THE MAIZE TRUST

FINAL REPORT FOR COMPLETED MYCOTOXIN RESEARCH PROJECTS

Project no: MTM15-05

Closing date :	**30 June 2020**

1. Title of research project

Phenotypic and genotypic criteria for selecting maize plants resistant to *Fusarium verticillioides*

2. Personal details *(refer to application)*

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4. Project duration and funding overview *(refer to application)*

Duration of project:	2015 to 2019 (5 years)	
Total budget of project:	3 000 000,00	
Amount received from Maize Trust:	1 925 000, 00	
Other sources of funding for this project:	**Contributor**	**Amount received**
	NRF (Thuthuka)	R720 000,00 (2016 - 2018)

	Stellenbosch University (Subcom B)	R150 000 (2016 - 2018)

5. Summary *(description of the project, capturing the main findings; maximum 250 words)*

Resistance to *Fusarium verticillioides*, the fungus that causes Fusarium ear rot (FER) of maize and produces harmful mycotoxins most notably fumonisins (FUM), is mediated at different levels. These include phenotypic (structural), physico-chemical (biochemical) and genetic properties of the maize plant, all of which were investigated in this study. Inbred lines characterised for their response to *F. verticillioide*s/FUM and uncharacterised commercial cultivars were used in this study.

Disease-related indicators (FER severity, FUM and fungal accumulation) did not correlate with structural, biochemical or genetic properties, when compared as groups. However, individual, biochemical traits (pH, carbon-nitrogen ratio) may serve as resistance indicators due to their negative correlation with disease-indicators. The results strongly suggest that gene-based selection for resistance could be an efficient strategy as *peroxidase* and *PR5* gene expression had significant positive correlations with disease-related indicators in greenhouse and field trials and the *PR1* gene in the inbred line trial.

The physiological and biological maturity-stages of kernels were determined to be the most vulnerable to FUM contamination, irrespective of whether grain infection occurred during early or later kernel-developmental stages. Modification of FUM, as a detoxification strategy and potential indicator of resistance, remains unclear and warrants further investigation.

Enhanced plant resistance was demonstrated in F_1 hybrids, generated from resistant and susceptible inbred lines, although the level of resistance did not differ significantly from their parental inbred lines. Inheritance of resistance was found to be additive and the generation of resistant hybrids would be more efficiently achieved by screening and using resistant parental inbred lines.

6. Objectives *(refer to application)*

6.1 Strategic objectives *(alignment with Maize Trust objectives)*

Strategic objectives	Yes/No
To support the establishment of the magnitude of mycotoxin contamination of maize during the stages of its production, storage, and processing in South Africa.	
To support the regular monitoring of the occurrence of the fumonisins, aflatoxins, zearalenone, and trichothecenes (DON and NIV) in locally produced and imported maize.	
To support the determination of the factors which contribute to mycotoxin contamination during the production (pre-harvest), storage (post-harvest) and processing of maize.	**Yes**

To support the development of practical, affordable and environmentally sound methods to manage toxigenic fungi in maize, with particular emphasis on the introduction of resistance in local maize cultivars.	**Yes**
To support the development of sound mycotoxin risk management practices in the maize supply chain to ensure the delivery of safe products to the consumer.	

6.2 Project objectives *(list main objectives)*

1. To correlate *F. verticillioides* colonisation and fumonisin contamination with pericarp thickness, kernel hardness and husk tightness in maize inbred lines resistant and susceptible to *F. verticillioides* colonisation and fumonisin accumulation

2. To assess the physico-chemical factors pertaining to kernel micro-environment (moisture content, pH, sugar content and nutrient status) for its suitability and potential regulation of fumonisin deposition in maize inbred lines resistant and susceptible to *F. verticillioides* colonisation and fumonisin accumulation

3. To determine the expression of defence-related genes identified by RNA-seq in MT project 12-01, Italy and China in maize inbred lines resistant and susceptible to *F. verticillioides* colonisation and fumonisin accumulation

4. To use phenotypic, physico-chemical and molecular indicators to screen South African maize inbred lines and commercial cultivars for resistance to *F. verticillioides* colonisation and fumonisin accumulation

5. Determine the general and specific combinability of resistant and susceptible inbred lines toward understanding the inheritance of resistance in maize

7. Work plan *(refer to application)*

7.1 Work plan as stated in the application *(list main tasks)*

Project 1: Correlation of *F. verticillioides* colonisation and fumonisin contamination with pericarp thickness, kernel hardness, presence of pericarp phenolics, and husk tightness in maize inbred lines resistant and susceptible to *F. verticillioides* colonisation and fumonisin accumulation

Project 2: To assess the physico-chemical factors pertaining to kernel micro-environment (moisture content, pH, sugar content and nutrient status) for its suitability and potential regulation of fumonisin deposition in maize inbred lines resistant and susceptible to *F. verticillioides* colonisation and fumonisin accumulation

- **Sub-project 2.1:** The effect of inoculation time vs. kernel developmental stage on fumonisin production by *F. verticillioides*
- **Sub-project 2.2:** The effect of physico-chemical changes during kernel maturation on fungal infection and fumonisin deposition

Project 3: Determining the expression of defence-related genes identified by RNA-seq in MT project 12-01, Italy and China in maize inbred lines resistant and susceptible to *F. verticillioides* colonisation and fumonisin accumulation

- **Sub-project 3.1:** Determine the extent of fumonisin-derivatives and other types of masked fumonisins to identify additional plant mechanisms involved in resistance to fumonisin deposition.

Project 4: The association of structural, biochemical and molecular characteristics with resistance to *Fusarium verticillioides* and fumonisin contamination

Project 5: Determining the general and specific combinability of resistant and susceptible inbred lines toward understanding the inheritance of resistance in maize

7.2. Achieved tasks according to the stated work plan *(list measurable units as milestones and provide an indication of progress made, e.g. tasks achieved or not [add additional rows if necessary])*

Milestones	Achievements
PROJECT 1: Evaluation of phenotypic traits on inbred lines	Project completed. • Resistant and susceptible inbred lines to *F. verticillioides*/FUM were evaluated during the 2016/17 season. • Significant differences between lines were found for the structural properties evaluated in this study (silk length, husk coverage, pericarp thickness 100-kernel mass and kernel hardness). • However, none of these correlated with disease-related indicators namely FER severity, fungal or FUM contamination.
PROJECT 2: Evaluation of physico-chemical factors regulating fumonisin deposition **Sub-project 2.1:** The effect of inoculation time vs. kernel developmental stage on fumonisin deposition by *F. verticillioides*	Project completed. Sub-project completed. • The physiological and biological maturity stages of kernels were determined to be most vulnerable to FUM contamination. • This result was consistent irrespective of whether infection occurred at flowering or later during kernel development.

Sub-project 2.2: The effect of physico-chemical changes during kernel maturation on fungal infection and fumonisin deposition	Sub-project completed: • Biochemical properties including pH and carbon to nitrogen ratio correlated negatively with disease-related indicators and could be used to indicate potential resistance. • Kernel micro-environmental changes including total sugar content correlated with FUM content; and potentially contribute to increased mycotoxins deposition observed at later kernel maturity stages. • The correlation of amylose and amylopectin, as sugar monomers, with disease-related indicators are currently being determined.
PROJECT 3: Expression of defence-related genes in resistant and susceptible inbred lines	Project completed. • The expression of *PR1*, *PR5* and a putative peroxidase gene was determined and correlated with phenotypic traits of inbred lines.
Sub-project 3.1: Determine the extent of fumonisin-derivatives and other types of masked fumonisins to identify additional plant mechanisms involved in resistance to fumonisin deposition.	Sub-project completed: • Free, hydrolysed fumonisins (HFUM) were found in low quantities in maize grain. • It did not correlate significantly with free FUM in maize grain. • The reason why plants modify FUM in different ways is unclear; and the detoxification of these fungal metabolites remains to be demonstrated.
PROJECT 4: Structural, biochemical and molecular criteria for resistance	Project completed (November 2018): • The criteria for resistance to *F. verticillioides*/FUM was evaluated on 15 maize cultivars. • The expression of *PR1*, *PR5* and a putative peroxidase gene was determined and correlated with disease-related indicators. • pH and the carbon to nitrogen content of grain correlated negatively with

	disease-related indicators and may also be easy, cost-effective measurements of potential resistance in material. • Structural properties did not correlate with disease-related indicators. Although these may contribute to the overall observed resistance, the do not present a formidable means of resistance once breached. • Resistance to FER/FUM is rather dependant on the genetic properties of the maize plant and thus enhancing resistance remains the most effective management strategy.
PROJECT 5: General and specific combinability for resistance	Project completed. • F_1 maize hybrids, generated from inbred lines resistant to FER/FUM and *Aspergillus flavus*/aflatoxins, were evaluated for improved resistance to *F. verticillioides*/FUM. • Generally, the resistance levels of hybrids did not differ significantly from their resistant parental inbred lines. • Additive gene effects were predominant in the inheritance of resistance in this set of hybrids. • Parental inbred line performance was indicative of F1 hybrids performance indicating that hybrids with improved resistance are more efficiently generated from resistant inbred lines.

8. Detailed report covering the research during the full grant period *(introduction, methods, results, tables, figures and discussion)*

INTRODUCTION

Host-plant resistance provides a convenient, environmentally sound and economically viable option for managing the fungus *F verticillioides* that causes Fusarium ear rot (FER) of maize and produces fumonisins (FUM) with noxious effects on humans and animals. While resistance to *F. verticillioides* is not readily available in South African commercial maize cultivars, local maize inbred lines with resistance to FER and FUM accumulation has been identified (Janse van Small et al., 2012; Rensburg et al., 2015; Rose et al. 2016; 2017). Resistance to *F. verticillioides* in maize is complex, quantitative and numerous physical, biochemical and molecular factors can contribute to the observed resistance (Chen et al., 2012; Lanubile et al. 2010; 2012; 2017). Moreover, understanding the inheritance of resistance in maize would be invaluable for the breeding of resistance to FER and/or FUMS.

