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Determining resistance to *Fusarium verticillioides* and fumonisin accumulation in African maize inbred lines resistant to *Aspergillus flavus* and aflatoxins

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Abstract *Fusarium verticillioides* and *Aspergillus flavus* cause Fusarium ear rot (FER) and Aspergillus ear rot (AER) of maize, respectively. Both pathogens are of concern to producers as they reduce grain yield and affect quality. *F. verticillioides* and *A. flavus* also contaminate maize grain with the mycotoxins fumonisins and aflatoxins, respectively, which has been associated with mycotoxicosis in humans and animals. The occurrence of common resistance mechanisms to FER and AER has been reported. Hence, ten Kenyan inbred lines resistant to AER and aflatoxin accumulation were evaluated for resistance to FER, *F. verticillioides* colonisation and fumonisin accumulation; and compared to nine South African lines resistant to FER and fumonisin accumulation. Field trials were conducted at three localities in South Africa and two localities in Kenya. FER severity was determined by visual assessment, while *F. verticillioides* colonisation and fumonisin content were quantified by real-time PCR and liquid chromatography tandem mass spectrometry, respectively. Significant genotype x environment interactions was determined at each location ($P \leq 0.05$). Kenyan inbred CML495 was most resistant to FER and *F. verticillioides* colonisation, and accumulated the lowest concentration of fumonisins across localities. It was, however, not significantly more resistant than Kenyan lines CML264 and CKL05015, and the South African line RO549 W, which also exhibited low FER severity ($\leq 5\%$), fungal target DNA (≤ 0.025 ng μL^{-1}) and fumonisin levels (≤ 2.5 mg kg^{-1}). Inbred lines resistant to AER and aflatoxin accumulation appear to be promising sources of resistance to *F. verticillioides* and fumonisin contamination.

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Introduction

Fusarium ear rot (FER) and Aspergillus ear rot (AER) are important diseases of maize in Africa as they reduce both grain yield and quality. The diseases are caused by

the fungal pathogens *Fusarium verticillioides* (Sacc.) Nirenberg and *Aspergillus flavus* Link:Fr., respectively. These fungi produce toxic secondary metabolites in maize grain, called mycotoxins, which pose a serious threat to human and animal health. Fumonisins, produced by *F. verticillioides*, has been associated with oesophageal cancer of human adults in South Africa, Italy, Iran and China (Franceshi et al. 1990; Rheeder et al. 1992; Shephard et al. 2000; Li et al. 2001) and neural tube defects in infants (Hendricks 1999; Missmer et al. 2006). Aflatoxins, produced by *A. flavus*, are potent carcinogens and have resulted in severe cases of aflatoxicosis in Kenya which led to human fatalities (Williams et al. 2004). It also reduced the height of children in Benin and Togo (Gong et al. 2002) and suppressed the immune system of both humans and animals (Williams et al. 2004). Fumonisins and aflatoxins are poisonous to livestock, with fumonisins causing equine leukoencephalomalacia (Kellerman et al. 1990) and porcine pulmonary oedema syndrome (Colvin and Harrison 1992), while aflatoxins cause aflatoxicosis in cattle and sheep. Mycotoxin levels in food and feed are strictly regulated in the USA and European Union (FDA 2000; European Commission, 2007). In South Africa, the maximum tolerable level for aflatoxin levels in foods is 10 µg/kg (Government Gazette 2004). Recently, South Africa has amended regulations regarding the tolerances for fungus-produced toxins in foodstuffs by limiting fumonisins in maize grain, intended for further processing, to 4000 µg/kg while processed products, ready for human consumption, may not contain more than 2000 µg/kg of fumonisins (Goverment Gazette 2016). Studies conducted in rural areas of South Africa indicated that fumonisin levels in maize grain often exceed tolerable limits enforced in Europe and USA (Shephard et al. 2007; Shephard 2008; Ncube et al. 2011).

Fumonisins and aflatoxins can co-occur on maize grain (Fandohan et al. 2005), but the nature of the interaction between their respective producers, *F. verticillioides* and *A. flavus*, is still unclear. It has been suggested that kernels infected by *F. verticillioides* may become resistant to subsequent infection by *A. flavus* (Wicklow et al. 1988). Marin et al. (1998) found that *F. verticillioides* outgrew *A. flavus* over a range of temperatures and reduced its growth in vitro. This supports the hypothesis of a competitive relationship between the two species, possibly for the same resources or niche on maize ears (Marin et al. 1998). Giorni et al.

(2009), however, demonstrated that these fungi occupied different ecological niches based on carbon source utilization patterns in vitro. When plants were co-inoculated with *F. verticillioides* and *A. flavus*, their grain contained significantly less aflatoxins than grain from plants that were inoculated with *A. flavus* only (Zummo and Scott 1992). Conversely, Abbas et al. (2006) reported that fumonisin and aflatoxin levels in hybrids naturally infected with *F. verticillioides* and *A. flavus* were positively correlated. These results suggest that both species can grow on the same plant without necessarily competing for the same resources if host plants are highly susceptible.

The planting of resistant maize cultivars, as part of an integrated disease management strategy, is considered an efficient approach to reduce FER and AER and minimize the risk of mycotoxin accumulation in maize. Zummo and Scott (1992) found that infection with *F. verticillioides* did not prevent the identification of genotypes resistance to *A. flavus*. The strong positive correlations between FER and AER severity, and also between AER and aflatoxin accumulation (Robertson-Hoyt et al. 2007), suggest that selecting maize germplasm for reduced FER should reduce AER and aflatoxin contamination. Quantitative trait loci (QTLs) associated with resistance to FER, AER, fumonisin and aflatoxin accumulation suggest that resistance to FER, AER and their associated mycotoxins may be genetically linked (Robertson-Hoyt et al. 2007). Xiang et al. (2010) further revealed that QTLs associated with resistance to FER, AER and Gibberella ear rot (GER), caused by *Fusarium graminearum* Schwabe, clustered in the same chromosome regions while Wisser et al. (2006) also reported QTLs to different diseases clustered in the maize genome. In a study evaluating the defense-responses of other FER-causing *Fusarium* spp. and *A. flavus*, Lanubile et al. (2015) demonstrated the overlap or community of genes and regulation pathways activated in response to infection by these pathogens.

Enhancing host-plant resistance in crops can be expedited by the identification of sources of resistance, preferably in locally-adapted breeding materials. Resistance, if durable under different growing conditions and in different maize production areas, can then be introduced into high-yielding and agronomically superior planting material that is susceptible to FER and fumonisin contamination. Plant resistance provides a number of advantages compared to other disease management options, such as ease of handling,

affordability and being environmentally friendly. Maize inbred lines and hybrids have thus been evaluated worldwide for resistance to either FER/fumonisin contamination or AER/aflatoxin contamination (Clements and White 2004; Robertson et al. 2006; Afolabi et al. 2007; Eller et al. 2010; Henry et al. 2009; Parsons and Munkvold 2012), but seldom against both.

FER and fumonisin contamination is considered an important constraint to maize production in South Africa. AER and aflatoxin contamination, on the contrary, poses a limited threat in temperate maize production areas, and affects only subsistence farmers in northern KwaZulu-Natal where maize-growing conditions are subtropical. In eastern Africa, however, plant protection efforts are focused almost entirely on AER and aflatoxin contamination of maize, with little to no information available on other ear rot pathogens and their mycotoxins, including *F. verticillioides*. The objectives of this study, therefore, were to evaluate Kenyan inbred lines resistant to AER and aflatoxin accumulation for resistance to FER and fumonisin accumulation in South Africa and Kenya and to compare resistant Kenyan inbred lines to well-characterised FER/fumonisin-resistant South African inbred lines.

