The association of maize characteristics with resistance to *Fusarium verticillioides* and fumonisin accumulation in commercial maize cultivars

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Abstract

Fusarium verticillioides is the primary fungus that causes Fusarium ear rot (FER) of maize. Infection results in reduced grain yield and quality due to moulding and the contamination of grain with toxic compounds namely fumonisins. Resistance to fungal infection and fumonisin accumulation in maize and maize grain is governed at different levels. In this study, the structural, physico-chemical and genetic basis of resistance to *F. verticillioides* was investigated in two, replicated field trials at Potchefstroom and Vaalharts in South Africa. Phenotypic data (silk length, husk coverage, pericarp thickness hundred-kernel mass and kernel hardness), physico-chemical data (kernel pH, moisture content, total nitrogen and carbon as well as phenolic acid content) and the expression of pathogenesis-related-5 gene (*PR5*) and peroxidase gene expression was evaluated in 15 commercial cultivars under artificially inoculated and natural infection conditions. The data were correlated to FER severity, fumonisin accumulation and fungal DNA (referred to as infection indicators). Disease development and fumonisin contamination in Vaalharts was significantly more than in Potchefstroom. There were no significant correlations ($r \geq 0.60$) between phenotypic characteristics and infection indicators. Kernel pH was the most important trait associated with disease development and was negatively correlated (between $r = -0.58$ and $r = -0.75$) to all infection indicators. *PR5* gene expression had significant positive correlations ($r = 0.69$ and $r = 0.72$) with the fungal and fumonisin levels, respectively. This study presents of the first data demonstrating the use of gene expression in identifying FER/fumonisin-resistant plant material and could aid breeders and growers in selecting resistant material more effectively.

Keywords: *Fusarium verticillioides*, fumonisin, infection indicator, gene expression

1. Introduction

Fusarium ear rot (FER) of maize is caused by *Fusarium verticillioides* (Saccardo) Nirenberg, *Fusarium proliferatum* (Matsushima) Nirenberg, *Fusarium subglutinans* (Saccardo) Nirenberg and *Fusarium temperatum* (Scauflaire and Munaut) (Schoeman *et al.*, 2018). These are all mycotoxigenic pathogens that affect maize (*Zea mays* L.) at all stages of development (Foley, 1962; Munkvold, 2003a). Of the FER pathogens, *F. verticillioides* is considered to be the most important in Southern Africa (Boutigny *et al.*, 2012; Schoeman *et al.*, 2018).

The contamination of maize grain with *F. verticillioides* is a major constraint to maize production resulting in a reduction of yield and quality (Gerber *et al.*, 2010; Presello *et al.*, 2008). *F. verticillioides* causes rotting of maize kernels and produces a range of toxic secondary metabolites, called mycotoxins, of which fumonisins are the most important (Gelderblom *et al.*, 1988). The ingestion of fumonisin-contaminated maize cause health implications in humans and animals including oesophageal cancer in humans, leukoencephalomalacia in horses as well as liver and kidney damage in sheep (Kriek *et al.*, 1981; Marasas *et al.*, 1981, 2004).

The success of the pathogen is largely attributed to the hemi-biotrophic nature that allows it to colonise plant tissue endophytically or later become a necrotroph (Bacon and Hinton, 1994; Bacon *et al.*, 2008). From the anthesis period the kernel microenvironment favours the endophytic development of the pathogen, allowing it to proliferate without necessarily causing cell death (Wicklow *et al.*, 2005). Induction of the necrotrophic phase, although not fully understood, is related to biotic and abiotic stress endured by the pathogen (Bacon and Hinton, 1994; Bacon *et al.*, 2008). These stress factors can include other microorganisms competing for nutrients, plant cell pH, water availability, plant drought or water logging (Bacon *et al.*, 2001, 2008). Although fumonisins have been shown to accumulate during the endophytic phase, it is strongly associated with the necrotrophic phase (Bacon and Hinton, 1994; Bacon *et al.*, 2008). Furthermore, the disease is also strongly driven by environmental conditions (Pérez-Brito *et al.*, 2001; Rose *et al.*, 2016). Warmer, dryer conditions, especially around silking, increase the rate of successful infection, the pathogen breaching host-defence mechanisms, and, therefore, increasing the likelihood of disease development and fumonisin accumulation down the line (Cao *et al.*, 2014).