The first line of defence in a maize plant to *F. verticillioides* infection involves structural barriers. These barriers include pericarp thickness, silk detachment and husk tightness (Headrick and Pataky, 1991; Hoenisch and Davis, 1994; Warfield and Davis, 1996; Fandohan et al., 2003; Munkvold, 2003b; Anderson et al., 2004; Sampietro et al., 2013; Cao et al., 2014). Several views on the role of pericarp thickness in mediating resistance to *F. verticillioides* have been reported (Hoenisch and Davis, 1994; Ivic et al., 2008; Sampietro et al., 2009; Cao et al., 2014). A thicker pericarp was thought to offer more resistance to breaking and to insect damage, and was related to less kernel infection by *F. verticillioides* (Hoenisch and Davis, 1994). Contrary, a thicker pericarp favoured FUM accumulation in kernels in a study by Cao et al. (2014) who concluded that a thicker pericarp could slow down kernel drying, resulting in moisture conditions within the kernel being longer favourable for fungal growth and FUM production. No correlation between pericarp thickness and resistance to FER was found in Croatian inbred lines and hybrids (Ivic et al., 2008). Sampietro and co-workers (2009) however, suggested that the pericarp and its wax content are resistance factors to FUM accumulation in most genotypes assayed. Nevertheless, other kernel factors including the presence of phenolic could not be excluded.

Little information is available on the relationship between kernel hardness and *F. verticillioides* infection and FUM contamination. With rapid, simple technologies like near infrared (NIR) hyperspectral imagining optimised to determine kernel hardness (Williams et al., 2009) it offers a cost-effective technique to determine correlations between kernel hardness and fungal and FUM contamination. In a study by Cao et al. (2014), genotypes exhibiting less husk tightness due to low relative humidity conditions at silking, became more susceptible to kernel infection by *F. verticillioides*.

Fumonisin production by *F. verticillioides* is regulated by numerous biological and physicochemical changes taking place during kernel maturation. *In vitro* studies have shown that changes involving pH and kernel substrates (carbon, nitrogen, sugar, and starch) regulate FUM production by *F. verticillioides* (Marín et al., 1995; Keller et al., 1997;

Marín et al., 1999a; Flaherty et al., 2003; Bluhm et al., 2008; Jurado et al., 2008; Schmidt-Heydt et al., 2008; Marín et al., 2010; Picot et al., 2010; Smith et al., 2012). As maize kernels mature the amount of water available for fungal metabolism (water activity (aw)) decreases which induces FUM gene expression by *F. verticillioides* (Jurado et al., 2008; Schmidt-Heydt et al., 2008). Acidic pH conditions enhance FB1 biosynthesis by *F. verticillioides* and alkaline pH conditions repress FB1 biosynthesis (Keller et al., 1997; Flaherty et al., 2003; Kim and Woloshuk, 2008). Furthermore, high levels of pericarp phenylpropanoids (phenolics) have been associated with less disease severity and FUM accumulation (Sampietro et al., 2012). Similarly, several researchers have found significant inhibitory effects of phenolics on *Fusarium* spp including *F. verticillioides* (Assabgui et al., 1993; Samapundo et al., 2007; Boutigny et al., 2009; Ferrochio et al., 2013). Therefore, physico-chemical factors may serve as indicators of resistance and correlating them to traditional disease severity and FUM contamination may help determine this.

Genes involved in the resistance response of maize to *F. verticillioides* infection have been identified in China, Italy and South Africa (Campo et al., 2004; Gao et al., 2007; Lanubile et al., 2010; 2012a; 2012b; 2014 Campos-Bermudez et al., 2013; Sampietro et al., 2013; van Zyl, 2015). Resistant and susceptible maize genotypes transcribe the same defence-related genes to *F. verticillioid*es, but expressions differ in speed and intensity (Lanubile et al., 2010). The activation of pathogen-associated molecular pattern triggered immunity (PTI) to *F. verticillioides* corresponds well with reports that resistance in maize, to multiple ear rot pathogens such as *F. graminearum*, *Aspergillus flavus*, and *F. verticillioides*, are common (Farrar and Davis, 1991; Presello et al., 2004; Presello et al., 2006; Robertson-Hoyt et al., 2007; Henry et al., 2009; Presello et al., 2011; Mesterházy et al., 2012; Rose et al., 2017). However, recently, Lanubile et al. (2014) reported evidence of Resistance-gene mediated resistance and suggested that gene-based selection for resistance is possible. Determining differential expression of defence-related genes in resistant and susceptible inbred lines could provide useful markers for the screening of potentially resistant breeding material.

Understanding the contribution of each level of resistance and how these (structural, physico-chemical and genetic make-up) relate to one another will provide a powerful predictive ability of resistance. Finally, breeding remains the cornerstone in plant improvement and the occurrence common resistance mechanisms to different ear rot pathogens provide evidence of developing resistant cultivars to multiple mycotoxigenic fungi and their mycotoxins. In a study by Hung and Holland (2012) their results suggested that the most efficient way to improve resistance to FER and FUM contamination in hybrids is to evaluate and select among resistant inbred lines before using resources to create and evaluate hybrids. Knowledge on the inheritance of resistance in maize to *F. verticillioides* and FUMS will be of great benefit to maize breeders in developing resistance maize cultivars.

MATERIALS AND METHODS (for Projects 1-5; as applicable)

***Inbred line greenhouse trial*:** Well-characterised maize inbred lines were used for the greenhouse study namely two lines resistant to FER/FUM (CML 390 and CML 444), one susceptible line (R2565y), and one line susceptible to FER but resistant to FUM accumulation (CB 222) (Table 1). The trial was conducted at the Welgevallen research farm, Stellenbosch University during 2017/18 after a larger field trial was lost to severe drought (2016/17) with no results obtained (APR 2016; MTM15-05). Plants were manually pollinated at flowering. A randomised complete block design (RCBD) was used with three biological replicates per treatment. The treatments consisted of *F. verticillioides* artificially inoculated using the silk-channel method and water inoculation as uninoculated control. The primary ears were harvested at biological maturity, threshed and the grain stored for further analyses.

***Cultivar field trial*:** Fifteen commercial cultivars, planted in the national cultivar trial conducted by the ARC-GC during the 2016/17 season, were randomly selected for this study (Table 2). The cultivars were planted in two localities namely Potchefstroom and Vaalharts. Each cultivar was planted in three field plots in a RCBD at each locality. A field plot consisted of two 10-m rows with 1 m spacing between rows and each row consisted of 25-30 plants. One row was artificially inoculated with *F. verticillioides* while the other plot served as the uninoculated control. Maize plants were allowed to dry naturally infield for 4 to 6 weeks before being manually harvested per row. The maize ears were rated for FER severity following harvest, threshed per row and the grain stored for further analyses.

***Diallel trial*:** Three maize inbred lines, characterised for their response to FER/FUM (Small et al., 2012; Rose et al., 2016); and two lines characterised for their response to Aspergillus ear rot (AER) and aflatoxins (Rose et al., 2017; Okoth et al., 2017a; b) were used in this study. Lines were crossed using method 1 of Griffing's diallel mating system and resulted in 18 hybrids (Table 3). The parental inbred lines and F_1 hybrids were planted in the 2014/15 seasons at three locations namely Potchefstroom, Makhatini and Vaalharts. Field plots were 10 m long (~ 33 plants) and replicated three times in each environment. The parental inbred lines and hybrids were planted in a RCBD and pollination occurred naturally. Artificially inoculated trials were evaluated for FER severity, *F. verticillioides* DNA and FUM content in resultant maize grain.

***Structural trait assessments*:** Silk length, husk coverage, kernel hardness and pericarp thickness were assessed for both field and greenhouse trials unless stated otherwise.

Silk length and husk coverage: The silk length of maize plants was measured at anthesis for the cultivar trials only. The silks of 25 primary ears were measured per plot and measured from the point of silk emergence through the husk leaves to the tip of the silks and rounded off to the nearest half cm. Husk coverage was visually scored as either open or closed, depending on the visibility of the maize ear. For the inbred lines, husk coverage was scored at anthesis and at harvest for each biological replicate. For each cultivar, 25 primary ears were scored per field plot.

Kernel mass and hardness: Hundred-kernel mass (HKM) was determined according to Guelpa et al. (2015) and near-infrared (NIR) spectroscopy was used to determine kernel hardness (Downey et al., 1986). HKM was determined by counting 100 intact kernels for each field (cultivars) or biological (inbred lines) replicate and weighed in grams.

Kernel hardness assessment by NIR spectroscopy, performed by placing samples in a glass Petri plate, was 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 and absorbance measurements were calculated by the NIR LabWare software and used in a formula proposed by Downey et al. (1986) to obtain index values for hardness. For the inbred line trial, kernel hardness via NIR spectroscopy was assessed for flour samples and the cultivar trials was assessed using kernel and flour samples.

Pericarp thickness: The thickness of the kernel pericarp was measured microscopically. Three intact kernels were soaked for 4 hours in sterile deionised H_2O and cut longitudinally from the dent to the tip cap using a sterile scalpel. One half of each kernel was used to measure the thickness of the pericarp with two measurements recorded per kernel. Three kernels were assessed for each field (cultivar trial) or biological (inbred line trial) replicate with the measurements recorded in mm.

***Physico-chemical properties assessments*:** Physico-chemical properties including moisture content, kernel pH, carbon and nitrogen content as well as phenolic acid content were assessed for both inbred lines and cultivar assessments, unless stated otherwise.

Moisture content and kernel pH: The moisture content of maize grain was measured directly after harvest. For the inbred line trial, 5 to 10 kernels were used with three technical replications performed for each biological replicate. The moisture content was determined as a percentage at 20-25ºC. About 25 g of intact kernels were used per measurement and three observations taken for each field plot.

The pH was assessed by mixing 500 mg of finely milled flour with 5 mL of sterile deionised H_2O. Three technical replications for each field (cultivar trial) or biological (inbred line trial) replicate was assessed.