Materials and methods

Plant material evaluated

Ten Kenyan inbred lines, previously characterised as resistant to AER and aflatoxin accumulation (Dr. D. Makumbi, personal communication), and nine maize inbred lines from South Africa, previously characterised as resistant to FER and fumonisin accumulation (Small et al. 2012; Rose et al. 2016) were used in this study (Table 1). Additionally, two South African inbred lines susceptible to FER and fumonisin accumulation were included as susceptible checks. The South African inbred lines were provided by the Agricultural Research Council—Grain Crops Institute (ARC-GCI) in Potchefstroom, South Africa, while the Kenyan inbred lines were provided by the International Maize and Wheat Improvement Center (CIMMYT) in Nairobi, Kenya.

Field trials

Field trials in South Africa were conducted at Potchefstroom (grid ref.: 26°73′S, 27°07′E; altitude,

1349 m) in the Northwest province and Vaalharts (grid ref.: 27°95′S, 24°83′E; altitude, 1180 m) in the Northern Cape province during the 2012/13 maize growing season. A trial was also conducted at Makhatini (grid ref.: 22°39′S, 32°17′E; altitude, 77 m) in the KwaZulu-Natal province of South Africa during 2013, as this locality represents the most tropical maize-growing site in the country. In Kenya, screening trials were conducted in 2013 and performed at the Kenya Agricultural and Research Institute (KARI) stations of Katumani (grid ref.:1°35′S, 37°14′E; altitude 1600 m) and Kiboko (grid ref.:2°15′ S, 37°75′ E; altitude 975 m). These stations lie within the Machakos County in the semi-arid eastern Kenya, and has been shown to be an aflatoxin hot spot (Lewis et al. 2005).

Standard procedures to prepare fields were followed at all the trial locations. Maize kernels were manually planted (two seeds per hill) in double-row 10-m plots, with an intra-row spacing of 0.3 m and an inter-row spacing of 1 m. The trials were planted using a randomised complete block design and replicated three times, with experimental plots thinned to 33 plants per plot 3 weeks after emergence. The Potchefstroom and Makhatini trials were conducted under dryland conditions and supplemented with overhead irrigation when required, while the trial conducted at Vaalharts was flood irrigated weekly. The trials conducted at Katumani and Kiboko were drip irrigated.

Fungal isolates and production of inoculum

Country-specific, well-characterised toxigenic isolates were used to generate inoculum. Isolates obtained from the areas where the field trials were conducted were used wherever possible. Isolates of *F. verticillioides* used to inoculate South African trials consisted of GCI 315 and GCI 790, which were originally isolated from infected maize in Ndwedwe (KwaZulu-Natal) and Rushof (Northern Cape), respectively. The prolific fumonisin B_1 producer MRC 826 (Rheeder et al. 1992), isolated from infected maize in the Transkei region (South Africa), was also included to ensure a high level of pathogenicity and fumonisin production. Three Kenyan isolates of *F. verticillioides*; UONK005, UONK038 and UONK048 were used to inoculate the Kenyan field trials and are held in the culture collection of the University of Nairobi,

Table 1 Maize inbred lines evaluated for resistance to Fusarium ear rot, *Fusarium verticillioides* colonisation and fumonisin accumulation in South Africa and Kenya during the 2012/13 maize growing seasons

Inbred line name	Line code[a]	Institution[b]	FER/fumonisin status	AER/aflatoxin status
CB-222	1	ARC-GCI—South Africa	Resistant[c]	Unknown
CB-248	2	ARC-GCI—South Africa	Resistant[c]	Unknown
CKL05003	3	CIMMYT—Kenya	Unknown	Resistant
CKL05015	4	CIMMYT—Kenya	Unknown	Resistant
CKL05019	5	CIMMYT—Kenya	Unknown	Resistant
CKL05022	6	CIMMYT—Kenya	Unknown	Resistant
CML182	7	CIMMYT—Zimbabwe	Resistant[c]	Unknown
CML247	8	CIMMYT—Kenya	Unknown	Resistant
CML264	9	CIMMYT—Kenya	Unknown	Resistant
CML390	10	ARC-GCI—South Africa	Resistant[c]	Unknown
CML444	11	ARC-GCI—South Africa	Resistant[c]	Unknown
CML495	12	CIMMYT—Kenya	Unknown	Resistant
I137tnW	13	ARC-GCI—South Africa	Susceptible[c]	Unknown
La Posta Seq C7-F103-2-1-1-1xMIRTC5 Bco F80-4-2-1-1-1-3-1-B–B-B (La Posta)	14	CIMMYT—Kenya	Unknown	Resistant
MIRTC5 Bco F78-2-2-1-1-1xDERRc2 15-3-7-1-1-B–B-B–B (MIRTC5)	15	CIMMYT—Kenya	Unknown	Resistant
P502c2-185-3-4-2-3-B-2-B–B-B–B-B–B (P502c2)	16	CIMMYT—Kenya	Unknown	Resistant
R119 W	17	ARC-GCI—South Africa	Resistant[c]	Unknown
R2565y	18	CIMMYT—Zimbabwe	Susceptible[c]	Unknown
RO549W	19	CIMMYT—Zimbabwe	Resistant[c]	Unknown
US2540W	20	ARC-GCI—South Africa	Resistant[c]	Unknown
VO617y-2	21	CIMMYT—Zimbabwe	Resistant[c]	Unknown

[a] Designated line code for the inbred lines used in this study

[b] Agricultural Research Council—Grain Crops Institute (ARC-GCI), CIMMYT—International Maize and Wheat Improvement Center

[c] According to Small et al. (2012) and Rose et al. (2016)

School of Biological Sciences. These isolates were obtained from infected maize from the highly contaminated Nandi district.

Inoculum for disease screening trials was prepared by growing the *F. verticillioides* isolates in Armstrong liquid medium (Booth 1971). Conidial suspensions were further prepared according to Small et al. (2012) and the concentration was adjusted to 1×10^6 spores mL^{-1} for each *F. verticillioides* isolate. Equal volumes of the three conidial suspensions per country were then combined to produce the final inoculum. The inoculum was kept at 4 °C prior to and during the inoculation process, and inoculum viability was confirmed by fungal growth on potato dextrose agar following field inoculations.

Inoculation and assessment of disease severity

Maize ears were inoculated at the R2 blister stage (Ritchie et al. 1993) according to the method described by Small et al. (2012). Once the inoculated ears reached physiological maturity and the grain dried to 12–18% moisture they were manually harvested, de-husked and FER symptoms visually assessed. Disease severity was determined by the percentage of each ear covered by visible symptoms of FER (Clements and White 2004). Maize ears were then mechanically shelled and bulked per experimental plot. A 250-g kernel sample from each bulked plot was ground to produce fine maize flour. Flour samples were stored at

−20 °C until the extraction of fumonisins and genomic DNA was performed.

Quantification of *Fusarium verticillioides* in maize grain

DNA was isolated from both fungal cultures (0.02 g) and milled maize samples (2.0 g), according to the methods described by Boutigny et al. (2012). Freeze-dried mycelia of MRC 826, GCI 315, GCI 790, the Kenyan isolates (UONK005, UONK038 and UONK048), as well as the maize samples were stored at −20 °C until genomic DNA was extracted. Additional DNA extractions were performed on water-inoculated maize for use as target-DNA-free host DNA for the preparation of matrix matched standard curves. DNA was isolated using the commercially available DNeasy® Plant Mini kit (QIAGEN), with some modifications. The initial DNA isolation step consisted of the CTAB/PVP lysis method, and an additional phenol extraction step was performed on fungal cultures prior to the use of the commercial kit (Boutigny et al. 2012). The quantity and purity of the DNA yield of each fungal and grain sample was determined with a NanoDrop ND-1000 spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa).