Management of FER/fumonisins is particularly challenging (Munkvold, 2003a). Successful practices for managing the disease are limited. Cultural practices are aimed at creating an environment that is less conducive for infection and disease development in the field (Munkvold, 2003b). Practices such as fertilisation methods, biocontrol agents, application of insecticides, tillage, crop rotation and adjusting planting and harvesting dates have been investigated (Flett *et al.*, 1998; Mabuza *et al.*, 2018; Munkvold, 2003b; Parsons and Munkvold, 2010; Pereira *et al.*, 2007). Cultural practices are by large ineffective in controlling *F. verticillioides* in the field especially when environmental conditions are conducive for disease and mycotoxin development (Cao *et al.*, 2014; Munkvold, 2003c). Plant resistance, therefore, presents the most feasible method to control FER and fumonisin accumulation (Maschietto *et al.*, 2017; Munkvold, 2003b). The strong environmental effect and polygenic nature of resistance to *F. verticillioides* complicate breeding endeavours (Lanubile *et al.*, 2017; Pérez-Brito *et al.*, 2001). A number of factors are documented to contribute to FER/fumonisin-resistance within maize inbred lines such as phenotypic, physico-chemical and genetic factors (Mesterházy *et al.*, 2012; Picot *et al.*, 2010). However, the relative importance of all these factors and how they correlate to each other are not well characterised.

Resistance in maize to *F. verticillioides* is mediated at different levels. Initially successful infection of maize kernels depends on the fungus crossing certain barriers. These include the length of maize silks, duration of silking,

silk wetness and husk coverage (Maiorano *et al.*, 2009; Parsons and Munkvold, 2010; Reid *et al.*, 1992; Warfield and Davis, 1996). Susceptibility of maize has been documented to increase with shorter silks, faster silk senescence and incomplete husk coverage. The maize kernel has certain structural features that can contribute to resistance, i.e. the pericarp layer. Studies have shown that maize kernels with a thicker pericarp had lower fumonisin levels than ones with thinner pericarps (Sampietro *et al.*, 2013). Additionally, the pericarp has different layers contributing to the overall hardness of the kernel (Fox and Manley, 2009). Furthermore, hardness can also be indicated by several other kernel factors such as the vitreous and starchy endosperm, size, weight and density (Guelpa *et al.*, 2015). Tolerance to cracking of kernels indirectly contributes to resistance to pathogens by preventing wounds that can act as infection sites for *F. verticillioides* (Sampietro *et al.*, 2013) and other mycotoxigenic fungi.

Following successful breach of structural barriers, *F. verticillioides* interacts with the kernel microenvironment where resistance to the fungus may be mediated at a physico-chemical level (Picot *et al.*, 2010). Developing kernels initially has an alkaline pH and high moisture content (Warfield and Gilchrist, 1999). Reports suggest that the fungus has mechanisms in place, such as the PAC1 protein, to allow fungal growth under these conditions (Flaherty *et al.*, 2003). Fumonisin production, however, is reportedly suppressed at alkaline conditions (Shim *et al.*, 2003). As kernel development progresses, nitrogen and free water becomes more limiting and kernel pH becomes more acidic (pH 3.0–4.0) (Keller *et al.*, 1997). This is correlated with an increase in fumonisin production (Jurado *et al.*, 2008; Keller *et al.*, 1997; Shim *et al.*, 2003). Furthermore, maize kernels inoculated with *F. verticillioides* has been shown to have a lower pH than water inoculated kernels (Van Zyl, 2015). Therefore, maize genotypes that are predisposed to factors such as higher kernel pH or nitrogen content during kernel development could also indirectly contribute to resistance to FER (Marín *et al.*, 2004). Phenolic compounds in maize kernels may play an active role in resistance to fungal pathogens (Salinas-Moreno *et al.*, 2017; Zabka and Pavela, 2013). Phenolic compounds are secondary metabolites produced by plants both constitutively and in response to external stimuli (Ponts *et al.*, 2011). They limit the growth of *F. verticillioides* as well as break down fungal cell membranes (Boutigny *et al.*, 2008; Ponts *et al.*, 2011). Furthermore, they have been shown to detoxify fumonisins *in vitro* (Beekrum *et al.*, 2003; Ferrochio *et al.*, 2013).

The molecular basis of resistance to FER and fumonisin accumulation has been extensively investigated in maize (Lanubile *et al.*, 2010, 2014, 2017; Maschietto *et al.*, 2016). This includes the induction of pathogenesis-related (PR) genes following pathogen infection (Breen *et al.*, 2017).

Effective at low concentrations, *PR* genes are expressed in all plant-pathogen interactions in an attempt to inhibit pathogen colonisation (Maschietto *et al.*, 2016; Sinha *et al.*, 2014). Lanubile *et al.* (2010) reported that *PR1*, *PR5*, *PRm3* and *PRm6* were found in a maize inbred line susceptible to *F. verticillioides* when compared to a tolerant line prior to inoculation, but that their expression was increased 48 hours after inoculation (Lanubile *et al.*, 2010). Genes that protect maize kernels from oxidative stress, i.e. peroxidase have also been associated with resistance in maize to *F. verticillioides* (Lanubile *et al.*, 2017; Maschietto *et al.*, 2016).