Carbon and nitrogen analysis: The total soluble nitrogen and carbon in kernels was assessed using the TruSpec® Micro at the Central Analytical Facility (CAF) at Stellenbosch University. Ten mg of dried, homogeneous maize flour was placed into an aluminium foil weighing boat and the sample was subjected to combustion at 1050ºC. The elements within the sample formed the following gases: carbon dioxide, H_2O, nitrogen and nitrogen oxides as well as sulphur dioxide and sulphur trioxide. These gases were carried to the absorption columns where it was desorbed and quantified.

Phenolic acid extractions and quantification: Several phenolic acids were extracted from milled maize flour (1 g) according to the optimised protocol developed by Cassiem (2018). Free and bound phenolics were extracted from the maize samples. Phenolic acids quantified represent the major phenolic acids found in the maize kernel pericarp layer. The quantification procedure employed an external standard dilution series using purified

commercial products trans-ferulic, caffeic, ρ-coumaric, sinapic and ρ-hydroxybenzoic acids. The standard curve consisted of a 10-point dilution series ranging from 0.0125–200 mg kg-1. Quantification was performed at CAF, Stellenbosch University.

Gene expression of defence-related genes

Total RNA was isolated as described by Wang et al. (2011). The obtained RNA concentration was determined using a ND-1000 NanoDrop Spectrophotometer where after complementary DNA (cDNA) was synthesised using the iScript cDNA synthesis kit (Bio-Rad Laboratories, USA). Samples were prepared according to the manufacturer's protocol

Reverse transcription quantitative PCR (RT-qPCR) was performed to quantify the relative expression of peroxidase, PR1 and PR5 in the fungus-inoculated and water inoculated kernels according to Lanubile et al. (2010). The elongation factor (EF)-1α was used as reference gene. Validation experiments were performed to show that efficiencies of the target genes and reference gene amplifications were equal (Bustin et al., 2009; Arnold, 2016 Shaikh, 2018). For the inbred line trial, biological replicates were analysed separately, with three technical replications. Cultivar field replications were pooled, and three technical replicates were performed for each cultivar. Template-free samples were included in each run that served as the no template control.

Disease-related indicators**:** Disease severity, fungal content and FUM contamination was assessed as disease-related indicators and were performed for both the field and greenhouse trials unless stated otherwise.

Visual disease rating: At harvest, the cultivar trial was assessed for visual disease severity. Severity assessments were performed on an arbitrary continuous scale from 1-10, with 1 being no visual symptoms and 10 being very severe. The primary ears of 15 plants were assessed for each field plot and expressed as a percentage. Visual disease rating was only scored for cultivar trial (field trials).

Fumonisin extraction and quantification: Fumonisins were extracted from maize flour using a methanol/water (70:30 vol/vol) extraction buffer according to Rose et al. (2016). The analyses was performed at 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-1 for FB1 and FB2 and 0,005 to 2,08 mg kg-1 for FB3.

Quantitative PCR of Fusarium verticillioides *in maize kernels*: Genomic DNA of grain samples and *F. verticillioides* MRC 826 mycelia was extracted according to Boutigny et al. (2012). For the preparation of standard curves for quantitative PCR (qPCR), DNA from pathogen-free maize kernels and *F. verticillioides* MRC 826 was used. A 5-point matrix-matched dilution series was created with the fungal DNA diluted in pathogen-free maize DNA (10 ng μL^{-1}). The quantity of *F. verticillioides* DNA was determined by using primers Fver356 forward/Fver412 reverse (Nicolaisen et al., 2009) as described by Boutigny et al. (2012). Quantification was performed using the Rotor-Gene™ 6000 (Corbett Life Science, Mortlake, Australia). Standard curves met the acceptance criteria with a

correlation coefficient (R^2) > 0.98, a slope (M)-value of between -3.2 and -3.4, and a reaction efficiency (E-value) of 0.98-1.05 (Bustin et al., 2009).

***Data analyses*:** Analysis of variance (ANOVA) was performed to assess the response of inbred lines and cultivars for the parameters evaluated in this study. Where variation deviated from normality, log-transformation was performed to improve homogeneity. For the cultivar trial, ANOVA revealed significant differences between localities and, therefore, ANOVA was then performed per locality. Pearson's correlation was performed for each locality to determine correlations between resistance characteristics and disease-related indicators. Multifactor analysis (MFA) was assessed for all trials to determine whether significant correlations exist between plant traits and the disease-related indicators. For the MFA structural characteristics, physico-chemical properties and genetic responses were grouped and compared to the group of disease-related indicators. Partial least squares (PLS) regression was performed to test correlations between individual characteristics and the disease-related indicators group. Within these multivariate analyses, both the log-transformed and non-log-transformed data was included.

RESULTS

Project 1: *Correlation of* F. verticillioides *colonisation and fumonisin contamination with pericarp thickness, kernel hardness, presence of pericarp phenolics, and husk tightness in maize inbred lines resistant and susceptible to* F. verticillioides *colonisation and fumonisin accumulation*

Project 2: *To assess the physico-chemical factors pertaining to kernel micro-environment (moisture content, pH, sugar content and nutrient status) for its suitability and potential regulation of fumonisin deposition in maize inbred lines resistant and susceptible to* F. verticillioides *colonisation and fumonisin accumulation*

Project 3: *Determining the expression of defence-related genes identified by RNA-seq in MT project 12-01, Italy and China in maize inbred lines resistant and susceptible to* F. verticillioides *colonisation and fumonisin accumulation*

***Disease-related indicators*:** The FUM levels of the fungal-inoculated grain of CB 222 (456.1 mg kg^{-1}) and R2565y (829.6 mg kg^{-1}) was significantly higher than all other samples recorded (Table 4). Similarly, the fungal-inoculated grain of CB 222 (8.21 ng μL^{-1}) and R2565y (11.16 ng μL^{-1}) had the highest levels of fungal target DNA measured (Table 4).

***Phenotypic characteristics*:** The ears of all inbred lines were closed by husk leaves; with the exception of inbred line CB 222 that had one open ear (data not shown). Kernel hardness, assessed on flour samples for inbred lines, indicated that the resistant line CML 390 (51.8) had the highest hardness index and did not differ significantly from CML 444 (39.8). Conversely, FUM-resistant line CB 222 (19.0) had the lowest hardness index (Table 5). Resistant line CML 390 (0.137 mm) had the thickest pericarp layer and it

differed significantly from susceptible line R2565y (0.115 mm) and resistant line CML 444 (0.097 mm) which had the thinnest pericarp layer (Table 5). Line R2565y (51.0 g) had a significantly greater HKM than the other inbred lines, whereas CML 444 (26.3 g) had the lowest HKM.

***Physico-chemical*:** The moisture content of fungal-inoculated grain from resistant line CML 390 (23.4%) was significantly higher compared to all other samples evaluated, with the exception of susceptible line R2565y (Inoculated; 22.8%). Conversely, resistant line CML 444 (Inoculated; 13.7%) had the lowest moisture content and did not differ significantly from water-inoculated grain of CML 444 (15.5%) (Table 6). The fungal-inoculated grain of line CML 390 was the least acidic (pH 6.7) and only differed significantly from the water-inoculated grain of line CB 222 (pH 6.3).

Line CB 222 (Inoculated; 138.2 mg kg^{-1}) had the greatest levels of free phenolic acids, not differing significantly from CB 222 (Control; 57.2 mg kg^{-1}) and R2565y (Control; 60.5 mg kg^{-1}) (Table 6; Fig. 1). CML 444 (Inoculated; 8.6 mg kg^{-1}) had the lowest levels of free phenolic acids and did not differ statistically from several other samples, including CML 390 (Inoculated; 14.9 mg kg^{-1}; control; 17.7 mg kg^{-1}) and R2565y (Inoculated; 17.3 mg kg^{-1}). Bound phenolic acids was the most abundant in water-inoculated grain of line CML 444 (232,1 mg kg-1) and did not differ statistically from CML 390 water- (155.8 mg kg^{-1}) and- fungal-inoculated (190.2 mg kg^{-1}) grain (Table 6; Fig. 2). The water-inoculated grain of line CML 444 (244.3 mg kg^{-1}) also had the highest levels of total phenolic acids, not differing significantly from several other samples, including CML 390 (Inoculated; 205.0 mg kg^{-1}) and CB 222 (Inoculated; 232.8 mg kg^{-1}). R2565y (Inoculated; 73.7 mg kg^{-1}) had the lowest levels, not differing significantly from several others, including CML 444 (Inoculated; 100.5 mg kg^{-1}) and CB 222 (Control; 105.6 mg kg^{-1}).

The fungal-inoculated grain of CML 444 (2.3%) had the highest total nitrogen percentage, not differing significantly from CML 444 (Control; 2.1%) (Table 6). The lowest nitrogen percentage was observed in R2565y (Control; 1.3%) and did not differ significantly from R2565y (Inoculated; 1.6%) and CML 390 (Inoculated; 1.7%). Line CB 222 (Control; 39.2%) contained the highest carbon percentage and only differed significantly from line R2565y (Control; 28.1% and Inoculated; 31.5%). The water-inoculated grain of CB 222 and R2565y (22.3) had the greatest C/N ratio and only differed significantly from CML 444 (Inoculated; 16.9) and (Control; 17.7) (Table 6).

***Genetic response*:** The relative expression of peroxidase was the highest in water-inoculated grain of susceptible line R2565y (9.1 fold change) and only differed significantly from the water- (0.11 fold change) and- fungal-inoculated (0.07 fold change) grain of resistant line CML 390 (Fig. 3). The relative expression of PR1 was greatest in FUM-resistant line CB 222 (fungal-inoculated; 0.60 fold change) which did not differ significantly from a few other samples including the fungal- (0.04 fold change) and- water-inoculated (0.14 fold change) grain of R2565y (Fig. 4). The water-inoculated grain of CML 390 had the lowest expression (<0.00) similar to both fungal- and- water-inoculated grain of CML 444 (0.001 fold change). The water-inoculated sample of R2565y (2.98 fold change) had the highest relative expression of PR5 but did not differ significantly from R2565y (fungal-inoculated; 2.56 fold change) and CB 222 (fungal-inoculated; 1.67 fold

change) (Fig. 5). The lowest expression of PR5 was seen in CML 390 (water-inoculated; 0.002 fold change), not differing significantly from CB 222 (water-inoculated; 0.04) and CML 444 (fungal-inoculated; 0.01 fold change).

