Fusarium verticillioides infection and fumonisin synthesis as affected by maize plant stressors

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Declaration

I, Jane Baile Ramaswe declare that the dissertation hereby submitted by me for this degree of Magister Scientiae in Environmental Science at the Potchefstroom campus of the North-West University, is my own independent work and has not previously been submitted by me at another University. All sources of materials and financial assistance used for the study have been duly acknowledged. I cede copyright of this dissertation to the North-West University.

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To God be the Glory.

I deserve to see what my life would look like if I give it a 100%. "The choice I had was either to give up or keep going however I rather give it a year and put in work and fail than not to try at all".

ABSTRACT

Fusarium verticillioides is an important ear rot pathogen of maize that can lead to economic losses due to yield and grain quality reduction. Symptoms vary depending upon genotype, environment and disease severity. *F. verticillioides* can produce fumonisin B_1, B_2 and B_3 that can cause mycotoxicoses in animals and are also statistically linked with oesophageal cancer in humans. It has been shown that abiotic factors such as substrate, temperature and water activity can have a profound effect on fumonisin synthesis. The aim of this study is to elucidate the potential effect of plant density on *F. verticillioides* infection and fumonisin production in maize grain. Plant density field trials (2011-2014) with a progressive decline of soil nitrogen were planted at the experimental farm of the ARC-GCI in Potchefstroom. Plant density treatments of 10 000, 20 000, 30 000, 40 000 and 50 000 plants.ha^{-1} were planted using CRN3505 and PAN6P-110 in a completely randomized block design, replicated three times. As nitrogen and plant density appeared to be obscuring the effects of each other, a separate plant density field trial with adequate nitrogen was planted (2015) in Potchefstroom using 8 cultivars in a split plot design, replicated three times. Naturally infected ears were harvested at 12% moisture, threshed and grain milled and subjected to qPCR (*F. verticillioides* target DNA) and HPLC (fumonisin levels). During the 2011/2012 and 2012/2013 seasons, chlorophyll fluorescence parameters were measured at different days after plant (DAP) to determine plant vitality at different plant densities. Leaf material from experimental plots were sampled and analysed by the Eco-Analytica laboratory of the North-West University for total nitrogen (N), carbon (C) and sulphur (S). Three pathogenesis-related proteins, chitinase, peroxidase and β-1,3-glucanase were measured at different stages of plant development to determine their role during fungal infection and fumonisin production. This study showed that under nitrogen poor conditions, cultivar choice, environment and low plant densities could lead to elevated fungal infection and fumonisin production in maize grain, placing subsistence and small scale farmers at risk. In farming systems with adequate soil nitrogen, as plant density increase, grain moisture decrease and target DNA and fumonisins increase. Applications of LAN can influence target DNA in maize grain. Only trace amounts of fumonisins were quantified and the effect of LAN is inconclusive at this stage. This study further demonstrated an increase in the available leaf nutrients (N, S and C) as well as PR proteins (chitinase and β-1,3-glucanase) to correlate with a decrease in fumonisin levels. The increase of PR proteins during critical

infection stages of the maize plant (silk and milk) is a significant finding, as maize ears are susceptible to fungal infection and fumonisin production can occur as soon as fungal infection commenced. It was unexpected though, that available leaf nutrients as well as PR proteins did not affect fungal infection, but fumonisin levels. Currently, an integrated approach is taken to manage fungal infection and subsequent fumonisin production in maize grain. Even though fungal infection can be managed, fumonisin production can be unpredictable due to genotype, environment and substrate. Chitinase and β-1,3-glucanase response to fumonisins in this study can be used in breeding programmes to improve resistance to specifically fumonisin production in maize grain. This study re-iterated the importance of appropriate management practices such as obtaining environmentally adapted seed, applying fertilizers and using the correct planting methods to improve maize yields but also manage the mycotoxin threat to end users. This study also contribute to a better understanding of maize plant defence mechanisms and the aspects of maize physiological processes and nutritional values can effectively contribute to improved management strategies of *F. verticillioides* fungal infection and contamination by fumonisins.

KEY WORDS: Maize, *Fusarium verticillioides*, Fumonisins, management practices, plant stressors.

OPSOMMING

Fusarium verticillioides is ‘n ekonomiese belangrike mielie-kopvrot patogeen wat tot ‘n verlaging in graankwaliteit en oesverliese kan lei. Simptome kan varieer na gelang van genotipes, omgewings toestande en siektegraad. Hierdie swam beskik oor die vermoe om mikotoksiene genaamd fumonisiene te produseer en hierdie fumonisiene kan dan ook verskeie siektesimptome in diere en mense veroorsaak. Fumoniene word in verskillende analoë opgedeel op grond van hulle molekulêre samestelling. Fumonisien B_1, B_2 en B_3 kom die meeste in die natuur voor en FB_1 word statisties verbind met slukdermkanker in mense. Daar is verskeie verwysings in die literatuur wat bewys dat abiotiese stremmingsfaktore soos substraat, temperatuur en beskikbare water ‘n effek op fumonisien produksie in mieliegraan het. Die doel van die studie was dus om die potensiële effek van plantdigtheid en die gepaardgaande stremmingsfaktore (nutrient en waterbeskikbaarheid) op *F. verticillioides* infeksie en fumonisien produksie te bepaal. Eksperimentele plantdigtheids veldproewe met ‘n afname in grondnutriente was gedurende 2011-2014 geplant op die navorsings-plaas van die Landbounavorsingsraad – Instituut vir Graangewasse. Plantdigthede was 10 000, 20 000, 30 000, 40 000 en 50 000 plante ha^{-1}. Die cultivars CRN3505 en PAN6P-110 was in ‘n totale gerandomiseerde blokontwerp geplant, en elke behandeling is drie keer herhaal. Aangesien die gesamentlike effek van plantdigtheid en ‘n afname in grond nutriente moontlik resultate kon verdoesel is ‘n aparte plantdigtheidsproef met voldoende grondnutriente asook ‘n aparte nutrientproef gedurende 2015 by die LNR-IGG geplant. Om die effek van cultivars uit te skakel, was daar 8 kultivars geplant in ‘n gesplete blokontwerp en elke behandeling was drie keer herhaal. Mieliekoppe was natuurlik ge-infekteer met *F. verticillioides* en proewe was ge-oes teen 12 % graanvog. Individuele behandelings was gedors, en die graan was gemaal en gebruik in kwantitatiewe polisikliese kettingreaksies (ook bekend as qPCR in engels) om die hoeveelheid *F. verticillioides* DNA te kwantifiseer. Daar is gebruik gemaak van hoëprestasie vloeistofchromatografie (ook bekend as HPLC in engels) om die hoeveelheid fumonisiene te kwantifiseer. Gedurende die 2011/2012 en 2012/2103 seisoene is chlorofil parameters getoets verskeie dae na plant om vas te stel of die metode gebruik kan word om plantstremming as gevolg van verskillende plantdigthede te meet. Blaar materiaal is deur die Eco-Analytica Laboratorium van die Noordwes-Universiteit ge-analiseer vir beskikbare stikstof, koolstof en swael. Drie proteine (chitinase, peroksidase en ß-1,3-

glukanase) wat bekend is in plant weerstands reaksies teenoor swaminfeksie is gekwantifiseer om te bepaal of hulle ‘n rol speel in swaminfeksie en fumonisien produksie in mieliegraan. Hierdie studie het gewys dat onvoldoene nutriente, kultivarkeuses, omgewingstoestande asook lae plantdigthede, swaminfeksie en fumonisienvlakke kan verhoog. Onder sulke omstandighede is bestaansboere en opkomende boere blootegestel aan ongewenste vlakke van kopvrot- en fumonsienbesmetting. Die studie het getoon dat boerderystelsels (met voldoende grondnutriente) met hoë plantdigthede ook ‘n verhoging in swaminfeksie en fumonsienproduksie toon. Daar is klein hoeveelhede fumonsiene in die mieliegraan gekwantifiseer uit die aparte plantnutrientproef en resultate is onbeslis in die stadium. Daar was ‘n omgekeerde korrelasie tussen die beskikbare blaarnutriente (N, C en S) asook chitinase en ß-1,3-glukanase met fumonisiene. Die toename in chitinase en ß-1,3-glukanase met kritieke groeistadiums (baardvorming en melkstadium) van die mielieplante is ‘n belangrike bevinding aangesien mielieplante tydens die stadiums uiters vatbaar is vir swaminfeksie asook fumonisienproduksie. Aangsien al die literatuurstudies aandui die die proteine verantwoordelik is vir weerstand teen swaminfeksie was die bevinding dat daar ‘n verlaging van fumonisiene en nie swaminfeksie was nie, onverwags. Produsente maak tans gebruik van geintegreerde beheermaatreëls om swaminfeksie en fumonisien produksie in mieliegraan te beperk. Daar is verskeie beheermaatreëls om swaminfeksie te beperk, maar die beheer van fumonisienproduksie is meer gekompliseerd en fumonisien produksie is onvoorspelbaar as gevolg van die genotipe, omgewing en substraat asook die vermoë van isolate om fumonsiene te produseer. Die chitinase en ß-1,3-glukanase proteine kan in telingsprogramme gebruik word om weerstand teen spesifiek fumonisien produksie te verbeter. Hierdie studie het die belangrikheid beklemtoon in die selektering van geskikte genotipes (wat by die omgewing aangepas is), toediening van nutriente en die korrekte plantdigthede om nog steeds goeie opbrengste te verseker maar om ook die mikotoksien-bedreiging te verminder. Hierdie studie dra by tot kennis rakende mielieplant verdediging-meganismes en fisiologiese prosesse wat ‘n bydrae maak tot verbeterde beheermaatreëls om sodoende *F. verticillioides* infeksie en fumonisien produksie te beperk.

SLEUTELWOORDE: Mielies, *Fusarium verticillioides*, fumonisiene, bestuurspraktyke, plant stremmingsfaktore.

LIST OF ABBREVIATIONS

Companies and departments

ARC-GCI	Agricultural Research Council – Grain Crops Institute
CAST	Council for Agricultural Science and Technology
DAFF	Department of Agriculture Forestry and Fisheries
FDA	Food and Drug Administration of America
FAO	Food and Agriculture Organization
IARC	International Agency for Research on Cancer
NDA	National Department of Agriculture
SADC	Southern African Development Community
SAGIS	South African Grain Information Services
SAGL	South African Grain Laboratories

Chemicals and reagents

Al	Aluminium
BSA	Bovine Serum Albumin
C	Carbon
Ca	Calcium
CLA	Carnation Leaf Agar
EDTA	Ethylene diamine tetraacetic acid
HCl	Hydrochloric acid
H_2O	Water
H_2O_2	Hydrogen peroxide
KCl	Potassium chloride
K_2HPO_4	Dipotassium phosphate
KH_2PO4	Monopotassium dihydrogen phosphate
LAN	Limestone ammonium nitrate
Mg	Magnesium
N	Nitrogen
Na_2HPO4	Sodium phosphate dibasic
OPA	o-phthalylaldehyde

PDA	Potato Dextrose Agar
P	Phosphorus
S	Sulphur

Terms and equipment

ANOVA	Analysis of variance
a_w	water availability
CULT	Cultivars
DAP	Days after plant
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunosorbent Assay
ELEM	Leucoencephalomalacia
F_o	Fluorescence
F_m	Maximal fluorescence
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
F_v/F_m	Maximum quantum yield
g	Grams
GC/MS	Gas Chromatography-Mass Spectroscopy
HPLC	High Performance Liquid Chromatography
LC/MS	Liquid Chromatography-Mass Spectroscopy
LSD	Least Significant Differences
PCR	Polymerase chain reaction
PD	Plant Density
PI_{ABS}	Performance index based on absorption
PPE	Pulmonary oedema
PR-proteins	Pathogenesis related proteins
PSI	Photosystem I
PSII	Photosystem II
Q_A	Quinone A
q-PCR	quantitative Polymerase Chain Reaction
r	Correlation coefficient
R^2	Regression coefficient

REP	Replicate
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-qPCR	Real Time quantitative Polymerase Chain Reaction
TLC	Thin-layer chromatography

SI units

ºC	Degrees Celsius
%	Percentage
ha	Hectare
ha^{-1}	Per hectare
K	Potassium
Kg	Kilogram
kg ha^{-1}	Kilogram per hectare
L	Litre
mg mL^{-1}	Milligram per milliliter
mL	Milliliter
mm	Millimeter
mM	Millimolar
ng μL^{-1}	Nanogram per microliter
nm mg^{-1} Prot	Nano moles per milligram proteins
Pg	Picogram
pg ug^{-1}	Picogram per microgram
ppm	Parts per million
rpm	Revolutions per minute
µL	Microliter
µM	Micromolar

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INTRODUCTION AND PROBLEM STATEMENT

Maize (Zea *mays* L.) is a major food crop and source of carbohydrates particularly in the rural population of South Africa (Walker & Schulze, 2006). It is estimated that about 8000 commercial maize producers are responsible for the major part of maize production in South Africa while subsistence farmers (DAFF, 2011) produce the rest. The production of the crop depends on the correct application of management practices ensuring both environmental and agricultural sustainability (Nape, 2011). By understanding how plants interact morphologically and physiologically in a community and identifying management practices, which allow them to maximize the use of growth resources in their environment, is very important (Arif, 2013). The productivity of maize could also be attributed to a combination of factors such as low soil fertility, unfavourable environmental conditions, poor agricultural management as well as pests and diseases (Tisdale *et al*., 1990; Major *et al*., 1991). Therefore, plant density, fertilizer source and rate of application, watering regimes and fungal infection by *Fusarium verticillioides* and subsequent fumonisin synthesis were factors studied in this project.

Fusarium verticillioides is the most commonly isolated pathogen from maize kernels in South Africa and worldwide. *F. verticillioides* and *F. proliferatum* are responsible for production of mycotoxins including fumonisin B_1, B_2, and B_3. Fumonisins are associated with various animal diseases and human oesophageal cancer. Incidence of *F. verticillioides* is higher in warmer climates under dry conditions (Janse van Rensburg *et al*., 2015). No fungal control measures have been developed for maize and natural *F. verticillioides* infection depends on climatic factors such as temperature (Janse van Rensburg, 2012; Parsons & Munkvold, 2012), genotypes (Miller, 2001) and inoculum present (Blandino *et al*., 2008). The most plausible solution seems to be prevention in the field through crop techniques that will lessen conditions for fungal infection and subsequent fumonisin synthesis (Nicholson *et al*., 2004).

Plant density influences on fungal infection and fumonisin synthesis in maize kernels

Since high yields are important to South African maize producers, it is imperative that these are in accordance with accepted food safety limits, including fungal contamination and mycotoxin production. Plant density recommendations for maize production have

increased to a point at which they are now double those recommended in the 1950's. Excessive plant populations could induce moisture and nutrient stress on individual maize plants, which could increase their susceptibility to mycotoxin producing fungi (Trento *et al*., 2002; Bruns, 2003). On the other hand, factors that can reduce plant density include soil surface residues that interfere with germination in no-till agriculture or the usage of lower seeding densities to minimize yield reduction associated with dry conditions. Tokatlidis & Koutroubas (2004) stated that tolerance to high plant populations, along with tolerance to other biotic and abiotic stresses, has in recent years constituted the determinant parameters that contribute to improved maize productivity. On the other hand, only a few studies have been conducted on the influence of plant density concerning sanitary aspects, such as mycotoxin contamination. Bilgrami & Choudhary (1998) reported lower aflatoxin levels in densely cultivated plants whereas Rodriguez-Del-Bosque (1996), Bata *et al*. (1997) and Bruns & Abbas (2005) reported that plant populations had no effect on aflatoxin, zearalenone or fumonisin contamination of maize kernels. Abbas *et al*. (2012) reported there was no evidence that lowered seedling density (to reduce stress) reduced aflatoxin or fumonisins in maize research trials in America. According to Logrieco *et al.* (2002) very little and unclear information has been recorded about the effect of plant population on the contamination of mycotoxins such as fumonisins. Research results thus far indicate a decrease in *F. verticillioides* infection and fumonisins in field trials as plant densities increase (with a decline of nitrogen from one season to another). A possible explanation could be that at lower plant densities, cultivars respond by producing more kernels and therefore also an increased number of silks which provides additional avenues for fungal infection. Higher plant densities will have a denser canopy that could restrict fungal infection via the silks. Within the field trials completed thus far, the declining nitrogen and plant densities could have obscured the effects of each of these two factors on one another and on fungal infection and fumonisin synthesis. It is therefore imperative to determine the separate effects of nitrogen and plant density on fungal infection and fumonisin synthesis within different environments and increased genotypes.

The influence of nitrogen on fungal infection and fumonisin synthesis in maize kernels

F. verticillioides infection and ear colonization is favoured by high levels of moisture and high relative humidity, from silking to the end of the maturation period (Reid *et al.,* 1999, Logrieco *et al*., 2002). These favourable conditions could be prolonged as a consequence of rich nitrogen (N) fertilizer applications, which would lead to longer vegetative growth and higher leaf expansion (Blandino & Reyneri, 2007). On the other hand, maize plants exposed to drought or fertility stress are also more susceptible to infection by microorganisms than plants not under stress. Elevated aflatoxin levels have been associated with fertility-related stresses, particularly n deficiency (Lisker & Lillehoj, 1991). Anderson *et al*. (1975), Jones & Duncan (1981) and Munkvold (2008) reported that a higher rate of n fertilizer application consistently resulted in reduced aflatoxin rates. Blandino *et al.* (2008) related higher fumonisin contamination with high n rates and in the presence of n deficiencies. Marocco *et al.* (2008) reported that n fertilisation significantly increased fumonisins levels, the authors however only applied two nitrogen treatment rates (0 kg N ha^{-1} and 270 N ha^{-1}). It is necessary to understand and quantify the effect of nitrogen levels on *F. verticillioides* and resultant fumonisin production under local conditions.

AIM

The aim of this study is to determine the effect of maize plant stressors on *Fusarium verticillioides* infection and fumonisin production in maize plants.

OBJECTIVES

1a) To identify the effect of increased plant densities (with a gradual depletion of soil nitrogen) on *F. verticillioides* infection of maize kernels and subsequent fumonisin production by means of field trials (Potchefstroom) using two cultivars.

1b) To identify the effect of increased plant densities (with adequate soil nitrogen) on *F. verticillioides* infection of maize kernels and subsequent

fumonisin production by means of field trials (Potchefstroom) using 8 cultivars.

1c) To identify the effect of nitrogen source (Urea and LAN) and rate (25, 50, 100, 150 and 175 kg ha^{-1}) on *F. verticillioides* infection of maize kernels and subsequent fumonisin production by means of field trials (Potchefstroom) using eight cultivars planted at a density of 30 000 plants ha^{-1}.

2a) Establish if Chlorophyll fluorescence can be used as measurement of photosynthetic performance in maize plants as early indicator of plant stress.

2b) Elucidate the role of available maize leaf nutrients on *F. verticillioides* infection of maize kernels and subsequent fumonisin.

2c) Investigate the potential role of Pathogenesis Related (PR) Proteins associated with plant defence mechanisms against *F. verticillioides.*

These objectives were achieved by a sequence of experiments that are outlined in separate chapters:

Chapter 1: includes a literature overview of *F. verticillioides* and the resultant fumonisins that can be produced in maize grain. This chapter includes a discussion regarding the factors that influence maize production and the possibility of reducing stress factors such as plant density and nitrogen applications to reduce fungal infection and fumonisin production in maize grain. Chlorophyll fluorescence as measurement of photosynthetic performance in plants as early indicator of plant stress as well as the potential role of Pathogenesis Related (PR) Proteins associated with plant defence mechanisms against *F. verticillioides* were also studied.

Chapter 2: the effect of five different plant densities (with a progressive decline of nitrogen in the soil) on *F. verticillioides* infection of maize ears and subsequent fumonisin production under field conditions were studied. As the effects of the declining nitrogen and plant densities obscured the effects of each other, a separate plant density trial with adequate nitrogen was also planted to further study the reaction of *F. verticillioides* infection of maize ears and subsequent fumonisin production. Quantification of *F.*

verticillioides target DNA was achieved using qPCR while the quantification of fumonisins (FB_1, FB_2 and FB_3) levels were done using High Performance Liquid Chromatography (HPLC).

Chapter 3: investigated chlorophyll fluorescence as measurement of photosynthetic performance in plants as early indicator of plant stress. The role of available leaf nitrogen (N), carbon (C) and suphur (S) on fungal infection and fumonisin production in maize grain as well as the role of PR proteins (chitinase, peroxidase and β-1,3-glucanase) on fungal infection and fumonisin production in maize grain was investigated.

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CHAPTER 1

REVIEW OF *FUSARIUM VERTICILLIOIDES* INFECTION AND FUMONISIN PRODUCTION IN MAIZE GRAIN.

1.1 The importance of maize production in South Africa

A variety of grain crops are produced in South Africa and maize is regarded as one of the most important crops followed by wheat, oats, and sunflower (NDA, 2005). Maize (*Zea mays* L.) belongs to the grass family Poaceae in the genus *Zea* (Park, 2001). It is cultivated globally as one of the most important cereal crops over a diverse range of climatic conditions (Dowswell *et al.,* 1996). Maize is also regarded as a major food crop and source of carbohydrates for the majority of the South African population (Walker & Schulze, 2006). In addition, its production is an important source of job creation not only for labour on farms, but also in certain economic sectors (Vink & Kristen, 2003). It also serves as a raw material for manufactured products such as paper, medicine and food (DAFF, 2012) and ensures food security in South Africa and the Southern African Development Community (SADC) region (NDA, 2011). In South Africa, commercial maize producers are responsible for the major part of maize production while subsistence producers are mostly based in the rural areas (Bankole & Adebanjo, 2003).

In South Africa, maize is grown in almost all provinces with the Free State, North-West and Mpumalanga being the largest production areas (Du Plessis, 2003). About 48 % of white maize produced in South Africa is mainly used for human consumption and 52 % of yellow maize is used as animal feed (DAFF, 2013). Agriculture in the 21st century faces many challenges that it has to produce food to feed a growing population (World Bank, 2009). The world population, which was estimated at 7.2 billion in 2013, is estimated to reach 8.1 billion by 2025 and 9.6 billion by 2050 (UN Population Division, 2013). In South Africa, mid-year population was estimated at 52.98 million in 2013 and have been found to increase to 54 million in 2014 and 54.96 million for 2015 (Stats SA, 2013, 2014 & 2015). To meet the food requirements and achieve food security agricultural production has to increase with the growing population (Inocencio *et al.,* 2003).

Consumption levels of maize by humans and animals in South Africa can be between 400 to 500 g per person per day (Shephard, 2008). In a study conducted by Sydenham

et al. (1991), most South Africans consume maize in the form of maize meal, corn flakes and snacks as an ingredient of many different food products. Maize consumed by animals also end up in the human food chain through eggs, cheese, meat and dairy products. Therefore, the quality of maize in South Africa has a direct impact on the health of humans and animals who consumes maize products on a regular basis (Dawlal, 2010).

According to the Department of Agriculture Forestry and Fisheries (DAFF, 2013), the total estimated commercial maize production in 2012 was 11.72 million tons with an estimated yield of 4.21 t/ha and this production decreased by 3.3% from 2011, which was estimated at 12.12 million tons. In recent years, drought had an adverse effect on maize production with a decline in yield in the 2013 to 2015 seasons. Total maize production was 9.95 million tons in 2014/2015, the lowest since 2006/2007 when the yield was 7.12 million tons (SAGIS, 2015).

1.1.1 Factors that influence maize production

Maize production depends on management practices that will ensure environmental and agricultural sustainability (Du Plessis, 2003; Nape, 2011). Major constraints that influence maize yield includes abiotic and biotic factors. Abiotic factors include inadequate temperatures, moisture, soil pH, nutrient supply, and light intensity as well as agricultural practices such as plant populations.

1.1.2 Abiotic factors

1.1.2.1 Temperature

Temperature is described as a measure of the intensity of heat and a primary factor affecting the rate of plant development (Hatfield *et al.,* 2011). Temperature has become a major concern for plant scientists worldwide due to climatic changes. The change in temperature (low and high) can lead to substantial crop losses (Xin & Browse, 2000; Sanghera *et al.,* 2011) and the current challenge is to consider the potential future impact on agriculture (Watanabe & Kume, 2009; Shah *et al.,* 2011). Maize is a tropical grass that is well adapted to many climates (Belfield & Brown, 2008). Germination and emergence of maize require a minimum temperature of 10°C and an optimum soil temperature of 17°C. The optimum temperature for maize growth and development is 19 to 32°C, with temperatures of 35°C and above considered inhibitory (Baloyi, 2012). Maize is not well

adapted to low temperatures and lack mechanisms to acclimatize to cold (Sanghera, 2011). Temperatures above 35°C and low humidity during flowering have an adverse effect on pollination, fertilization and grain formation (Hatfield *et al.,* 2011). Maize pollen viability decreases with exposure to temperatures above 35°C (Du Plessis, 2003).