There has been much progress in terms of understanding the maize – *F. verticillioides* pathosystem and how individual characteristics relate to resistance in maize inbred lines. However, there is limited understanding on how these characteristics relate to each other or how the collective knowledge can be applied to identify resistant genotypes. The purpose of this study was to investigate host resistance traits at the structural, physico-chemical and genetic level of uncharacterised commercial maize cultivars resistant or susceptible to FER and fumonisin accumulation. Information obtained in this study would help breeders and producers to select FER/fumonisin-resistant material more efficiently.

2. Materials and methods

Plant material and artificial inoculation

Fifteen commercial cultivars, planted during the 2016/17 season, were randomly selected for this study (Table 1). The cultivars were planted in two localities; Potchefstroom (grid ref.: 26730 S, 27070 E; altitude, 1,349 m) and Vaalharts (grid ref.: 27950 S, 24830 E; altitude, 1,180 m) representing dryland and irrigation production, respectively. Each cultivar was planted in three field plots in a randomised complete block design at each locality. Field plots consisted of two 10-m rows, 1 m spacing between rows and maize kernels were planted with an intra-row spacing of 0.3 m.

The primary maize ears were inoculated at anthesis with the highly pathogenic, highly virulent and high fumonisin producing *F. verticillioides* isolate MRC 826 (Programme on Mycotoxins and Experimental Carcinogenesis, Medical Research Council (PROMEC-MRC), Tygerberg, South Africa). Silk channel inoculation was performed (Reid *et al.*, 1999) using a spore concentration of 2×10^6 spores/ml (Booth, 1971). The control (further referred to as naturally infected) maize ears were inoculated with 1 ml sterile deionised H₂O in each plot.

Table 1. Fifteen commercial maize cultivars evaluated at Potchefstroom and Vaalharts during the 2016/17 season.

Cultivar number	Grain colour	GM/non-GM ¹
1	yellow	GM
2	yellow	GM
3	yellow	Non-GM
4	yellow	GM
5	yellow	Non-GM
6	yellow	GM
7	yellow	Non-GM
8	white	GM
9	white	GM
10	white	GM
11	white	GM
12	white	Non-GM
13	white	Non-GM
14	white	GM
15	white	Non-GM

¹ GM = genetically modified; Non-GM = non-genetically modified.

Grain processing

Maize plants were allowed to dry naturally in field for 4 to 6 weeks before being manually harvested (primary ear only) per row. The primary ear of each cultivar was pooled per row with fungal- and water-inoculated ears kept separately. Ears were threshed, and the grain was pooled. A 250 g sample of kernels per field replicate (Janse van Rensburg *et al.*, 2011) was milled and stored at -20 °C, while an additional 50 g was milled and stored at -80 °C for further analyses.

Structural trait assessments

The silks of 25 primary ears per cultivar were measured at anthesis for each field plot. Measurements were taken from the point of silk emergence from the husk leaves to the tip of the silks (Table 2). Husk coverage was visually scored as open or closed depending on the visibility of kernels, at anthesis and again at harvest (Table 2).

Kernel hardness was assessed using two methods: hundred-kernel mass (HKM) (Guelpa *et al.*, 2015) and near-infrared (NIR) spectroscopy (Downey *et al.*, 1986) (Table 2). NIR spectroscopy was performed using the NIRFlex N-500 Frontier Transformed (FT-NIR) spectrophotometer (BÜCHI Labortechnik GmbH, Flawil, Switzerland) and NIR LabWare version 3.0 software (BÜCHI Labortechnik GmbH) and evaluated in the diffuse reflectance mode. Samples were subjected to NIR light at a resolution of 32 cm⁻¹ from 1,100 to 2,500 nm. Absorbance measurements

Table 2. Structural, physico-chemical and genetic factors measured.

Structural	Physico-chemical	Gene expression
Silk length	moisture content	PR5
Husk coverage (at anthesis and at harvest)	pH	peroxidase
100-Kernel mass	phenolic acids (free, bound and total)	
Kernel hardness (NIR) ¹	carbon to nitrogen ratio	
Pericarp thickness		
¹ NIR = near-infrared spectroscopy.		

were calculated by the NIR LabWare software based on the light reflected. Absorbance measurements ($1/R$) at 2,230 nm were used in a formula proposed by Downey *et al.* (1986) to obtain index values for hardness as follows, hardness = $\alpha + b (\log 1/R)$, where $\alpha = -40$ and $b = 100$. NIR spectroscopy was done for whole-kernel and flour samples.