***Correlations*:** Only correlations with a Pearson's correlation coefficient (r) of ≥ 0.60 were considered as noteworthy correlations. Fumonisins and fungal target DNA levels (r = 0.82) correlated when the inbred line trial data was analysed (Table 7). Fungal target DNA correlated (r = 0.61) with *PR1* gene expression while it correlated with PR5 (r = 0.69), however, it was not statistically significant at a 95% confidence level.

***Multivariate analysis*:** Principle component analysis (PCA) of inbred lines showed a clear separation of resistant lines CML 390 and CML 444 and FUM-resistant line CB 222 and susceptible line R2565y based on the first principle component (F1) (Fig. 6). The biplot accounted for 49.8% of the total variation, where F1 explained 33.4% and F2 explained 16.4% of the variation. Most of the characteristics and disease-related indicators evaluated made a significant contribution to F1, including HKM, free phenolic acids, PR1 and PR5. According to the multifactor analysis (MFA), the biplot represented 49.8% of the variation observed with principle component 1 (F1) representing 35.0% and F2 representing 14.8% of the total variation. Some of the groups of characteristics, representing different levels of resistance, associated together such as disease-related indicators and genetic response. However, no correlation between the different groups and disease-related indicators, based on the squared Pearson correlation coefficient (RV), could be determined (Fig. 7). Phenotypic characteristics (RV = 0.25), physico-chemical properties (RV = 0.15) and genetic responses (RV = 0.31) did not correlate with disease-related indicators. Partial least squares (PLS) analysis, however, revealed that certain characteristics within the different groups had significant correlations to disease-related indicators (as a group) based on the variable importance on the projection (VIP) value (Fig. 8). Structural characteristics included HKM (VIP = 1.7) and kernel hardness (NIR; VIP = 1.1), physico-chemical properties included moisture content (VIP = 1.6) and levels of free phenolic acids (VIP = 1.2). All three genes assessed had significant variable importance's in the projection (Peroxidase: VIP = 1.2; PR1: VIP = 1.6; PR5: VIP = 1.8).

<u>Sub-project 2.1:</u> The effect of inoculation time vs. kernel developmental stage on fumonisin production by *F. verticillioides*
<u>Sub-project 2.2:</u> The effect of physico-chemical changes during kernel maturation on fungal infection and fumonisin deposition

Free, total FUM in inbred line R2565y ranged from 0.01 to 156.6 mg kg^{-1} in grain inoculated 7 days after pollination; as opposed to 0.09 to 35.7 mg kg^{-1} FUM in grain inoculated 35 days after pollination (Fig. 1). Nonetheless, the trend of FUM deposition in maize kernels were the same between inoculation timepoints. Similarly, total FUM quantified in grain from line I-B ranged from 0.2 to 228 mg kg-1 (7 days after pollination) while 0.2 to 79.4 mg kg^{-1} FUM were quantified in grain inoculated 35 days after pollination (Fig. 2). Similar levels of free FUM were quantified in maize grain inoculated 7 days after pollination (0.3 to 23.6 mg kg^{-1}) or 35 days after pollination (0.02 to 124.5 mg kg^{-1}) of line CB-222 (Fig. 3). The lowest levels of free FUM were quantified in grain from line CML 444 inoculated 7 days after pollination (0.1 to 5.8 mg kg^{-1}) while grain inoculated 35 days after

pollination contained FUM levels ranging from 0.5 to 39.8 mg kg^{-1} (Fig. 4). The trend of FUM deposition in maize grain over different sampling times (7, 28, 42 and 52 days after infection) were the same between inoculation timepoints (1 and 2). The result indicate irrespective of when infection occurs (early kernel-maturity or late kernel maturity) that the physiological and biological kernel maturation stages are the most vulnerable to FUM deposition by *F. verticillioides*.

No correlation could be determined between total FUM, hydrolysed FUM and biochemical properties such as fatty acid or moisture content in any of the inbred lines evaluated (Fig. 13 and 14). The analyses of sugar components amylose and amylopectin are underway, in a current MSc study, to determine whether the change in these can be correlated with the deposition of FUM in kernels as they mature.

***Project 4:* The association of structural, biochemical and molecular characteristics with resistance to Fusarium verticillioides and fumonisin contamination**

***Disease-related indicators*:** Disease severity in Potchefstroom (17.6%) was significantly lower than levels in Vaalharts (26.8%). In Potchefstroom, FER severity of cultivar 4 (12.7%) was the lowest but only differed significantly from three other cultivars namely cultivar 10 (28.4%), 9 (20.7%) and 7 (20.7%) (Table 8). No significant differences in total FUM levels between cultivars were determined in grain inoculated with *F. verticillioides*. There was, however, significantly more FUM in cultivar 15 (33.6 mg kg^{-1}) 3 (25.0 mg kg^{-1}) and 8 (23.0 mg kg^{-1}) in the water-inoculated grain. Similarly, fungal target DNA levels in fungal-inoculated grain were not significantly different between cultivars. Slightly more variation was observed in the water-inoculated grain of cultivar 3 (0.013 ng μL^{-1}) and 15 (0.009 ng μL^{-1}) having the highest levels of fungal target DNA (Table 8).

In Vaalharts cultivars 1 (15.1%) and 2 (16.4%) had significantly lower FER severity lower disease severity than all other cultivars evaluated (Table 9). Cultivar 11 (35.1%) had significantly higher disease symptoms when compared to other cultivars and did not differ significantly from several other cultivars including cultivar 12 (30.4%) and 4 (30.2%). Cultivar 1 (0.5 mg kg^{-1}) contained significantly less FUM in fungal-inoculated grain compared to all other cultivars, except cultivars 2 (1.6 mg kg^{-1}) and 9 (6.2 mg kg^{-1}). Generally, control grain had higher levels of FUM compared to that measured in the fungal-inoculated samples. Cultivar 5 (594.2 mg kg^{-1}) had the highest levels of FUM in the control grain and was statistically similar to several other cultivars, including cultivars 10 (360.4 mg kg^{-1}), 11 (177.6 mg kg^{-1}) and 13 (481.5 mg kg^{-1}) (Table 9).

No fungal target DNA was detected in the fungal-inoculated grain of cultivar 1 and the content did not differ significantly from cultivars 2 (0.006 ng μL^{-1}) and 9 (0.025 ng μL^{-1}) (Table 9). Furthermore, the inoculated grain of cultivar 5 (0.311 ng μL^{-1}) had the highest levels of fungal target DNA and did not differ significantly from several other cultivars, including cultivars 10 (0.145 ng μL^{-1}), 11 (0.114 ng μL^{-1}) and 13 (0.174 ng μL^{-1}). Similarly, in the water-inoculated grain, no target DNA was detected in cultivar 9 and this did not differ significantly from cultivars 1 (0.01 ng μL^{-1}) and 2 (0.003 ng μL^{-1}). Cultivar 5 (0.243 ng μL^{-1}) had the highest levels in the control samples, not differing significantly from

several other cultivars including cultivars 10 (0.121 ng μL^{-1}), 11 (0.091 ng μL^{-1}), and 13 (0.191 ng μL^{-1}) (Table 9).

***Phenotypic characteristics*:** Cultivar 4 (11.9 cm) had the longest exposed silks and did not differ significantly from a number of other cultivars, including cultivars 5 (11.6 cm) and 3 (9.7 cm) when evaluated at Potchefstroom (Table 10). Cultivar 1 (7.6 cm) had the shortest exposed silks with the length measured being statistically similar to most of the other cultivars, including cultivars 15 (7.7 cm), 2 (9.5 cm) and 13 (9.5 cm). At anthesis cultivars 1 (0.0%) and 11 (0.0%) displayed no open ears and these did not differ statistically from other cultivars including cultivars 4 (2.7%) and 13 (1.3%). Cultivar 10 (56.6%) had the highest percentage exposed maize ears but it did not differ significantly from several other cultivars, including cultivars 6 (49.3%) and 9 (38.7%). At harvest, cultivar 11 (17.3%) still displayed the lowest percentage of open ears and did not differ significantly from several other cultivars including cultivars 1 (21.3%) and 4 (20.0%). Cultivars 12 (74.7%), 9 (70.7%) and 10 (70.4%) had the highest percentage of exposed maize ears at harvest. Little variation was observed between cultivars when the 100-kernel mass (HKM) was evaluated (Table 10). Cultivars 5 (31.2 g), 9 (31.8 g) and 10 (31.4 g) had the greatest mass, respectively, but only differed significantly from cultivar 1 (23.1 g).

In Potchefstroom, kernel hardness, based on flour samples, revealed cultivar 9 (12.1) had the highest hardness index, not differing significantly from several other cultivars, including cultivars 5 (11.1) and 7 (10.0) (Table 10). Cultivar 6 (5.3) had the lowest hardness index and did not differ significantly from numerous other cultivars, including cultivars 1 (5.8) and 4 (5.6). When hardness was assessed on whole kernel samples, cultivar 7 (51.1) had the greatest hardness index and was statistically similar to several other cultivars, including cultivars 8 (47.3) and 9 (46.5). Cultivar 6 (35.3) again had the lowest hardness index and did not differ significantly from other cultivars including cultivar 3 (39.7) and 4 (39.6). Cultivar 1 (0.103 mm) had the thinnest pericarp but only differed significantly from cultivar 2 (0.167 mm), 3 (0.149 mm), 10 (0.140 mm) and 11 (0.139 mm) that had the thickest pericarp (Table 10).

Significant differences between cultivars were determined for the different structural characteristics evaluated at Vaalharts (Table 11). Cultivar 2 (9.6 cm) had the longest silks, however, its length did not differ significantly from several other cultivars, including cultivar 9 (9.1 cm) and 10 (9.2 cm). Cultivar 15 (5.7 cm) had the shortest exposed silks and did not differ from cultivars 6 (6.2 cm), 11 (6.7 cm) as well as 3 and 14 (6.8 cm).