1.1.2.2 Moisture

Maize requires a significant amount of moisture and an optimal range of 500 to 600 mm of well distributed rain is conducive to proper growth. Under rain fed conditions, a yield of 3 152 kg ha^{-1} requires between 350 and 450 mm of rain per year (Du Plessis, 2003). After germination and up to flowering stage, maize tolerates less moisture. More moisture is required during the reproductive period and less moisture towards maturity (Kumar *et al.,* 2012). Approximately 10 to 16 kg of grain are produced for every millimeter of water used. At maturity, each plant will have used 250 litres of water in the absence of moisture stress (Du Plessis, 2003). Maize has adequate tolerance to waterlogging, but this tolerance is higher when the growing point is below the ground and lowest at the flowering stage, especially when combined with high temperatures (Belfield & Brown, 2008; Baloyi, 2012).

1.1.2.2 Soil pH

Soil acidity increases as pH drops below 7 (neutral pH) and soil alkalinity increases as pH increases above 7 and this pH range can affect nutrient availability to maize plants resulting in a reduction in plant growth (Lafitte, 1994). Soil acidity limits the uptake of basic plant nutrients such as calcium (Ca), potassium (K) and magnesium (Mg), while aluminium (Al) toxicity in the soil also damage plant roots (English & Cahill, 2005). Maize is moderately well adapted to a soil pH of 6.0 to 7.5 (FSSA, 2007). Soil acidity in South Africa is the main cause of soil degradation and reduces small and large-scale maize production significantly (Materechera & Mkhabela, 2002). Soil salinity reduces uptake of nutrients and decreases total dry matter production (Ayad *et al.,* 2010).

1.1.2.4 Plant and soil nutrients

Maize production requires soil that has balanced quantities of plant nutrients (Du Plessis, 2003) and it is therefore essential to analyse the fertility of the soil (Ofori & Kyei-Baffour, 2004) and to apply appropriate nutrient sources at a meaningful rate and at the right time. The focus of maize producers is usually on three of the six macronutrients, nitrogen (N), potassium (K) and phosphorus (P) as these nutrients give the largest response to good

yield (Hani *et al.,* 2006). According to Du Plessis (2003), the estimated amount of nutrients removed from the soil for each ton of grain produced is 15.0 to 18.0 kg of N, 2.5 to 3.0 kg of P and 3.0 to 4.0 kg of K. Each of these nutrients has a critical function that is required in varying quantities in plant tissue (Hani *et al.,* 2006). Insufficient N is the second biggest constraint after drought in tropical maize production, since maize has a strong growth response to nitrogen supply (Lafitte, 2000). Maize therefore require N as nutrient source in large quantities (Muzilli & Oliveira, 1992), to ensure high yields (Arif, 2013). Nitrogen stress reduces photosynthesis by reducing leaf area and accelerates leaf senescence. Maize plants that suffer from a lack of nitrogen are weaker and slower growing; predisposing them to infection by pathogens (Agrios, 2010).

Potassium (K) increases disease resistance and drought tolerance, essential for photosynthesis and regulate many other metabolic processes required for plant growth (Tucker, 1999). Potasium activates enzymes to metabolise carbohydrates for the manufacturing of amino acids and proteins. It also enhances stalk and stem rigidity and the regulation of the opening and closing of stomata (Imas & Magen, 2000). In most South African soils, phosphorus (P) is the most deficient nutrient; therefore, yields would largely increase when phosphorus is added to the soil as it enhances seed germination and early plant growth (van Averbeke & Yoganathan, 2003). As phosphorus is required for healthy root development, it should be applied at planting (PDA, 2008). Phosphorus also plays an important role in increased disease resistance through the improved balance of nutrients in the plant or by accelerating the maturity of the plant and allowing it to escape infection by pathogens that prefer younger tissues (Agrios, 2010).

1.1.2.5 Plant density

Plant density is regarded as one of the most important cultural practices that determines grain yield (Randhawa *et al*., 2003). Higher plant densities endorse inter plant competition for natural resources such as light and water, thereby decreasing the number of ears plant and kernel set per ear produced (Sangoi, 2000). Photosynthesis will be impaired due to less penetration of light to the crop canopy thereby increasing the competition between plants for available nutrients, which will affect grain yield (Sharifi *et al.,* 2009).

Liu *et al.* (2006) reported that maize yield differs significantly under varying plant density levels due to difference in genetic potential. Row width also plays an important role in

determining the plant density. Inter-row spacing can increase competition between plants and affect the yield if it is too narrow. At favourable environmental conditions, higher plant populations will produce smaller ears or few kernels per plant, but the greater numbers of plants will still result in a higher yield (ARC-GCI, 2002).

Olson & Sanders (1988) reported at low densities, many modern maize hybrids do not grow effectively and quite often produce only one ear per plant. Maize population for maximum economic grain yield varies between 30,000 to over 90,000 plants per hectare. Under cooler and warmer regions, the plant densities required to produce maize yields of 3000 kg ha^{-1} are 19 000 plants ha^{-1}, 16 000 plants ha^{-1} and 14 000 plants ha^{-1}, respectively. For a yield of 6 000 kg ha^{-1} under cooler and warmer regions the plant population densities are 37 000 plants ha^{-1}, 31 000 plants ha^{-1} and 28 000 plants ha^{-1}, respectively (Du Plessis, 2003). According to Tollenaar *et al.* (1997) maize grain yield declines when plant density is increased beyond the optimum plant density, primarily because of the decline in the harvest index and increased stem lodging.

1.1.3 Biotic factors

1.1.3.1 Fungal diseases

Yield reduction due to biotic factors is the consequence of a parasitic relationship where the pathogen, herbivore or insect derives food from its host (Ransom *et al.,* 1993; Vega *et al.,* 2001). In maize, the most common diseases are caused by *Pythium* spp*., Fusarium* spp*., Gibberella* spp*., Trichoderma* spp*. and Penicillium* spp*.*, but other fungi such as *Stenocarpella maydis* and *Rhizoctonia spp.* can also be involved. Seed, seedlings and roots infected by *Pythium* spp. are most often soft (wet) and dark coloured, as opposed to roots infected with *Fusarium* spp*., Gibberella* spp*., S. maydis and Rhizoctonia* spp*.*, which are firm or leathery. *Fusarium* spp, *Gibberella* spp. and *S. maydis* can colonize roots and lower stems of plants as well as maize ears and grain causing severe yield losses (Agrios, 2010).

The main *Fusarium* spp. isolated from maize are *F. verticillioides*, *F. proliferatum*, *F. subglutinans* and *F. temperatum*. The most important producers of fumonisins are *F. verticillioides* and *F. proliferatum* because of their overall high levels of production, wide

geographical distribution, frequent occurrence in maize, and association with known animal mycotoxicoses (Fumero *et al*., 2016).

1.1.3.2 Weeds

According to the Food and Agriculture Organization (FAO), 13% global loss of agricultural production is due to weed infestation. In Africa, it is reported that more than 50% of crop losses are due to weeds (Sibuga, 1997), which is higher than the sum of the potential losses due to insect and pathogens (Oerke, 2006). Maize is most sensitive to weed competition during the early developmental stages. The growth of maize plants in the first week is rather slow and it is during this period that weeds establish rapidly and become competitive. Maximum weed competition in maize occurs during the period of 2 to 6 weeks after planting. Weeds compete directly with maize crops for nutrients, space, light, and water thus reducing maize yield. This suggests the importance of maintaining the field free of weeds during this critical period of weed competition (Zanine & Santos, 2004). If weeds are left uncontrolled in fields, they are capable of reducing yields by more than 80% (Karlen *et al*., 2002; Farai *et al.,* 2014). Therefore, integrated measures such as controlling weeds through seed bed preparation, seed treatment, improved fertilizer practices and chemical control methods could ensure good yields (Khatri, 2012).

1.1.3.3 Insects

The larvae of the lepidopterous stem borers *Busseola fusca* and the spotted stem borer, *Chilo partellus* are generally considered the most damaging insect pests of maize in Africa (Overholt *et al.,* 2001). Dejen *et al*. (2014) states that their distribution and pest status vary according to environmental conditions. These stem borers are known to attack maize during the first eight weeks after planting and late damage leads to stem lodging (Bosque-Perez & Eigenbrode, 2011). Stem borers interfere with the movement of nutrients through the plants vascular system and can reduce grain weight and kernel numbers, thereby reducing yields (ISU, 2012). *C. partellus* can spread rapidly by means of displacing indigenous species of stem borers, thereby becoming the most damaging stem borer in Arica (Kfir *et al.,* 2002). For example, in the eastern Highveld region of South Africa, *C. partellus* partially displaced *B. fusca* over a period of seven years. Within two years, it became the prevalent stem borer; constituting 90% of the total stem borer populations. The possible reasons for the displacement of the indigenous species is that hibernating

larval populations of *C. partellus* terminate diapause and develop a month earlier than *B. fusca* (Dejen *et al.,* 2014). Damage by *B. fusca* and *C. partellus* result in economic loss to maize in many African countries, with smallholder farmers suffering more severe losses than commercial farmers (Mushore, 2005; Mutyambai *et al.,* 2014). In South Africa, annual yield loss caused by stem borers to maize is 10% although previous losses of 25-75% have also been recorded (Sylvain *et al.,* 2015). Control options for managing stem borers include a combination of chemical-, biological- and cultural-controls as well as plant resistance (*Bt*-gene technology).

1.2 Taxonomy and identification of fumonisin producing *Fusarium* spp.

Seven mating populations A, B, C, D, E, F, and G were proposed for *G. fujikuroi.* Mating population A is most often associated with maize in which *F. verticillioides* is characterized (Nirenberg, 1976). Based on the structure in or on which conidiogenous hyphae are borne, *Fusarium* spp. are classified under the Hyphomycetidae sub-class of the Deuteromycetes (Agrios, 2010). The fumonisin producing *F. verticillioides* (Saccardo) Nirenberg and *F. proliferatum* (Matsushima) Nirenberg, belong to teleomorph *Gibberella moniliformis* and *Gibberella intermedia*, respectively (Leslie & Summerell, 2006).

On potato dextrose agar (PDA) medium, *F. verticillioides* produce white mycelium that may become violet as cultures age (Desjardins, 2006). *F. verticillioides* produces microconidia that is oval to club shaped with a flattened base and contains no septa (Leslie & Summerell, 2006). Long chains of microconodia in the aerial mycelium are common. Sometimes these chains occur in pairs and can give a 'rabbit ear' appearance (Leslie & Summerell, 2006). Macroconidia is relatively long and slender, slightly falcate or straight and thin walled. The apical cell is curved and often tapered to a point with the basal cell being notched or foot shaped. Three to five septa can be present in macroconidia. Sporodochia may be tan or orange. *F. verticillioides* is morphologically identical to isolates of *F. thapsinum* (Klittich, Leslie, Nelson & Marasas) that do not produce the diagnostic yellow pigment. *F. verticillioides* is also very similar to *F. andiyazi* but does not form pseudochlamydospores; however, it can produce swollen cells in hyphae that may be difficult to differentiate from pseudochlamydospores (Leslie & Summerell, 2006; Nelson *et al.,* 1983).

F. proliferatum correspond to mating population C or D (Desjardins, 2006). On potato dextrose agar (PDA) medium, *F. proliferatum* produce white mycelium but may become purple-violet with age. Blue-black sclerotia may develop in some isolates. Microconidia is slender, thin walled and relatively straight with a curved apical cell and poorly developed basal cell (Leslie & Summerell, 2006). Macroconidia is club shaped with a flattened base and zero septate. Aerial mycelium may be found in chains of varying, but usually moderate length, false heads. These chains are generally shorter than those of *F. verticillioides* and the chlamypdospores are absent.

1.3 Symptoms caused by *F. verticillioides*

F. verticillioides symptoms vary depending on genotype, the environment and disease severity. They can range from non-symptomatic infections to severe rotting of all plant parts (Munkvold & Desjardins, 1997). Early infections can cause plant malformation and deformation of kernel shape and size (Headrick *et al.,* 1990). Infected ears can show a white to pink mycelium on random kernels, or on a group of kernels (Figure 1). Fungal growth can be found alongside (Figure 2) insect feeding channels (Koehler, 1959; Farrar & Davis, 1991; Miller, 1994). The infection may occur internally causing invisible symptoms, and produce `starburst` symptoms (Figure 3), which are characterized by white or pink streaks from the silk insertion causing kernel rot (Payne, 1999; Duncan & Howard, 2010).

Figure 1: Infected maize ear covered with white-pink mycelium.

Figure 2: Fusarium ear rot in maize alongside insect feeding channel.

Figure 3: “Starburst” symptoms on infected maize kernels.

1.4 Symptomless infection

F. verticillioides is one of the most common fungi found symptomless colonising seeds of maize and teosinte (Desjardins *et al.*, 2005) and is in many cases ignored because it does not cause visible damage to the plant (Munkvold & Desjardins, 1997). Symptomless infection of kernels is often very high, but fumonisin levels may be very low (Bush *et al.*, 2004). *F. verticillioides* may remain undetected in kernels until germination, when it infects the emerging seedlings (Bacon & Hinton, 1996). Under plant stress conditions, the symptomless endophytic relationship may convert to a disease and/or mycotoxin producing interaction (Abbas *et al*., 2006). Yield can be reduced by endophytic *F. verticillioides* infected plants, due to deterioration of the stalk parenchyma tissue and gradual dehydration of the plant (Foley, 1962).

1.5 Epidemiology of *F. verticillioides*

Doohan *et al.* (2003) reported that climatic factors throughout plant development determine the presence of fungal infection on maize kernels and subsequent mycotoxin production in grain. In line with this finding, Janse van Rensburg (2012) reported higher

infection rates of *F. verticillioides* and fumonisin levels with warmer maize production areas such as the Northern Cape, some areas of the Free State and drier areas of the North-West province of South Africa. *F. verticillioides* infection is favoured by temperatures higher than 28°C and dry conditions during flowering stage of plant development (Shelby *et al.,* 1994) as well as rainfall before harvest (Pascale *et al.,* 1997; Fandohan *et al.,* 2003; de la Campa *et al.,* 2005). Marin *et al.* (1999) reported that *F. verticillioides* on maize plants grows well at optimum temperatures of 30°C and 0.97 a_w in vitro. In a study done by Rossi *et al.* (2009), it was reported that sporulation by *F. verticillioides* progressively increased between 5°C and 27°C and then declined rapidly with temperatures higher than 30°C.

F. verticillioides has a saprophytic and parasitic stage and may infect maize at all stages of plant development, either via the silk channel, infected seed or wounds (Reid *et al*., 1999) and can also grow systemically in the plant (Figure 4). Under favourable conditions, *F. verticillioides* produces a large number of micro- and macroconidia, which colonizes the soil and survives on plant residues (Sikora *et al*., 2003). *F. verticillioides* decreases more slowly in fields with surface residue than in fields with buried residues (Cotton & Munkvold, 1998). *F. verticillioides* conidia as primary source of inoculum and can be dispersed by wind, insects and rain (Bergstrom & Shields, 2002), thereby infecting maize plants in the new growing season. Schaafsma *et al*. (1993) and Munkvold *et al*. (1997) reported that silks are most susceptible to infection during the first week of silking and moisture on the silks favours infection.

Another proposed infection pathway is systemically from the seeds (Oren *et al.,* 2003). As described by Munkvold *et al.* (1997), transmission of *F. verticillioides* from seeds to kernels of maize can be divided in four steps: 1) seed to seedling transmission 2) colonization of the stalk 3) movement into the ear and 4) spread within the ear. Seeds provide one of the most efficient methods of pathogen dissemination at great distances and allow pathogen introduction into new areas (Leslie & Summerell, 2006; Wilke *et al.,* 2007). Seed infection by *F. verticillioides* is of major concern because it can reduce seed quality and result in contamination of grain with fumonisins (Munkvold & Desjardins, 1997). It is reported that *F. veriticilllioides* can also reduce seed germination and vigour at variable levels, but no reliable data exist to support this effect (Machado *et al*., 2013).

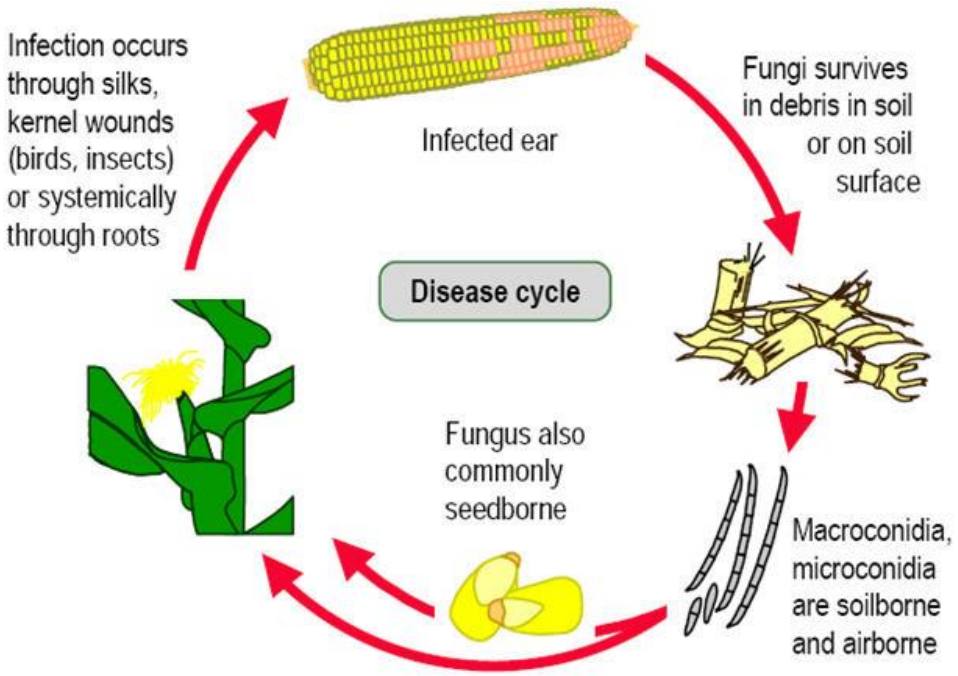


Figure 4: The disease cycle of *F. verticillioides* in maize (source: www.pioneer.com)

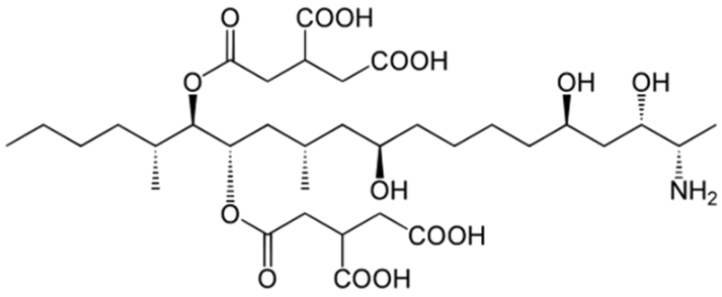
1.6 An overview of fumonisins

Fumonisins are secondary metabolites, which were first isolated from MRC826 strain *F. verticillioides* MRC826 in 1988 (Gelderblom *et al.,* 1988). Fumonisins can be produced by almost all strains of *F*. *verticillioides*. They are sphinganine analog mycotoxins bearing a structural resemblance to free sphingoid bases found in all classes of sphingolipids (Williams *et al.,* 2007). The toxicity of fumonisins is enhanced by the inhibition of the enzyme ceramide synthase in the sphingolipid biosynthetic pathway, resulting in the depletion of the sphingolipids and finally disruption of the biological membranes (Riley *et al.,* 1994). Sphingolipids play a critical role in cell regulation and a variety of cell signaling pathways. Thus, the disruption of sphingolipid metabolism may account for the multiple carcinogenic diseases in humans and animals associated with fumonisins (Soriano *et al.,* 2005).

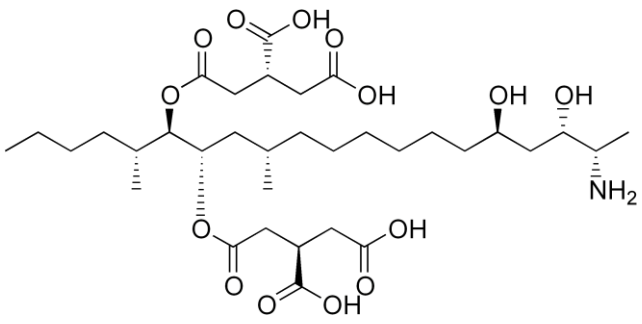
To date, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) are the most important analogues (Scott, 2011) (Figure 5). FB_1 usually constitutes about 70% of the

total FB content found in naturally contaminated food and feed (Milicevic *et al.,* 2010) and is believed to be the most toxic and is considered by the International Agency for Research on Cancer (IARC) to be a possible carcinogen to humans (class 2B) (IARC, 2002).

Environmental factors such as variations in pH, water availability, temperature and inadequate plant nutrition have been identified as regulators of fumonisin production (Sanchis & Magan, 2004; Mogensen *et al.,* 2009). The fumonisin biosynthetic genes are clustered (Proctor *et al.,* 2003), and one of these genes, *FUM1* gene plays a crucial role in fumonisin production since it encodes the polyketide synthase that catalyses the first step of the biosynthetic pathway (Seo *et al.,* 2001). Marín *et al.* (1999) & Samapundo *et al*. (2005) reported that fumonisin production increased with increasing water activity at 0.92–0.98 a_w and at optimal temperatures between 20°C and 30°C depending on the isolate. It was confirmed by Jurado *et al.* (2008) that the expression of the *FUM1* gene was significantly increased under water stress conditions (0.95 a_w) which may increase FB_1 production by *F. verticillioides*. Under field conditions, it was reported that dry environments, during pollination, affect fumonisin production (Shelby *et al.,* 1994).



a) FB_1



b) FB_2

Figure 5: The structure of Fumonisin B_1 and B_2 adapted from Barna-Vetro *et al.* (2000).

1.7 Health implications of fumonisins to humans and animals

Fumonisins were given attention due to their carcinogenic nature to humans and animals (Hussein & Brasel, 2001). Fumonisins have been linked to diseases such as Leucoencephalomalacia (ELEM) in horses where extensive necrosis of brain tissue takes place and porcine pulmonary oedema (PPE) in pigs where the chronic accumulation of fluid in the lungs occurs (Yates *et al*., 2005). Field outbreaks of ELEM have been reported since 1891 (Haliburton & Buck, 1986) and since then reported sporadically in countries such South Africa, China, Egypt, South America, and Germany (Haliburton & Buck, 1986; Kellerman *et al.,* 1990). During 1988, ELEM was reproduced using pure cultures of *F. verticillioides* in horses in South Africa (Marasas, 1976) and showed typical characteristics of liquid necrosis of the white matter of the brain (WHO, 2000). Kriek *et al*. (1981) induced PPE in pigs and liver cancer in rats fed with maize contaminated with *F. verticillioides*. PPE is characterized by yellow fluid in the pleural cavity (WHO, 2000). The clinical signs usually include decreased feed consumption, weakness, cyanosis and death (JECFA 56th, 2001).

The prevalence of oesophageal cancer in humans has been reported in certain parts of South Africa (Rheeder *et al.,* 1992; Marasas *et al.,* 2004), China (Wang *et al.,* 2000), Italy (Franceschi *et al.,* 1990) and Iran (Shepherd *et al.,* 2000), where high levels of fumonisins in maize have been found. Based on studies reported on the involvement of fumonisins causing abnormalities in mice, it was suggested that the consumption of these toxins may be related to human neural tube defects and associated birth defects (Marasas *et al.,* 2004). Fumonisin B_1 reduced the uptake of folate in different cell lines, which is of great concern as fumonisins, during pregnancy, could result in elevated risk of neural tube birth defect in the developing foetus (Marasas *et al.,* 2004).

Since the discovery of fumonisins, there has been increased interest in the health concerns associated with the consumption of maize contaminated with fumonisins. In the absence of fumonisin regulation in South Africa, the guidelines of the Food and Drug Administration of America (FDA) were followed. In 2016, the South African health ministry published regulations regarding tolerable fumonisin levels in food and feed (Table 1).

Table 1: Regulation of fumonisins as published in the South African Government Gazette, Section 15 (1) of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972).