The thickness of the kernel pericarp was measured using the ZEISS Stemi 508 Greenough Stereo Microscope (Carl Zeiss AG, Oberkochen, Germany) and visualised with the Axiocam ERc 5s camera (Carl Zeiss AG) (Table 2).

Physico-chemical properties assessments

The moisture content of maize grain was measured directly after harvest using the Draminski TwistGrain moisture meter (Draminski Electronics, Olsztyn, Poland). The pH of maize grain was measured using the Jenway 3510 pH meter (Bibby Scientific Limited, Stone, UK). The pH was assessed by mixing 500 mg of finely milled flour with 5 ml of sterile deionised H₂O (Table 2).

The total soluble nitrogen and carbon in kernels was assessed using the TruSpec[®] Micro (LECO Corporation, St Joseph, MI, USA) at the Central Analytical Facility (CAF) at Stellenbosch University. Carbon and nitrogen content were assessed by Vario EL Cube elemental analysis.

Total phenolic acids were extracted as described by Cassiem (2018) by first extracting the free phenolic acids followed by the ester-bound phenolic acids. The quantification procedure employed an external standard dilution series using purified commercial products trans-ferulic, caffeic, p-coumaric, sinapic and p-hydroxybenzoic acids (Sigma-Aldrich, St. Louis, MO, USA). The standard curve consisted of a 10-point dilution series ranging from 0.0125–200 mg/kg. Quantification was performed at CAF, Stellenbosch University, using a Synapt G2 quadrupole time of flight (Q-TOF) mass spectrometer (Waters, Milford, MA, USA)

with a chromatograph of Waters Ultra pressure liquid and a photo-diode array detection. Separation was accomplished using the High Strength Silica technology T3 operated in a negative electrospray ionisation mode (Waters) (Table 2).

Gene expression of defence-related genes

Total RNA was isolated as described by Wang *et al.* (2011). Flour, stored at -80 °C (0.2 g) was used for RNA isolation using TRIS-HCl (pH 9.0) (Merck, Darmstadt, Germany) and TRIzol Reagent (Sigma-Aldrich). RNA was treated with RNase-free DNase set (QIAGEN, Hilden Germany) according to the manufacturer's recommendations and the RNA concentration determined using a ND-1000 NanoDrop Spectrophotometer (Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa).

Complementary DNA (cDNA) was synthesised using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. Reverse transcription quantitative PCR (RT-qPCR) was performed to quantify the relative expression of peroxidase and PR5 in the fungus-inoculated and water-inoculated kernels while the elongation factor (*EF*)-1 α (Nicolaisen *et al.*, 2009) was used as reference gene. Validation experiments were performed to ensure that the efficiencies of the target genes and reference gene amplifications were equal (Bustin *et al.*, 2009). The RT-qPCR reactions were carried out on the CFX96[™] Real-Time system (Bio-Rad Laboratories) according to the method described by Lanubile *et al.* (2010). A melt curve analysis ranging from 60–95 °C was included in each run to ensure amplicon specificity. Cultivar field replications were pooled, and three technical replicates were performed for each cultivar. Template-free samples were included in each run. The relative expression of the defence-related genes was calculated for the fungus-inoculated and naturally infected samples using the 2^{- $\Delta\Delta C_t$} method (Livak and Schmittgen, 2001), where ΔC_t is the threshold cycle value difference between the target and reference gene of each reaction. The standard deviation (SD) of the C_t values between replicates was less than 5% (Table 2).

Climate data

Supplemental irrigation was supplied as needed at both localities. In addition, the climatic data is given for the production season (December 2016 – August 2017). Potchefstroom had a minimum of -4.1 °C and a maximum temperature of 36.5 °C. During this period the accumulated rainfall amounted to 439.7 mm, relative humidity ranged between 7.7 and 98.1% and the total heat units were 1,477. In Vaalharts, temperatures ranged between -4.7 and 43.4 °C, rainfall totalled 365.8 mm, relative humidity ranged between 5.2 and 96.7% and there was a total of 2,260 heat units.

Infection indicators

At harvest, the trials were assessed for visual disease severity. Severity assessments were performed on a continuous scale from 1-10, with 1 being no visual symptoms and 10 being completely colonised. The primary ears of 15 plants were assessed for each field plot and expressed as a percentage.