The fungal target DNA in maize samples was determined by qPCR according to Boutigny et al. (2012). The sensitivity of the qPCR primer pair to detect and quantify the isolates used in the South African and Kenyan trials was also confirmed prior to the analyses of maize samples. For direct comparisons with inoculated grain samples, a mixed DNA standard curve was prepared by diluting the *F. verticillioides* isolate MRC 826 DNA ($\sim$20 ng μL^{-1}) 4^1–4^5-fold in water-inoculated maize DNA (10 ng μL^{-1}). Real-time PCR reactions for each dilution were performed in triplicate and the standard curve was analysed using the Rotor-GeneTM 2.0.2.4 software (Corbett Life Science, Whitehead Scientific (Pty) Ltd., South Africa). It plotted the cycle threshold (C_t) values of the diluted standards against the logarithm of the known DNA concentration (Nanodrop Spectrophotometer measurement). Dynamic tube normalisation, starting at Cycle 1 and noise slope correction for standard curve analysis was selected on the Rotor-GeneTM 2.0.2.4 software programme for all runs performed.

To assess the linearity and presence of inhibitors, the correlation coefficient (R^2), slope (M-value) and reaction efficiency (E-value) were noted after the analysis of the standard curve. Stringent parameters were set to ensure accurate, inhibition-free qPCR assays with $R^2 > 0.99$, M-values of −3.4, and an efficiency of 0.99 (Corbett Life Science 2006; European Commission 2008; Bustin et al. 2009). The melting temperature (T_m) of *F. verticillioides* target DNA was evaluated to confirm analytical specificity *in planta*.

To quantify *F. verticillioides* target DNA in inoculated maize samples, the qPCR assays contained a duplicate of each sample and standard *F. verticillioides* DNA (4^2, 4^3 and 4^4-fold dilution of pathogen DNA in maize DNA free of *F. verticillioides* infection) were analysed in triplicate. All the assays contained a no template control (NTC). Using the matrix specific standard curves, the C_t values were transformed into DNA concentrations using the Rotor-Gene™ 2.0.2.4 software.

Fumonisin quantification from maize grain

Fumonisin levels in maize samples were determined by the dilute-and-shoot method using liquid chromatographic tandem mass spectrometry (LC–MS/MS). Fumonisins were extracted from milled maize samples (5 g) according to Rose et al. (2016). The samples were diluted in a 1:1 ratio with HPLC-grade water and submitted to the Central Analytical Facility (CAF) at Stellenbosch University, South Africa for the detection and quantification of FB_1, FB_2 and FB_3. Fumonisin standards (B_1 [10 mg], B_2 [10 mg], and B_3 [1 mg]), guaranteed 95% pure, were obtained from the Medical Research Council—Programme on Mycotoxins and Experimental Carcinogenesis (MRC-PROMEC), Tygerberg, South Africa. A dilution series that ranged between 0.05 and 20 mg kg^{-1} for FB_1 and FB_2, and between 0.005 and 2 mg kg^{-1} for FB_3 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 of the calibration curve limit were appropriately diluted using HPLC-grade water and re-analysed. After adjustment for the volume of extract used in the purification procedure, the minimum limits of quantification for FB_1, FB_2 and FB_3 were 0.02, 0.002 and 0.02 mg kg^{-1}, respectively.

Data analysis

The data obtained from the visual assessment of FER symptoms, fumonisin accumulation and *F. verticillioides* target DNA determination 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 FER symptoms, total fumonisin accumulated and fungal target DNA contamination using the CORR procedure in SAS employing the untransformed means of variables.

The data was furthermore subjected to additive main effects and multiplicative interaction (AMMI) analysis of variance (Gauch and Zobel 1996) 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 (PC1), located on the *X*-axis, indicates the level of resistance where inbred lines with higher PC1 values (positive or negative) are considered low risk to FER disease severity, *F. verticillioides* colonisation or fumonisin accumulation. The second principle component (PC2), located on the *Y*-axis, represents performance stability of genotypes where PC2 values near zero demonstrate greater adaptability of genotypes to different environments (Yan and Kang 2003).

The stability of the genotypes across locations was determined by the AMMI stability values (ASV) (Purchase et al. 2000), which is 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 was graphically represented in GGE biplots. The GGE biplots were generated in GenStat 15th edition (Payne et al. 2012) by using the singular value decomposition (SVD) of the first two principle components (PC1 and PC2), according to Yan and Rajcan (2002). The GGE biplot graphically represented the genotype main effect and the G × E interaction (Yan 2001; Yan et al. 2000). The method is based on evaluating genotypes, firstly by considering only the effects of the genotype and G x 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 (Gabriel 1971). Mega-environments 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 consistently across years.

The estimation of resistance and stability of genotypes to FER, *F. verticillioides* colonisation and fumonisin accumulation was evaluated by an average environment coordination (AEC) method (Yan et al. 2000; Yan 2001; Yan and Rajcan 2002). It used the average principal components in all environments, represented as a circle, with a line drawn through the average environment and the biplot origin called the average environment axis (AEA). The perpendicular line passing through the AEA and the biplot origin, called the average ordinate environment (AEO), divided the genotypes into those exhibiting above-average resistance (to the right of the AEO) and those showing below-average resistance (to the left of the AEO). Genotypes were projected on the AEA and ranked on resistance (low FER, *F. verticillioides* target DNA, fumonisins), with increased resistance in the direction of the arrow. The arrow points to a greater genotype main effect, while the AEC ordinate, and either direction away from the biplot origin, indicates greater G × E interaction effect and reduced stability.

The PC1 and PC2 were also used to obtain the ideal test environment, characterised by large PC1 scores (more power to discriminate genotypes in terms of the genotypic main effect) and small (absolute) PC2 scores (more representative of the overall environments) (Yan and Rajcan 2002). GGE biplots were constructed with genotype-focus and symmetrical scaling.

Results

Fusarium ear rot severity

Maize inbred lines evaluated in South Africa and Kenya differed significantly in their expression of FER

Table 2 Mean Fusarium ear rot, *Fusarium verticillioides* colonisation and total fumonisin content of maize inbred lines evaluated in South Africa and Kenya during the 2012/2013 maize growing seasons

Inbred line name	FER severity (%)[1]	*F. verticillioides* colonisation (ng/uL)[2]	Fumonisin accumulation (mg/kg^{-1})[3]
CB-222	10.2bc	0.031d–h	3.2c–g
CB-248	6.3b–g	0.038d–f	3.8cd
CKL05003	6.3b–g	0.024e–h	2.1e–g
CKL05015	3.3fg	0.037d–g	2.7d–g
CKL05019	7.8b–f	0.019f–h	2.4d–g
CKL05022	5.7b–g	0.024e–h	2.2e–g
CML182	5.1d–g	0.021f–h	2.8d–g
CML247	6.0b–g	0.066bc	3.4c–f
CML264	2.5g	0.024e–h	2.2e–g
CML390	5.7b–g	0.036d–g	2.6d–g
CML444	9.4b–d	0.035d–g	3.5c–e
CML495	2.0g	0.012h	2.0fg
I137TNW	19.0a	0.039d–f	4.3bc
La Posta	10.5b	0.019f–h	3.2c–g
MIRTC5	4.9d–g	0.017gh	2.1e–g
P502c2	5.9b–g	0.043de	3.0c–g
R119 W	20.8a	0.139a	9.3a
R2565y	8.3b–e	0.076b	5.6b
RO549W	3.5e–g	0.017gh	1.9g
US2540W	5.4c–g	0.049cd	2.5d–g
VO617y-2	2.7g	0.026e–h	4.2bc
Mean	7.2	0.038	3.3