Human Intake (ppm)	**Total Fumonisins (FB_1 + FB_2 + FB_3) (ppm)**
Raw maize grain intended for further processing	4 ppm
Maize flour and Maize meal ready for consumption	2 ppm
Animal Feeds (ppm)	**Total Fumonisins (FB_1 + FB_2 + FB_3) (ppm)**
Equids and rabbits	5 ppm (≤20% of diet)**
Beef and poultry	50 ppm (≤50% of diet) **
All other species or classes of life stock and pet animals	10 ppm (≤50% of diet) **

** Dry weights

1.8 Detection and quantification of fumonisin producing *Fusarium* spp.

Detection and quantification of fumonisin-producing fungi in maize has involved plate counts and infection rates (percentage of seed that show fungal growth after surface disinfection) as an indication of fungal biomass (Schwadorf & Müller, 1989; Saxena *et al.*, 2001). However, these conventional methods are time consuming and in the case of infection rate, not applicable to milled samples. Additionally, microscopic identification of fungi *in planta* requires technical skill. A number of culture media have been used to grow and identify *F. verticillioides*, which includes carnation leaf agar (CLA) and potato dextrose agar. Identification of this *Fusarium* spp. by morphological characters and mating type are reported to be important methods used to identify the fungal species. However, the genus Fusarium contains a large group of species, which makes it difficult to depend only on morphological characters of *Fusarium* spp. (Leslie & Summerell 2006; Jurado *et al.,* 2008). Ergosterol is a component specific to fungal cell membranes that may be influenced by high levels oxygen and water activity. Ergosterol assays has proven to be useful in the early detection of fungi in grain such as wheat (Saxena *et al.,* 2001) and maize (De Castro *et al.,* 2002). The ergosterol method has limitations in that ergosterol extract of fungi growing within a natural substratum may be a recovery of a variety of different fungi embedded therein (Padgett & Posey, 1993). Therefore, the

quantification of total ergosterol from maize kernels may not give an accurate account of the fumonisin producing fungal mass.

Since *F. verticillioides* is morphologically similar to *F. thapsinum*, *F. proliferatum* and *F. nygamai,* identification can be problematic (Leslie & Summerell, 2006). Aiming to overcome the obstacles of identification as well as developing rapid tools for detection, nucleic acid based methods have been developed for the identification and quantification of mycotoxigenic fungi. Various quantitative PCR assays have been developed for the identification of toxigenic *Fusarium* spp., using species specific primers (Reid *et al.,* 1999; Waalwijk *et al.,* 2004). Sequencing of species-specific primers and the translocation elongation 1-α (*TEF1*) gene are being used to overcome limitations of morphological characterizations. Data from sequencing can be used to separate *Fusarium* spp. into phylogenetic species (O'Donnell *et al*., 2000).

1.9 Detection and quantification of fumonisins

Analytical methods for rapid, sensitive, and accurate determination of mycotoxins in cereals and cereal-based products are highly required in order to evaluate the risk of exposure to humans and animals. Accurate results will ensure that enforced regulatory levels can be met (Pascale, 2009). These methods require three major steps 1) extraction of toxins from the sample, 2) clean-up of the extract to remove impurities and 3) detection and quantification of mycotoxins using the relevant analytical instruments and technologies (Shephard, 1998; Rahmani *et al.,* 2009).

There are several chromatographic methods that are used for the quantitative detection of mycotoxins in cereals (Shephard, 2008). These methods include analytical methods such as thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography(HPLC), liquid secondary-ion mass spectroscopy (LC/MS), gas chromatography-mass spectroscopy (GC/MS), ultra high-performance liquid chromatography tandem mass spectrometry (Ren, 2011; Shephard, 2012).

TLC methods are used for the quantitative measurements of common mycotoxins (aflatoxins and fumonisin) with detection by visual procedures based on silica gel or silica gel infused with organic acid (Li *et al.,* 2010). This method cost less, is simple and suitable for rapid screening, but lack the specificity needed for a quantitative method, therefore

the TLC were replaced by other techniques (Roseanu *et al.,* 2010). GC methods has been used to measure trichothecenes in fungal cultures in tandem with MS (Nielsen & Thrane, 2001). Fumonisin analysis using GC requires multiple time consuming sample handling steps such as sample hydrolysis, clean up and derivatization prior to analysis and so has found little application for fumonisin detection (Shephard, 1998).

HPLC is a widely used method for the detection and determination of toxins form cereals (Edwards *et al.,* 2002). Since fumonisins do not fluorescence or contain a UV absorbing chromophore, most HPLC methods measure fumonisins after derivatization of their free amino group by employing o-phthalylaldehyde (OPA) reagent (Shephard, 2000). Even though this method is accurate and specific, most of the assays are expensive, time-consuming and require expensive extraction methods and clean-up procedures (Ndube, 2011).

LC/MS is a useful technique to quantify different mycotoxins, including those that do not absorb UV light (Lattanzio *et al.,* 2009; Pascale, 2009). Measurements of mycotoxins by LC-MS is often complicated due to matrix effects and ion suppression. It is therefore necessary to purify extracts prior to detection (Lattanzio *et al.,* 2009). The LC/MS technique is expensive and necessitates the use of high level professionals for operation (Sforza *et al*., 2006). GC/MS is a widely used method for the qualitative and quantitative determination of mycotoxins (Rahmani *et al.,* 2009). It separates a mixture of compounds on a stationary column using a carrier gas such as helium, and the mycotoxins are detected and quantified using a mass spectrometer. This method require expensive instrumentation and kits. They are less sensitive than LC/MS and they offer a detection limit of less than 0.05 ppm for many mycotoxins (Schmale & Munkvold, 2009).

1.10 Management options to reduce *F. verticillioides* infection and fumonisin contamination

1.10.1 Agronomic practices

The contamination of maize grain by fumonisins mainly occur during crop development in the field (Wild & Gong, 2010), but can also continue during harvest, processing, transport and storage. Therefore, it is crucial to apply good agronomic practices to manage conditions conducive for fungal infection and fumonisin production contamination

(Jouany, 2007; Blandino *et al.,* 2009). These strategies should involve an appropriate choice of planting dates in temperate areas to avoid adverse conditions during critical periods such as flowering and kernel drying. Early planting reduces the risk of flowering coinciding with drier and warmer weather, when environmental conditions are most favourable for fungal infection (Parsons & Munkvold, 2010) and fumonisin production (Abbas & Shier, 2007). Seedbed preparation and crop rotation with non-host crops will reduce the build-up of primary inoculum (Munkvold, 2003).

Plant density recommendations for maize production have increased to a point at which they are now double those recommended in the 1950's. Excessive plant populations could induce moisture and nutrient stress on individual maize plants (Mukanga *et al.,* 2011), which could increase their susceptibility to mycotoxin producing fungi (Trento *et al*., 2002; Bruns, 2003). According to Logrieco *et al.* (2002) very little and unclear information has been recorded about the effect of plant population on the contamination of maize with mycotoxins. It is well known that drought stress impairs numerous metabolic and physiological processes in plants (Levitt, 1980). It leads to growth reduction, reduction in the content of chlorophyll pigments and water and changes in fluorescence parameters (Lu & Zhang, 1999; Colom & Vazzana, 2003; Li *et al.,* 2006). Nutrient uptake by plants is decreased under drought stress conditions due to reduced transpiration, impaired active transport and membrane permeability resulting in reduced root absorbing power. Most of the damaging effect of drought is associated with the photosynthetic process of the plant. One of the earliest responses to drought is stomatal closure. It was reported by Schmidt-Heydt *et al*. (2008) that under field conditions, a progressive increase in water stress during kernel maturation might be a critical factor affecting fumonisin accumulation by *F. verticillioides*.

Maize plants exposed to drought or fertility stress are also more susceptible to infection by microorganisms than plants not under stress (Blandino e*t al.,* 2008). Elevated aflatoxin levels have been associated with fertility-related stresses, particularly N deficiency (Lisker & Lillehoj, 1991). Jones & Duncan (1981) and Munkvold (2003) reported that a higher rate of N fertilizer application consistently resulted in reduced aflatoxin rates. Blandino *et al.* (2008) related higher fumonisin contamination with high N rates and in the presence of N deficiencies. Marocco *et al.* (2008) reported that N fertilisation significantly increased fumonisins levels, the authors however only applied two N treatment rates (0 kg N ha^{-1}

and 270 N ha^{-1}). It is necessary to understand and quantify the effect of N levels on *F. verticillioides* and resultant fumonisin production under local conditions.

Application of insecticides and herbicides is important to control infection routes created by certain insects and to reduce plant stress due to competition with weeds for nutrients, space, water and light (Torelli *et al.,* 2012). Time of harvest can have major consequences on the levels of contamination by fumonisins. In general, earlier harvest results in lower concentrations of mycotoxin production (Jones *et al.,* 1981). Maize plants are usually left in the field to dry slowly, however, the moisture percentage may remain high enough to allow continued fungal infection and the production of fumonisins in kernels. Therefore, taking into consideration the environment, crop size and producer's needs (Kikafunda-Twine *et al.,* 2001), a proper harvesting period and moisture content of less than 15% have to be considered in order to minimize continued fungal growth and fumonisin production in maize kernels (Maiorano *et al.,* 2009).

F. verticillioides infection can still arise in storage because of moisture variability in the grain. Therefore, storage facilities should be thoroughly cleaned before the new crop is stored, because grain residue often harbour large populations of storage moulds. This storage structures should also protect the grain from rain, ground water, rodents, birds, and temperature fluctuations (Munkvold, 2003). Temperature (<25°C) during storage should be maintained, in order to prevent fungal growth and mycotoxin production as this can reduce grain quality and safety, resulting in serious economic losses and health hazards (FAO, 2004).

During transportation, kernel damage occurs either by bruising or by breaking (Wagacha & Muthomi, 2008). Further spoilage of maize kernels can occur when transported over long distances and where temperatures are favorable for fungal infection and fumonisin production in maize ears (Dawlal, 2010).

1.10.2 Resistant genotypes

Resistant genotypes are regarded as the most effective strategy to control *Fusarium* spp. infection and resultant mycotoxin production in maize (Russell *et al.,* 2010). There has recently been an increase in breeding programs at both public and private institutions to develop resistant genotypes for *F. verticillioides* infection. This would provide producers

with environmentally and economically sound measures to control fumonisin accumulation (Small *et al.,* 2012). The development of resistant cultivars has been difficult due to environmental influences, ineffective disease screening methods, unwanted traits coupled with highly resistant germplasm and the polygenetic nature of resistance (Mouton, 2014). A wide range of maize genetic diversity was evaluated under artificial inoculated experimental trials in Africa, Europe and America and sources of resistance to Fusarium ear rot and fumonisin contamination have been found among hybrids (Santiago *et al.,* 2013).

Complete resistance to *F. verticillioides* and fumonisin contamination in maize cultivars planted in southern Africa has not yet been identified (Rheeder *et al.,* 1990; Schjøth *et al.,* 2008). However recently, Small *et al.* (2012) have identified maize inbred lines with high resistance levels to *F. verticillioides* and fumonisin contamination. These lines were selected for their agronomic characteristics and are used extensively at the Agricultural Research Council – Grain Crops Institute in Potchefstroom (breeding programme) (Mouton, 2014).

1.10.3 Biological control

Biological control methods to reduce fungal infection (He & Zhou, 2010; Alberts *et al*., 2016) of crops is being increasingly recognized as a viable, eco-friendly alternative that limits the massive use of synthetic chemical pesticides (Charan, 2010). This method involves an interaction between the host, the pathogen and the antagonistic biocontrol microorganism (Larkin & Fravel, 1998; Alabouvette *et al.,* 2009). Microorganisms naturally associated with and adapted to the vegetative parts of a specific plant, sharing the ecological niche with the pathogen, could hold advantages as biocontrol agents. For example, Bacon *et al.* (2001), has described *Bacillus subtilis* as one of the microorganisms that occupies the same ecological niche as *F. verticillioides* within the maize plant and effectively inhibits growth of the fungus, based on competitive exclusion. *Trichoderma harzianum* is one of the promising biological control agents, which can colonize roots and compete for space and nutrients with plant pathogenic fungi (Elad, 1996). However, information on its ability to induce systemic resistance in maize against *F. verticillioides* is still lacking (Ferrigo *et al.,* 2014).

1.11 Stress based disease detection technique: Chlorophyll fluorescence

Photosynthesis is the process whereby light energy is converted into chemical energy to synthesize carbohydrates and oxygen (O_2). This process takes place within the chloroplasts, which contain disc-like structures called thylakoids, where the conversion of light energy to chemical energy are initiated. The thylakoid membrane contains specialised complexes named photosystem I and photosystem II (Baker, 2004). These photosystems consists of chlorophyll pigments that are responsible to absorb light energy. The light energy absorbed by the chlorophyll molecules are used to drive photosynthesis, excess energy can be dissipated as heat or it can be re-emitted as light at shorter wavelengths (chlorophyll fluorescence) (Maxwell & Johnson, 2000). By measuring the intensity of chlorophyll fluorescence, which is only observed in chlorophyll *a* in photosystem II, the functioning of the plant can be investigated (Baker, 2004).

Therefore, chlorophyll fluorescence has become a tool in plant physiology that is used to measure the photosynthetic efficiency of the plant and for detailed information on the photochemistry of photosystem II (PSII) at a relatively low cost. It has had a major role in understanding the fundamental mechanisms of photosynthesis and the drivers of it. Environmental factors such as climate change, genetic variation, and ecological diversity (Murchie & Lawson, 2013) have an influence on the photosynthetic efficiency of the plant. The increased interest in the use of chlorophyll fluorescence has been due to research in crop improvement and in particular for the screening of desirable plant traits and linking genomic information with phenological responses (Baker & Rosenqvist, 2004).

Research interest has been based on PSII because it emits high levels of variable chlorophyll fluorescence (Schreiber, 2004). PSII is also accepted to be the most vulnerable part of the photosynthetic apparatus to light-induced damage. The damage to PSII is the first indicator of stress in a leaf (Maxwell & Johnson, 2000).

Changes in the photochemical yield of chlorophyll fluorescence were first observed as early as 1960 by Kautsky (Kautsky *et al*., 1960) and was then formulated as the Kautsky effect. They found that, upon transferring photosynthetic material from the dark into the light, an increase in the yield of chlorophyll fluorescence occurred over a period of time. In the PSII, quinone A (Q_A) absorbs an electron; however, it is not able to accept another until it has passed the first electron onto a subsequent electron carrier (Q_B). During this

period, the reaction centre is closed. During this time, the closed reaction centres leads to a reduction in the efficiency of photochemistry and so to a corresponding increase in the yield of fluorescence (Maxwell & Johnson, 2000).

An increase in the minimum fluorescence (F_o) is a result of a higher number of Q_B^- non-reducing reaction centres, which ultimately results in a decrease of F_v/F_m (Zhu *et al*., 2011). F_v/F_m is maximum quantum yield of PSII, which gives the information about the proportion of the light absorbed by chlorophyll in PSII that is used in photochemical processes (Strasser *et al*., 2001). Unlike F_v/F_m which utilizes only extreme values of chlorophyll fluorescence, namely minimal (F_o) and maximal (F_m) fluorescence, PI_{abs} appeared to be very suitable and sensitive parameter to investigate plant overall photosynthetic performance under different abiotic and biotic stresses as well as during development of photosynthetically active plant organs (Appenroth *et al.,* 2001). These parameter takes into account all of the main photochemical processes, such as absorption and trapping of excitation energy, electron transport and dissipation of excess excitation energy.

1.12 Pathogenesis Related (PR) Proteins associated with plant defence mechanisms

Plants possess a variety of defence mechanisms to protect themselves against microbial and pathogen invasion through the activation of complex defence strategies, which includes physical and chemical barriers as well as inducible defence mechanisms (Guest & Brown, 1997; Delledonne *et al.,* 2001; Hematy *et al.,* 2009). These responses also involve changes in the expression of plant genes, which are regulated in the plant. The production and accumulation of pathogenesis-related (PR) proteins is one of the most common responses in plants. Some of these proteins are expressed in response to chemicals that act in a similar way as pathogen infection (Delaney *et al.,* 1994). Apart from infectious pathogens triggering the synthesis of PR proteins, their expressions are dependent on internal developmental stages of the plant (Sinha *et al.,* 2014).

PR proteins are grouped into 17 different families; some of these families have chitinase, β-1, 3-glucanase and peroxidase activities that contribute to the defence of plants against fungal pathogens (Jackson & Taylor, 1996; Eulgem, 2005). Chitinases are reported to be a group of enzymes that hydrolyse the glyosidic bonds in chitin and exhibit antifungal

activity (Sahai & Manocha, 1993; Jackson & Taylor, 1996). Plants that express high levels of chitinase show a decreased susceptibility to infection by fungi with cell walls composed of chitin (Broglie *et al.,* 1991). β-1,3-glucanases are highly regulated enzymes which catalyse the hydrolytic cleavage of β-1,3-glucans present in plant cell walls (Hoj & Fincher, 1995). They respond to pathogenic attack and are involved in several physiological and developmental processes, such as germination and fertilization (Meikle *et al.,* 1991). Peroxidases is expressed to limit cellular spreading of the infection through the establishment of structural barriers or by producing reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Passardi *et al.,* 2005). Peroxidases act in cell wall reinforcement by catalysing lignification (Passardi *et al.,* 2004) and enhance resistance against multiple pathogens. PR proteins play an important role in breeding programmes to ensure resistance in genotypes against fungal infection and subsequent mycotoxin production (Sinha *et al.,* 2014).

1.13 CONCLUSIONS

Maize is an important crop that is grown worldwide and serves different functions of economic value, particularly as a staple diet for millions of people in South Africa especially in the rural populations (Walker & Schulze, 2006). South Africa is the second largest maize production region in Africa, producing an estimated 10-12 million tons of maize annually. It is produced throughout the South African community under various agro ecological conditions and is grown under commercial, small-scale and subsistence farming levels. Maize production depends on the correct application of management practices ensuring both environmental and agricultural sustainability (Nape, 2011). Its productivity could be limited due to a combination of factors such as low soil fertility, unfavourable environmental conditions, poor agricultural management as well as pests and diseases (Tisdale *et al*., 1990). Maize also serves as a convenient substrate for naturally occurring fungal pathogens, which may result in mycotoxin contamination when environmental conditions are suitable. Economically important maize fungal ear rots are the *Fusarium spp.*: *F. graminearum* (*sensu lato*), *F. verticillioides*, *F. proliferatum* and *Stenocarpella maydis. F. verticillioides* and *F. proliferatum* can produce fumonisins, B_1, B_2, and B_3, which are toxic secondary, possible carcinogenic metabolites that occur naturally as contaminants of agricultural products such as maize. The distribution and predominance of this *Fusarium* spp. and their concomitant fumonisin production varies

depending on season, geographic locality, climatic factors such as temperature and moisture, host genotype and agricultural practices (Nyaka *et al*., 2010).

Commercial maize in South Africa are subjected to a grading system which ensures that contaminated maize do not enter the food and feed market. There are no systems in place to monitor subsistence and small scale farmer's maize and they are often at risk of consuming contaminated maize (Marasas *et al.* 2008) and most people are not aware of the health risks imposed by it (Kpodo *et al.,* 2000; Gamanya & Sibanda, 2001; Ngoko *et al.,* 2001). The possible consumption of contaminated maize by humans and animals provide enough pressure to research *F. verticillioides* infection of maize plants and subsequent fumonisin production as well as the management thereof to sustain maize production in South Africa (Gerber, 2010).

The aim of this study was to determine the effect of maize plant stressors on *F. verticillioides* infection and fumonisin production in maize plants. Stressors such as plant density, soil nitrogen availability, nitrogen source (Urea and LAN) and nitrogen rate (25, 50, 100, 150 and 175 kg ha^{-1}) will be studied under field conditions. Chlorophyll fluorescence will be used as a possible tool to quantify the photosynthetic efficiency in maize plants as early indicator of plant stress. In field trials, maize will be evaluated for the available maize leaf nutrients as well as the expression of PR protein activity during different growth stages of the maize plant have an effect on *F. verticillioides* infection and fumonisin production.

The findings of this study will be used in an integrated management system to minimize *F. verticillioides* infection and fumonisin production in maize kernels. It will aid farmer's understanding of maize production and enable them to reduce grain contamination and prevent such grain being used for food or feed, thus improving human and animal food safety measures. PR protein information can add value to breeding programs as genotypes with improved defence mechanisms against *F. verticillioides* infection can be developed.

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CHAPTER 2

THE EFFECT OF PLANT DENSITY AND NITROGEN RATES ON *FUSARIUM VERTICILLIOIDES* INFECTION AND FUMONISIN PRODUCTION IN MAIZE GRAIN UNDER FIELD CONDITIONS.

2.1 ABSTRACT

Fusarium verticillioides is an important ear rot pathogen of maize that can lead to economic losses due to yield and grain quality reduction. Symptoms vary depending upon genotype, environment and disease severity. *F. verticillioides* can produce fumonisin B_1, B_2 and B_3 that can cause mycotoxicoses in animals and are also statistically linked with oesophageal cancer in humans. It has been shown that abiotic factors such as substrate, temperature and water activity can have a profound effect on fumonisin synthesis. The aim of this study was to elucidate the potential effect of plant density on *F. verticillioides* infection and fumonisin production. Plant density field trials (2011-2014) with a progressive decline of soil nitrogen were planted at the experimental farm of the ARC-GCI in Potchefstroom. Plant densities of 10 000, 20 000, 30 000, 40 000 and 50 000 plants ha^{-1} were planted using cultivars CRN3505 and PAN 6P-110 in a completely randomized block design, replicated three times. As nitrogen and plant density appeared to be obscuring the effects of each other, a separate plant density field trial with adequate nitrogen and a separate nitrogen trial (using two sources at 5 application rates) was planted (2015-2016) in Potchefstroom using 8 cultivars in a split plot design, replicated three times. Naturally, infected ears were harvested at 12-14% moisture, threshed and grain milled and subjected to qPCR (*F. verticillioides* target DNA) and HPLC (fumonisin levels). From the 2011-2014 field trials, ANOVA indicated plant density x season x cultivar to have a highly significant effect on target DNA quantified from maize grain ($P<0.0001$). Target DNA was significantly higher in the 2013-2014 seasons for both PAN6P-110 and CRN3505. Highest target DNA levels for the 2013-2014 season was quantified at 10 000, 20 000 and 30 000 plants ha^{-1}. Plant density x season had a significant effect on fumonisins in grain ($P<0.01$). Fumonisins were significantly higher in the 2011-2012 season at 10 000, 20 000, 30 000 and 40 000 plants ha^{-1}. Fumonisins were most prominent in the 10 000 plants ha^{-1} density for seasons 2011/2012, 2012/2013 and 2013/2014. In the separate plant density field trial of 2015, plant density had a significant

effect on target DNA ($P<0.05$) and fumonisin ($P<0.05$) production in grain. Regression analyses show an increase in target DNA ($R^2=0.97$) and fumonisins ($R^2=0.86$) as plant densities increase. ANOVA indicated plant density to have a highly significant effect on moisture percentage in grain ($P<0.0001$). Regression analyses show a decrease in grain moisture percentage as plant densities increase ($R^2=0.98$). Correlation analyses to quantify the relationship between grain moisture percentage and target DNA was inversely significant ($r=-0.97$). Grain moisture percentage and fumonisin had a moderate inverse correlation ($r=-0.47$). In the separate nitrogen trial, nitrogen source and rate of application had a significant effect regarding target DNA and fumonisins in maize grain ($P<0.0001$ and $P<0.01$ respectively). Regression analyses show a strong relationship between the rate of LAN application and target DNA in maize grain ($R^2=0.79$). Rate of UREA application had a non-significant relationship on target DNA in maize grain. Regression analyses showed a weak relationship between the rate of LAN application and fumonisins ($R^2=0.58$) and a non-significant relationship between the rate of UREA application and fumonisins. This study showed that under nitrogen poor conditions, cultivar choice, environment and low plant densities could lead to elevated fungal infection and fumonisin production in maize grain, placing subsistence and small scale farmers at risk. In farming systems with adequate soil nitrogen, as plant density increase, grain moisture decrease and target DNA and fumonisins increase. Applications of LAN can influence target DNA in maize grain. Only trace amounts of fumonisins were quantified and the effect of LAN is inconclusive at this stage. This study re-iterated the importance of appropriate management practices such as obtaining environmentally adapted seed, applying fertilizers and using the correct planting methods to improve maize yields but also manage the mycotoxin threat to end users.

2.2 INTRODUCTION

Maize (*Zea mays* L.) is one of the most important sources of carbohydrates produced worldwide. It ranks first in South Africa with an estimated total yield of 7 536 875 million ha^{-1} planted (crop estimates committee, 2016). The bulk of maize used in South Africa is for animal feed (38.4%) and food (36.1%) products (SAGL, 2015). With an increase in the human population, the South African agricultural sector is under pressure to increase maize production (Chincholkar & Mukerji, 2007). Maize production depends on the correct application of management practices ensuring both environmental and agricultural

sustainability (Nape, 2011). By understanding how plants interact morphologically and physiologically in a community and identifying management practices, which allow them to maximize the use of growth resources in their environment, is very important (Arif *et al.,* 2013). Maize productivity constraints could be attributed to a combination of factors such as low soil fertility, unfavourable environmental conditions, poor agricultural management as well as pests and diseases (Tisdale *et al*., 1990; Major *et al*., 1991).