Total fumonisins were extracted from maize flour using a methanol/water (70:30, v/v) extraction buffer according to Rose *et al.* (2016). Fumonisin extracts were submitted to CAF, Stellenbosch University, for LC-MS/MS. Fumonisin standards FB₁, FB₂ and FB₃ was prepared as described by Small *et al.* (2012). A 6-point standard curve was set up ranging from 0.05 to 20.16 mg/kg for FB₁ and FB₂ and 0.005 to 2.08 mg/kg for FB₃.

Genomic DNA of grain samples and *F. verticillioides* MRC 826 mycelia was extracted according to Boutigny *et al.* (2012). Quantitative (q)PCR was performed to quantify the amount of *F. verticillioides* target DNA in the maize kernels as described by Boutigny *et al.* (2012). The melting temperature (T_m) of the amplicons was assessed to confirm analytical specificity. The SD of the C_t values for a sample was less than 5%. Standard DNA (16 times dilution) was included in the qPCR assay and analysed in triplicate. Using the matrix specific standard curves, the C_t values of sampled evaluated were transformed into DNA concentrations using the Rotor-Gene™ 2.0.2.4 software.

Data analysis

One-way analysis of variance (ANOVA) was performed to assess the response of cultivars for the parameters evaluated in this study. Where variation deviated from normality, log-

transformation was performed to improve homogeneity. ANOVA revealed significant differences between localities and, therefore, ANOVA was subsequently performed per locality (Results not shown). Pearson's correlation was performed for each locality to determine correlations between resistance characteristics and infection indicators. Multifactor analysis (MFA) was assessed for all trials to determine whether significant correlations exist between plant traits and the infection indicators. For the MFA, individual parameters were placed in groups as follows: structural characteristics, physico-chemical properties and genetic responses and compared to the group of infection indicators. Partial least squares regression was performed to test correlations between individual characteristics and the group of infection indicators. Within these multivariate analyses, both the log-transformed and non-log-transformed data was included.

3. Results

No gene expression analyses were performed on cultivars evaluated at Potchefstroom due to the low levels of infection indicators. Gene expression could not be obtained for cultivar 15 (Vaalharts). Relative peroxidase expression (fold change) in the fungal-inoculated grain was higher in cultivar 14 (0.297-fold) than any other cultivar evaluated (Figure 1). Alternatively, cultivars 1, 3, 6 (0.034-fold) and 4 (0.043-fold) had the lowest expression of peroxidase. In the water-inoculated grain, cultivar 14 (1.182-fold) still had the greatest expression (Figure 1). Cultivars 1 (0.059-fold) and 13 (0.062-fold) had the lowest relative expression. In terms of the fungal-inoculated grain, expression of *PR5* was highest in cultivar 5 (0.143-fold), not differing significantly from cultivar 14 (0.137-fold) (Figure 2). In the water-inoculated grain, cultivars 11 (0.057-fold) and

Figure 1. Expression of peroxidase gene commercial maize cultivars grown in Vaalharts. Bars marked with the same superscript do not differ significantly ($P > 0.05$).

Figure 2. Expression of pathogenesis-related 5 (*PR5*) relative in commercial maize cultivars grown in Vaalharts. Bars marked with the same superscript do not differ significantly ($P>0.05$).

14 (0.055-fold) had the lowest expression. There was no detectable expression of *PR5* in the water-inoculated grain of cultivar 4, other than that, cultivar 9 (0.001-fold) had the lowest expression (Figure 2).

Pearson's correlations between phenotypic and physico-chemical characteristics with infection indicators

Only correlations with a Pearson's correlation coefficient (r) of ≥ 0.60 were considered as noteworthy correlations. In Potchefstroom, there were significant positive correlations between fumonisin levels and fungal target DNA levels with both inoculated ($r=0.70$) and naturally infected ($r=0.74$) samples (Table 3). In Vaalharts, the kernel pH had an inverse

correlation with infection indicators including visual disease severity ($r=-0.73$), total fumonisins ($r=-0.72$) and fungal target DNA ($r=-0.75$) of inoculated grain (Table 4). The expression of the *PR5* gene in inoculated samples had significant positive correlations with different infection indicators, including total fumonisins of naturally infected ($r=0.72$) and inoculated ($r=0.71$) grain samples as well as with the fungal target DNA levels of the inoculated grain ($r=0.69$). Infection indicator, FER severity, had significant correlations with other infection indicators including total fumonisins of inoculated samples ($r=0.69$). Fumonisin and fungal target DNA levels of inoculated grain ($r=0.94$) had a strong positive correlation (Table 4). A similar trend was seen in the naturally infected samples, where

Table 3. Pearson's correlation matrix of physico-chemical properties with infection indicators of maize cultivars evaluated in Potchefstroom during the 2016/17 season.^{1,2}

