermore, we identified inbred lines CML495, CKL05019, LaPosta, and MIRTC5 as the most adaptable to the test environments and resistant genotypes for the three traits tested. These germplasm can be included in maize breeding programs for developing resistant lines to AER and aflatoxin accumulation.

Acknowledgments: The MAIZE Competitive Grants Initiative, International Maize and Wheat Improvement Centre (CIMMYT), and CGIAR, the National Commission for Science, Technology and Innovation (NACOSTI) of Kenya, the South African Maize Trust and the National Research Foundation (NRF) of South Africa (South Africa—Kenya Research Partnership Programme Bilateral; RPPB13102856988) are acknowledged for funding this research. We also thank D. Makumbi from CIMMYT, Kenya for providing the inbred line seed; and M. van der Rijst and M. Booyse from ARC-Infruitec, South Africa for statistical analyses.

Author Contributions: A.V. and S.O. conceived and designed the experiments; L.J.R., S.O., A.O., H.S., and N.E.I.N. conducted all field trials and generated the data; S.O. and L.J.R. analy zed the data; S.O and L.J.R. wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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