In maize, *Fusarium* spp. are the most common toxigenic fungi in the field and cause ear rot disease Marocco *et al*. (2008). Ear rot of maize caused by *Fusarium verticillioides* can infect maize kernels through silks and through kernels damaged by insects (Headrick & Pataky, 1991). Symptoms can be observed on random kernels, groups of kernels or physically injured kernels (Marocco *et al*., 2008) as a white to light pink fungal growth. *F. verticillioides* can also be found endophytically and not show any symptoms. In addition, *F. verticillioides* can produced mycotoxins known as fumonisins, which are harmful to humans and animals (CAST, 2003; Pasquali *et al.,* 2010). Fumonisins are linked to numerous diseases in humans and animals (Marasas *et al.,* 2004; Kimanya *et al.,* 2010) and are classified as Group 2B carcinogens (IARC, 2002). The fumonisin analogs FB_1, FB_2, and FB_3, are the most abundant naturally occurring fumonisins, with FB_1 predominating and usually being found at the highest levels. Maize grown domestically has been known to be frequently contaminated with unacceptable levels of FB_1 in the Transkei regions of South Africa (Burger *et al.,* 2010; Shephard *et al.,* 2013). Recently, the South African health ministry has amended the regulations that enforces the tolerable intake levels for fungus-produced toxins in foodstuffs in terms of section 15 (1) of the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act No. 54 of 1972) (Government gazette, 2012). Of which, raw maize grain that is intended for further processing, should contain more not than 4000 pg kg^{-1} of $FB_1 + FB_2$, and maize ready for human consumption should contain no more than 2000 pg kg^{-1} of $FB_1 + FB_2$. Methods for prevention of exposure to the fumonisins, particularly in rural communities, remain important (Alberts *et al.,* 2016). Only a limited number of developing countries have legislative maximum levels for fumonisins and implementation of these is often very poor. These regulations are not applicable in rural areas and exposure to mycotoxins on a daily basis is common (Pitt *et al.,* 2012).

It is reported that substances or conditions that impose stress on the fungus also have an influence on mycotoxin biosynthesis. Abiotic factors such as substrate, temperature and water activity have been reported to have a profound effect on fumonisin synthesis. Plant density recommendations for maize production have increased to a point at which they are now double those recommended in the 1950's. Excessive plant populations could induce moisture and nutrient stress on individual maize plants, which could increase their susceptibility to mycotoxin producing fungi (Trento *et al*., 2002; Bruns, 2003). Tokatlidis & Koutroubas (2004) stated that tolerance to high plant populations, along with tolerance to other biotic and abiotic stresses, has in recent years constituted the factors that contribute to improved maize productivity. On the other hand, factors that can reduce plant density include soil surface residues that interfere with germination in no-till agriculture or the usage of lower seeding densities to minimize yield reduction associated with dry conditions.

Only a few studies have been conducted on the influence of plant density concerning sanitary aspects, such as mycotoxin contamination. Bilgrami & Choudhary (1998) reported lower aflatoxin levels in densely cultivated plants whereas Rodriguez-Del-Bosque & Eigenbrode (1996), Bata *et al*. (1997) and Bruns & Abbas (2005) reported that plant populations had no effect on aflatoxin, zearalenone or fumonisin contamination of maize kernels. Abbas *et al*. (2012) reported there was no evidence that lowered seedling density (to reduce stress) reduced aflatoxin or fumonisins in maize in research trials in Los Angeles. According to Logrieco *et al.* (2002) very little and unclear information has been recorded about the effect of plant population on the contamination of mycotoxins such as fumonisins.

It is well established that drought stress impairs numerous metabolic and physiological processes in plants (Levitt, 1980). It leads to growth reduction, reduction in the content of chlorophyll pigments and water and changes in fluorescence parameters (Lu & Zhang, 1999; Colom & Vazzana, 2003 and Li *et al.,* 2006). Nutrient uptake by plants is decreased under drought stress due to reduced transpiration, impaired active transport and membrane permeability resulting in reduced root absorbing power. Most of the damaging effect of drought is associated with the photosynthetic efficiency of the plant. One of the earliest responses to drought is stomatal closure. It was reported by Schmidt-Heydt *et al*. (2008) that under field conditions, a progressive increase in water stress during kernel

maturation might be a critical factor affecting fumonisin accumulation by *F. verticillioides*. Studies by Jurado *et al.,* (2008) indicated that expression of the *FUM1* gene was significantly increased under water stress conditions (0.95 a_w), which may indicate an increase in FB production by *F. verticillioides* under such conditions.

F. verticillioides infection and ear colonization is favoured by high levels of moisture and high relative humidity, from silking to the end of the maturation period (Reid *et al.,* 1999, Logrieco *et al*., 2002). These favourable conditions could be prolonged as a consequence of rich nitrogen (N) fertilizer applications, which would lead to longer vegetative growth and higher leaf expansion (Blandino & Reyneri, 2007). On the other hand, maize plants exposed to drought or fertility stress are also more susceptible to infection by microorganisms than plants not under stress. Elevated aflatoxin levels have been associated with fertility-related stresses, particularly N deficiency (Lisker & Lillehoj, 1991). Anderson *et al*. (1975) and Jones *et al*. (1981) reported that a higher rate of N fertilizer application consistently resulted in reduced aflatoxin rates. Blandino *et al.* (2008) related higher fumonisin contamination with high N rates and in the presence of N deficiencies. Marocco *et al.* (2008) reported that N fertilisation significantly increased fumonisins levels, the authors however only applied two N treatment rates (0 kg N ha^{-1} and 270 N ha^{-1}).

The aim of this study was to 1) identify the effect of increased plant densities (with a gradual decline of soil nitrogen) on *F. verticillioides* infection of maize kernels and subsequent fumonisin production by means of field trials (Potchefstroom) using two cultivars (2011-2014). 2) To identify the effect of increased plant densities (with adequate soil nitrogen) on *F. verticillioides* infection of maize kernels and subsequent fumonisin production by means of field trials (Potchefstroom) using 8 cultivars (2015-2016). 3) To identify the effect of two nitrogen types applied at 5 rates on *F. verticillioides* infection of maize kernels and subsequent fumonisin production by means of field trials (Potchefstroom) using 8 cultivars (2015-2016).

2.3 MATERIALS AND METHODS

2.3.1 Plant density trial with a gradual decline in soil nitrogen

Field trials were conducted at the Agricultural Research Council experimental farm in Potchefstroom for three seasons (2012-2015). According to the maize production

guideline of the ARC-GCI, a plant population of 24 000 plants ha^{-1} will yield 5 tons ha^{-1} (CRN3505 and PAN6P-110 used in this study). The experiments were laid out in a randomized complete block design, replicated three times. Plot sizes were 12 m x 8 m and comprised five plant densities that included 10 000 plants ha^{-1}, 20 000 plants ha^{-1}, 30 000 plants ha^{-1} (control), 40 000 plants ha^{-1} and 50 000 plants ha^{-1} using maize cultivars CRN3505 and PAN6P-110. The desired plant densities were achieved with row spacing of 0.9 m (inter-row) x 15 cm (intra-row), 0.9 m x 30 cm, 1.0 m x 30 cm, 1.2 m x 30 cm and 2.0 m x 30 cm, respectively. After emergence, maize seedlings were counted in each respective block to determine actual plant densities. The land was irrigated when necessary to maintain a water holding capacity of between 33 and 200 kPa. Fertilizers were not applied to ensure gradual decline of nitrogen in the soil. Prior to planting, soil samples from each experimental plot were collected with a soil auger in a zigzag pattern. Individual soil samples from the top- and sub-soil were placed in polyethylene bags and sent to the Agricultural Research Council – Industrial Crops in Rustenburg for nutrient analyses. To control weeds, 2-3 L ha^{-1} of Bateleur gold herbicide (20 g L^{-1}) was applied as a pre-emergence control of annual grasses. For post- emergence control, 2.3 L ha^{-1} of Basagran (480 g L^{-1}) and 1 L ha^{-1} of Servian (50 g L^{-1}) were incorporated using a high volume boom sprayer with a flat fan-type spray nozzle. Manual weeding was implemented throughout the season until plant maturation.

2.3.2 Separate plant density field trial with adequate soil nitrogen

An additional plant density field trial was planted at the Agricultural Research Council experimental farm in Potchefstroom (2015/2016) to separately determine the effect of plant density from that of plant density with a decline in soil nitrogen, as these appeared to be obscuring the effects of each other and the effect on the two cultivars in the initial trials planted. Additional cultivars (Table 1) and the same plant densities as described above, were used in a split plot design, replicated three times to investigate the possible effect of environment, cultivar and plant density on *F. verticillioides* infection and fumonisin production in maize grain. The whole plot factor was plant density and the sub-plot factor was cultivars.

Table 1: Selected cultivars used to determine the effect of plant density on *F.verticillioides* infection and fumonisin contamination in maize grain

Cultivar	Grain colour	Growth period
CRN3505	White	Medium
PAN6P-110	Yellow	Medium
SC 608	Yellow	Medium-slow
LS8537	White	Medium
PAN6Q 245	White	Medium
LS8518	Yellow	Medium
IPM52-11R	White	Medium
P2432R	Yellow	Medium

2.3.3 Separate nitrogen (type and rate) field trial

An additional nitrogen field trial (2015-2016) was planted at the Agricultural Research Council experimental farm in Potchefstroom using the same eight cultivars described above in a split block design. The whole plot factor was nitrogen application (type and rate) and the sub-plot factor was cultivars. Two fertilizers Limestone Ammonium Nitrate (LAN) containing 28% nitrogen and UREA containing 46% nitrogen were used as treatments. Based on the results of soil analysis prior to planting, the starting concentration of N in the soil was 25 kg ha^{-1}. Applications rates were calculated to obtain rates of 50, 100,150 and 175 kg ha^{-1} of fertilizer. Six weeks after planting, fertilizers were incorporated immediately into the soil in burrows barrows 10 cm away from the plant roots to reduce ammonia toxicity released by UREA. Precautions were taken when applying UREA in order to avoid loss of nitrogen. The plants were irrigated after application to make sure that the fertilizers blended into the soil.

2.4 Harvest and sample preparation

Maize ears were manually harvested at 12-14% moisture, according to their treatment plots and placed in 50 kg polypropylene bags. Ear weight, kernel weight and moisture percentage of individual treatment plots were captured.

2.4.1 Laboratory analysis

A 1 kg grain sample from each cultivar and treatment was collected and stored in a cold-room at 4 °C for a maximum of 1 week prior to milling and further analysis. Sub-samples of 250 g from the initial 1 kg maize kernel samples were individually milled in a Cyclotech 1093 sample mill with a 1 mm mesh sieve. The mill was thoroughly cleaned with high-pressure air between each sub-sample to minimize cross contamination. The milled samples were used for DNA and fumonisin extractions and subjected to quantitative Real-time PCR (qPCR) and High Performance Liquid Chromatography (HPLC) to quantify target DNA and fumonisins.

2.4.1.1 DNA extraction and quantitative Real-Time PCR (qPCR) analysis for *Fusarium verticillioides* target DNA

Three hundred milligram maize powder of each individual sample was added into 2 mL homo-polymer cap micro tubes from Axygen, before isolating the target DNA using a DNeasy Plant Mini kit (QIAGEN, Germany). An amount of 500 µL buffer AP1 and 4 µL of RNaseA were added to the sample material; the aliquot was vortexed and incubated for 10 minutes at 65 °C. The samples were then mixed by adding 130 µL of AP2 buffer and each of the samples was incubated in ice for 5 minutes. The samples were centrifuged for 5 minutes at 14 000 rpm and the supernatant pipetted into a 2 mL QIAshredder mini spin column. The samples were then centrifuged for 2 minutes at 14 000 rpm. The flow-through was then transferred into a new tube and it was mixed with 1.5 µL of buffer AP3/E. Six hundred and fifty micro litres of the mixture was transferred into a 2 mL DNeasy mini spin column tube and centrifuged for 1 minute at 8 000 rpm. The flow-through was discarded. The spin column was placed in a new 2 mL collection tube. The DNA was washed with 500 µL AW buffer and centrifuged for 1 minute at 8 000 rpm. DNA material was washed again with 500 µL AW buffer and centrifuged for 2 minutes at 14 000 rpm. The spin column containing the DNA was placed on a new 2 mL micro-centrifuge tube and 100 µL of AE elution buffer was added, and samples were incubated for 5 minutes at room temperature and then centrifuged for 1 minute at 8 000 rpm. The DNA concentration was measured by using a nanodrop 2000c spectrophotometer (Thermo Scientific) and each individual DNA sample was diluted to 10 ng with autoclaved molecular grade water. The samples were stored in the -20 ºC freezer prior to qPCR analysis.

To test for linearity and the presence of inhibitors in the pathogen DNA, standard curves were prepared from 10 ng μL^{-1} (in triplicate) diluted 10-, 100-, 1000-, 10 000- and 100 000-fold in maize DNA that was free of fungal contamination (10 ng μL^{-1}). To assess the presence of inhibitors in maize, the method described by Boutigny *et al*. (2012) was followed. qPCR reactions were performed in a MyiQTM2 Two-Colour Real-Time PCR detection system (Bio-Rad, Hercules, USA) with a 96-well reaction plate and Tungsten halogen optical lamp. The primers Taqfum-2 F and Vpgen-3R in combination with the FUM-probe 1 as tested by Waalwijk *et al*. (2008) were used in this study. The primer/probe set had the following nucleotide sequence: Taqfum-2 F, 5′-ATGCAAGAGGCGAGGC AA-3′; Vpgen-3R, 5′-GGCTCTCRGAGCTTGGCAT- 3′ and FUM-probe 1, 5′-/56-FAM/CAATGCCATCTT CTTG/36-TAMSp/-3′. The sensimix reagent kit (sensimixTMno ref QT 505–05) from Celtic (Bioline) was used for qPCR. For each reaction, 4 µL of the DNA sample was mixed with 12.5 µL sensimix, 2.125 µL Fumprobe 1 (1 µM), 0.875 µL Taqfum-2 F (333 nM), 0.875 µL Vpgen-3R (333 nM) and 4.625 µL Melford molecular grade water. No template controls were subjected to the same procedure to exclude or detect any possible contamination or carryover. The 96-well plate was incubated for 10 minutes at 95 °C and thereafter, each of the 40 PCR cycles was performed according to the following temperature regime: 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s. The Bio-Rad iCyclerTmiQ Optical System Software Version 3.0a was used to calculate the concentrations of target DNA of *Fusarium* spp. that produce fumonisins (picogram fungal DNA 300 mg ground maize kernels) present in a sample. Standard curves from runs yielded a highly significant relationship (R^2= 0.99). Slopes were within the acceptance criterion (between −3.1 and −3.6) and efficiencies ranged from 95 to 105 %.

2.4.1.2 Extraction and quantification of fumonisins

Fumonisin levels were analysed according to the Vicam method. A 50 g aliquot from each of the 250 g milled sub-samples was mixed with 5 g of sodium chloride prior to extraction. Fumonisin were extracted in 100 mL methanol: water (80:20, v/v) for five minutes using industrial blenders. The extract was then filtered through Whatman No.5 filter paper. A volume of 10 mL aliquot was diluted with 40 mL phosphate-buffered saline (1x PBS) (8.0 g Sodium Chloride, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, dissolved in 990 mL HPLC purified water with pH adjusted to 7.0 with Hydrochloric acid). Diluted samples were

extracted through microfiber filters (0.45 µM) and 10 mL of the diluted filtrate was passed through FumoniTest affinity columns from Vicam at a flow rate of 1 drop per second. The column was then washed with10 mL of PBS. A volume of 1.5 mL of HPLC grade methanol was used to elute the material at a rate of 1 drop per second into a glass cuvette. Methanol eluate was dried in a TurboVap LV (Caliper Sciences) with the aid of a slow stream of high purity Nitrogen. Samples were re-dissolved in 200 µL methanol and purified water (50:50 v/v). Each sample (50 µL) was transferred to 250 µL conical inserts. Each insert was placed into a 2.5 mL glass vial, which was then placed into a carousel. The first position of the carousel had a 2.5 mL glass vial with o-phthaldialdehyde (OPA from Sigma) which is the derivatisation reagent. The Waters 717 plus auto sampler was set up to mix 100 µL of the OPA with the 50 µL of sample in the conical insert. Twenty microliters of this mixture was injected after a delay time of 1 minute. Fluorescence was performed at excitation and emission wavelengths of 335 and 440 nm respectively using a Waters 2475 multi λ fluorescence detector. The analytical column, Symmetry C18, 5 µM 3.9 x 150 mm from Waters was used. The detection limit of the method used was 0.016 ppm. Recovery data were obtained in triplicate by fortifying clean maize samples (Vicam) with 5 ppm fumonisin B_1, B_2 and B_3.

2.5 Field trial data analysis

All data was subjected to analysis of variance (ANOVA), and the means were compared with the least significant difference test with GenStat (2010) 13th edition software. The residuals were tested for deviation from normality. In cases where deviation was significant (skew), data was log transformed. Fisher's protected LSD (Least Significant Difference) was calculated at a 5% significance level to compare means of treatment effects (Montgomery, 1984).

2.6 RESULTS

2.6.1 Plant density trial with a gradual decline in soil nitrogen

The highest order interaction between plant density x season x cultivar had a highly significant effect regarding target DNA quantified from maize grain ($P<0.0001$, Table 2). Target DNA concentrations were significantly higher in the 2013/2014 season for CRN3505 at all plant densities compared to the other seasons and plant densities (Table

3). In CRN3505, the highest target DNA concentrations were quantified at 10 000 and 20 000 plants ha^{-1} with 626.66 pg μg^{-1} and 834.16 pg μg^{-1}, respectively.

Similarly, target DNA concentrations were significantly higher in the 2013/2014 season for PAN6P-110 compared to the other seasons and plant densities (Table 3). In PAN6P-110, the highest target DNA concentrations were quantified at 20 000 and 30 000 plants ha^{-1} with 127.25 pg μg^{-1} and 267.08 pg μg^{-1}, respectively. Overall, cultivar CRN3505 was more susceptible compared to PAN6P-110 regarding fungal infection (target DNA) with a mean target DNA of 106.32 pg μg^{-1} and 58.36 pg μg^{-1}, respectively.

Season as main variable had a highly significant effect on fumonisins quantified from maize grain ($P<0.001$, Table 4). Fumonisin levels were significantly higher in the 2011/2012 season (Table 5) with a mean value of 2.09 ppm compared to the other seasons. An interaction between plant density x season had a highly significant effect on fumonisins quantified from maize grain ($P<0.01$, Table 4). Fumonisins were significantly higher in the 2011/2012 season at 10 000, 20 000, 30 000 and 40 000 plants ha^{-1} (Table 5). Fumonisins were most prominent at 10 000 plants ha^{-1} for seasons 2011/2012, 2012/2013 and 2013/2014.

Table 2: Analysis of variance of the effects of plant density (PD), cultivar and season on target DNA (pg μg^{-1}) in maize grain.

Source	d.f.	s.s.	m.s.	v.r	F pr.
REP	2	2678.147	1339.073	0.40	0.6716
CULT	1	68766.852	68766.852	20.58	< 0.0001
PD	4	161062.185	40265.546	12.05	< 0.0001
PD*CULT	4	154014.575	38503.644	11.52	< 0.0001
REP(PD*CULT)	8	67380.077	3743.338	1.12	0.3569
SEASON	3	500047.473	500015.824	49.64	< 0.0001
SEASON*CULT	3	310967.198	103655.733	31.02	< 0.0001
PD*SEASON	12	551470.014	45955.835	13.75	< 0.0001
PD*SEASON*CULT	12	461959.994	38496.666	1.52	< 0.0001

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 3: The effect of plant density x season x cultivar interaction on target DNA (pg µg^{-1}) in maize grain.

	2011-2012		2012-2013		2013-2014		2014-2015	
Plants ha^{-1}	**PAN6P-110**	**CRN3505**	**PAN6P-110**	**CRN2505**	**PAN6P-110**	**CRN3505**	**PAN6P-110**	**CRN3505**
10 000	15.03^{gh}	3.45^{h}	101.48^{defg}	6.52^{gh}	127.25^{def}	626.66^{b}	68.46^{fgh}	42.10^{fgh}
20 000	3.66^{h}	2.91^{h}	4.24^{h}	12.47^{gh}	189.43^{cd}	834.16^{a}	5.96^{h}	0.70^{h}
30 000	8.55^{gh}	5.23^{h}	6.81^{gh}	6.90^{gh}	267.08^{c}	184.46^{cd}	18.27^{gh}	4.99^{h}
40 000	5.91^{h}	3.69^{h}	71.30^{fgh}	3.24^{h}	133.28^{def}	132.06^{def}	12.63^{gh}	2.48^{h}
50 000	5.50^{h}	4.93^{h}	39.25^{fgh}	24.28^{gh}	79.26^{efgh}	166.46^{de}	26.79^{gh}	58.72^{fgh}

Different letters shown indicate significant differences (P<0.05) between mean target DNA at different plant densities x season x cultivar based on Fisher's protected LSD.

Table 4: Analysis of variance of the effects of plant density (PD), cultivar and season on fumonisin production in maize grain.

Source	d.f.	s.s.	m.s.	v.r	F pr.
REP	2	0.049	0.025	0.01	<0.001
PD	4	8.562	2.141	1.09	0.389
CULT	1	0.980	0.980	0.50	0.488
PD*CULT	4	4.304	1.076	0.55	0.701
SEASON	2	26.239	13.120	11.42	<0.001
PD*SEASON	8	26.173	3.272	2.85	0.013
CULT*SEASON	2	1.050	0.525	0.46	0.637
PD*CULT*SEASON	8	7.326	0.916	0.80	0.609

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 5: The effect of plant density x season on fumonisins (ppm) in maize grain.

Season x plant density	2011-2012	2012-2013	2013-2014	2014-2015
10 000 plants ha^{-1}	1.95^{abc}	1.38^{cde}	1.26^{cdef}	0.56^{efg}
20 000 plants ha^{-1}	2.81^{a}	1.09^{cdefg}	0.45^{fg}	0.72^{efg}
30 000 plants ha^{-1}	2.77^{a}	0.41^{fg}	0.50^{efg}	1.76^{bcd}
40 000 plants ha^{-1}	2.37^{ab}	0.62^{efg}	1.10^{cdefg}	0.62^{efg}
50 000 plants ha^{-1}	0.85^{defg}	0.66^{efg}	0.67^{efg}	0.28^{g}
Mean fumonisins	2.09^{a}	0.83^{b}	0.79^{b}	0.78^{b}

Different letters shown indicate significant differences (P=<0.05) between mean target DNA at different plant densities x cultivar based on Fisher's protected LSD.

2.6.2 Separate plant density field trial with adequate soil nitrogen

After log-base-10 transformation of data, ANOVA indicated plant density as main variable to have a significant effect on target DNA in maize grain (P=0.05, Table 6). Regression analyses showed a strong relationship between plant density and target DNA quantified from grain with an increase in target DNA as plant densities increase (R^2=0.97, Figure 1).

After log-base-10 transformation of data, plant density as main variable had a significant effect on fumonisins in maize grain (P<0.05, Table 7). Regression analyses showed a strong relationship between plant density and fumonisins quantified from grain with the highest mean fumonisins of 1.3 ppm at 30 000 plants ha^{-1} (R^2=0.86, Figure 2).

Plant density as main factor had a highly significant effect on moisture percentage in grain (P<0.0001, Table 8). Regression analyses showed a strong relationship between plant density and moisture percentage with a decrease in grain moisture percentage as plant densities increase (R^2=0.98, Figure 3).

Correlation analyses to quantify the relationship between grain moisture percentage and target DNA was inversely significant (r=-0.97, Table 9). Grain moisture percentage and fumonisin had a moderate inverse correlation (r=-0.47, Table 9). The correlation between *F. verticillioides* target DNA and fumonisin was moderately correlated (r=0.43, Table 3).

Table 6: Analysis of variance of the effects of plant density (PD) and cultivar on target DNA production in maize grain.