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 ($P \leq 0.05$)

[1] The percentage of maize ears covered with visual symptoms of Fusarium ear rot

[2] The absolute concentrations of *F. verticillioides* target DNA

[3] Total fumonisin content as the sum of FB$_1$, FB$_2$ and FB$_3$ for three field plots

symptoms. Lines designated as resistant accumulated ≤5.0% FER symptoms across localities and included Kenyan lines CML495 (2.0%), CML264 (2.5%), CKL05015 (3.3%) and MIRTC5 Bco F78-2-2-1-1-1xDERRc2 15-3-7-1-1–B–B-B–B (MIRTC5; 4.9%) as well as South African lines VO617y-2 (2.7%) and RO549 W (3.5%) (Table 2). FER symptoms were significantly (P ≤ 0.05) influenced by the environment, genotype and the interactions between these factors (Table 3). The genotype x environment interactions (GEI) was responsible for the largest variation and explained 57.9% of the variation observed, followed by the genotype (25.0%) and environment (17.1%) effect. The first and second interaction principle component axis (IPCA) were both

significant, with IPCA1 accounting for 25.4% of the total G × E variation and IPCA2 for 17.1% of the variation observed (Table 3). In total, the two PCA's accounted for 42.5% of the variability in FER disease symptom expression. Stability of inbred line's response varied across locations, with ASV ranging from 0.35 to 1.69, and a mean of 0.82 (Table 4). Inbred lines CML264 (0.35), MIRTC5 (0.45), CML495 (0.46), P502c2-185-3-4-2-3-B-2-B–B-B–B-B–B (P502c2; 0.48), CKL05003 (0.52), VO617y-2 (0.53), La Posta Seq C7-F103-2-1-1-1xMIRTC5 Bco F80-4-2-1-1-1-3-1-B–B-B (La Posta; 0.54), US2540 W (0.61), CML390 (0.68) and I137tnW (0.69) were the most stable in their response to FER across locations. Conversely, inbred lines CML444

Table 3 Additive main effects and multiplicative interaction analysis of variance of Fusarium ear rot (FER), *Fusarium verticillioides* colonisation and total fumonisin content of maize inbred lines evaluated in South Africa and Kenya

Source of variation	d.f.	FER severity[a]				*F. verticillioides* colonisation[b]				Fumonisin accumulation[c]			
		s.s.	m.s.	F pr	Variation (%)	s.s.	m.s.	F pr	Variation (%)	s.s.	m.s.	F pr	Variation (%)
Total	314	500	1.592			13.801	0.044			350.6	1.117		
Treatments	104	337.4	3.244	<0.001		12.343	0.1187	<0.001		312	3	<0.001	
Genotypes	20	84.4	4.218	<0.001	25.0	1.906	0.0953	<0.001	15.4	26.1	1.304	<0.001	8.4
Environments	4	57.7	14.425	<0.001	17.1	3.931	0.9828	<0.001	31.8	224.4	56.104	<0.001	71.9
Block	10	8.2	0.825	0.3884		0.039	0.0039	0.8535		1.1	0.107	0.8357	
Interactions	80	195.3	2.442	<0.001	57.9	6.506	0.0813	<0.001	52.7	61.5	0.769	<0.001	19.7
IPCA 1	23	85.8	3.73	<0.001	25.4	5.752	0.2501	<0.001	46.6	30.5	1.325	<0.001	9.8
IPCA 2	21	57.7	2.749	<0.001	17.1	0.514	0.0245	<0.001	4.2	23.9	1.136	<0.001	7.7
Residuals	36	51.8	1.439	0.0039		0.24	0.0067	0.5696		7.2	0.199	0.3873	
Error	200	154.4	0.772			1.418	0.0071			37.5	0.188		

IPCA interaction principle component axis scores

[a] The percentage of maize ears covered with visual symptoms of Fusarium ear rot

[b] The absolute concentrations of *F. verticillioides* target DNA

[c] Total fumonisin content as the sum of FB_1, FB_2 and FB_3

Natural log transformation used in analyses

Table 4 Additive main effects and multiplicative interaction analysis stability values (ASVs) and interaction principle component axes (IPCA) values for Fusarium ear rot (FER), *Fusarium verticillioides* colonisation and total fumonisin content of maize inbred lines evaluated in South Africa and Kenya

Inbred line name	FER severity[a]			*F. verticillioides* colonisation[b]			Fumonisin accumulation[c]		
	IPCA1	IPCA2	ASV	IPCA1	IPCA2	ASV	IPCA1	IPCA2	ASV
CB-222	0.6	−0.54	1.04	0.05	−0.22	0.58	−0.38	−0.18	0.51
CB-248	0.71	0.21	1.08	−0.11	−0.12	1.19	−0.37	0.23	0.53
CKL05003	0.3	0.27	0.52	0.15	−0.03	1.64	−0.1	−0.19	0.23
CKL05015	0.01	−1.12	1.12	0.17	−0.13	1.86	−0.48	−0.47	0.77
CKL05019	−0.85	−0.18	1.27	−0.02	0.14	0.25	0.35	−0.12	0.46
CKL05022	−0.69	0.03	1.02	0.02	0.14	0.26	0.25	0.05	0.32
CML182	0.58	0.03	0.87	0.13	0	1.41	−0.43	−0.64	0.84
CML247	−0.55	−0.05	0.83	0.17	0.13	1.88	0	−0.48	0.48
CML264	−0.19	0.21	0.35	−0.04	0.1	0.45	0.18	0.22	0.32
CML390	0.12	−0.66	0.68	0.17	−0.19	1.87	−0.27	−0.15	0.37
CML444	−1.14	0.06	1.69	0.19	0.29	2.1	0.89	−0.73	1.35
CML495	−0.31	−0.09	0.46	0.12	0.07	1.37	−0.15	−0.18	0.26
I137TNW	0.18	0.63	0.69	−1.06	0.04	11.88	0.21	0.7	0.75
La Posta	0.31	−0.27	0.54	0.06	0.04	0.63	0.13	0.17	0.24
MIRTC5	−0.22	0.31	0.45	0.08	0.1	0.86	0.17	0.11	0.24
P502c2	−0.26	0.29	0.48	−0.01	0.1	0.18	0.8	0.36	1.08
R119 W	0.23	0.86	0.92	−0.19	−0.12	2.16	−0.41	0.29	0.6
R2565Y	0.6	−0.24	0.92	−0.06	−0.08	0.72	−0.59	0.56	0.94
RO549W	0.66	0.38	1.05	0.01	0.02	0.13	0.29	0.05	0.38
US2540W	0.05	−0.6	0.61	0.09	−0.32	1.07	−0.21	0.03	0.27
VO617y-2	−0.15	0.48	0.53	0.11	0.04	1.22	0.11	0.35	0.38
Mean			0.82			1.61			0.54

[a] The percentage of maize ears covered with visual symptoms of Fusarium ear rot

[b] The absolute concentrations of *F. verticillioides* target DNA

[c] Total fumonisin content as the sum of FB_1, FB_2 and FB_3 for three field plots

(1.69), CKL050019 (1.27), CKL050015 (1.12), CB-248 (1.08), RO549 W (1.05), CB-222 (1.04) and CKL05022 (1.02) were least stable in their expression of FER symptoms across locations (Table 4).