	FER severity	Total fumonisins (N)	Total fumonisins (I)	Fungal target DNA (I)	Fungal target DNA (N)
pH	-0.49*	-0.10	-0.16	-0.09	-0.11
Phenolic acids (free)	0.32*	-0.17	0.00	0.28	0.06
Phenolic acids (total)	0.33*	-0.25	-0.08	0.23	0.03
Total nitrogen	0.33*	0.11	-0.35*	-0.31*	0.05
Total carbon	0.17	-0.11	-0.05	-0.03	-0.06
C/N	0.33	-0.12	0.41*	0.35*	-0.02
Total fumonisins (N)	0.23	1.00	0.24	0.11	0.70*
Total fumonisins (I)	0.10	0.24	1.00	0.75*	-0.10
Fungal target DNA (I)	0.23	0.11	0.75*	1.00	-0.12
Fungal target DNA (N)	0.05	0.70*	-0.10	-0.12	1.00

¹ * means significant P -value with 95% confidence level ($P<0.05$).
² Fungal target DNA = *Fusarium verticillioides* target DNA in maize grain samples; I = inoculated; N = naturally infected.

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Table 4. Pearson's correlation matrix of structural characteristics, physico-chemical properties and genetic responses with infection indicators of maize cultivars evaluated in Vaalharts during the 2016/17 season.^{1,2}

	FER severity	Total fumonisins (N)	Total fumonisins (I)	Fungal target DNA (I)	Fungal target DNA (N)
pH	-0.73*	-0.69*	-0.72*	-0.75*	-0.58*
Phenolic acids (bound)	0.32*	0.16	0.30*	0.24	0.03
Total nitrogen	0.34*	0.29	0.30*	0.32*	0.25
Total carbon	0.01	0.05	0.13	0.11	0.00
C/N	-0.37*	-0.41*	-0.31*	-0.36*	-0.36*
Relative <i>peroxidase</i> expression (N)	0.10	0.37*	0.28	0.24	0.35*
Relative <i>peroxidase</i> expression (I)	0.18	0.39*	0.40*	0.36*	0.34*
Relative <i>PR5</i> expression (N)	0.46*	0.59*	0.59*	0.53*	0.60*
Relative <i>PR5</i> expression (I)	0.47*	0.72*	0.71*	0.69*	0.62*
FER severity	1.00	0.58*	0.69*	0.63*	0.55*
Total fumonisins (N)	0.58*	1.00	0.71*	0.75*	0.89*
Total fumonisins (I)	0.69*	0.71*	1.00	0.94*	0.65*
Fungal target DNA (I)	0.63*	0.75*	0.94*	1.00	0.67*
Fungal target DNA (N)	0.55*	0.89*	0.65*	0.67*	1.00

¹ * means significant *P*-value with 95% confidence level (*P*<0.05).

² Fungal target DNA = *Fusarium verticillioides* target DNA in maize grain samples; I = inoculated; N = naturally infected.

fumonisin and fungal target DNA levels (*r*=0.89) had a strong positive correlation. The fumonisin levels measured in naturally infected grain was also significantly correlated to fumonisin levels measured in inoculated grain (*r*=0.71) as well as fungal target DNA levels (*r*=0.75) of inoculated grain (Table 4).

Multivariate analysis

Principle component biplot of cultivars response supported ANOVA analysis with the first principal component (F1) separating most of the variables based on locality (Figure 3). The biplot explained 57.1% of the variation, with F1

Figure 3. Principle component biplot of commercial maize cultivars showing all structural, physico-chemical and genetic characteristics evaluated.

responsible for 46.9% and F2 accounting for 10.3% of the variation observed. Most variables made a significant contribution to variation on F1, including infection indicators, genetic variables as well as pH and moisture content. Based on F1, most variables were positively associated with samples in Vaalharts and negatively associated with samples in Potchefstroom, with the exception of pericarp thickness, silk length, kernel pH and C/N. Only free phenolic acids significantly separated samples on F2 (Figure 3). MFA was only performed for Vaalharts. even though most of the variables evaluated in this study were associated with each other, no correlations between the different groups of variables (levels of resistance), including structural characteristics (squared Pearson's correlation coefficient (RV)=0.08), physico-chemical properties (RV=0.21) or genetic responses (RV=0.30), with infection indicators could be determined

(Figure 4). The biplot explained 41.9% of the variation observed where 24.1% of the variation was explained by F1 and 17.8% explained by F2. Although most of the variables were positively associated, there were no significant correlations between the groups of factors. The group of infection indicators were, however, strongly associated with each other (Figure 4). Since MFA could not determine a clear correlation of the groups of variables, partial least squares regression analysis was performed for the group of infection indicators vs individual resistance characteristics (Figure 5). Certain characteristics correlated with infection indicators namely physico-chemical properties including pH (Variable importance of the projection (VIP)=2.3), N (VIP=1.2) and C/N (VIP=1.3), genetic response of *PR5* in inoculated grain (VIP=2.2), *PR5* expression of the naturally infected grain (VIP=1.9) as well as peroxidase activity in the inoculated grain (VIP=1.2) (Figure 5).