Source	d.f.	s.s.	m.s.	v.r	F pr.
REP	2	4.0748	2.0374	5.05	< 0.001
CULT	7	2.9224	0.4175	1.04	0.413
PD	4	8.0248	2.0062	4.98	0.001
CULT*PD	28	10.9802	0.3921	0.97	0.515

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

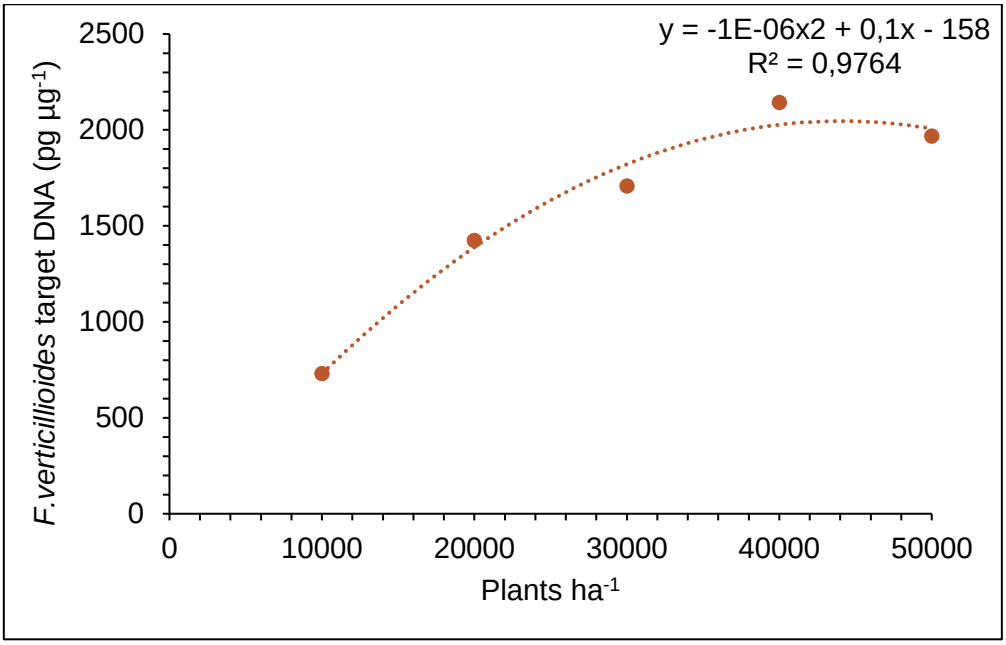

Figure 1: The effect of plant density on target DNA (pg μg^{-1}) quantified from maize grain.

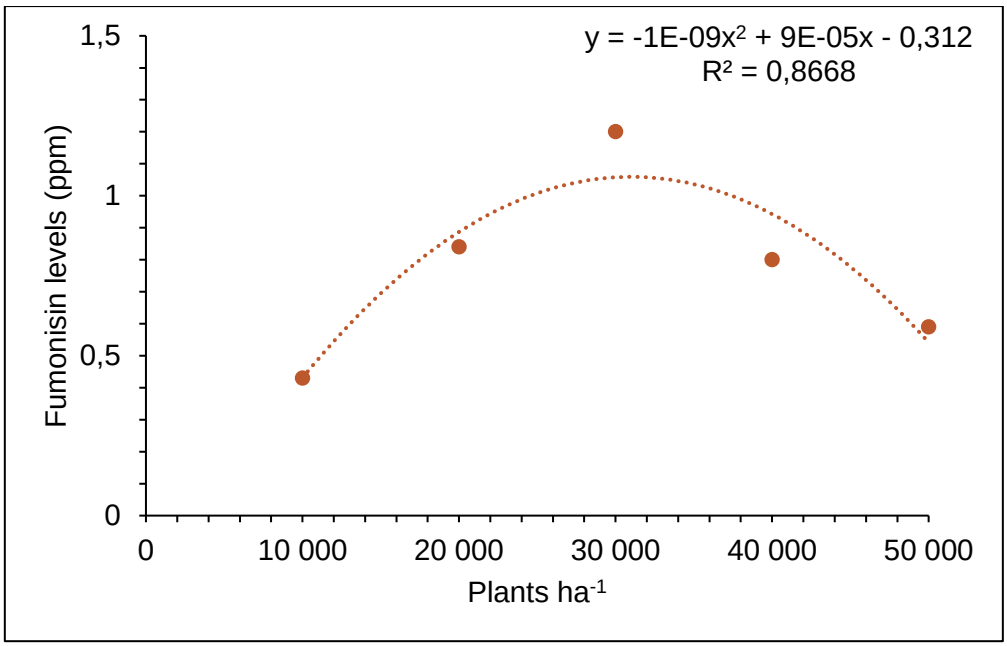

Figure 2: **The effect of plant density on fumonisins in maize grain measured in parts per million.**

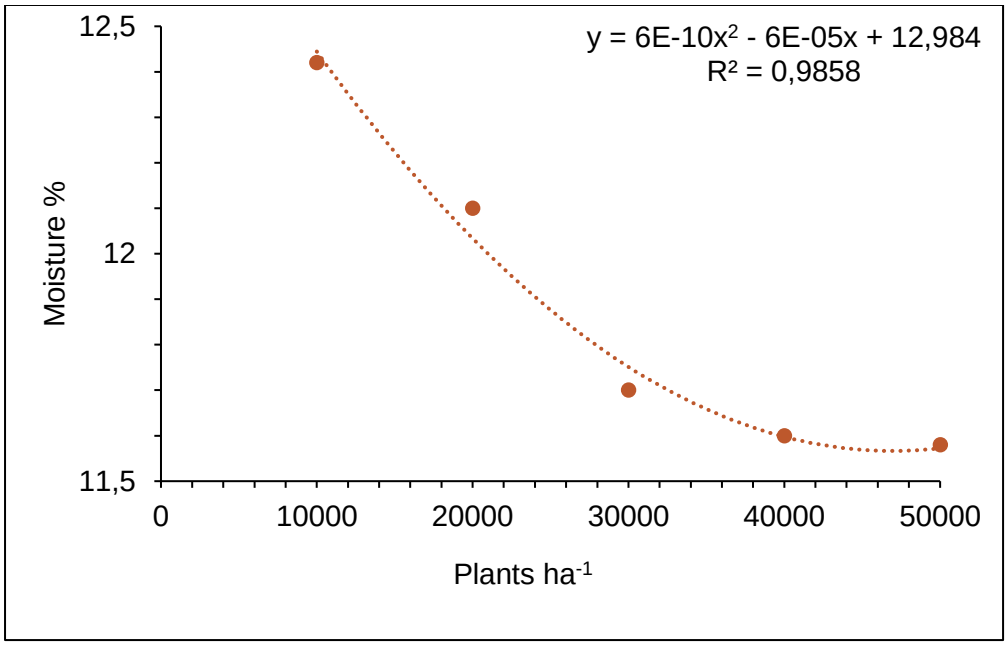

Figure 3: **The effect of plant density on moisture percentage of maize grain at harvest.**

Table 7: Analysis of variance of the effects of plant density (PD) and cultivar on fumonisin production in maize grain.

Source	d.f.	s.s.	m.s.	v.r	F pr.
REP	2	5.5039	2.7519	4.65	< 0.001
CULT	7	6.6588	0.9513	1.61	0.146
PD	4	7.3517	1.8379	3.11	0.020
CULT*PD	28	24.8241	0.8866	1.50	0.084

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 8: Analysis of variance of the effects of plant density (PD) and cultivar on moisture percentage of maize grain directly after harvest.

Source	d.f	s.s.	m.s.	v.r	F pr.
REP	2	0.956	0.478	1.65	0.1988
PD	4	12.867	3.217	11.13	<.0001
REP (PD)	8	3.332	0.416	1.44	0.1957
CULT	7	0.612	0.087	0.30	0.9503
CULT*PD	28	14.373	0.513	1.78	0.0683

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 9: Correlation analyses to show the relation between variables (Moisture percentage, target DNA and fumonisins).

	Moisture %	Target DNA	Fumonisins
Moisture %	1,00		
Target DNA	-0,97	1,00	
Fumonisins	-0,47	0,43	1,00

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

2.6.3 Separate nitrogen (type and rate) field trial

An interaction between nitrogen source x rate of application had a significant effect regarding target DNA in maize grain ($P<0.0001$, Table 10). A regression analyses indicated a strong relationship ($R^2=0.79$) between the rate of LAN application and target

DNA with the highest target DNA quantified (849 pg μg^{-1}) at 25 kg ha^{-1} LAN and the lowest at 160 pg ug^{-1} at 100 kg ha^{-1} LAN (Figure 4). There was a non-significant relationship between the rate of UREA application and target DNA (R^2=0.13, Figure 4).

Nitrogen source x rate of application had a significant effect on fumonisins (P<0.01, Table 11). Regression analysis (Figure 5) indicated a weak relationship (R^2=0.58) between the rate of LAN application and fumonisins with the highest fumonisins (0.22 ppm) quantified at 175 kg ha^{-1} and the lowest at 25 kg ha^{-1} and 150 kg ha^{-1} LAN (0.08 ppm). There was a non-significant relationship between the rate of UREA application and fumonisins DNA (R^2=0.02, Figure 5).

Table 10: Analysis of variance of the effects of nitrogen source (UREA/LAN), rate (25, 50, 100,150 and 175 kg ha^{-1}) and cultivar on target DNA in maize grain.

Source	d.f.	s.s.	m.s.	v.r	F pr.
REP	2	6971827.47	3485913.73	11.40	<.0001
SOURCE	1	28404.02	28404.02	0.09	0.7610
RATE	4	4827553.30	1206888.33	3.95	0.0046
SOURCE*RATE	4	9793216.65	2448304.16	8.01	<.0001
REP(SOURCE*RATE)	18	20860325.49	1158906.97	3.79	<.0001
CULT	7	1497148.33	213878.33	0.70	0.6726
CULT*SOURCE	7	3248334.43	464047.78	1.52	0.1662
CULT*RATE	28	8109660.27	289630.72	0.95	0.5474
CULT*SOURCE*RATE	28	6937370.00	247763.21	0.81	0.7370

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 11: Analysis of variance of the effects of nitrogen source (UREA/LAN), rate (25, 50, 100, 150 and 175 kg ha^{-1}) and cultivar on fumonisins in maize grain.

Source	df	s.s	m.s	v.r	F pr.
REP	2	0.767	0.383	7.73	0.0007
SOURCE	1	0.003	0.003	0.07	0.7991
RATE	4	0.181	0.045	0.91	0.4600
SOURCE*RATE	4	0.668	0.167	3.37	0.0115
REP(SOURCE*RATE)	18	0.863	0.048	0.97	0.5017
CULT	7	0.324	0.046	0.93	0.4835
CULT*SOURCE	7	0.620	0.089	1.78	0.0948
CULT*RATE	28	0.949	0.034	0.68	0.8807
CULT*SOURCE*RATE	28	0.694	0.025	0.50	0.9829

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

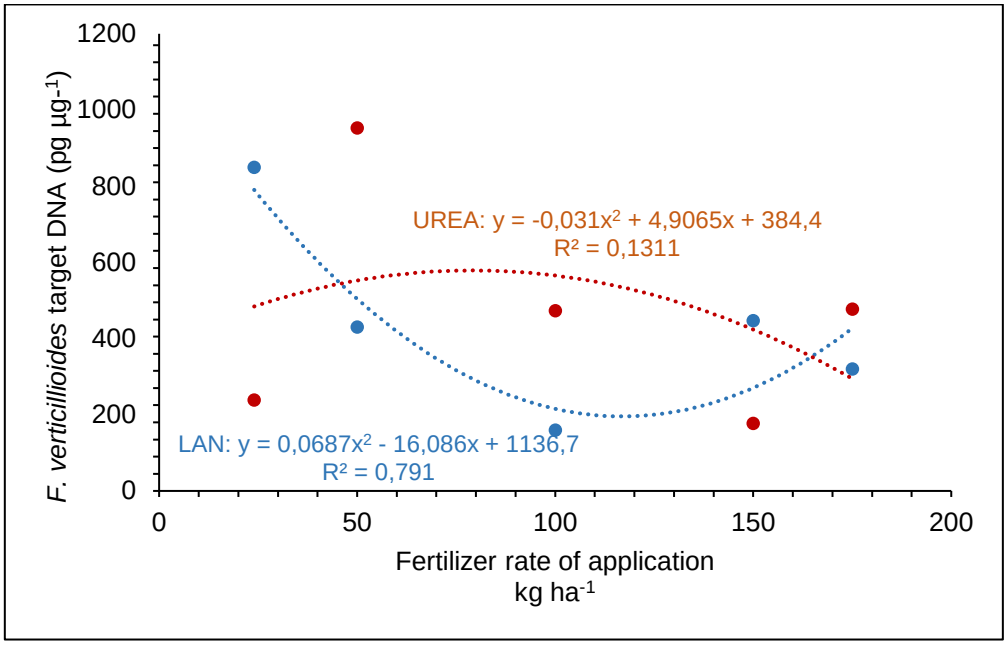

Figure 4: The effect of nitrogen source x rate of application on *F. verticillioides* target DNA (pg µg^{-1}) quantified in maize grain.

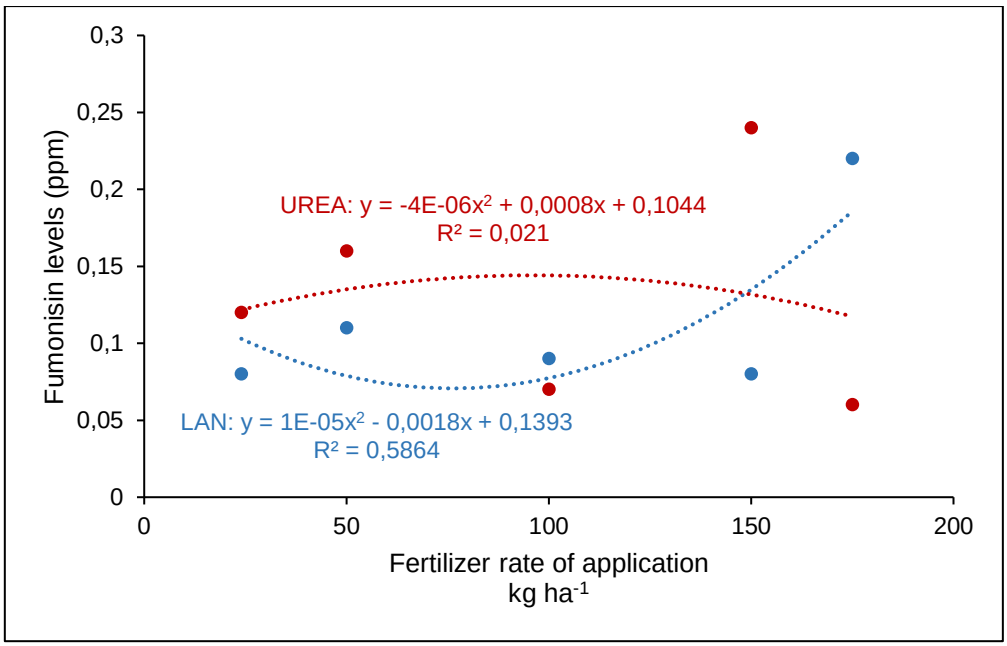

Figure 5: The effect of nitrogen source x rate of application on fumonisins (ppm) quantified in maize grain.

2.7 DISCUSSION AND CONCLUSIONS

Optimum plant densities for maximum economic yield varies with the cultivar and environment (Bruns & Abbas, 2005). It was reported that maize plant populations above the optimum, waste plant nutrients and water as water- and nutrient stress will be induced, thereby increasing their susceptibility to infection by mycotoxigenic fungi (Bruns, 2003). Alvarado-Carillo *et al*. (2010) confirmed this finding by reporting increased aflatoxin content and *Macrophomina phaseolina* (charcoal rot disease) at high maize-plant densities in Mexico. Blandino *et al*. (2008) reported that experimental field plots with higher plant populations had higher values of Fusarium kernel infection (+24%) and higher fungal ear rot severity (+43%) than plots with lower plant densities. In contradiction with these findings, Bilgrami & Choudhary (1998) reported that the natural occurrence of aflatoxin was lower in densely cultivated plants, while in other experiments, plant density had no effect on aflatoxin, zearalenone or fumonisin production in maize kernels (Rodriguez-Del-Bosque, 1996; Bata *et al*., 1997, Bruns & Abbas, 2005).

Blandino *et al*. (2004) reported an increase in fumonisin levels with an increase in N fertilization as well as in the presence of N deficiency. There is a paucity regarding information of the effect of declining nitrogen and plant densities below the optimum on *Fusarium verticillioides* infection and fumonisins in maize grain. A decline in plant

populations can be due to diseases causing seedling rot, the wrong cultivar selection (not adapted to the area) and limited access to pesticides and fertilizers (Bryceson, 2000; 2002). Furthermore, maize planting methods (seed broadcasting and planting in the plough furrow) result in poor and scattered maize stands (Du Toit & Nemadodzi, 2008). Smallholder and developing farmers often make use of mechanical planters operated by contractors. These contractors are not always aware of the correct implementation of planting practices (Du Toit & Nemadodzi, 2008), thereby putting the farmer's yield at risk due to incorrect planting depths and row spacing. In the rural areas of South Africa, most of the maize produced is used for home consumption and these people are mainly at risk to consume fumonisin contaminated maize.

Plant density trials with a gradual decline in soil nitrogen

This study showed that season x cultivar x plant densities below the optimum have an effect on target DNA, with the highest levels quantified in the 2013/2014 season. Bruns (2003), Alvarado-Carillo *et al*. (2010) and Blandino *et al*. (2008) reported that maize plant densities above the optimum, increase fungal infection and mycotoxin production and this study reports that maize plant densities below the optimum (with a decline in nitrogen) can increase fungal infection in maize grain depending on the environment (season) and cultivar. Overall, CRN3505 was more susceptible than PAN6P-110 and elevated target DNA levels in CRN3505 at the 10 000 and 20 000 plants ha^{-1} can lead to a reduction in grain quality and yield loss.

Fumonisins in maize grain was affected by plant density and season, with fumonisin levels above the allowable 2 ppm level at 20 000, 30 000 and 40 000 plants ha^{-1} in the 2011/2012 season, regardless of cultivar. Inconsistent responses between the amount of target DNA and actual fumonisin production could be due to variation in naturally occurring fungal isolates and differences in weather patterns from one season to another (Miller, 2001).

Separate plant density field trial with adequate soil nitrogen

The results of the one year study suggest that plant density influenced target DNA and fumonisins quantified from maize grain. Mean target DNA levels quantified at different plant densities ranged from 750 – 2125 pg ug^{-1} and fumonisins ranged from 0.42 – 1.3 ppm. It was reported by Doko *et al*. (1995) that inoculum of *F. verticillioides* is generally

abundant in temperate areas and therefore it is not significantly diluted by increasing plant density. In this study, experimental blocks with higher plant densities had higher fungal target DNA in maize grain. According to Munkvold (2003), higher plant populations lead to a lower airflow in the row-space with higher relative humidity and promote fungal infection and colonization of the maize ears. The substantial amount of target DNA quantified in maize grain at all plant densities in this study is of concern, as it will lead to a reduction in grain quality.

Fumonisins increased as plant densities increased, up to 30 000 plants ha^{-1} and then decreased. This is in line with studies from that reported an increase in disease severity and fumonisins at higher plant densities in three years of a four year study. The fumonisins quantified in this study however, is not of concern, as it is below the legal limit of 2 ppm (for human intake). Similar to this study, Blandino *et al*. (2008) also found no significant differences between hybrids or for the interaction between plant density and hybrid.

Contrary to Blandino *et al*. (2008) who found no significant differences on grain moisture percentage at harvest, this study showed a highly significant effect of plant density on grain moisture percentage at harvest. Furthermore, the significant inverse correlation between grain moisture percentage and *F. verticillioides* target DNA suggest that microclimate differences during the first part of grain maturation (Munkvold, 2003) may not be the only variable influencing fungal infection, but that grain moisture percentage can have an additive effect regarding increasing fungal levels and fumonisin production in maize grain at harvest. Warfield & Gilchrist (1999) reported an increase in fumonisin B_1 levels on kernels cultured at lower moisture content. They hypothesized that kernels with a lower moisture content have an increased substrate concentration conducive to fungal infection and fumonisin production and that excessive kernel moisture could inhibit fungal growth and/or fumonisin B_1 production.

This study has shown that the described plant density of 30 000 plants per ha^{-1} exhibited high target DNA and fumonisin levels in maize grain. A plant density of 10 000 plants per ha^{-1} is preferred to higher plant densities to obtain lower target DNA and fumonisin levels in maize grain, this however, will not produce desired crop yields. This study is being repeated in the 2016/2017 growing season at Bethlehem, Potchefstroom and Cedara to

further observe the effect of environment (season), cultivar and plant densities on fungal infection and fumonisin production in maize grain.

It is evident from the study that more research is needed regarding recommended plant densities to find the best compromise between yield, fungal infection and fumonisin production in maize grain. The results of this study re-iterate the importance of selecting the correct plant density according to the environment and soil type and to harvest kernels at the correct moisture percentage. In South Africa, maize is usually left in the field until a seed moisture percentage of 12.5 to 14.0 has been reached (Maize production guide, ARC-GCI). It is then harvested and delivered directly to the silo. Should drying facilities be available, it can be harvested at a higher moisture percentage. The moisture percentage of maize kernels at harvest and/or storage should be revisited to establish the possible additive effect of increasing fungal levels and fumonisin production in maize grain after harvest.

Separate nitrogen (type and rate) field trial

In order to achieve a yield of 5 tonnes with the selected cultivars in Potchefstroom, soil N must be 75 kg ha^{-1}. In this study, the starting concentration of N in the soil was 25 kg ha^{-1} and LAN and UREA as nitrogen sources were applied at 50, 100,150 and 175 kg ha^{-1}. The results of the one year study suggest that only LAN had a significant effect on target DNA and fumonisins in maize grain at different application rates. Ramirez *et al*. (1997) evaluated the effects of different plantation areas and found no effect using UREA and dibasic ammonium phosphate on fumonisin production.

The highest amount of target DNA in maize grain was quantified at 25 kg ha^{-1} (849 pg) and the lowest at 100 kg ha^{-1} LAN application. The highest amount of fumonisins (0.22 ppm) were quantified at 175 kg ha^{-1} and the lowest at 100 kg ha^{-1} (0.08 ppm). It is more likely that other variables influenced target DNA and fumonisin levels. The high amount of target DNA at 25 kg ha^{-1} in the LAN experimental blocks does not compare to that of the UREA experimental blocks when taken into consideration that 25 kg ha^{-1} was the starting concentration of N in the soil and that LAN and UREA was not added at this stage. Only trace amounts of fumonisins were quantified from this study and therefore results are inconclusive at this stage. This study is being repeated in the 2016/2017 growing season at Bethlehem, Potchefstroom and Cedara to further observe the effect of

nitrogen type and rate as well as cultivar on fungal infection and fumonisin production in maize grain.

The study showed that under nitrogen poor conditions, cultivar choice, environment and low plant densities could lead to elevated fungal infection and fumonisin production in maize grain, placing subsistence and small scale farmers at risk. In farming systems with adequate soil nitrogen, an increase in target DNA and fumonisins were observed as plant densities increase. Although information regarding nitrogen type and application rate regarding fungal infection and fumonisin production in maize grain is inconclusive at this stage, it was reported by Blandino *et al*. (2004) that a decrease of fumonisins in maize grain can be observed at increased levels of nitrogen applications. In agreement with Blandino *et al*. (2004), Hassegawa *et al*. (2008) reported a combination of 100 kg ha^{-1} N with zinc (Zn) (1.0 kg ha^{-1}) and boron (B) (0.5 kg ha^{-1}) resulted in reduced FB_1 and FB_2 levels. Blandino *et al*. (2008) emphasized the importance of a balanced N fertilizer application due to an increase in FB_1 at lower N fertilizer applications as well as at higher N applications.

The study re-iterated the importance of appropriate management practices such as obtaining environmentally adapted seed, applying fertilizers and using the correct planting methods to improve maize yields but also manage the mycotoxin threat to end users.

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CHAPTER 3

CHLOROPHYLL FLUORESCENCE, LEAF NUTRIENTS AND PATHOGENESIS RELATED PROTEINS IN *FUSARIUM VERTICILLIOIDES* INFECTED AND FUMONISIN CONTAMINATED MAIZE GRAINS UNDER NATURAL FIELD CONDITIONS.