The genotype main effect and GEI was visualised by GGE biplot analysis (Fig. 1). In this study, the first two principal components accounted for 72.6% (PC1 = 47.2% and PC2 = 25.4%) of the total GGE variation for FER severity (Fig. 1). The genotype-ranking biplot revealed CML495 (#12), CML264 (#9) and VO 617y-e (#21) as the most resistant to FER, with CML495 (#12) being the most stable (Fig. 1a). The genotype-comparison biplot supported this result, with CML495 (#12) being found the ideal genotype with the lowest FER severity and greatest stability

across localities (Fig. 1b). Inbred lines CKL05015 (#4), CML264 (#9) and US2540 W (#20) were also identified as ideal genotypes, based on their proximity to CML495. Lines I137tnW (#13) and R119 W (#17) were the most susceptible genotypes (Fig. 1a, b). The polygon view, displaying the "which-won-where" pattern of the GGE biplot, revealed that inbred line CKL05015 (#4) was most resistant to FER at Makhatini, Potchefstroom and Kiboko, resulting in a mega-environment (Fig. 1c). Inbred lines US2540 W (#20) and CML390 (#10) were also resistant to FER. Inbred CML444 (#11) was most resistant to FER at Katumani, while CB-222 (#1) was most resistant in Vaalharts. Inbred lines CKL05015 (#4), CML444 (#11), R119 W (#17) and CB-222 (#1) served as
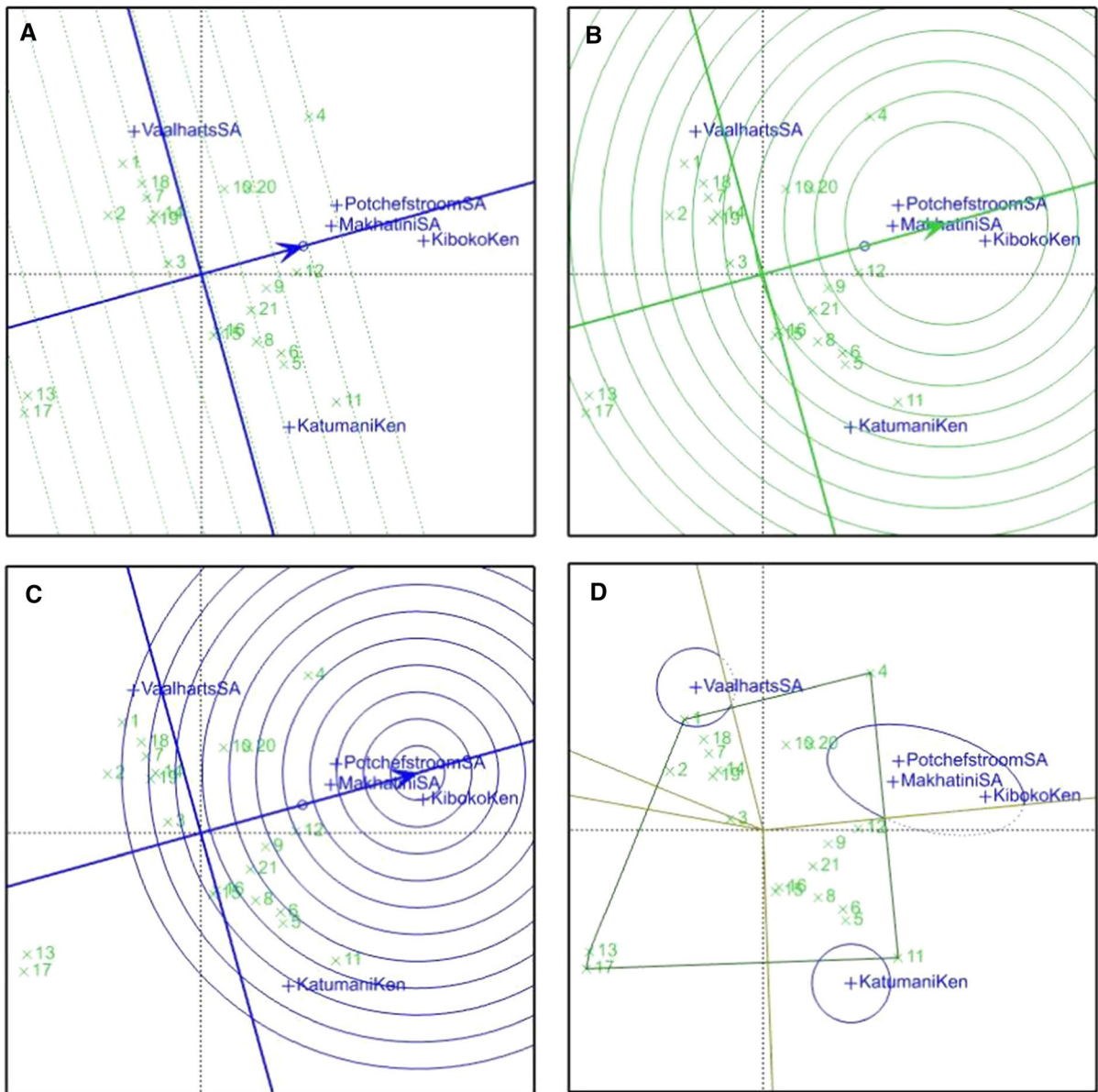
Fig. 1 Genotype main effect and genotype by environment interaction biplot for Fusarium ear rot severity in 21 maize inbred lines tested, based on environment-focused scaling for **a** the genotype ranking, **b** genotype comparison, **c** environment comparison and **d** the polygon view exhibiting mega- vertex hybrids forming the polygon, and were the most affected by GEI due to their distance from the biplot origin. The high PC1 value and low PC2 score of the environment comparison biplot revealed Kiboko as the ideal test environment, followed by Potchefstroom and Makhatini (Fig. 1d).

environments. *Green* and *blue numbers* represent genotypes and environments, respectively, with localities represented as: Potchefstroom (SA), Makhatini (SA), Kiboko (KEN) and Katumani (KEN). (Color figure online)

Fusarium verticillioides colonisation

Grain colonisation by *F. verticillioides*, as determined by the quantity of fungal target DNA, differed significantly in maize inbred lines across localities (Table 2). Resistant lines accumulated ≤0.025 ng μL^{-1} fungal

target DNA and included Kenyan lines CML495 (0.012 ng μL^{-1}), MIRTC5 (0.017 ng μL^{-1}), La Posta and CKL05019 (0.017 ng μL^{-1}) as well as CKL05003, CKL05022 and CML264 (0.024 ng μL^{-1}). South African lines RO549 W (0.017 ng μL^{-1}) and CML182 (0.021 ng μL^{-1}) were also resistant to fungal colonisation across localities (Table 2). The fungal DNA was significantly (P ≤ 0.05) influenced by the inbred line (genotype), locality (environment) and the interaction between the two (Table 3). The G × E interaction was responsible for 52.7% of the variation, while the environment and genotype accounted for 31.8 and 15.4% of the variation, respectively. Further analysis of the GEI revealed that IPCA 1 and IPCA2 were both significant and explained 46.6 and 4.2% of the variation observed, respectively. In total the IPCAs accounted for 50.8% of the total G × E variation influencing fungal colonisation. The stability of inbred line response to *F. verticillioides* colonisation, as determined by the ASV, ranged between 0.13 and 11.88 with a mean of 1.61 (Table 4). Inbred line RO549 W (0.13), P502c2 (0.18), CKL05019 (0.25), CKL05022 (0.26) and CML264 (0.45) were the most stable in their response to fungal colonisation across localities, while I137tnW (11.88) was the least stable (Table 4).