At anthesis, cultivar 1 had no open ears and was statistically similar to cultivars 2 (1.3%), 5 (9.3%), 8 (12.0%) and 10 (16.0%) while cultivars 3 and 4 (76.0%) had the highest percentage of open ears (Table 11). At harvest, cultivar 1 (4.0%) still had significantly lower levels of open ears compared to all other cultivars except cultivar 10 (20.0%). Cultivar 14 (73.3%) had the highest percentage of ears not covered by husk leaves but this did not differ significantly from several other cultivars, including cultivars 4 (73.1%), 2 (66.7%) and 15 (56.0%).

Cultivar 14 (40.9 g) had the greatest HKM measured whereas cultivar 15 (25.2 g) had the lowest mass with cultivar 12 (26.8 g) not differing significantly from cultivar 15 (Table 11). In Vaalharts, cultivar 14 (15.8) had the highest hardness index when flour samples were evaluated while 11 (56.6), 14 (50.8) and 1 (50.0) had amongst the highest hardness indices for kernels. Cultivar 8 (5.8) had the lowest hardness index and did not differ significantly from cultivars 3 (7.1) and 15 (8.5) (Flour samples). Cultivar 10 (40.0) had the lowest hardness index but only differed significantly from cultivars 1 (50.0), 14 (50.8), 13 (49.0) and 11 (56.6) (whole kernel samples).

The thinnest pericarp layer was recorded for cultivar 12 (0.094 mm) and it only differed significantly from a few cultivars including 7 (0.146 mm), 14 (0.143 mm), 2 (0.128 mm) and 1 (0.116 mm) (Table 11).

***Physico-chemical properties*:** In Potchefstroom, cultivar 5 (pH 6.40) and 4 (pH 6.39) had the highest pH measured in fungal-inoculated grain (Table 12). The grain of cultivar 10 (pH 5.99) was the most acidic and did not differ statistically from several other cultivars, including cultivars 11 (pH 6.04) and 13 (pH 6.05). Cultivars 3 and 10 (11.6%) had the lowest grain moisture content when fungal-inoculated grain was evaluated. These cultivars, however, only had significantly lower moisture levels than cultivar 8 (12.6%), 9 (12.2%) and 11 (12.3%). Similarly, cultivar 10, 2, 13 and 14 (11.7) had the lowest grain moisture when water-inoculated grain was evaluated and only differed significantly from cultivar 9 (12.6) (Table 12).

In Potchefstroom, little variation was determined between cultivars when free, bound and the total phenolic acid content was evaluated (Table 12). Cultivar 10 (0.95 mg kg^{-1}) had the highest amount of free phenolic acids, however, it only differed significantly from cultivar 5 (0.01 mg kg^{-1}) and 8 (0.04 mg kg^{-1}) that contained the lowest levels of free phenolic acids quantified (Fig. 15). Cultivar 14 (0.22 mg kg^{-1}) had significantly higher levels of bound phenolic acids quantified when compared to all the cultivars evaluated except cultivars 5 (0.09 mg kg^{-1}), 1 (0.05 mg kg^{-1}) and 15 (0.04 mg kg^{-1}) (Fig. 16). No significant differences between cultivars could be determined when the total phenolic acid content of the fungal-inoculated grain were evaluated.

Total nitrogen was the highest in cultivar 10 (1.43%) and its content did not differ significantly from cultivars 13 (1.3%), 14 (1.3%), 7 (1.3%) and 3 (1.3%) (Table 12). Cultivar 11 (1.0%) had the lowest nitrogen level but did not differ significantly from several other cultivars including cultivars 6 (1.0%) and 9 (1.1%). Cultivar 1 (39.1%) contained the least amount of total carbon when compared to all the cultivars evaluated except cultivars 2 (38.9%) and 3 (38.9%). Cultivar 15 (42.2%) had the highest level of total carbon, however, this was statistically similar to most of the other cultivars evaluated. In terms of the carbon:nitrogen ratio (C/N), cultivar 11 (44.2) had the highest ratio while cultivar 10 (29.5) and 3 (30.1) had the lowest ratio (Table 12).

In Vaalharts, cultivar 1 (pH 6.06) had the highest pH assessed in fungal-inoculated grain, statistically similar to cultivar 2 (pH 5.95) (Table 13). Cultivar 12 (pH 4.96) was the most acidic, not differing significantly from cultivars 11 (pH 5.07) and 13 (pH 4.97). Cultivar 10 (13.5%) had the greatest grain moisture content. It did not differ significantly from a

number of other cultivars, including cultivars 9 (13.0%), 11 (13.0%) and 14 (13.1%). Alternatively, cultivar 8 had the lowest grain moisture content, and did not differ significantly from several other cultivars including cultivars 1 (12.3%) and 15 (12.3%) (Table 13).

The lowest free phenolic acid levels were recorded in cultivars 5 and 14 (0.02 mg kg^{-1}) and only differed significantly from cultivars 4 (2.14 mg kg^{-1}), 3 (0.92 mg kg^{-1}) and 8 (0.52 mg kg^{-1}) which had the highest free phenolic acid content (Table 13; Fig. 17). The greatest levels of phenolic acids recorded were bound phenolics in cultivars grown in Vaalharts (Fig. 18). Cultivar 12 (71.35 mg kg^{-1}) had the greatest levels, statistically similar to several other cultivars, including cultivar 15 (42.39 mg kg^{-1}). Cultivar 3 (0.02 mg kg^{-1}) had the lowest bound phenolic acid content, not differing significantly from several other cultivars including cultivars 1 (0.10 mg kg^{-1}) and 6 (0.05 mg kg^{-1}). Like bound phenolic acid content, cultivar 12 (71.42 mg kg^{-1}) had the greatest levels of total phenolic acids and did not differ significantly from several other cultivars, including cultivar 15 (42.5 mg kg^{-1}). Overall, cultivar 6 (0.10 mg kg^{-1}) had the lowest phenolic acid content, similar to cultivars 1 and 5 (0.24 mg kg^{-1}), 2 (0.30 mg kg^{-1}) and 7 (0.46 mg kg^{-1}) (Table 13).

Cultivar 7 (1.4%) contained the greatest amount of nitrogen and only differed significantly from cultivars 1 (1.1%), 3 (1.2%) and 8 (1.1%) which contained the lowest nitrogen levels (Table 13). Cultivar 7 (45.5%) had a significantly greater carbon content that all other cultivars. Cultivar 3 (41.6%) however, had the lowest total carbon percentage, not differing significantly from cultivars 5, 8 and 10 (42.0%). In terms of carbon:nitrogen ratio (C/N) cultivar 1 (40.2) had the greatest ratio, statistically similar to several other cultivars, including cultivar 8 (39.3). Cultivar 6 (29.3) had the lowest ratio, statistically similar to several other cultivars, including cultivar 7 (32.3).

***Genetic response*:** No gene expression analyses were performed on cultivars evaluated at Potchefstroom due to the low levels of disease-related indicators. Gene expression could not be obtained for cultivar 15 (Vaalharts). The fungal-inoculated grain, expression of PR5 was highest in cultivar in cultivar 5 (0.143 fold change), not differing significantly from cultivar 14 (0.137 fold change) (Fig. 19A). In the water-inoculated grain, cultivars 11 (0.057 fold change) and 14 (0.055 fold change) had the lowest expression. There was no PR5 expression in the water-inoculated grain of cultivar 4, other than that, cultivar 9 (0.001 fold change) had the lowest expression (Fig. 19A). Relative peroxidase expression (fold change) in the fungal-inoculated grain was higher in cultivar 14 (0.297 fold change) than any other cultivar evaluated (Fig. 19B). Alternatively, cultivars 1, 3, 6 (0.034 fold change) and 4 (0.043 fold change) had the lowest expression of peroxidase. In the water-inoculated grain, cultivar 14 (1.182 fold change) still had the greatest expression (Fig. 19B). Cultivars 1 (0.059 fold change) and 13 (0.062 fold change) had the lowest relative expression.

***Locality-specific correlations*:** In Potchefstroom, there were significant positive correlations between FUM levels and fungal target DNA levels with both inoculated (r = 0.70) and uninoculated (r = 0.74) samples (Table 14). In Vaalharts, pH had an inverse correlation with disease-related indicators including visual disease severity (r = -0.73), total FUM (r = -0.72) and fungal target DNA (r = -0.75) of inoculated grain (Table 15). The

expression of the PR5 gene in inoculated samples had significant positive correlations with different disease-related indicators, including total FUM of uninoculated (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). Disease-related indicators including FER severity had significant correlations with other disease-related indicators including total FUM of inoculated samples (r = 0.69). Fumonisins and fungal target DNA levels of inoculated grain (r = 0.94) had a strong positive correlation (Table 15). A similar trend was seen in the uninoculated samples, where FUM and fungal target DNA levels (r = 0.89) had a strong positive correlation. The FUM levels measured in uninoculated grain was also significantly correlated to FUM levels measured in inoculated grain (r = 0.71) as well as fungal target DNA levels (r = 0.75) of inoculated grain (Table 15).

***Multivariate analyses*:** Principle component biplot of cultivars response supported ANOVA analysis with the first principal component (F1) separating most of the variables based on locality (Fig. 20). The biplot explained 57.1% of the variation, with F1 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 disease-related 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 (Fig. 20).

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 (RV = 0.08), physico-chemical properties (RV = 0.21) or genetic responses (RV = 0.30), with disease-related indicators could be determined (Fig. 21). 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 disease-related indicators were, however, strongly associated with each other (Fig. 21). Since MFA could not determine a clear correlation of the groups of variables, partial least squares regression (PLS-R) analysis was performed for the group of disease-related indicators vs individual resistance characteristics (Fig. 22). Certain characteristics correlated with disease-related indicators namely physico-chemical properties including pH (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 control grain (VIP = 1.9) as well as peroxidase activity in the inoculated grain (VIP = 1.2) (Fig. 22).