3.1 ABSTRACT

Maize (*Zea mays* L.) is a host to numerous pathogenic species that can cause root, stalk, leaf and ear rots, which can have a negative impact on grain quality and yield. Furthermore, an economically important fungal pathogen, *Fusarium verticillioides,* can *produce* secondary metabolites called fumonisins in maize grain. *F. verticillioides* can infect maize plants systemically, through insect channels and through silks. It can also be an endophyte, present in asymptomatic maize tissue. Integrated management practices need to be applied to limit fungal infection and fumonisin production in maize grain. It was reported that substances or conditions that impose stress on the fungus also have an influence on mycotoxin biosynthesis and therefore our research focus is on the effect that plant stressors have on *F. verticillioides* infection and fungal biomass and fumonisin synthesis. The aim of the study was to 1) assess chlorophyll fluorescence measurements as an indication of photosynthetic performance to indicate stress due to plant density, 2) to elucidate the role of available leaf nitrogen (N), carbon (C) and suphur (S) on fungal infection and fumonisin production in maize grain and 3) to elucidate the role of PR proteins (chitinase, peroxidase and β-1, 3- glucanase) on fungal infection and fumonisin production in maize grain. Field trials were planted using two cultivars (CRN3505 and PAN6P-110) in a randomised complete block design, comprising of five different plant density treatments (10 000, 20 000, 30 000, 40 000 and 50 000 plants ha^{-1}) during 2011/2012 and 2012/2013. Chlorophyll fluorescence parameters were measured at different days after plant (DAP) to determine plant vitality at different plant densities. Leaf material from experimental plots were sampled and analysed by the Eco-Analytica Laboratory of the North-West University for total nitrogen (N), carbon (C) and sulphur (S). Three pathogenesis-related proteins, chitinase, peroxidase and β-1,3-glucanase were measured at different stages of plant development to determine their role during fungal

infection and fumonisin production. Results in this study indicated that photosynthetic performance (PI_{abs}) can be used to measure plant stress due to plant density, although the sampling stage (DAP) is of importance. The best photosynthetic performance was observed at 30 000 plants ha^{-1}. This was the recommended plant density for the cultivar and environment and therefore used as control in this study. In the 2012 season, in increase in plant density significantly decreased N and C but S increased and was significantly higher at 50 000 plants ha^{-1}. In the 2013 season, only DAP had variable effects on N and C %. Plant density had a significant effect on S %, although results were variable. A moderate inverse correlation (P=0.01, r=-0.46) was observed between fumonisins and N % in maize leaves during the 2013 season at 70 DAP. Similarly, a moderate inverse correlation (P=0.01, r=-0.46) was observed between fumonisins and C % in maize leaves during 2012 season at 70 DAP. Moderate inverse correlations (P=0.01, r-0.45; P=0.008, r=-0.51 and P=0.03, r=-0.42 respectively) were observed between fumonisins and S % in maize leaves during 2012 season at 70, 95 and 123 DAP. A weak correlation (P=0.05, r=0.36) was observed between target DNA and S % in the 2013 season. DAP had a highly significant effect on total proteins with a decrease in total proteins as plants matured. Chitinase activity was significantly higher at 95 DAP (2012) and 57 as well as 95 DAP (2013). Peroxidase activity levels was not affected by DAP, plant density or cultivar. A weak inverse correlation (P=0.04, r-0.37 and P=0.04, r=-0.38 respectively) was observed between fumonisin levels and chitinase activity in the 2013 season. A moderate correlation was observed between fumonisin levels and peroxidase activity at 83 DAP (P=0.04, r=0.40) in the 2013 season. A weak inverse correlation was observed at 97 DAP (P=0.04, r=-0.38) between fumonisin levels and β-1,3-glucanase activity. This study show an increase in the available leaf nutrients (N, S and C) as well as PR proteins (chitinase and β-1,3-glucanase) to correlate with a decrease in fumonisin levels. The increase of PR proteins during critical infection stages of the maize plant (silk and milk) is a significant finding, as maize ears are susceptible to fungal infection and fumonisin production can occur as soon as fungal infection commenced. It was unexpected though, that available leaf nutrients as well as PR proteins did not impact fungal infection, but fumonisin levels. Currently, an integrated approach is taken to manage fungal infection and subsequent fumonisin production in maize grain. Even though fungal infection can be managed, fumonisin production can be unpredictable due to genotype, environment and substrate. Chitinase and β-1,3-glucanase response to

fumonisins in this study can be used in breeding programmes to improve resistance to specifically fumonisin production in maize grain. This study contribute to a better understanding of maize plant defence mechanisms and the aspects of maize physiological processes and nutritional values can effectively contribute to improved management strategies of *F. verticillioides* fungal infection and contamination by fumonisins.

3.2 INTRODUCTION

Maize is an important nutrient source to humans and animals and is mainly used as a staple diet for millions of people, especially in Africa. In spite of transgenic crop technologies that have evolved over the years, maize remains susceptible to *Fusarium* spp. infection and colonization. *F. verticillioides* is a prevalent soil borne and air borne pathogenic fungus of maize that can cause maize seedling blight, root rot, stalk rot, kernel rot, ear rot, or systemic infection of maize (Danielsen & Jensen, 1998; Munkvold, 2003).

Research shows that two-thirds of maize loss is caused by *F. verticillioides* (Rifat *et al.,* 2013) and that it can lead to a reduction in the quality of maize grain as well as contamination of maize grain with fumonisins, thereby posing a threat to the economy and food supply (Pechanova & Pechan, 2015). Primary *F. verticillioides* inoculum can colonize plant debris (Cotton & Munkvold, 1998) and infect maize in the new growth season when environmental conditions such as temperature and moisture are favourable (Cotton & Munkvold, 1998). *F. verticillioides* infection can grow systemically within a maize plant, moving from the roots to the stalk and finally to the maize ear and kernels. Although systemic infection may result from inoculum that survives in maize residues in the soil, the relative importance of soil and seed borne inoculum as the cause of systemic infections is not clear (Oren *et al.,* 2002). Another infection pathway is through airborne conidia (micro- and macroconidia, abundant in maize fields during a growing season) that land on silks channels (Munkvold & Desjardins, 1997) and progress with silks into the ear, thereby infecting and colonizing maize grain. Janse van Rensburg, *et al*. (2012) suggested two phases in the development of fumonisins in maize kernels i.e. colonisation of maize tissues during the early post-silking stage followed by fumonisin production during the dough stage of grain fill.

Furthermore, studies have shown that the incidence of the infection can depend on the environmental conditions and the agronomic practices (Santiago *et al.,* 2015). Appropriate application of suitable agronomic practices such as planting and harvest dates, irrigation regimes, nitrogen management, and plant density (Ariño *et al.,* 2009; Abbas *et al.,* 2012) can be a tool for modulating the effect of environmental conditions that are conducive to the infection and fumonisin contamination (Blandino *et al.,* 2009). Plant density has been reported as an important agronomic factor that influence the micro-environment of the maize plant which affect the growth and yield formation (Rahman & Hossain, 2011). However, plant densities varies depending on the maize genotype, soil fertility and planting patterns (Sangoi, 2000). At some instances, maize is sensitive to variations in plant densities. At low plant densities, it can produce low yields and on the other hand, high plant densities may increase plant shading, which causes a decline in photosynthesis, and greatly enhance interplant competition for nutrients and water (Marchiori *et al.,* 2014). Photosynthesis has been explored as a guide of adverse conditions that can affect the photosynthetic efficiency of the plants (Perrault *et al.,* 2011). Photosynthesis can however be indirectly measured through the chlorophyll *a* fluorescence (Strasser *et al.,* 2000). Chlorophyll *a* fluorescence is a non-invasive way that monitors the activity of photosystem II, at the levels of photon absorption, excitation trapping and electron transport (Berger *et al.,* 2007). The assessment of chlorophyll *a* fluorescence is a quantitative measure of both photochemical and non-photochemical energy dissipation processes occurring in the leaves (Rohácek, 2002; Kramer *et al.,* 2004). The parameters are considered an important approach to evaluate the health of the photosynthetic apparatus within a leaf, and provides a rapid and accurate technique for detecting and quantifying the interaction among and tolerance of plants to stress (Maxwell & Johnson, 2000; Lichtenthaler *et al.,* 2005). Santos *et al*. (2006) reported that the photosynthetic performance of a plant reduces under adverse conditions therefore, in the study chlorophyll *a* fluorescence measurements taken from maize leaves were used to possibly quantify stress caused by plant density.

The adequate supply of micro- and macro-nutrients can protect maize against fungal infection and mycotoxin contamination (Hassegawa *et al*., 2008). Maize plants exposed to fertility stress are more susceptible to infection by microorganisms than plants not under fertility stress (Blandino *et al*., 2008). Nitrogen is one of the most important macronutrients in maize production and excess nitrogen can cause prolonged vegetative

growth of maize plants (Aulakh *et al.,* 2000). Fungal infection and ear rot colonization can be favoured by these conditions that can impose high moisture levels and high relative humidity from the silking period to the maturation stage (Reid *et al.,* 1999; Logrieco *et al.,* 2002. Carbon (C) is derived from the atmosphere and soil water. Nitrogen (N) is biologically combined with C, H, O and S to create amino acids which are the building blocks for proteins in grain crops (Hani *et al.,* 2006). Phosphorus (P) plays a major role in energy storage and transfer as ADP and ATP and been shown to reduce disease incidence in some plants and has been found to improve the quality of certain crops (van Averbeke & Yoganathan, 2003). Potassium (K) has been shown to improve disease resistance in maize, improve the size of grains and seeds, and improve the quality of fruits and vegetables. Potassium is also vital for plant growth because potassium is known to be an enzyme activator that promotes metabolism (Tucker, 1999). Sulphur (S) is essential in forming plant proteins because it is a constituent of certain amino acids. It also aids in seed production, chlorophyll formation, nodule formation in legumes, and stabilizing protein structure (Agrios, 2010).

Incorporating plant residues into agricultural soils also enhances organic carbon content and increases nutrient availability for plant uptake in the future (Cayuela *et al.,* 2009). However, it is more difficult to predict how much nutrient will become available at a specific time due to the complex nature by which the nutrients in the residues are recycled in the soil (Schoenau & Campbell, 1996). Soil microorganisms like fungi plays a significant role to the soil habitat and aids with cycling of nutrients and the decomposition of organic matter (Bridge & Spooner, 2001; Newton *et al*., 2003). Certain fungi are known to be antagonistic to other plant pathogens by competing with these pathogens to decompose plant debris in the soil. Some of these pathogens may survive and supply the plant with nutrients to stimulate growth and reduce stress (Newton *et al*., 2003). However, many pathogenic fungi available in the soil are reservoirs of inoculum that infect living plants or they may survive on dead plant residues whereby they infect and invade host plant tissue (Sabatini & Innocenti, 1999).

For years, researchers have been investigating the essential processes of the maize recognition to pathogen and the induction of effective defence mechanisms. By involving the most advanced system biology approaches, that include disciplines, such as proteomics, they have moved towards a better understanding of the many processes

occurring during maize interactions with its pathogens (Mehta *et al.,* 2008). A thorough understanding of how plants and pathogens recognize each other and differentiate to establish either a successful or an unsuccessful relationship is crucial in this field of investigation and for further explanation of a genetic basis of host resistance (Pechanova & Pechan, 2015). The ability of the plant to recognise the presence of a pathogen is the first step that leads to the activation of the plant defence response, which triggers a nonspecific mechanism consisting of plant cell wall thickening, signal transduction and transcription of defence related genes such as pathogenic related (PR) proteins (Alfano & Collmer, 2004). PR proteins have previously been reported to be involved in antifungal activity, energy metabolism, photosynthesis, protein folding and degradation indicating a common pathway for both stress and non-stress plant functions (Mehta *et al.,* 2008). PR proteins accumulate at primary infection sites as well as in tissues showing systemic acquire resistance (SAR) and prevent infection of plant cells (Stintzi *et al.*, 1993). Some of these PR proteins include β-1,3-glucanase, chitinase and peroxidise (Sels *et al*., 2008). β-1,3-glucanase are induced following viral, microbial or fungal infection. The major function of β-1,3-glucanase is to hydrolyze β-1,3-glucans that composes the cell walls of pathogens (Beffa *et al*., 1996). Chitinases are digestive enzymes that hydrolyse glycosidic bonds in chitin that composes the cell walls of fungi. Chitinases are normally expressed at low levels in plants, but are dramatically enhanced when exposed to biotic and abiotic stresses (Punja & Zhang, 1993). Peroxidases increase in plants in response to a great variety of stresses including viral, microbial and fungal infections. Peroxidases are enzymes able to oxidise different substrates in the presence of hydrogen peroxide (H_2O_2) (Passardi *et al.,* 2004). Peng & Kuc (1992) showed that H_2O_2 inhibits the germination of spores of a variety of fungal pathogens. Peroxidases limit cellular spreading of the infection through the generation of highly toxic environments by producing reactive oxygen species (ROS) (Passardi *et al.,* 2005; van Loon *et al.,* 2006). During stress including pathogenic attack, peroxidases are the first to alter their activity (Christensen *et al.*, 1998). The quantification of PR proteins can provide us with insight on defence mechanisms in cultivars that display susceptibility/resistance to fungal infection and colonization as well as fumonisin production. Genes responsible for the expression of pathogenesis related proteins have been given much attention and has been significantly improved to pose resistance against various pathogens in maize (Lambais *&* Mehdy, 1998; Petruzzelli *et al*., 1999).

This study the aimed to 1) investigate if chlorophyll fluorescence as measurement of photosynthetic efficiency can be used as an indicator of maize plant stress in the field due to plant density, 2) determine if available leaf nutrients affect *F. verticillioides* infection and fumonisin contamination of maize grain and 3) to quantify the activity of three pathogenesis-related proteins during fungal colonization and fumonisin contamination under field conditions.

3.3 MATERIALS AND METHODS

3.3.1 Chlorophyll *a* fluorescence kinetics

Chlorophyll *a* fluorescence was measured at 39, 70, 95 and 123 days after plant (DAP), which corresponds to 8 leaf, silk, milk and soft dough stages in the 2012 season. Senescence of plant leaves at soft dough stage (123 DAP) in the 2012 season, restricted chlorophyll *a* fluorescence measurements and therefore sampling dates were adjusted for the 2013 season. Sampling dates comprised of 42, 57, 70, 83 and 97 DAP, which correspond to the 8 leaf, 12 leaf, 2 silk stages, and milk stage (DAP may vary with ± 3 days from that of 2012, due to environmental conditions). The unfolded upper leaf was cut from the collar and placed in a brown paper bag and then into a cooler box. Prior to measurements, leaves were kept in the dark for 60 minutes, a time sufficient for all reaction centers of PSII to become open and chlorophyll migration is stagnant (Zouni *et al.,* 2001). At this stage, the electron acceptor side of PSII is in the oxidized state, as there is no electron flow in the photosynthetic electron transport chain and water oxidation by PS II. To ensure maximum fluorescence emissions (F_m), a pulse of saturating light at an intensity of 3200 mmol m^{-2} s^{-1} (650 nm peak wavelength) was applied on the leaf for 1 s, using fluorescence levels t= 50ms, t= 100ms, t= 300ms, t= 2ms, t= 30ms immediately after the dark adaption. Chlorophyll *a* fluorescence measurements was taken with a PEA fluorimeter (Hansatech instruments Ltd., King's Lynn, Norfolk, United Kingdom) using the method of Strasser *et al.* (2000).

To investigate the PSII behaviour, vitality and efficiency of photosynthesis, performance indexes (PI_{abs} and $PI_{abs,\ total}$) and PI_{total} was calculated. The PI index reflects the functionality of PSI and PSII and is a powerful tool to obtain quantitative information on the state of a plant's performance when experiencing stress. The PI_{total} is considered as a good indication of a plant's overall vitality (Strasser *et al.*, 2000).

3.3.2 Quantification of nutrient content (nitrogen, carbon and sulphur) in maize leaves

Approximately 15 - 30 cm of leaf material was collected randomly from each plot at 39, 70, 95 and 123 DAP, in the 2012 season and at 42 DAP, 57, 70, 83, 97 DAP in the 2013 season. Only the fully developed upper leaf blade was cut from each plant and placed inside a size 12 labeled brown paper bag. Samples were dried in a forced-air oven at 60°C for 24 hours and was ground with a pestel and motar to pass through a 0.5 mm sieve. About 2 g of leaf material was analysed by the North-West University, Eco-Analytica Laboratory (Potchefstroom campus, South Africa) for total carbon, nitrogen and sulphur content using the combustion method (Campbell, 1992). This method detects low levels of nitrogen, carbon and sulphur (Campbell, 1992) using a CN analyzer (Truspec CN determinator, LECO Cooperation, St. Joseph, Michigan, USA).

3.3.3 Determination of protein content and enzyme activity

Total protein content was quantified to determine chitinase, β-1, 3-glucanase and peroxidase activities in maize. Leaf material was collected in the 2012 and 2013 season at the DAP explained above. Leaf material were removed from the fully unfolded upper leaves of plants, taking care not to sample from plants where leaf samples were taken for chlorophyll *a* fluorescence quantifications, as there could have already be a PR protein response in these plants due to wounding of the leaves. Leave samples were placed in 2 mL cryovial tubes and immediately these tubes were immediately immersed in liquid nitrogen (N_2) in order to stop deterioration of proteins in the leaves. Samples were stored at -70°C until further analyses. Total proteins was extracted by using a stock solution of 10 mM phosphate buffer (pH=8.5) containing 12.14 g of trizma base dissolved in 1 L of distilled water and the pH adjusted to 8.5. Before the extraction, 0.045 g Ethylenediaminetetraacetic acid (EDTA) and 6 mL of 10% glycerol was added to 60 mL of the phosphate buffer. The frozen leaf material was weighed and grinded with 5 mL extraction buffer and 0.1 g of acid purified sand and 0.25 g of Poly-vinylpolypyrrolidone (Sigma-aldrich, USA) in a chilled pestle and mortar. The material was transferred into 50 mL centrifuge tubes and centrifuged at 15 000 rpm at 4 ºC for 15 minutes. The supernatant was kept at -20°C in 2 mL tubes for analyses.

3.3.3.1 Total Protein quantification

Total proteins were quantified using the Bio-Rad microplate reader and the assay was performed according to Bradford, 1976. Bovine Serum Albumin (BSA) - Fraction V lyophilized powder (0.5 mg mL^{-1}) from Sigma-Aldrich, was dissolved in 50 µL distilled water to make aliquots of standards. The standard wells of each plate contained 10 µL BSA, 150 µL distilled H_2O and 40 µL of Bio-Rad dye. The blank wells contained 160 µL distilled H_2O and 40 µL Bio-Rad dye. The assay mixture contained 10 µL of protein extract, 150 µL distilled H_2O and 40 µL Bio-Rad dye. Each sample was incubated at room temperature for 5-10 minutes. The absorbance of each sample was measured at 595 nm using an ELX 800 universal microplate reader (Bio-Tek instruments, USA). Individual samples were replicated five times.

3.3.3.2 Chitinase activity

Chitinase activity was determined according to the method of Wirth & Wolf (1990) with minor modifications. Na-acetate buffer (66 mM, pH=0.5) was prepared by dissolving 0.546 g in 100 mL distilled water. Protein extracts were diluted 80 times and replicated three times. The reaction mixture consisted of 60 µL sample, 840 µL 66 mM Na-acetate and 300 µL CM-chitin-RBV. Blank samples contained 60 µL distilled water, 840 µL 66 mM Na- acetate and 300 µL CM-Chitin-RBV. These mixtures were incubated at 37ºC for 30 minutes. To stop the reaction, 300 µL of 2N HCl was added to the sample mixtures. The mixture was cooled on ice for total precipitation and centrifuged at 14 000 rpm for 7 minutes. Samples were then diluted 4 times with distilled water before being measured with a photometric programme (spectrophotometer-uv probe version 2.43, Shimadzu, Thermo Scientific, USA) at an absorbance wavelength of 550 nm. Chitinase activity was expressed as $nm.mg^{-1}$ protein.

3.3.3.3 Peroxidase activity

Peroxidase activity was determined according to the method of Zieslin & Ben-Zaken (1991). A mass of 0.0146 g EDTA was added to 20 mL potassium buffer (40 mM) consisting of 3.48 g K_2HPO_4 and 2.72g KH_2PO_4 dissolved in 500 mL H_2O (pH =5.5) to make up the extraction buffer. The reaction mixture contained 500 µL extraction buffer, 340 µL distilled H_2O, 100 µL guaiacol (5 Mm), 50 µL hydrogen peroxide (H_2O_2, 8.2 mM) and 10 µL protein extract. The blank contained 500 µL extraction buffer, 340 µL distilled

H_2O, 100 µL guaiacol (5 Mm) and 50 µL hydrogen peroxide (H_2O_2, 8.2 mM). The absorbance was read at 470 mm using a kinetic programme (spectrophotometer-uv probe version 2.43, Shimadzu, Thermo Scientific, USA) at 30°C for 200 seconds. Each sample was replicated three times.

3.3.3.4 β-1, 3-glucanase activity

β-1, 3-glucanase activity was determined using the method of Nelson, 1944 & Somogyi, 1952. A reaction mixture made up of 15 µL protein extract, 250 µL Laminarin (2 mg mL^{-1}) and 235 µL of 15 mM Na-acetate buffer was incubated in test tubes at 37°C for 10 minutes. A volume of 500 µL somogyi reagent containing 0.4 g copper sulphate ($CuSO_4$), 18 g sodium sulphate (Na_2SO_4), 2.4 g sodium carbonate (Na_2CO_3), 1.6 g sodium hydrogen carbonate (Na_2HCO_3) and 1.2 g potassium sodium tartrate tetrahydrate ($KNaC_4H_4O_6.4H_2O$) was added to the reaction mixture to measure the reducing sugars. The solution was incubated for 10 minutes in water at 100 °C. After cooling over tap water, 500 µL Nelson's reagent 5.3 g ammonium molybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}.4H_2O$), 4 mL sulphuric acid (H_2SO_4) and sodium arsenate dibasic heptahydrate ($NaHAsO_4.7H_2O$) was added and the absorbance was measured at a wavelength (λ) of 540 nm using a photometric programme (spectrophotometer-uv probe version 2.43, Shimadzu, Thermo Scientific, USA). The β-1,3-glucanase activity was calculated using the standard curve of glucose (2, 4, 6, 8 and 10 mg. mL^{-1}, series).

3.4 Data analysis

Data was analysed by the Biometry unit of the Agricultural Research Council in Pretoria, South Africa. All data was subjected to analysis of variance (ANOVA), and the means were compared with the least significant difference test with GenStat (2015) 18th edition software. The residuals were tested for deviation from normality. In cases where deviation was significant (skew), data was log transformed. Fisher's protected LSD (Least Significant Difference) was calculated at a 5% significance level to compare means of treatment effects (Montgomery, 1984).

Pearson's correlations were used to establish possible correlations between:

- Chlorophyll *a* fluorescence and *F. verticillioides* target DNA in maize grain
- Chlorophyll *a* fluorescence and fumonisins in maize grain
- PR- proteins and *F. verticillioides* target DNA in maize grain
- PR- proteins and fumonisins in maize grain
- Plant leaf nutrients and *F. verticillioides* target DNA in maize grain
- Plant leaf nutrients and fumonisins in maize grain

F. verticillioides target DNA and fumonisin data from the 2012 and 2013 seasons in Chapter 2, were separately correlated to chlorophyll *a* fluorescence, PR protein and plant leaf nutrient data in this chapter. Janse van Rensburg, *et al*. (2012) suggested two phases in the development of fumonisins in maize kernels i.e. colonisation of maize tissues during the early post-silking stage followed by fumonisin production during the dough stage of grain fill therefore data corresponding to 70, 95 and 123 DAP during 2012 and at 70, 83, 97 DAP during 2013 was used to determine possible correlations.

3.5 RESULTS

3.5.1 Chlorophyll a fluorescence measurements in the 2012 and 2013 seasons respectively.

3.5.1.1 *Photosynthetic index (PI_{abs}) of maize plants*

Plant density and DAP as main variables had a highly significant effect on the photosynthetic efficiency of the maize plant during the 2012 season ($P<0.001$ and $P<0.001$, Table 1). The photosynthetic efficiency significantly decreased as plant densities increased, with the highest value at 10 000 plants ha^{-1} (18.5) and the lowest value at 50 000 plants ha^{-1} (13.45, Figure, 1). There were significant differences observed between DAP with the higher PI_{abs} measurement noticed at 70 and 123 DAP and significantly lower PI_{abs} at 39 and 95 DAP (Figure 2). DAP x cultivar interaction significantly affected the PI_{abs} in maize leaves, with significantly higher PI_{abs} at 70 and 123 DAP in both PAN6P-110 and CR3505 (Table 2).

During the 2013 season, Plant density x DAP x cultivar interaction significantly influenced the photosynthetic performance of maize plants ($P=0.01$, Table 3). Although there were

significant interactions between variables, cultivars PAN6P-110 and CRN3505 had a significantly lower PI_{abs} mean values at 97 DAP at all the plant density treatments. However, the highest PI_{abs} mean value in regards to plant densities, could be observed at 30 000 plants ha^{-1}, respectively (Table 4).