The first two principal components of the GGE biplot analysis accounted for 95.2% (PC1: 87.2% and PC2: 8.0%) of the total GGE variation for fungal colonisation (Fig. 2). Genotype ranking and genotype comparison biplots were congruent in determining CML495 (#12), CKL05019 (#5) and CML444 (#11) as ideal genotypes with stable resistance to fungal colonisation across localities, while I137tnW (#13) was the worst performing genotype (Fig. 2a, b). Inbred line CML444 (#11) was the best performing genotype across all environments, resulting in all the localities constituting a single mega-environment (Fig. 2c). Other inbred lines that were also resistant across localities included CML495 (#12), CKL05019 (#5), CML264 (#9) and CML247 (#8) and CKL05022 (#6). Vertex genotypes CML444 (#11), CKL05015 (#4), US2540 W (#20) and I137tnW (#13) delimited the polygon and differed most in their response to fungal colonisation (Fig. 2c). Environment-comparison biplot revealed Kiboko as the ideal test environment, followed by Katumani, Makhatini and Potchefstroom (Fig. 2d).

Fumonisin accumulation

Maize inbred lines accumulated significantly different levels of fumonisins across localities. Kenyan lines CML495 (2.0 mg kg^{-1}), MIRTC5 and CKL05003 (2.1 mg kg^{-1}), CKL05022 and CML264 (2.2 mg kg^{-1}) and CKL05019 (2.4 mg kg^{-1}) was considered highly resistant to fumonisin accumulation. South African lines RO549 W (1.9 mg kg^{-1}) and US2540 W (2.5 mg kg^{-1}) were also highly resistant to fumonisin accumulation (Table 2). The environment, genotype and the interaction between them (P ≤ 0.05) had a highly significant effect on the total fumonisins that accumulated in the grain of maize inbred lines (Table 3). The largest source of variation in fumonisin contamination was the environment, and it explained 71.9% of the total variation. The genotype and GEI further accounted for 8.4 and 19.7% of the variation, respectively. The IPCA1 and IPCA 2 were both significant and explained 9.8 and 7.7% of the total variation, respectively (Table 3). The stability of inbred line response to fumonisin accumulation across environments was determined by ASV, which ranged from 0.23 to 1.35 (Table 4). Inbred line CKL05003 (0.23), La Posta (0.24), MIRTC5 (0.24), CML495 (0.26), US2540 W (0.27), CKL05022 (0.32) and CML264 (0.32) were most stable in their response to fumonisin accumulation across localities, while CML444 (1.35) and P502c2 (1.08) were the least stable (Table 4).

The principal components of the GEI affecting fumonisin accumulation accounted for 79.8% (PC1: 45.1% and PC2: 34.7%) of the total GGE variation (Fig. 3a). Inbred line CML247 (#8) was the most resistant to fumonisin accumulation, along with CKL05003 (#3) and CML495 (#12). The ideal genotype based on mean and stability in the genotype-comparison biplot revealed CML247 (#8) as the ideal genotype, while CKL05003 (#3) and CML495 (#12) were desirable due to their proximity to CML247 (Fig. 3b). The inbred lines CML444 (#11), CKL05015 (#4), R2565y (#18), R119 W (#17), I137tnW (#13) and P502c2 (#16) formed the polygon and differed most in their response to fumonisin contamination (Fig. 3c). Line CML444 (#11) was the best performing genotype at Katumani, Makhatini and Potchefstroom, which resulted in a mega-environment for this inbred line. Line CKL05015 (#4) was the best
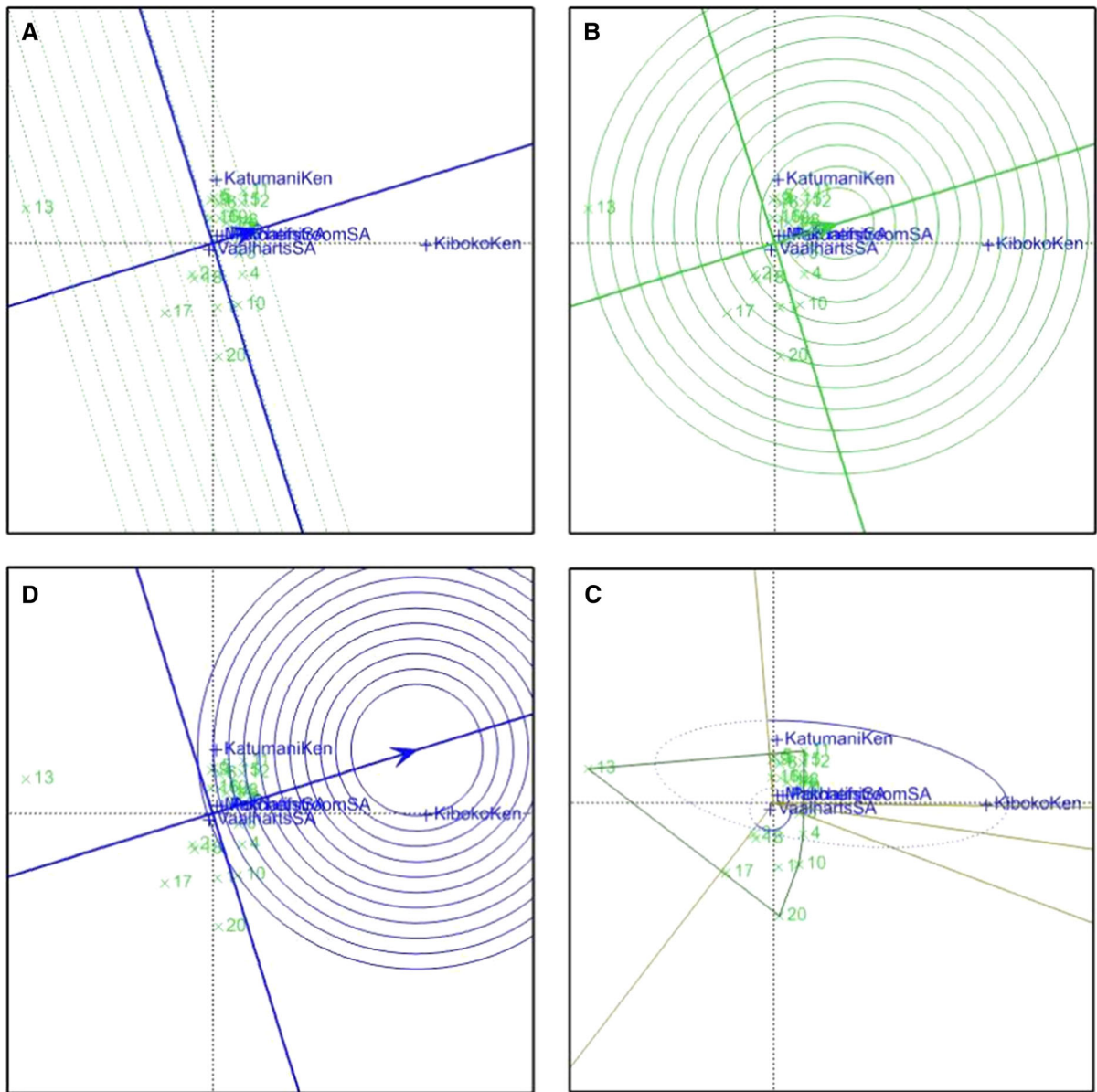
Fig. 2 Genotype main effect and genotype by environment interaction biplot for *Fusarium verticillioides* colonisation in 21 maize inbred lines tested, based on environment-focused scaling for **a** the genotype ranking, **b** genotype comparison, **c** environment comparison and **d** the polygon view exhibiting mega-environments. *Green* and *blue numbers* represent genotypes and environments, respectively, with localities represented as: Potchefstroom (SA), Makhatini (SA), Kiboko (KEN) and Katumani (KEN). (Color figure online)

performing genotype at Kiboko, while R2565y (#18) was the best performing genotype at Vaalharts. The localities evaluated were divided into different sectors, indicating significant crossover interaction, with Kiboko being the ideal test environment (Fig. 3d).