<u>Project 5:</u> Determining the general and specific combinability of resistant and susceptible inbred lines toward understanding the inheritance of resistance in maize

***FER severity assessment*:** The mean FER severity observed in Makhatini (12.7%) and Vaalharts (11.6%) did not differ significantly from each other, but differed significantly from the mean FER in Potchefstroom (4.4%) (Table 16). Disease severity ranged from 4.0%

to 31.6% in Makhatini, from 0.3% to 12.2% in Potchefstroom and 2.4% to 30.3% in Vaalharts. The hybrid R119W x CKL05015 exhibited the lowest FER symptoms (4.0%) in Makhatini and differed significantly from the worst performing inbred lines (CML444; 31.6%, CML390; 24.6% and R119W, 22.6%) but not from the best performing inbred lines (CML495; 9.7% and CKL05015; 11.6%) (Table 16). Several hybrids did not differ significantly from R119W x CKL05015, including CML444 x CML495 (4.6%), CML495 x CML390 (5.1%), CKL05015 x CML495 (6.4%), CML495 x CKL05015 (8.2%) and CKL05015 x R119W (11.6%). A hybrid resulting from the crossing of two Kenyan inbred lines (CKL05015 x CML495) had the lowest signs of infection (0.3%) in Potchefstroom, which differed significantly from FER severity in all parental lines but not in hybrids CML444 x CKL05015 (1.2%) and R119W x CKL05015 (1.2%) (Table 16). In Vaalharts, the hybrid CKL05015 x CML444 developed significantly less FER symptoms (2.4%) than the parental lines, but not significantly less than hybrids CKL05015 x CML390 (3.7%), R119W x CKL05015 (3.8%) and CKL05015 x R119W (5.2%). Inbred line CML390 developed most FER symptoms in Potchefstroom (12.2%) and Vaalharts (30.3%), which differed significantly from the susceptible check R119W (1.4% and 9.8%, respectively). CML390 did not have the most FER symptoms in Makhatini (24.6%) but it did not differ from the genotype with the most symptoms (CML444; 31.6%) (Table 16).

***Quantification of F. verticillioides DNA in maize grain*:** The mean *F. verticillioides* DNA concentrations in maize grain differed significantly between the three locations. Makhatini had the highest mean fungal DNA content of 0.038 ng μL^{-1} and differed significantly from Vaalharts (0.021 ng μL^{-1}) and Potchefstroom (0.012 ng μL^{-1}) (Table 16). The latter localities also differed significantly from each other. The hybrid, CML495 x CML390 was least colonised by F. verticillioides in Makhatini (0.011 ng μL^{-1}), which was significantly less than the other inbred lines apart from parental inbred line CML495 (0.025 ng μL^{-1}). The hybrids that were significantly more contaminated with *F. verticillioides* than CML495 x CML390 included CML444 x CKL05015 (0.055 ng μL^{-1}), R119W x CML495 (0.055 ng μL^{-1}) and CML390 x R119W (0.076 ng μL^{-1}). The fungal content in CML390 x R119W (0.076 ng µL-1) did not differ significantly to that measured in parental inbred lines CKL05015 (0.064 ng μL^{-1}), CML390 (0.063 ng μL^{-1}) and R119W (0.074 ng μL^{-1}) (Table 16).

Potchefstroom had very low levels of *F. verticillioides* colonisation. The hybrids CKL05015 x CML444, CKL05015 x R119W, CML495 x CKL05015, CML495 x CML444, CML495 x R119W and R119W x CML495 all had fungal DNA below the limit of detection in Potchefstroom (0.006 ng μL^{-1}). In Potchefstroom, the lowest detectable fungal DNA was observed in the cross R119W x CML444 (0.006 ng μL^{-1}). The fungal content measured in this hybrid only differed significantly from hybrids CML390 x CML444 (0.038 ng μL^{-1}) and R119W x CML390 (0.035 ng μL^{-1}) (Table 16). In Vaalharts, *F. verticillioides* target DNA could not be detected in hybrids CKL05015 x CML444, CML444 x CKL05015, CML495 x CKL05015, R119W x CKL05015 and R119W x CML444. The hybrids CML444 x CML495 and CML495 x CML390 had the lowest detectable fungal DNA in Vaalharts (0.007 ng μL^{-1}), which only differed significantly from hybrid CML495 x R119W (0.045 ng uL^{-1}) and inbred lines CKL05015 (0.087 ng μL^{-1}) and CML390 (0.082 ng μL^{-1}) (Table 16). All the hybrids differed significantly from inbred line CKL05015 (0.087 ng μL^{-1}) that contained the most fungal DNA in Vaalharts (Table 16). Hybrids CKL05015 x CML390 (0.009 ng µL-1)

and CML390 x CKL05015 (0.012 ng μL^{-1}) had significantly less fungal DNA compared to their parental lines (CKL05015; 0.087 ng μL^{-1} and CML390; 0.082 ng μL^{-1}) in Vaalharts.

***Fumonisin analysis*:** The means of total FUM concentration in maize grain differed significantly between locations, with the highest mean concentration measured in Makhatini (3.936 mg kg-1) followed by Vaalharts (1.198 mg kg^{-1}) and Potchefstroom (0.322 mg kg^{-1}) (Table 16). FUM concentrations ranged from 2.433 mg kg^{-1} to 6.199 mg kg^{-1} in Makhatini, from 0.027 mg kg^{-1} to 1.352 mg kg^{-1} in Potchefstroom and from 0.071 mg kg^{-1} to 6.470 mg kg^{-1} in Vaalharts. The hybrid CML495 x CML390 had the lowest FUM concentration (2.433 mg kg-1) in Makhatini, which differed significantly from that of the worst performing parental line (CKL05015; 6.199 mg kg^{-1}) as well as parental lines CML390 (5.786 mg kg^{-1}), CML444 (3.759 mg kg^{-1}), CML495 (5.725 mg kg^{-1}) and R119W (5.627 mg kg^{-1}). The FUM content of most hybrids; including CML495 x R119W (2.568 mg kg^{-1}), CML444 x CML495 (2.587 mg kg^{-1}), CKL05015 x R119W (2.757 mg kg^{-1}) and CKL05015 x CML495 (2.751 mg kg^{-1}); did not differ significantly from that of hybrid CML495 x CML390 (2.433 mg kg^{-1}) (Table 16). R119W x CML495 accumulated the highest FUM concentration (6.281 mg kg-1) among the hybrids in Makhatini, which did not differ significantly from the parental lines. Hybrids CML495 x CML390 (2.433 mg kg^{-1}), CML495 x R119W (2.568 mg kg^{-1}) and R119W x CML390 (2.831 mg kg^{-1}) contained significantly less FUM levels than their parental inbred lines (CML390; 5.786, CML495; 5.725 and R119W; 5.627 mg kg^{-1}) (Table 16).

Hybrid CML495 x CML390 (0.027 mg kg^{-1}) had the lowest detectable FUM content in Potchefstroom which it did not differ significantly from the FUM content in line CML495 (0.029 mg kg^{-1}) (Table 16). FUM concentrations in hybrids CKL05015 x CML444, CKL05015 x R119W, CML444 x CML495, CML495 x CKL05015, CML495 x R119W and R119W x CML495 were lower than the detectable limit of 0.02 mg kg^{-1}. In Potchefstroom, the FUM content of parental lines CML444 (0.573 mg kg^{-1}), CML495 (0.029 mg kg^{-1}) and CKL05015 (0.104 mg kg^{-1}) did not differ significantly from the best performing hybrid (CML495 x CML390; 0.027 mg kg^{-1}). In Vaalharts, lines CML 390 and CKL05015 accumulated a high FUM content of 6.470 mg kg^{-1} and 4.536 mg kg^{-1}, respectively (Table 16). Hybrid CML444 x CKL05015 had the lowest FUM (0.071 mg kg^{-1}), which did not differ significantly from that in hybrids such as CKL05015 x CML444 (0.083 mg kg^{-1}), R119W x CKL05015 (0.137 mg kg^{-1}) and CKL05015 x CML495 (0.661 mg kg^{-1}). In Vaalharts, hybrids CKL05015 x CML390 (0.581 mg kg^{-1}) and CML390 x CKL05015 (0.706 mg kg^{-1}) accumulated less FUM compared to their parental lines CML390 (6.470 mg kg^{-1}) and CKL05015 (4.536 mg kg^{-1}) (Table 16).

***AMMI analysis*:** Genotypes, environments and GEI were significant sources of variation for all three traits evaluated ($P < 0.05$) (Table 17). The genotype effect (22.29%) was the most important in explaining variation in the amount of fungal target DNA retrieved. The majority of the variation in FER severity (26.45%) and FUM concentrations (64.25%) was attributed to the environment. The first IPCA was important in explaining the GEI for all three traits ($P < 0.05$), especially FUM concentration ($P < 0.001$), while the second IPCA was not significant for all three traits ($P = 0.0783$) (Table 17). The AMMI analysis for FER severity revealed that 24.67% of the total variation was explained by the genotype effects, 26.45% by the environment and 17.28% by GEI (Table 4). The genotype effects,

environmental effects and GEI were accounted for 22.29%, 15.18% and 20.83% of the total variation observed in *F. verticillioides* colonisation, respectively. The total variation observed in accumulated FUM was largely attributed to the environment (64.25%) while the genotype effects and GEI explained 12.26% and 8.49% of the total variation, respectively (Table 17).

The three most stable genotypes for FER severity, *F. verticillioides* colonisation and FUM contamination were all hybrids (Table 18). Hybrid R119W x CKL05015 was most stable for FER symptom expression (0.0164), while CKL05015 x R119W (0.0068) and CKL444 x CML495 (0.0523) were most stable for *F. verticillioides* colonisation and FUM contamination, respectively. The parental lines CKL05015 (0.2018) and CML390 (1.615) had the least stability for *F. verticillioides* colonisation and FUM concentration, respectively. Hybrid CKL05015 x CML444 (1.1318) was the least stable for FER severity and was the only hybrid to be the least stable across all three parameters (Table 18).

***GGE biplot analysis*:** The first and second PC explained 59.0 and 23.4%, respectively, of the variation in FER severity, accounting for 82.4% of the total variation observed (Fig. 23). Hybrid R119W x CKL05015 (C16) had the overall highest level of resistance for FER severity, followed by CML390 x CKL05015 (C5). These hybrids (R119W x CKL05015 and CML390 x CKL05015) also had low PC2 scores, indicating good stability (Fig. 23A and Table 18). The parental line CML390 (L2) had the most FER symptoms, followed by CML390 x R119W (C7) (Fig. 23A).