Table 1: ANOVA table indicating main effects and interactions regarding the effect of different plant densities, DAP and cultivar on the photosynthetic performance (PI_{abs}) of maize plants during the 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	1.63	0.81	0.08	
CULT	1	2.63	2.63	0.24	0.622
PD	4	578.15	144.54	13.40	<.001
PD*CULT	4	17.11	4.28	0.40	0.811
DAP	4	213.88	53.47	4.96	0.001
DAP*CULT	4	114.66	28.66	2.66	0.037
PD*DAP	16	246.17	15.39	1.43	0.145
PD*DAP*CULT	16	200.05	12.50	1.16	0.314

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

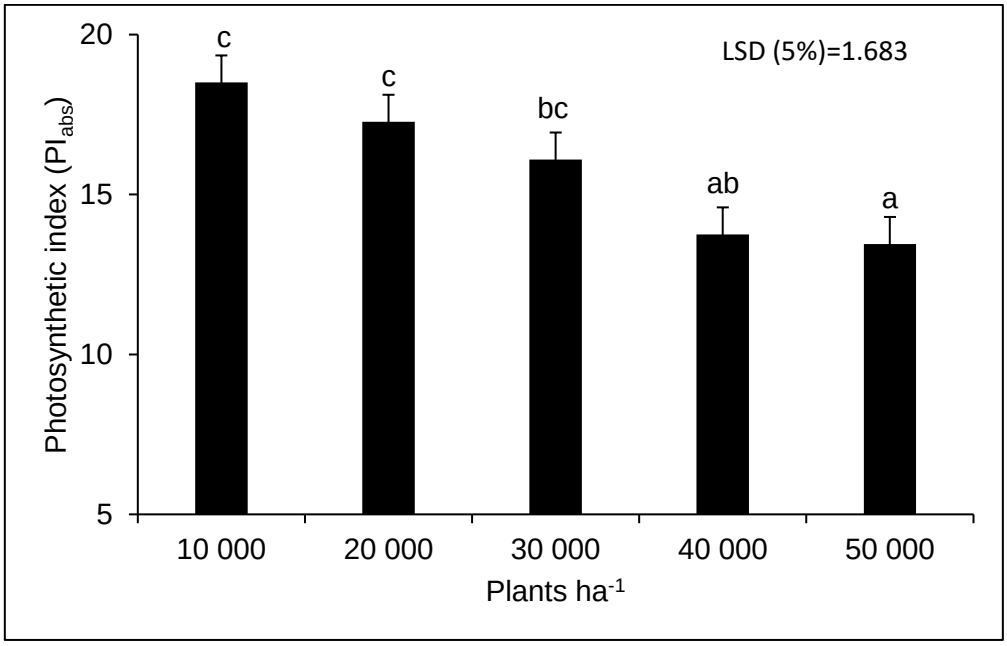

Figure 1: The effect of plant density on photosynthetic performance of maize during the 2012 season.

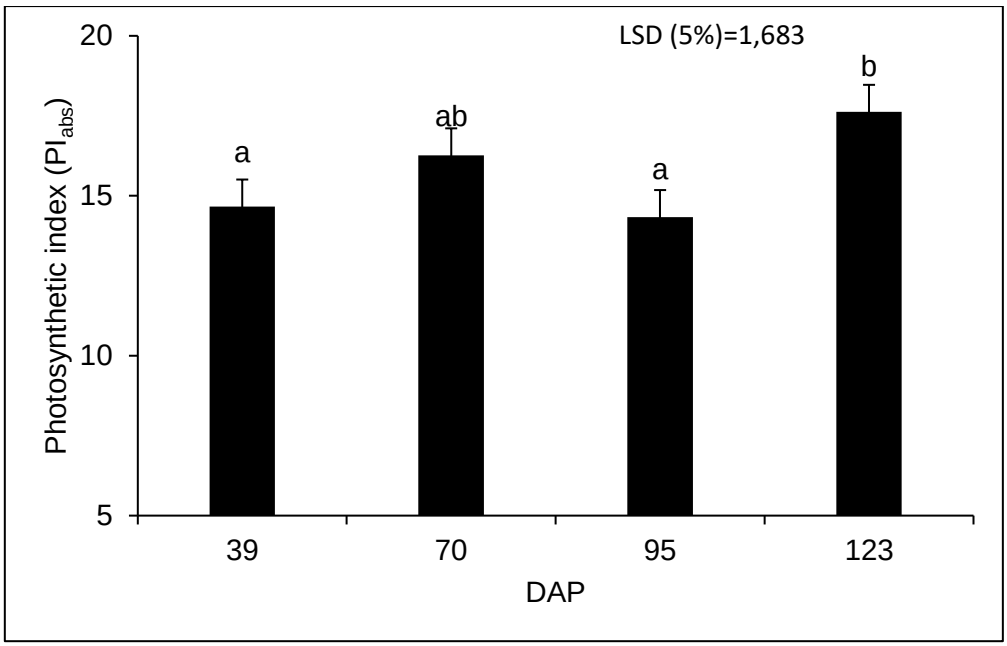

Figure 2: The effect of DAP on photosynthetic performance of maize during the 2012 season.

Table 2: The photosynthetic performance (PI_{abs}) measurements of maize cultivars PAN6P-110 and CRN3505 at DAP during the 2012 season.

DAP	PAN6P-110	CRN3505
39	15.77 ^{ab}	13.55 ^{a}
70	16.91 ^{ab}	15.61 ^{ab}
95	13.95 ^{a}	14.71 ^{ab}
123	16.69 ^{ab}	18.54 ^{b}

Different letters shown indicate significant differences (P=<0.05) between mean PI_{abs} of PAN6P-110 and CRN3505 based on Fisher's protected LSD.

Table 3: ANOVA table indicating main effects and interactions regarding the effect of different plant densities, DAP and cultivar on the photosynthetic performance (PI_{abs}) of maize plants during the 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	5.367	2.683	0.33	
CULT	1	53.077	53.077	6.60	0.012
PD	4	380.027	95.007	11.81	<.001
PD*CULT	4	33.130	8.282	1.03	0.396
DAP	4	2298.024	574.506	71.44	<.001
DAP*CULT	4	142.260	35.565	4.42	0.003
PD*DAP	16	138.217	8.639	1.07	0.390
PD*DAP*CULT	15	254.904	16.994	2.11	0.015

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 4: The effect of plant density, DAP and cultivar on photosynthetic performance (PI_{abs}) measurements of maize plants during the 2013 season.

DAP	***42***		***57***		***70***		***83***		***97***	
Plant ha^{-1}	**PAN6P-110**	**CRN3505**	**PAN6P-110**	**CRN3505**	**PAN6P-110**	**CRN3505**	**PAN6P-110**	**CRN3505**	**PAN6P-110**	**CRN3505**
10 000	17.1^{efgh}	2.7^{a}	14.4^{bcdefgh}	13.7^{bcdefgh}	13.9^{bcdefgh}	13.1^{bcdefgh}	14.8^{cdefgh}	16.6^{defgh}	4.3^{ab}	4.4^{ab}
20 000	16.7^{defgh}	12.9^{bcdefgh}	14.2^{bcdefgh}	16.1^{cdefgh}	14.9^{cdefgh}	14.5^{cdefgh}	14.5^{cdefgh}	16.5^{defgh}	6.8^{abcd}	7.3^{abcde}
30 000	16.8^{defgh}	15.9^{cdefgh}	19.0^{h}	13.5^{bdefgh}	16.2^{defgh}	16.9^{efgh}	19.9^{h}	20.1^{h}	7.4^{abde}	6.1^{abc}
40 000	15.7^{cdefgh}	13.0^{cdefgh}	17.3^{efg}	18.3^{fg}	18.8^{gh}	18.8^{gh}	19.8^{h}	15.6^{cdefgh}	7.5^{abcde}	8.5^{abcdef}
50 000	16.1^{defgh}	13.4^{bcdefgh}	18.9^{gh}	16.4^{defgh}	20.5^{h}	18.7^{gh}	18.9^{gh}	19.9^{h}	8.9^{abcdeg}	8.9^{abcdefg}
Mean	**16.48**	**11.58**	**16.76**	**15.60**	**16.86**	**16.40**	**17.58**	**17.74**	**6.98**	**7.04**

Different letters shown indicate significant differences (P=<0.05) between mean PI_{abs} at different plant density x cultivar x DAP based on Fisher's protected LSD.

3.5.1.2 *The total Photosynthetic performance ($PI_{abs, total}$) of maize plants*

There was a highly significant interaction regarding DAP and cultivar for the 2012 season (P<0.001, Table 5). A significantly higher level of $PI_{abs, total}$ at 39 DAP in PAN6P-110 (Table 6).

During the 2013 season, only DAP had a highly significant influence on the $PI_{abs,\ total}$ of maize plants (P<0.001, Table 7). A significant decrease of $PI_{abs,\ total}$ was recorded as maize plants matured, with the highest and lowest value observed at 42 and 97 DAP respectively (0.72 and 2.67, Figure 3).

Table 5: ANOVA table indicating main effects and interactions regarding the effect of plant densities, DAP and cultivar on the photosynthetic performance ($PI_{abs,\ total}$) of maize plants during the 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	3.954	1.977	0.25	
CULT	1	38.628	38.628	4.95	0.028
PD	4	4.639	1.160	0.15	0.963
PD*CULT	4	0.605	0.151	0.02	0.999
DAP	4	139.083	34.771	4.46	0.002
DAP*CULT	4	177.186	44.297	5.68	<.001
PD*DAP	16	3.140	0.196	0.03	1.000
PD*DAP*CULT	16	0.968	0.061	0.01	1.000

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 6: The photosynthetic performance ($PI_{abs,\ total}$) of maize cultivars PAN6P-110 and CRN3505 at different DAP during the 2012 season.

DAP	PAN6P-110	CRN3505
39	6.664 [b]	1.306 [a]
70	1.676 [a]	1.619 [a]
95	1.388[a]	1.386 [a]
123	1.641 [a]	1.819 [a]

Different letters shown indicate significant differences (P=<0.05) between mean $PI_{abs,\ total}$ of PAN6P-110 and CRN3505 based on Fisher's protected LSD.

Table 7: ANOVA table indicating main effects and interactions regarding the effect of different plant densities, DAP and cultivar on the photosynthetic performance ($PI_{abs, total}$) of maize plants during the 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	0.5663	0.2831	1.31	
CULT	1	0.0109	0.0109	0.05	0.823
PD	4	1.8518	0.4630	2.14	0.082
PD*CULT	4	0.2933	0.0733	0.34	0.851
DAP	4	72.6628	18.1657	83.89	<.001
DAP*CULT	4	1.0533	0.2633	1.22	0.309
PD*DAP	16	3.9979	0.2499	1.15	0.319
PD*DAP*CULT	16	3.2101	0.2006	0.93	0.542

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

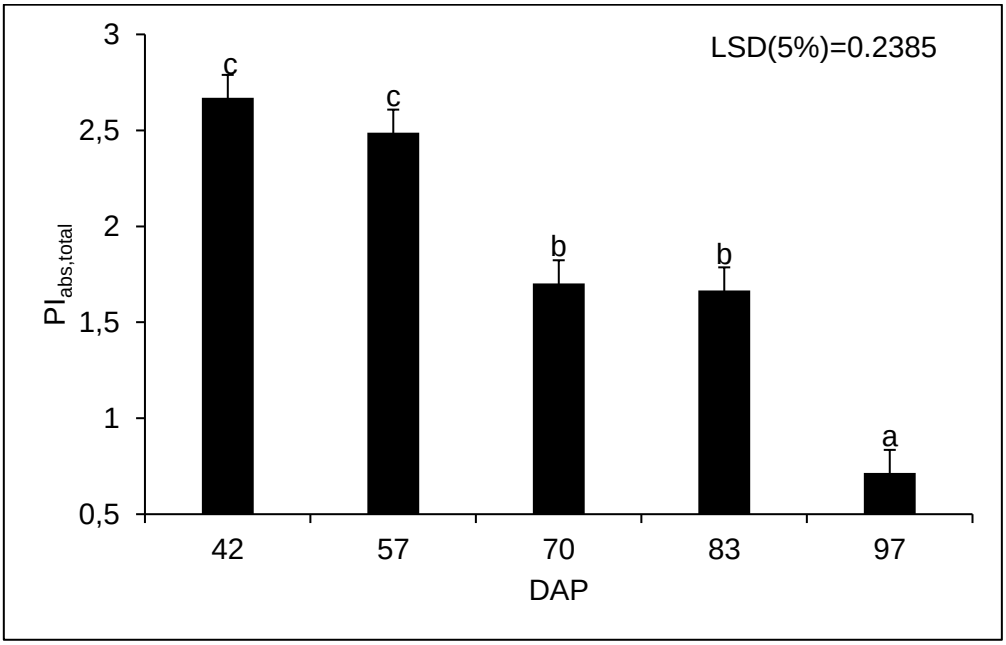

Figure 3: The effect of DAP on total photosynthetic performance of maize plants during the 2013 season.

3.5.2 The determination of nutrient availability in maize leaf samples.

3.5.2.1 *Nitrogen (N) content in the maize leaves*

During the 2012 season, plant density as main factor, had a significant influence on the nitrogen content in the maize leaves (P=0.003, Table 8). Mean nitrogen content was

significantly higher at a plant density of 10 000 plant ha^{-1} and significantly lower at a plant density of 50 000 plants ha^{-1} (Figure 4). An interaction between plant density and cultivar resulted in significantly higher nitrogen levels (P=0.02, Table 8) at 10 000 plants ha^{-1} for both PAN6P-110 and CRN3505 when compared to the other plant densities (Table 9).

In the 2013 season, ANOVA indicated that only DAP had a highly significant effect on nitrogen levels in maize leaves (P<0.0001, Table 10) with significantly higher levels of nitrogen observed at 42 and 57 DAP compared to significantly lower levels of nitrogen observed at 70, 83 and 97 DAP (Figure 5).

No correlation was recorded between nitrogen and target DNA during the 2012 and 2013 seasons. A moderate inverse correlation was recorded between nitrogen and fumonisin levels at 70 DAP (P=0.01, r=-0.46) for the 2013 season only.

Table 8: Analysis of variance of the effects of plant density (PD), cultivar and DAP on nitrogen content quantified from maize leaf samples during 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	40.518	20.259	2.71	0.0747
CULT	1	18.259	18.259	2.44	0.1233
PD	4	132.862	33.215	4.44	0.0033
PD*CULT	4	92.849	23.212	3.11	0.0217
REP(PD*CULT)	10	227.599	22.759	3.04	0.0035
DAP	3	28.791	9.597	1.28	0.2881
DAP*CULT	3	15.943	5.314	0.71	0.5492
PD*DAP	12	95.285	7.940	1.06	0.4072
PD*DAP*CULT	12	92.546	7.712	1.03	0.4327

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

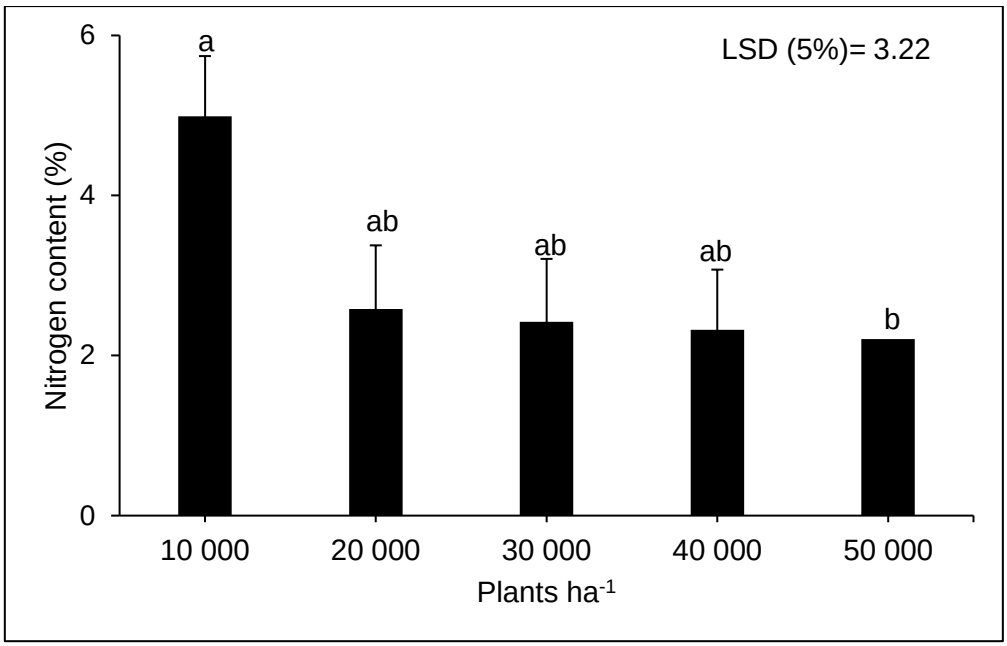

Figure 4: The effect of various plant densities on the nitrogen content quantified from maize leaves during the 2012 season.

Table 9: The effect of cultivar x plant density interaction on nitrogen content quantified from maize leaf during the 2012 season.

Plants ha^{-1}	PAN6P-110	CRN3505
10 000	7.14^{a}	2.85^{ab}
20 000	2.56^{b}	2.60^{b}
30 000	2.31^{b}	2.52^{b}
40 000	2.30^{b}	2.34^{b}
50 000	2.16^{b}	1.25^{b}

Different letters shown indicate significant differences (P=<0.05) between mean nitrogen content at different plant density x cultivar based on Fisher's protected LSD.

Table 10: Analysis of variance of the effects of plant density (PD), cultivar and DAP on nitrogen content quantified from maize leaf samples during the 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	1.668	0.834	1.74	0.1871
CULT	1	0.041	0.041	0.08	0.7726
PD	4	0.479	0.120	0.25	0.9084
PD*CULT	4	1.088	0.272	0.57	0.6881
REP(PD*CULT)	10	1.716	0.172	0.36	0.9588
DAP	4	24.139	6.035	12.57	<.0001
DAP*CULT	4	1.216	0.304	0.63	0.6413
PD*DAP	16	2.633	0.165	0.34	0.9889
PD*DAP*CULT	14	8.529	0.609	1.27	0.2614

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

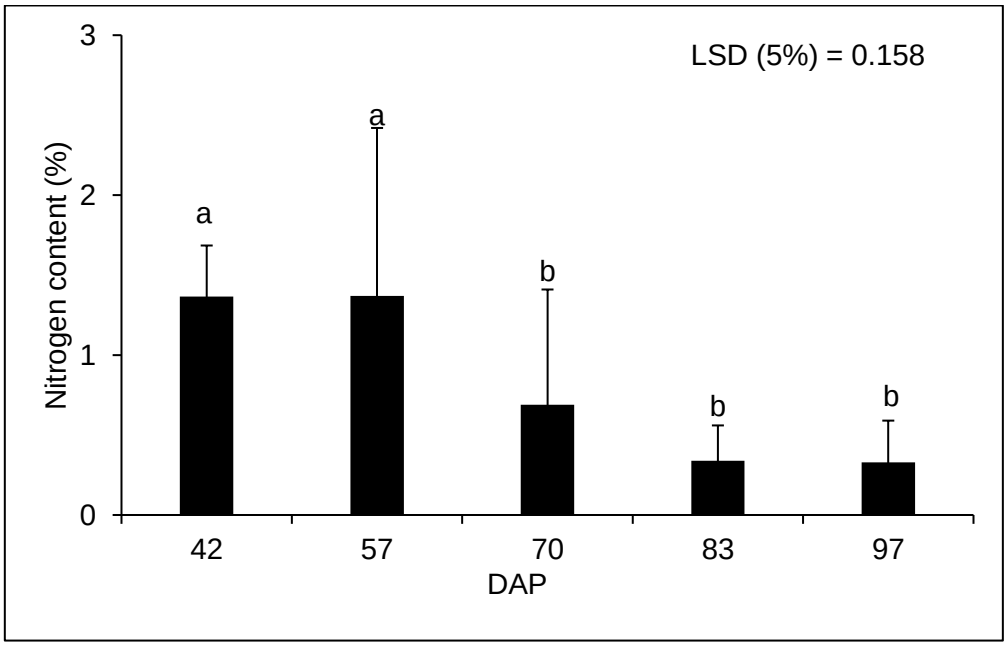

Figure 5: The effect of days after plant (DAP) on the nitrogen content quantified from maize leaf samples during the 2013 season.

3.5.2.2 *Carbon (C) content in the maize leaves*

DAP and plant density as main variables had a significant effect on carbon content in maize leaves (P=0.04 and P<0.0001 respectively, Table 11) in the 2012 season. Carbon content were significantly higher at 39 and 70 DAP (43.23% and 42.60%) compared to

95 and 123 DAP (39.30% and 38.9%, Figure 6). Carbon content was significantly lower at 50 000 plants ha^{-1} (39.4%), compared to the other plant densities with carbon content ranging from 41.1 to 41.7%, respectively (data not shown). Plant density x cultivar significantly influenced the carbon content in maize leaves (P<0.0001, Table 11) with a carbon content being significantly lower at 50 000 plants ha^{-1} in cultivar PAN6P-110 (Table 12).

During the 2013 season, ANOVA indicated that DAP as main variable had a highly significant influence on the carbon content in maize leaves (P<0.0001, Table 13). Carbon content was significantly higher at 57 DAP (44.29%) and significantly lower at 83 DAP (42.61%, Figure 7).

Although there was no correlation between target DNA and carbon in the 2012 season, a moderate inverse correlation was observed between fumonisin levels and carbon at 70 DAP (P=0.01, r=-0.46). There were no correlation between target DNA, fumonisins and carbon in the 2013 season.

Table 11: Analysis of variance of the effects of plant density (PD), cultivar and DAP on carbon content quantified from maize leaf samples during the 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	38.323	19.162	2.41	0.0985
CULT	1	22.611	22.611	2.84	0.0969
PD	4	82.415	20.601	2.59	0.0455
PD*CULT	4	117.389	29.347	3.69	0.0094
REP(PD*CULT)	10	204.565	20.456	2.57	0.0116
DAP	3	445.773	148.591	18.69	<.0001
DAP*CULT	3	32.064	10.688	1.34	0.2685
PD*DAP	12	121.746	10.145	1.28	0.2564
PD*DAP*CULT	12	81.244	6.770	0.85	0.5986

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

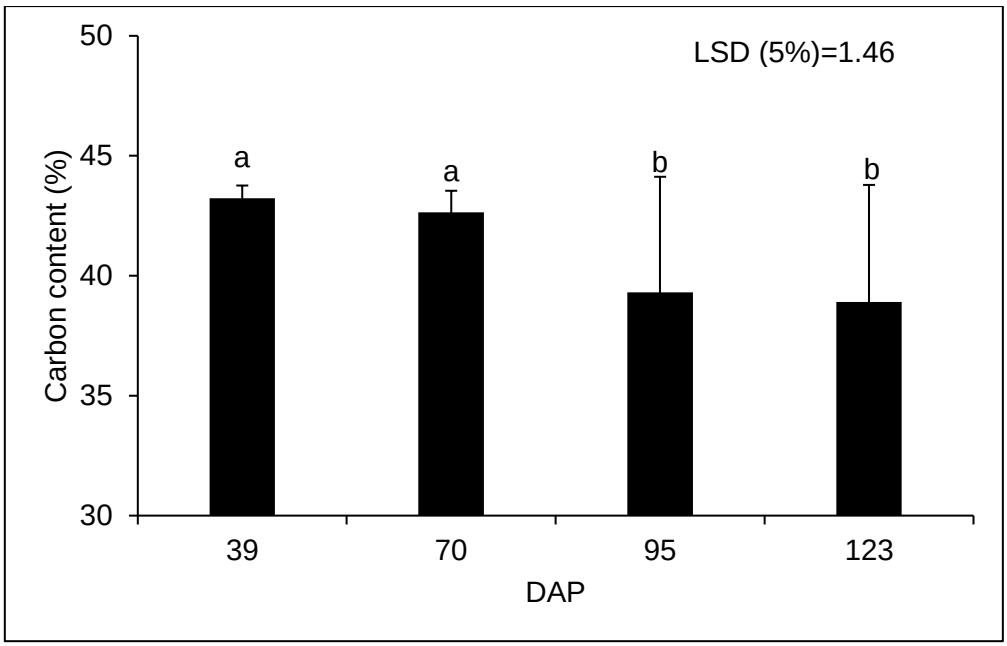

Figure 6: The effect of days after plant (DAP) on the carbon content quantified from maize leaf samples during the 2012 season.

Table 12: The effect of a cultivar x plant density interaction on carbon content quantified from maize leaf samples during the 2012 season.