Correlations between FER symptoms, *Fusarium verticillioides* target DNA and total fumonisins

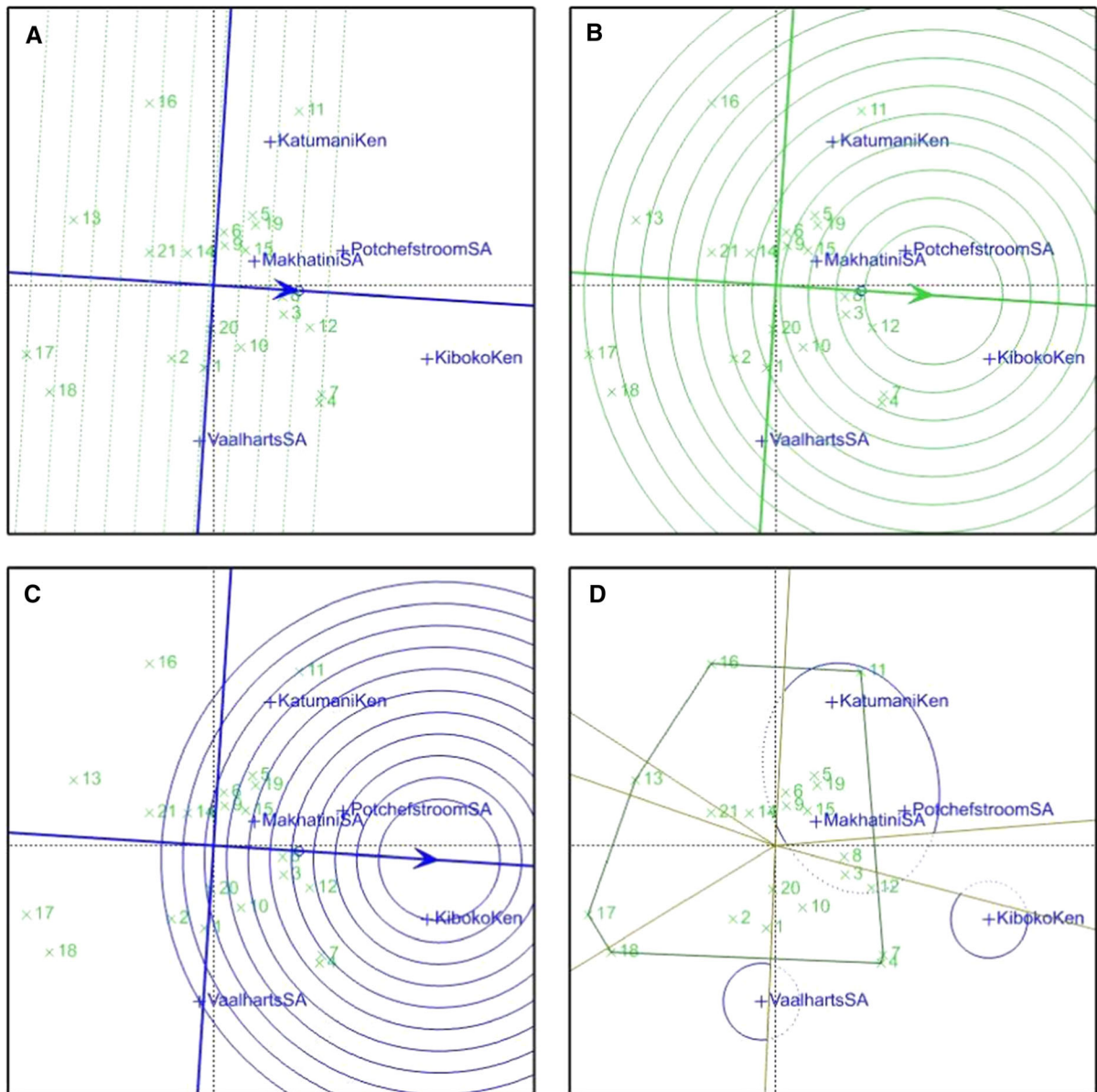Significant correlations were obtained between FER severity, fungal colonisation and total fumonisin

Fig. 3 Genotype main effect and genotype by environment interaction biplot for total fumonisin accumulation in 21 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. *Green* and *blue numbers* represent genotypes and environments, respectively, with localities represented as: Potchefstroom (SA), Makhatini (SA), Kiboko (KEN) and Katumani (KEN). (Color figure online)

content at all the test localities (Table 5). Disease severity had a significant but moderate correlation with *F. verticillioides* target DNA ($R = 0.54$; $P < 0.0001$), and a significant yet weak relationship with total fumonisins ($R = 0.38$; $P = 0.0019$) at Makhatini. Fungal target DNA and fumonisins showed a significant, strong correlation ($R = 0.66$;

$P < 0.0001$). At Potchefstroom, a significant but moderate correlation was found between FER severity and fungal target DNA ($R = 0.58$; $P < 0.0001$), while a significant, strong correlation was obtained between disease severity and total fumonisins ($R = 0.88$; $P < 0.0001$), as well as between fungal target DNA content and total fumonisin content ($R = 0.76$;

Table 5 Correlations between Fusarium ear rot severity, *Fusarium verticillioides* colonisation and total fumonisin content at field localities in South Africa and Kenya

Locality	Country	FER/*F. verticillioides* DNA	FER/Fumonisins	*F. verticillioides* DNA/Fumonisins
Makhatini	South Africa	0.54*	0.38 P = 0.0019	0.66
Potchefstroom	South Africa	0.58*	0.88*	0.76
Vaalharts	South Africa	0.82*	0.77*	0.81
Katumani	Kenya	0.40 P = 0.0011	0.39 P = 0.0015	0.55
Kiboko	Kenya	0.56*	0.55*	0.58
Combined	–	0.63	0.63	0.67

F. verticillioides DNA—fungal target DNA indicative of grain colonisation

Fumonisins—total fumonisins ($FB_1 + FB_2 + FB_3$)

FER fusarium ear rot

* $P < 0.0001$

$P < 0.0001$). Significant, strong relationships were found at Vaalharts between FER severity and *F. verticillioides* target DNA ($R = 0.82$; $P < 0.0001$), disease severity and total fumonisin content ($R = 0.77$; $P < 0.0001$), as well as between fungal target DNA and total fumonisins ($R = 0.81$; $P < 0.0001$) (Table 5).

FER severity at Katumani had a significant though moderate correlation with fungal target DNA content ($R = 0.40$; $P = 0.0011$) and total fumonisin content ($R = 0.39$; $P = 0.0015$) (Table 5). The *F. verticillioides* content of inbred lines also had a significant but moderate correlation with total fumonisin content ($R = 0.55$; $P < 0.0001$). At Kiboko, significant but moderate relationships were obtained between FER severity and fungal target DNA ($R = 0.56$; $P < 0.0001$), disease severity and total fumonisin content ($R = 0.55$; $P < 0.0001$) and fungal target DNA content and total fumonisins ($R = 0.58$; $P < 0.0001$). Across all localities, significant, strong correlations were found between FER severity and *F. verticillioides* target DNA ($R = 0.63$; $P < 0.0001$), disease severity and total fumonisin content ($R = 0.63$; $P < 0.0001$), as well as between fungal target DNA content and total fumonisins ($R = 0.67$; $P < 0.0001$) (Table 5).