The three test environments fell into different sectors of the polygon view, indicating crossover interactions (Fig. 23B). The hybrid CKL05015 x CML495 (C3) was the vertex genotype in the Potchefstroom sector, R119W x CKL05015 (C16) in Makhatini, and CKL05015 x CML444 (C2) in the Vaalharts sector (Fig. 2B). Hybrids CKL05015 x CML495 (C3), CML444 x CKL050105 (C8), CML444 x CML495 (C9), CML495 x CML390 (C12) and CML495 x R119W (C14) developed the least FER symptoms in Potchefstroom (Fig. 23B). The hybrids CML390 x CKL05015 (C5) and R119W x CKL05015 (C16) expressed the least FER symptoms in Makhatini, while CKL05015 (L1), CKL05015 x CML390 (C1), CKL05015 x CML444 (C2), CKL05015 x R119W (C4) and CML495 x CKL05015 (C11) had the least FER symptoms in Vaalharts. The hybrids CKL05015 x CML444 (C2) and CKL05015 x CML495 (C3) were the least stable as they were the farthest from the biplot origin. The inbred line R119W (L5) was located on the origin of the biplot (Fig. 23B) indicating average performance and its lack of response to the environment.

A GGE biplot on *F. verticillioides* colonisation of maize inbred lines and hybrids explained 91.6% of the total variation found under three different environments in South Africa (PC1 = 65.1.2% and PC2 = 26.5%) (Fig. 24). The hybrid CML495 x CKL05015 (C11) contained the least fungal DNA concentration followed by CML495 x CML390 (C12) and CML444 x CML495 (C9) (Fig. 24A). Hybrid CML495 x CKL05015 (C11) and CKL05015 x R119W (C4) were also close to the biplot origin indicating good stability. The worst performing genotypes in terms of *F. verticillioides* colonisation were inbred lines CKL05015 (L1) and CML390 (L2) (Fig. 24A). The test environments also fell into three different sectors for the *F. verticillioides* colonisation (Fig. 24B). In Makhatini, genotypes CKL05015 x CML495 (C3), CML444 x CML495 (C9), CML495 x CKL05015 (C11) and CML495 x CML390 (C12)

had the highest level of resistance to *F. verticillioides* colonisation. The majority of the genotypes performed relatively similar and had the lowest *F. verticillioides* target DNA concentrations in Potchefstroom and Vaalharts (Fig. 24B). The genotypes CML495 x R119W (C14), R119W (L5) and CML390 x R119W (C7) were the least stable genotypes with regards to fungal colonisation. All the inbred lines (L1 - L5) did not fall into the same segment as any test environment (Fig. 24B).

The PC1 accounted for 76.2% and PC2 for 12.9% of the FUM content variation, explaining 89.1% of the total variation (Fig. 25). The genotype CML495 x CML390 (C12), accumulated the lowest FUM concentration followed by CKL05015 x R119W (C4) although this genotype had a lower PC2 value (0.023) (Fig. 25A and Table 18). The inbred line CML390 (L2) accumulated the highest concentration of FUM followed by CKL05015 (L1) (Fig. 25A). The least stable genotypes were CML495 x R119W (C14) and R119W x CML495 (C15) (Fig. 25B). Potchefstroom and Vaalharts fell into the same sector of the polygon whereas Makhatini fell into a different sector. Several hybrids performed well in Makhatini including CKL05015 x CML495 (C3), CKL05015 x R119W (C4), CML444 x CML495 (C9) and CML495 x CML390 (C12). The inbred lines (L1 - L5) did not fall into the same segment as any test environment (Fig. 25B).

***Correlations*:** Significant correlations between FER severity, fungal target DNA and total FUM were determined ($P < 0.05$). Correlations between FER severity and *F. verticillioides* colonisation within environments were low to moderate, ranging from $r = 0.33 - 0.49$, while they ranged from $r = 0.36 - 0.48$ between FER severity and FUM contamination (Table 19). However, the correlation between FUM contamination and fungal biomass within environments was good, ranging from $r = 0.73 - 0.79$. A moderate but significant overall correlation of $r = 0.47$ was observed between FER severity and *F. verticillioides* colonisation (Table 19). FER severity had a moderate correlation with FUM contamination, with an overall Pearson correlation of $r = 0.53$. A high overall correlation between FUM contamination and fungal biomass was determined ($r = 0.71$) (Table 19).

***Diallel analysis*:** General and specific combining ability were significant for all three traits ($P < 0.05$) (Table 20). The GCA x environment and SCA x environment interactions were not significant except for the GCA x environment effect on FUM concentration ($P < 0.05$). The reciprocal, maternal and non-maternal effects were not significant for all three traits ($P < 0.05$) (Table 20). Baker's ratios of 0.83, 0.69 and 0.68 were observed for FER severity, *F. verticillioides* colonisation and FUM concentration, respectively. High broad sense heritability was observed for the evaluated traits and ranged from 0.95 to 0.97. Whereas the narrow sense heritability ranged from 0.65 to 0.81 for FER severity, *F. verticillioides* colonisation and FUM content, respectively (Table 20).

Inbred lines CKL05015 had the largest negative GCA estimate (-0.44) for FER severity ($P < 0.05$) (Table 21). The inbred line CML390 (0.24) and CML444 (0.15) had the largest positive and significant GCA estimates for FER severity ($P < 0.05$). The Kenyan inbred line CML495 had a negative GCA estimate (-0.01) for FER severity, which was not significant ($P < 0.05$). The significant GCA estimates for *F. verticillioides* colonisation observed in this study were very low, 0.009 (CML495) and -0.006 (CML390) ($P < 0.05$) (Table 21). Negative significant GCA estimates for resistance to FUM contamination were

observed in CKL05015 (-0.06) and CML495 (-0.10), with the latter having the largest value ($P < 0.05$). The inbred line CML390 had a significant positive GCA estimate (0.16) for FUM contamination ($P < 0.05$). The susceptible check, R119W, had no significant GCA for any of the traits evaluated ($P < 0.05$) (Table 21).

Hybrids CKL05015 x CML390 (-0.30), CKL05015 x R119W (-0.30) and CML390 x CML444 (-0.23) had the largest negative SCA estimates for FER severity, while CML390 x R119W (0.33), R119W x CKL05015 (0.32) and R119W x CML390 (0.21) had the largest positive estimates for the same trait (Table 22). The largest significant SCA estimates for *F. verticillioides* colonisation were obtained for hybrids CKL05015 x R119W (-0.01) and CKL05015 x CML390 (0.01) ($P < 0.05$). The hybrids CKL05015 x CML390 (-0.14) and CKL05015 x R119W (-0.18) exhibited the largest negative and significant SCA estimate for FUM content, while the highest positive and significant SCA estimate was observed on R119W x CML444 (0.18) (Table 22).

DISCUSSION

Plant defence mechanisms including physical attributes of the maize plant and kernel structure represents a formidable barrier to *F. verticillioides* infection and the accumulation of its mycotoxins, FUM. In this study, structural, physico-chemical and genetic characteristics of maize, potentially contributing to resistance to *F. verticillioides*, were explored in previously characterised maize inbred lines and uncharacterised commercial cultivars. This study is the first to report on the relationship between factors, representing different levels of resistance, to FER and FUM 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 disease-related indicators could be determined. Disease-related indicators including field evaluation for resistance, FUM and/or fungal quantification is the gold standard for determining plant response to *F. verticillioides*/FUM, however, the process is time consuming and labour intensive (Köppen et al., 2010; 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 disease-related indicators, some individual characteristics strongly correlated with these.

Defence-related genes, particularly pathogenesis-related (PR) genes were a good indicator of potential resistance to *F. verticillioides*, in this study. The association of genes, including PR5 and peroxidase, and disease-related indicators was observed in the inbred line and cultivar trials, respectively. This suggests that the genetic potential of the genotype is a key component of resistance to FER and FUM accumulation. A positive correlation was found between peroxidase and fungal- and- FUM contamination in both trials. Likewise, PR1 expression in the inbred line trial and PR5 in both trials also had positive correlations with fungal- and- FUM contamination. Defence-related genes were strongly induced in fungal-inoculated grain of FUM-resistant line CB 222 while it was down-regulated in the susceptible line R2565y. 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. Similar results were obtained for inbred lines where the expression of peroxidase, PR1 and PR5 was greater in R2565y (susceptible line) and CB 222 (susceptible to FER only) than in the resistant lines CML 390 and CML 444. 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. There are a range of other plant defence genes including PR genes and ROS genes that have been identified in the *F. verticillioides*-maize pathosystem that could be responsible for the resistant phenotype observed (Lanubile et al., 2010; Van Zyl, 2018).

Defence-related genes have been shown to be associated with resistance to FER and FUM 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*. 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 FUM accumulation. The kernel pH had a significant inverse relationship with disease-related indicators in the cultivar trial. Previous studies, such as Flaherty et al. (2003) determined that acidic conditions were associated with FUM accumulation in vitro. The kernel pH is a vital aspect during *F. verticillioides* growth and FUM production. Under alkaline kernel conditions, FUM production is suppressed (Picot et al., 2011). In our study, maize cultivars in Vaalharts, had significantly more fungal and FUM contamination and lower grain pH as compared to Potchefstroom. Furthermore, studies show that *F. verticillioides* growth is associated with higher pH and C/N during the earlier stages of kernel development, and more acidic and lower C/N conditions to be associated with FUM accumulation (Jimenez et al., 2003; Duncan and Howard, 2010). Within this study FER severity, FUM contamination and fungal target DNA levels were all negatively associated with pH and C/N because disease-related indicators were assessed in mature maize kernels.

We also demonstrated that FUM deposition is not dependant on the dent kernel-stage as suggested by Picot et al. (2010) but that maximum FUM contamination is observed at the physiological and biological kernel-stages, irrespective of whether infection occurred early (at flowering) or during later plant developmental phases. Moreover, the FUM and fungal contamination of maize grain followed a similar pattern regardless of when

infection occurred. This suggests that the maximum FUM contamination of maize grain is intrinsically linked to the progress of infection. Hydrolysed FUM contamination was low at all kernel developmental stages and did not correlate with free FUM measured at the same stage. Therefore, it cannot be considered as a potential indicator of resistance by means of plant detoxification.