Figure 4. Multifactor biplot of commercial cultivars in Vaalharts showing correlations between the different levels of resistance.

Figure 5. Partial least squares biplot of commercial cultivars in Vaalharts indicating correlations of infection indicators with individual phenotypic, physico-chemical and genetic characteristics.

4. Discussion

Structural, physico-chemical and genetic characteristics of maize, potentially contributing to resistance to *F. verticillioides*, were explored in uncharacterised commercial cultivars. This study, serving as preliminary data, is the first to report on the relationship between factors, representing different levels of resistance, to FER and fumonisin accumulation.

Resistance to *F. verticillioides* by maize is multifactorial; however, no association between structural, physico-chemical and genetic characteristics, representing different levels of resistance, and traditional infection indicators could be determined. Infection indicators including field evaluation for resistance, fumonisin and/or fungal quantification is the gold standard for determining plant response to *F. verticillioides*/fumonisins, however, the process is time consuming and labour intensive (Sampietro *et al.*, 2010). Therefore, the association of maize characteristics, that can be assessed much earlier and faster, would facilitate more efficient identification of resistant material. Although the different levels of resistance did

not correlate with infection indicators, some individual characteristics strongly correlated with these.

Defence-related genes were good indicators of potential resistance to *F. verticillioides*, in this study. The association of genes, including *PR5* and peroxidase, and infection indicators was observed in Vaalharts. This suggests that the genetic potential of the genotype is a key component of resistance to FER and fumonisin accumulation. A positive correlation was found between peroxidase and fungal- and fumonisin-contamination. This demonstrates the ability of the fungus to influence the expression of defence-related genes and the potential of the plant to defend itself.

Defence-related gene expression was low at harvest in the resistant cultivars. Resistant cultivars 1 and 9 had lower peroxidase expression levels than the moderately susceptible cultivar 14. Similarly, cultivars 1 and 9 also had lower *PR5* expression levels than susceptible cultivars 5, 10 and the moderately susceptible cultivar 14. These results suggest that resistant genotypes could have other genes that contribute to its observed resistance. Moreover, low gene expression at harvest potentially indicates that *F.*

verticillioides is no longer perceived as a threat, due to low levels, and thus the plant does not require the expression of defence-related genes at this point. Defence-related genes have been shown to be associated with resistance to FER and fumonisin accumulation in maize (Lanubile *et al.*, 2010, 2017; Maschietto *et al.*, 2016; Van Zyl, 2018). Most of these studies focused on gene expression at initial stages of kernel development; however, the results of this study are supported by the findings of Van Zyl (2018) who determined the genetic response of maize inbred lines as late as 52 days after inoculation with *F. verticillioides*. It is important to note that considering the difference between the water-inoculated and fungal-inoculated expression of peroxidase it may demonstrate, in cultivar 14, an example of the fungus' ability to modulate gene expression to ensure successful infection (Lanubile *et al.*, 2017). This is the first study that quantified defence-related gene expression, amongst other factors, following harvest and confirms that greater expression of defence related genes in susceptible genotypes after *F. verticillioides* infection.

Physico-chemical factors including kernel pH and the carbon:nitrogen ratio (C/N) strongly influenced the suitability of the kernel microenvironment for fungal infection and fumonisin accumulation. The kernel pH had a significant inverse relationship with infection indicators. Previous studies, such as Flaherty *et al.* (2003) revealed that acidic conditions were associated with fumonisin accumulation *in vitro*. The kernel pH is a vital aspect during *F. verticillioides* growth and fumonisin production. Under alkaline kernel conditions, fumonisin production is suppressed (Picot *et al.*, 2011). In our study, maize cultivars in Vaalharts, had significantly more fungal and fumonisin contamination and lower grain pH as compared to Potchefstroom. *F. verticillioides* growth is associated with higher pH and C/N during the earlier stages of kernel development, whereas more acidic and lower C/N conditions is associated with fumonisin accumulation (Duncan and Howard, 2010; Jiménez *et al.*, 2003). Within this study FER severity, fumonisin contamination and fungal target DNA levels were all negatively associated with pH and C/N because infection indicators were assessed in mature maize kernels.