Discussion

This study investigated Kenyan maize inbred lines, previously characterised for resistance to *A. flavus* and aflatoxin contamination, for *F. verticillioides* resistance for the first time, both under South African and Kenyan conditions. The Kenyan maize inbred line CML495 was found to be more resistant to FER, *F. verticillioides* colonisation and fumonisin accumulation across environments than any of the South African inbred lines previously characterised in South Africa. The inbred line's performance was stable across locations, even when evaluated under temperate conditions when compared to its adapted tropical and subtropical conditions. Therefore, CML495 is also considered to have broad adaptability. It has also been characterised as a late maturing line (CIMMYT 2005), which dries down slower than early maturing lines, thereby potentially extending the conditions conducive for fumonisin deposition. Nonetheless, CML495 demonstrated superior resistance to *F. verticillioides* infection and fumonisins. The maturity class of hybrids had the greatest effect on fumonisin contamination in a study in northern Italy (Battilani et al. 2008) while other studies noted that the maturity class did not influence the final fumonisin concentration in maize kernels (Ramirez et al. 1996; Blandino et al. 2009; Battilani et al. 2011). Hybrids with the slowest kernel drying rate, however, were most favourable for fumonisin accumulation irrespective of the maturity classification (Battilani et al. 2011). The use of CML495 in breeding programmes would not only provide a source of resistance to *A. flavus* and aflatoxins, but provide comprehensive resistance to *F. verticillioides* and fumonisin contamination too.

Two additional Kenyan inbred lines, CML264 and CKL05015, also developed less than 5% FER, with grain containing less than 0.025 ng μL^{-1} fungal target DNA and less than 2.5 mg kg^{-1} fumonisins. Inbred line CML264 was more stable in its response to disease expression, fungal colonisation and fumonisin accumulation. Inbred line CKL05015 was less stable in its response to FER severity across localities and showed specific adaptation at localities like Kiboko and Vaalharts where it showed resistance to fungal and fumonisin contamination. Inbred lines resistant to AER and aflatoxin accumulation, thus, appeared to be good sources of resistance to *F. verticillioides* and fumonisin accumulation. The identification of new sources of resistance to FER and fumonisin accumulation is of great importance, as all cultivars grown in South Africa are highly susceptible to FER and fumonisin contamination, with fumonisin levels often exceeding the tolerable limits enforced in Europe and the USA (Rheeder et al. 1990; Janse van Rensburg et al. 2015).

The response of inbred lines to FER, *F. verticillioides* colonisation and total fumonisin content was not always congruent. Inbred lines CKL05019, La Posta, CB-222 and CML444; which were all highly susceptible to FER, were intermediately resistant to fungal colonisation and fumonisins contamination. Inbred line CML247, which was intermediately resistant to FER, was susceptible to fungal colonisation but accumulated low levels of fumonisins. These results suggest that the genetic potential for resistance to FER (disease expression), fungal colonisation and fumonisin accumulation may exist independently from one another and is predominantly determined by the genetic composition of the maize line and the environment.

GEI significantly affected the performance of all inbred lines evaluated in this study. The GEI contributed more to the variation in FER severity and fungal colonisation than the genotype or environment, while the environment contributed the most to the variation observed in fumonisin content. These results indicate that inbred line response to *F. verticillioides* infection is dependent on the plant genotype to resist or succumb to infection under prevailing environmental conditions that support fungal development, inoculum pressure and insect infestation (Munkvold 2003).

The significant influence of the environment on fumonisin accumulation indicates the need to determine the suitability of an environment to promote the biosynthesis of fumonisins by *F. verticillioides*. Differential response of inbred lines to FER and fumonisin accumulation due to GEI has previously been documented (Clements and White 2004; Afolabi et al. 2007; Small et al. 2012; Rose et al. 2016), while fumonisin contamination was shown to be predominantly affected by the prevailing environmental conditions (Cao et al. 2014). This was also observed by de la Campa et al. (2005) when modelling the effects of environment, insect damage and *Bt* genotypes on fumonisin accumulation. The strong influence of environment on fumonisin accumulation accentuates the value of evaluating resistance to fumonisin accumulation in target environments. Fumonisin accumulation of maize inbred lines was particularly severe in Kiboko, Kenya. The contamination levels of maize grain obtained in this location far exceeded the 4 mg kg^{-1} fumonisin allowed for human and animal consumption as set by United States authorities (FDA 2000). The region is characterised by a bimodal rainfall with long rains occurring in March to May and short rains occurring from October to December/January. In 2013 the mean rainfall of 107.5 and 54.5 mm recorded at Kiboko during the long and short rainfall period, respectively, differed significantly from Katumani. The rainfall coincided with the grain filling period, while mean maximum temperature at Kiboko (31.3 °C) also differed significantly from Katumani (25.5 °C), creating a favourable environment for fumonisin production at Kiboko (Janse van Rensburg 2011). Temperature was shown to play a significant role in fumonisin production during the dough-stage of grain fill (de la Campa et al. 2005; Janse van Rensburg 2011) while drought-stress significantly affected FER and fumonisins (Parsons and Munkvold 2010). Kiboko provides the ideal environment for evaluating maize genotypes for resistance to *F. verticillioides* and more specifically fumonisin accumulation.

Significant, though inconsistent relationships between FER, *F. verticillioides* target DNA and total fumonisin content were obtained in this study. Significantly moderate correlations were obtained between FER and fungal target DNA at all localities except Vaalharts where a strong, positive correlation was obtained. Poor to moderate correlations were obtained between FER and fumonisin content at Makhatini, Katumani and Kiboko while strong,

positive correlations were obtained at Potchefstroom and Vaalharts. The relationship between fungal and fumonisin contamination was moderate to strong at all the localities. These results demonstrate the inconsistency of the relationship between FER/fungal target DNA and FER/fumonisins in selecting for reduced fumonisins, as they were significantly influenced by the production season (prevailing weather conditions and trial management practises). The relationship between fungal target DNA and fumonisins provided a more accurate indication of potential resistance to fumonisin accumulation (Janse van Rensburg et al. 2015; Rose et al. 2016). The inbred lines CKL05019, CB-222, La Posta and CML444 were highly susceptible to FER, with little fumonisin contamination, which reduced the relationship between these variables. These results emphasize the need for fumonisin analysis when evaluating resistance to FER and fumonisins (Afolabi et al. 2007; Small et al. 2012; Rose et al. 2016). The reduced relationship between FER severity, *F. verticillioides* and fumonisins could also be due to inaccurate visual assessment of disease symptoms and the ability of other *Fusarium* species to cause maize ear rot diseases. The cost and expertise required to quantify fungal target DNA or fumonisins may, however, be a deterrent in their implementation in resistance breeding programmes.

The use of resistant cultivars is considered the most economically viable, environmentally sound and convenient for producers to manage fumonisin contamination (Clements and White 2004). In this study, inbred lines resistant to AER and aflatoxin accumulation were more resistant to FER and fumonisin accumulation across localities when compared to South African FER/fumonisin-resistant inbred lines. These lines provide additional sources of resistance that could be used to improve resistance to FER and fumonisin in elite local lines or be used for the development of hybrids with improved resistance. The inbred lines are also a valuable resource that could be utilised in genomic and proteomic approaches to understand resistance mechanisms toward AER, FER and their associated mycotoxins.

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