The role of phenolic acids in the defence against *F. verticillioides* was unclear in this study. Inbred lines showed a correlation between free phenolic acids and disease-related indicators; however, this was not observed in the cultivars. 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 FUM 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 FUM accumulation in mature maize kernels. Phenolic compounds are not induced to inhibit *F. verticillioides* growth or FUM contamination in mature maize kernels (Cassiem, 2018). Other physico-chemical properties could also regulate fungal and FUM contamination in the grain, such as sugar and starch quantity, and should also be evaluated.

Kernel hardness and HKM correlated to disease-related indicators in the inbred line trial, however, when expanded to the cultivar trial, there was no correlation. This difference could be ascribed to the limited sample size of the inbred line trial, compared to the cultivar trial. Furthermore, structural characteristics and disease-related indicators, whether evaluated as a group or as individual characteristics, did not correlate. This could be due to the artificial inoculation employed that breeched certain structural barriers such as silk length and husk coverage. Nonetheless, there was also no strong correlation between disease-related indicators of uninoculated samples with structural characteristics. These results suggest that physical features are not good indicators of resistance to FER/FUM.

Commercial cultivars in this study showed varying degrees of resistance to FER and FUM accumulation. This was especially evident in Vaalharts where there was significant fungal and FUM contamination. FER and FUM accumulation occur most frequently in, dry conditions (Cao et al., 2014). Environmental conditions favoured disease development and FUM production in Vaalharts, as seen with the high levels of disease-related indicators in certain cultivars. The disease-related results in Vaalharts, therefore, suggest that cultivars 1, 2 and 9 have a high level of resistance to F. verticillioides and FUM accumulation. All three of these cutlivars are 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 FUM accumulation (Janse van Rensburg et al., 2015).

The genotypes most resistant to FER, fungal colonisation and FUM accumulation were hybrids R119W x CKL05015, CML495 x CKL05015 and CML495 x CML390, respectively. The level of resistance observed in these hybrids did not differ significantly from that of their respective parental inbred lines. The response of most other hybrids evaluated in this study to *F. verticillioides* and FUM accumulation was also comparable to that of their

parental inbred lines. This phenomenon was previously observed in diallel studies by King and Scott (1981) and Hung and Holland (2012), and emphasises the importance of using sources of resistance with desirable agronomic traits when initiating a resistance breeding programme. Although parental lines did not significantly different from their F_1 hybrids, certain hybrids exhibited improved resistance compared to their parental lines. This indicates that hybrids with better resistance than parental lines can be obtained by performing single crosses. Such hybrids, however, need to be further studied for resistance across locations and over several seasons. These should also be evaluated for resistance to other important diseases such as Gibberella ear rot, Diplodia ear rot and AER as well as their corresponding mycotoxins.

The principal gene action observed in this study was additive in nature for FER severity, fungal colonisation and FUM production in maize. Resistance to *F. verticillioides* and FUM contamination was highly heritable, with resistance in the hybrids highly similar to that of their parental inbred lines. It is, therefore, recommended to do resistance breeding with material that contains a certain level of resistance in inbred lines. This study has provided information regarding the inheritance of resistance to FER and FUM contamination. This is of utmost importance in conducting a successful resistance breeding programme for the development of commercial cultivars suited to both small and large scale farmers.

In conclusion, the evaluation of physical, biochemical and genetic characteristics, revealed key associations with traditional disease-related indicators. The genetic response of maize genotypes to *F. verticillioides* infection was paramount for resistance to FER and FUM 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 while providing keen insights into the inheritance of resistance. 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. 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*.

9. Scientific outputs

9.1 Outputs of project as stated in the application

Expected outputs	**Numbers**	**Achieved (yes/no or n.a.)**	**Nature of output e.g. title of papers, description, etc.**
Scientific papers:	4	Yes	Links, S., Flett, B.C., Viljoen, A. and Rose, L.J. 2020. Phenotypic, physico-chemical and genetic response of maize cultivars to infection by *Fusarium verticillioides*. World Mycotoxin Journal. DOI: 10.3920/WMJ2019.2537

			Ouko, A., Okoth, S, Netshifhefhe, N.E.I., Viljoen, A. and Rose, L.J. 2020. Tolerance to *Fusarium verticillioides* infection and fumonisin accumulation in maize F_1 hybrids and subsequent F_2 populations. Agronomy, DOI: https://doi.org/10.1002/agj2.20145 Karlien van Zyl, Lindy J. Rose and Altus Viljoen. 2019. *Fusarium verticillioides FUM1* and *FUM19* gene expression in maize kernels during early infection. Under review by Physiological and Molecular Plant Pathology. DOI: 10.1016/j.pmpp.2019.101430. Netshifhefhe, N.E.I., Flett, B.C., Viljoen, A. and Rose, L.J. 2018. Inheritance and genotype by environment analyses of resistance to *Fusarium verticillioides* and fumonisin contamination in maize F_1 hybrids. Euphytica 214: 235 (https://doi.org/10.1007/s10681-018-2310-4(0123456789).
Technical reports:			5 MT Annual Progress Reports and 1 Final report 3 Annual Progress Reports to the NRF and Subcom B for support funding.
Databases:			Differential gene expression database based on inbred lines resistant or susceptible to FER/FUM
Procedures/methods:			Different bioinformatics approaches to analyse transcriptome data. Optimisation and validation of fungal gene expression *in planta*
Human capacity development:			Please see point 12.
Technology transfer:			
Other outputs:			• A poster presentation will be made at the Mycokey

			Conference to be held from 19 to 22 October 2020. • An oral presentation presented at the World Mycotoxin Forum, 4 – 6 October 2019, Belfast, Ireland. • An oral and poster presentation, respectively, at the South African Plant Breeders Association (SAPBA) congress held in March 2016. • One poster presentation at 1st Mycokey congress in 2017 (Belgium). • One oral presentation at SAPBA congress held in March 2018. • One oral presentation at 14th European Fusarium Seminar held in April 2018 (Vienna). • Three oral presentations at 2nd African Symposium on Mycotoxicology (ASM) held in June 2018. • One poster presentation at 2nd ASM held in June 2018.

9.2 Reasons for outputs not achieved

N/A

10. Successful institutional and inter-institutional collaboration

Researcher	Institution	Role
Dr M. Stander	Stellenbosch University (Mass Spectrophotometry unit)	Phenolic compounds and free as well as hydrolysed FUM analyses
Dr P. Williams	Stellenbosch University (Dept of Food Science)	NIR analyses of kernel hardness

Prof B.C. Flett	ARC-GC	Management of cultivar field trials
Mrs M. Moolman	Stellenbosch University (ICP-MS and XRF unit)	Analyses of carbon, nitrogen and sulphur in grain
Drs M. Visser and B. Coetzee	Stellenbosch University (Department of Genetics)	Bioinformatic analyses of transcriptome data.
Dr M. Booyse and Ms M. van der Rijst	ARC-Infruitec (Biometry unit)	Statistical analyses

11. Benefit of the outputs to the maize industry

Maize is the most important food and feed crop produced in South Africa, and consumed by millions of people as a staple diet. Ear rot diseases and contamination of maize grain with toxic substances presents both a food security and food safety concern. Poor quality grain with high levels of mycotoxins could impede export while safe food, proper quality control and the availability of resistant maize will boost confidence levels in South African agriculture and agricultural products, nationally and internationally. The efficient selection of planting material with superior qualities, such as disease resistance, will provide South African producers; whether commercial, emerging or small-scale; an affordable and environmentally sound means to plant well-adapted material for food and feed production. The use of resistant breeding material provides the highest probability of developing resistant cultivars while obtaining resistance to multiple ear rot fungi and their mycotoxins has also been shown to be possible. Furthermore, the relatively easy evaluation of certain kernel properties like pH and carbon – nitrogen ratio may be used to preliminary to identify resistance while field evaluation and fumonisin analyses remains the most trustworthy tools for the identification and development resistant maize cultivars.

12. Progress with regards to human resource development *(e.g. training of post-graduate students in mycotoxin research)*

The following human resources were developed during the duration of the project (2016 – 2019)

- Dr Karlien van Zyl (female; white) obtained her PhD in December 2018 and, with the support of the Maize Trust and CONSOLIDOC programme at SU, she was retained as a postdoctoral fellow until July 2019. She is currently employed at Roche SA, an international biotechnology company

- Three Master's students graduated:
  - Mr Stefan Links (male; coloured) graduated with distinction in April 2019. He is currently employed as an intern with GrainSA.
  - Mrs Asheeqah Cassiem (female; coloured) graduated in December 2018. She is currently working for Roche SA.
  - Ms Nakisani Netshifhefhe (female; black) graduated in March 2017 and is currently employed as a Research Associate (Plant Health) with Bayer (Monsanto, Ltd – Petit, SA).

- Mr Fagrie Arnold (male; coloured), obtained his BScHons, with distinction, in December 2016. He's currently a MSc student (SU) continuing mycotoxin-related research on wheat in the Western Cape.

- Two final year BScAgric students, Ms Nokukhanya Mahlalela (female; black) and Ayesha Shaikh (female; coloured), executed their final year project within this MT project and graduated in December 2018.

- Ms Shaikh joined our research group in January 2019 as a MSc student and will complete her MSc this year with an envisioned graduation date of December 2020.

13. Statement whether funds were adequate to complete the project

Yes, funds were adequate.

14. Comments *(discuss anything you wish to share with The Maize Trust)*

I would like to take the opportunity to thank the MT for their funding and support of this project. Substantial contributions in knowledge, expertise and skills were obtained and it would be my hope that these will contribute positively to the maize industry.

15. Signature of the Project Leader

_____	Kuilsriver_____	23 June 2020
Project Leader	**Place**	**Date**

16. Signature of Responsible Authority

_____	Stellenbosch_____	23 June 2020_____
Name / Institution	**Place**	**Date**