The role of phenolic acids in the defence against *F. verticillioides* was unclear in this study. In a study by Ferrochio *et al.* (2013), low levels of ferulic acid were not associated with plant resistance. It was further suggested that for significant reduction of FER and fumonisin contamination to be achieved, an external application of ferulic acid should be applied (Ferrochio *et al.*, 2013). The results in this study, however, indicate no direct link between phenolic acid quantity and resistance to *F. verticillioides* or fumonisin accumulation in mature maize kernels. Phenolic compounds are not induced to inhibit *F. verticillioides* growth or fumonisin contamination in

mature maize kernels (Cassiem, 2018). The kernel moisture content ranged from 11.6-12.6% for grain evaluated in Potchefstroom and from 11.9-13.5% for grain evaluated in Vaalharts. Other physico-chemical properties could also regulate fungal and fumonisin contamination in the grain, such as sugar and starch quantity, and could also be evaluated in further studies.

Structural characteristics and infection indicators, whether evaluated as a group or as individual characteristics, did not correlate. This could be due to the artificial inoculation employed that breached certain structural barriers such as silk length and husk coverage. Nonetheless, there was also no strong correlation between infection indicators of naturally infected samples with structural characteristics. These results suggest that physical features are not sufficient indicators of resistance to FER/fumonisin accumulation. The importance of structural barriers can be further investigated using other inoculation techniques as the inoculation method used in this study breached certain factors such as the husk leaves.

Commercial cultivars in this study showed varying degrees of resistance to FER and fumonisin accumulation. This was especially evident in Vaalharts where there was significant fungal and fumonisin contamination. FER and fumonisin accumulation occur most frequently in, dry conditions (Cao *et al.*, 2014). Environmental conditions favoured disease development and fumonisin production in Vaalharts, as seen with the high levels of infection indicators in certain cultivars. The infection-indicator data in Vaalharts, therefore, suggest that cultivars 1, 2 and 9 are highly tolerant to *F. verticillioides* colonisation and fumonisin accumulation. All three of these cultivars are genetically modified (GM) cultivars. Conversely, cultivars 5 (non-GM), 10 (GM) and 13 (non-GM) are considered as highly susceptible cultivars. This supports findings that commercial cultivars in South Africa display varying degrees of resistance to FER and fumonisin accumulation (Janse van Rensburg *et al.*, 2015).

The water-inoculated grain samples were more contaminated with fumonisins than the *F. verticillioides*-inoculated samples in the cultivar trial in Vaalharts. The response of cultivars was, however, consistent between the two plots, further supporting the resistance status of the aforementioned cultivars. Field conditions present a variety of factors that could have contributed to this occurrence. The significant genotype by environment effect observed in this study can be ascribed to inherent differences in meteorological conditions at the localities. The maximum temperature and relative humidity at Vaalharts, during the maize-growing season (December to June) is generally higher compared to Potchefstroom (Rose *et al.*, 2016; 2017; Small *et al.*, 2012) providing ideal conditions for fumonisin contamination. In general,

Vaalharts also has a slightly higher rainfall during April and May, when the grain is drying; and combined with high relative humidity due to irrigation further enhances conditions for fumonisin production. Naturally occurring fumonisin-producing isolates may also have been present and established better under the prevailing conditions compared to the isolate used for inoculation. Other sources of inoculum such as from seed, soil or airborne inoculum could also have contributed to the increased fumonisin levels in naturally infected grain (Munkvold, 2003a). *F. verticillioides* is the most abundant fumonisin-producing species in South Africa with different isolates possessing highly variable toxigenic potential and generally similar virulence (Schoeman *et al.*, 2018). Although this study showed strong correlations between FER symptoms, fungal growth and fumonisin contamination, certain isolates could be present in very low quantities, proving symptomless yet having a very high toxigenic potential (Miedaner *et al.*, 2010; Schoeman *et al.*, 2018).

In conclusion, the evaluation of physical, biochemical and genetic characteristics, revealed key associations with traditional infection indicators. The genetic response of maize genotypes to *F. verticillioides* infection was paramount for resistance to FER and fumonisin contamination. Furthermore, conditions within the kernel microenvironment such as pH and C/N ratio could serve as indicators of potential resistance. The results from this study, therefore, provide breeders with additional tools for more efficient selection of resistant material. Multi-site, multi-year evaluation of the cultivars used in this study is warranted to determine the stability of their response to *F. verticillioides* infection. Finally, the expression of defence-related genes not only provided an indication of potential resistance but also provided more evidence for the molecular mechanisms governing resistance to *F. verticillioides*.

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Conflict of interest

The authors declare no conflict of interest.

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