Plants ha^{-1}	PAN6P-110	CRN3505
10 000	41.87 ^{a}	41.58 ^{a}
20 000	41.29 ^{a}	40.99 ^{ab}
30 000	41.20 ^{a}	41.90 ^{a}
40 000	41.53 ^{a}	41.04 ^{ab}
50 000	37.05 ^{b}	41.78 ^{a}

Different letters shown indicate significant differences (P=<0.05) between mean carbon content at different plant density x cultivar based on Fisher's protected LSD.

Table 13: Analysis of variance of the effects of plant density (PD), cultivar and DAP on carbon content quantified from maize leaf samples during the 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	1.386	0.693	1.47	0.2353
CULT	1	3.827	3.827	8.14	0.0800
PD	4	3.103	0.776	1.65	0.1702
PD*CULT	4	4.618	1.155	2.45	0.3901
REP(PD*CULT)	10	10.083	1.008	2.14	0.0301
DAP	4	86.151	21.538	45.78	<.0001
DAP*CULT	4	2.936	0.734	1.56	0.1930
PD*DAP	16	11.542	0.721	1.53	0.1085
PD*DAP*CULT	16	9.266	0.579	1.23	0.2638

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

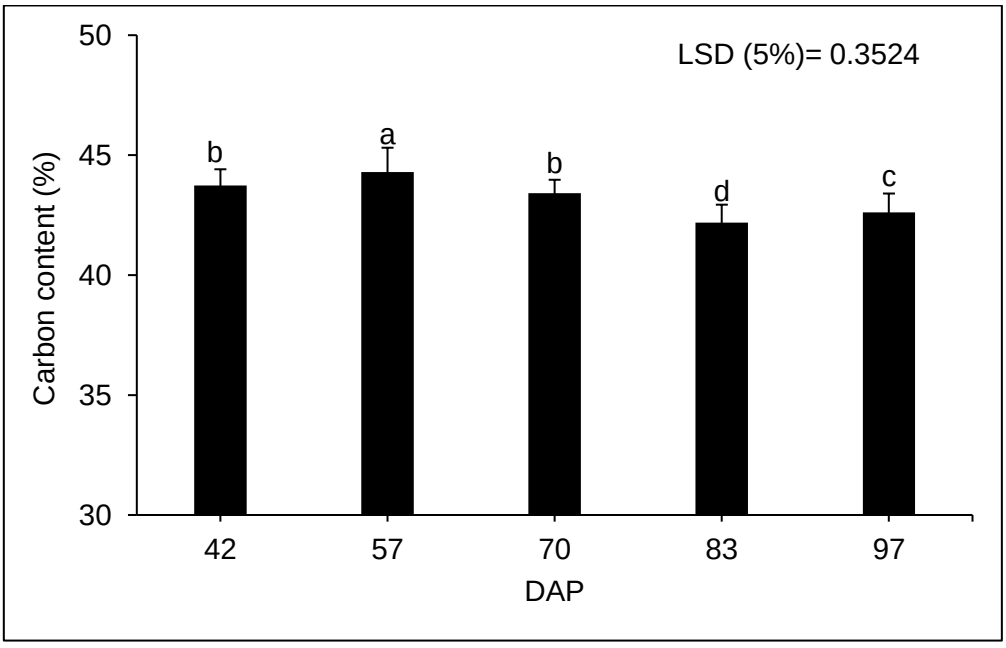

Figure 7: The effect of days after plant (DAP) on the carbon content quantified from maize leaf samples during the 2013 season.

3.5.2.3 *Sulphur (S) content in the maize leaves*

ANOVA showed that cultivar, plant density and DAP as main variables had highly significant effects on sulphur content in maize leaves for the 2012 (P=0.009, P<0.0001 and P<0.0001, Table 14) and 2013 season (P=0.004, P<0.0001 and P<0.0001, Table 15). During the 2012 season, sulphur content was the highest at 50 000 plants ha^{-1} compared to the other plant densities (Figure 8). Sulphur levels were significantly higher at 39 DAP and decreased as the plants matured (Figure 9). Sulphur content levels was significantly higher in cultivar CRN3505 (0.30%) when compared to cultivar PAN6P-110 (0.27%, data not shown).

During the 2013 season, sulphur content was significantly higher in maize leaves at 40 000 plants ha^{-1} and the lowest at 10 000 plants ha^{-1} (Figure 10). The sulphur content was significantly higher at 42 DAP (0.43%) compared to the other DAP (Figure 11). Highly significant differences between the cultivars showed CRN3505 (0.27%) with low levels of sulphur content when compared to PAN6P-110 (0.32%).

No correlation was recorded between target DNA and sulphur in the 2012 season. Moderate inverse correlations were recorded between fumonisin levels and sulphur content at 70, 95 and 123 DAP (P=0.01, r=-0.45, P=0.008, r=-0.51 and P=0.03, r=-0.42 respectively).

During the 2013 season, a weak correlation was recorded between target DNA and sulphur content at 70 DAP (P=0.05, r=0.36). No correlation was recorded between fumonisins and sulphur content.

Table 14: Analysis of variance of the effects of plant density (PD), cultivar and DAP on sulphur content quantified from maize leaf samples during the 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	0.058	0.029	5.52	0.0063
CULT	1	0.038	0.038	7.23	0.0093
PD	4	0.164	0.041	7.82	<.0001
PD*CULT	4	0.039	0.009	1.88	0.1252
REP(PD*CULT)	10	0.067	0.007	1.28	0.2636
DAP	3	0.786	0.262	50.11	<.0001
DAP*CULT	3	0.022	0.007	1.41	0.2498
PD*DAP	12	0.044	0.004	0.70	0.7468
PD*DAP*CULT	12	0.018	0.001	0.28	0.9898

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

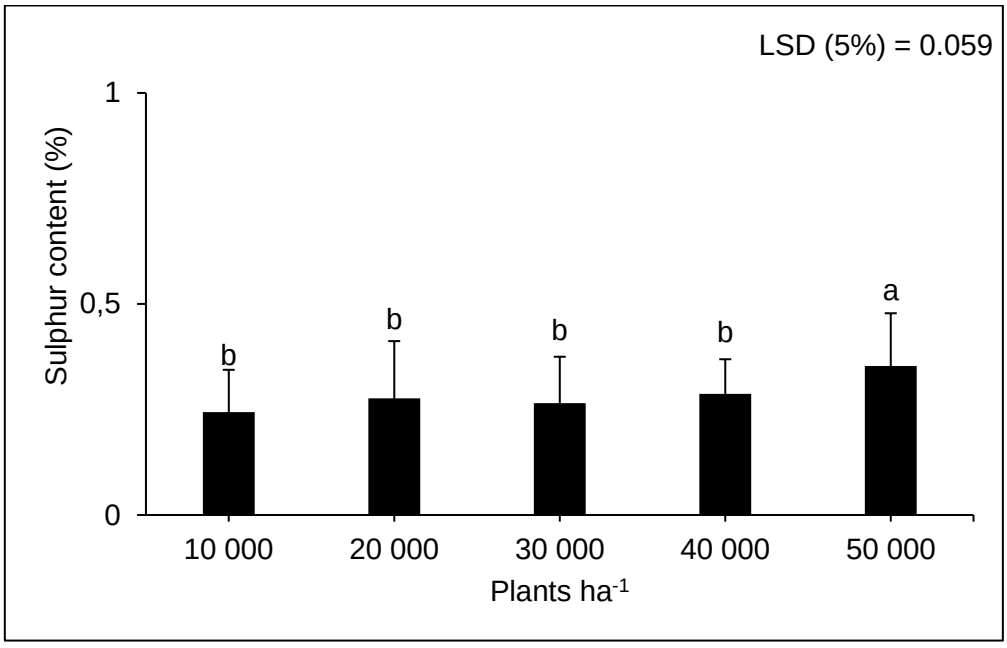

Figure 8: The effect of plant density on the sulphur content quantified from maize leaf samples during the 2012 season.

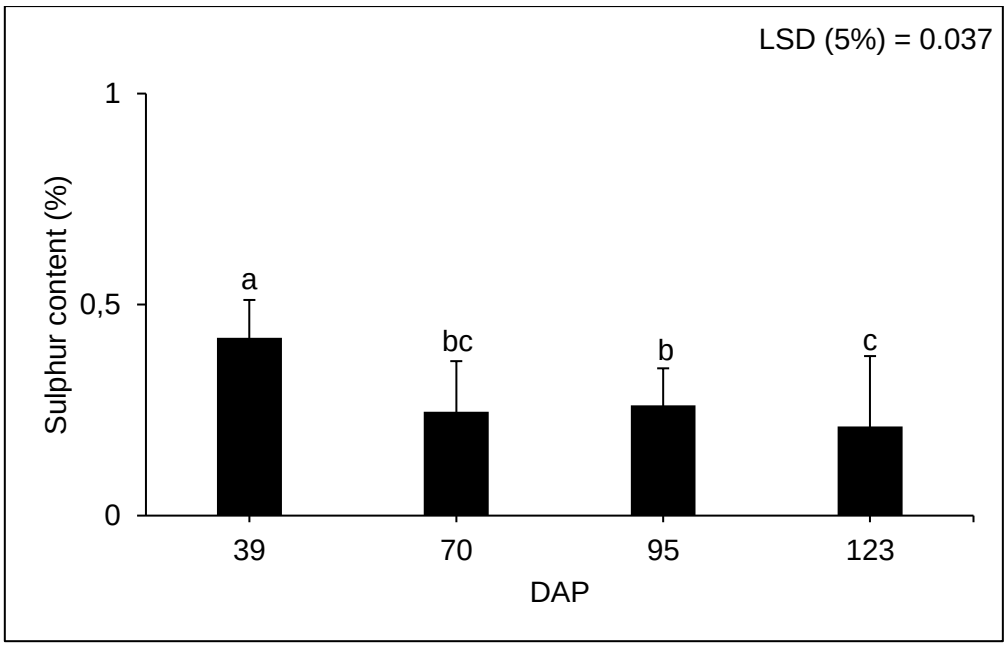

Figure 9: **The effect of days after plant (DAP) on the sulphur content quantified from maize leaf samples during the 2012 season.**

Table 15: **Analysis of variance of the effects of plant density (PD), cultivar and DAP on sulphur content quantified from maize leaf samples during the 2013 season.**

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	0.025	0.013	1.22	0.3002
CULT	1	0.091	0.091	8.74	0.0041
PD	4	0.323	0.081	7.74	<.0001
PD*CULT	4	0.051	0.013	1.27	0.2888
REP(PD*CULT)	10	0.152	0.0152	1.46	0.1722
DAP	4	0.891	0.223	21.36	<.0001
DAP*CULT	4	0.049	0.012	1.18	0.3239
PD*DAP	16	0.197	0.012	1.18	0.2998
PD*DAP*CULT	16	0.217	0.014	1.30	0.2165

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

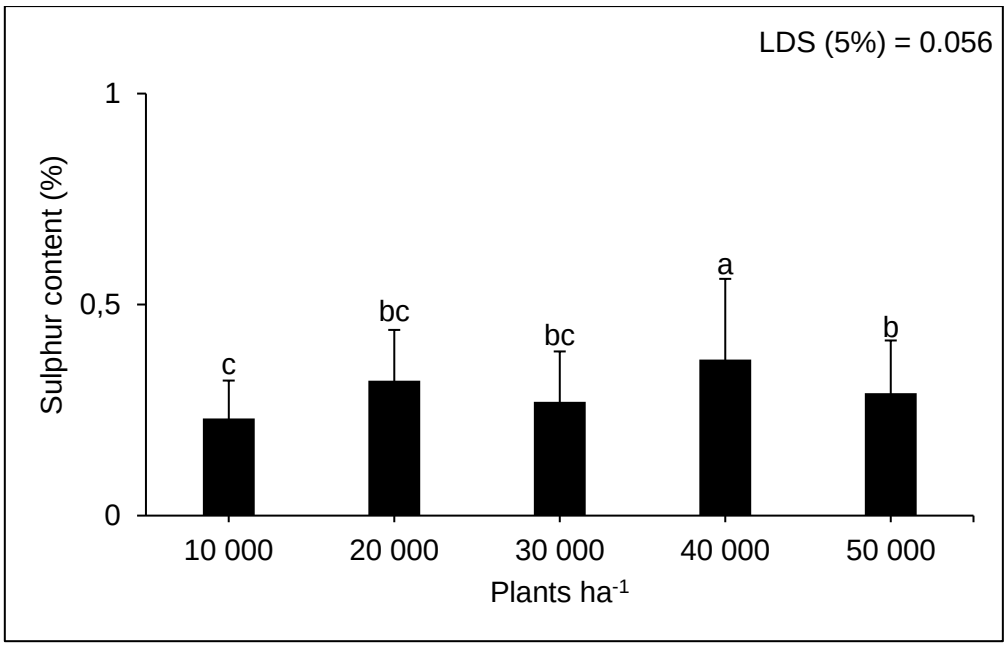

Figure 10: The effect of plant density on the sulphur content quantified from maize leaf samples during the 2013 season.

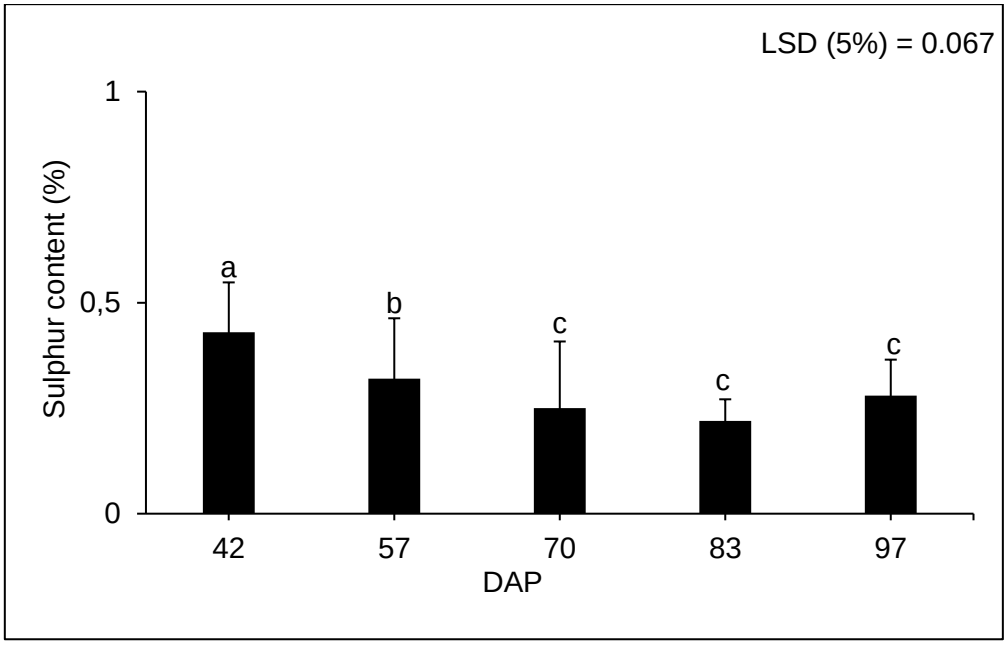

Figure 11: The effect of days after plant (DAP) on the sulphur content quantified from maize leaf samples during the 2013 season.

3.5.3 Determination of protein content and enzyme activity in the 2012 and 2013 seasons respectively

3.5.3.1 *Total leaf protein content*

During the 2012 season, DAP as main variable had a highly significant effect on total protein content quantified from maize leaves (P= 0.001, Table 16). Total leaf protein decreased as plants matured (Figure 12) with the highest value at 39 DAP (2.17 mg mL^{-1}) and the lowest value of 0.25 mg mL^{-1} at 123 DAP.

During the 2013 season, an interaction between plant density x DAP had a highly significant effect regarding total leaf protein (P<0.0001, Table 17). The total leaf protein was significantly higher at 42 DAP over all the plant densities and at 83 DAP at 40 000 and 50 000 plants ha^{-1} (Table 18).

There was no correlations recorded between target DNA,fumonisins and total leaf protein in the 2012 season. An inverse correlation was recorded at 83 DAP between fumonisins levels and total leaf proteins in the 2013 season (P=0.02, r^2=-0.42).

Table 16: Analysis of variance of the effects of plant density (PD), cultivar and DAP on total leaf protein during the 2012 season.

Source	d.f	s.s	m.s	v.r.	F pr.
REP	2	2.618	1.309	8.53	0.0007
CULT	1	0.099	0.099	0.65	0.4251
PD	4	0.840	0.210	1.37	0.2600
PD*CULT	4	0.243	0.0610	0.40	0.8108
REP(PD*CULT)	10	1.798	0.179	1.17	0.3348
DAP	3	45.103	15.034	97.93	<.0001
DAP*CULT	12	0.886	0.074	0.48	0.9154
PD*DAP	12	0.886	0.074	0.48	0.9154
PD*DAP*CULT	10	0.529	0.053	0.34	0.9632

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

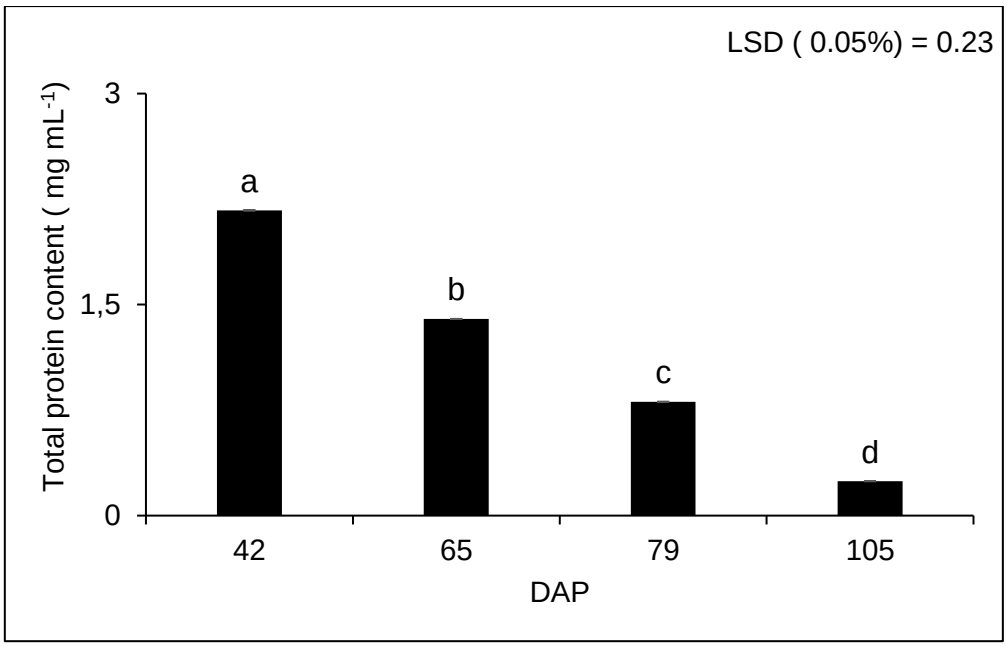

Figure 12: The effect of days after plant (DAP) on total protein content from maize leaf samples in the 2012 season.

Table 17: Analysis of variance of the effects of plant density (PD), cultivar and DAP on total leaf protein during the 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	0.040	0.020	1.00	0.3720
CULT	1	0.028	0.028	1.39	0.2417
PD	4	0.773	0.193	9.60	<.0001
PD*CULT	4	0.133	0.033	1.66	0.1686
REP(PD*CULT)	10	0.329	0.033	1.63	0.1122
DAP	4	1.350	0.338	16.76	<.0001
DAP*CULT	4	0.079	0.019	0.98	0.4240
PD*DAP	16	0.885	0.055	2.75	0.0016
PD*DAP*CULT	16	0.513	0.032	1.59	0.0898

d.f= Degree of Freedom, s.s= Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 18: The interaction effect of plant density (PD) and DAP on total leaf protein content during the 2013 season.

	Days after plant (DAP)				
	Mean total leaf protein expressed as mg mL^{-1}				
Plants ha^{-1}	**42**	**57**	**70**	**83**	**97**
10 000	1.262^{ab}	0.930^{ghij}	0.7568^{k}	0.918^{ghijk}	0.881^{hijk}
20 000	1.112^{bcdef}	1.010^{efghij}	0.900^{ghik}	0.857^{jk}	1.015^{efghij}
30 000	1.196^{abcd}	0.925^{ghij}	0.872^{ijk}	1.038^{defgh}	0.863^{jk}
40 000	1.203^{abc}	1.055^{cdefg}	0.972^{fghij}	1.267^{ab}	1.045^{cdefg}
50 000	1.143^{a}	1.015^{efghij}	1.032^{efghi}	1.303^{a}	1.105^{bcdef}

Different letters shown indicate significant differences (P=<0.05) between mean total leaf protein at different plant densities x DAP based on Fisher's protected LSD.

3.5.3.2 *Chitinase activity levels*

During the 2012 and 2013 seasons, only DAP significantly influenced chitinase activity (P=0.0004, Table 19 and P<0.0001, Table 20) in maize leaves. Significantly higher levels of chitinase activity were noticed at 95 DAP (1.67 nm mg^{-1} Prot) during 2012 (Figure 13) and 57 and 97 DAP during 2013 (0.80 nm mg^{-1} Prot and 0. 79 nm mg^{-1} Prot, respectively, Figure 14).

There was only a weak inverse correlation between fumonisin levels and chitinase activity in the 2013 season at 83 and 97 DAP (P=0.04, r=-0.37 and P=0.04, r=-0.38 respectively).

Table 19: Analysis of variance of the effects of plant density (PD), cultivar and DAP on chitinase activity during 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	0.848	0.424	6.00	0.0037
CULT	1	0.009	0.009	0.14	0.7133
PD	4	0.355	0.089	1.26	0.2937
PD*CULT	4	0.140	0.035	0.50	0.7381
REP(PD*CULT)	10	1.426	0.143	2.02	0.0418
DAP	4	8.067	2.017	28.56	<.0001
DAP*CULT	4	0.074	0.019	0.26	0.9008
PD*DAP	16	1.459	0.091	1.29	0.2232
PD*DAP*CULT	16	0.516	0.032	0.46	0.9601

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

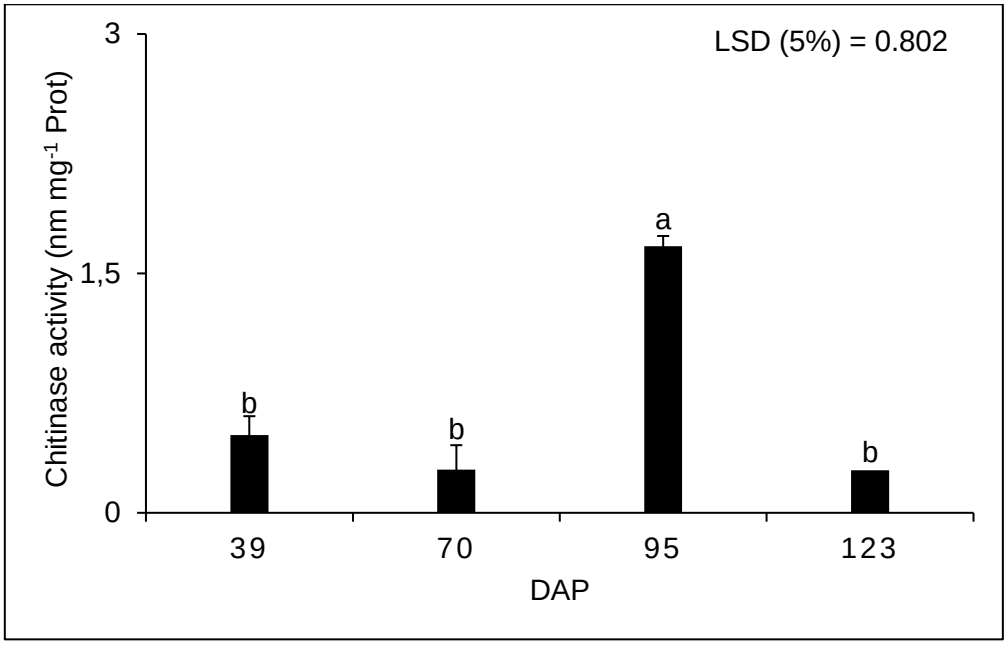

Figure 13: The effect of days after plant (DAP) on chitinase activity from maize leaf samples in the 2012 season.

Table 20: Analysis of variance of the effects of plant density (PD), cultivar and DAP on chitinase activity during 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	1.095	0.547	0.31	0.7385
CULT	1	1.588	1.588	0.89	0.3518
PD	4	3.846	0.961	0.54	0.7099
PD*CULT	4	6.599	1.649	0.92	0.4608
REP(PD*CULT)	10	14.984	1.498	0.84	0.5975
DAP	3	39.867	13.289	7.41	0.0004
DAP*CULT	3	4.054	1.351	0.75	0.5262
PD*DAP	12	13.249	1.104	0.62	0.8176
PD*DAP*CULT	10	19.390	1.939	1.08	0.3963

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

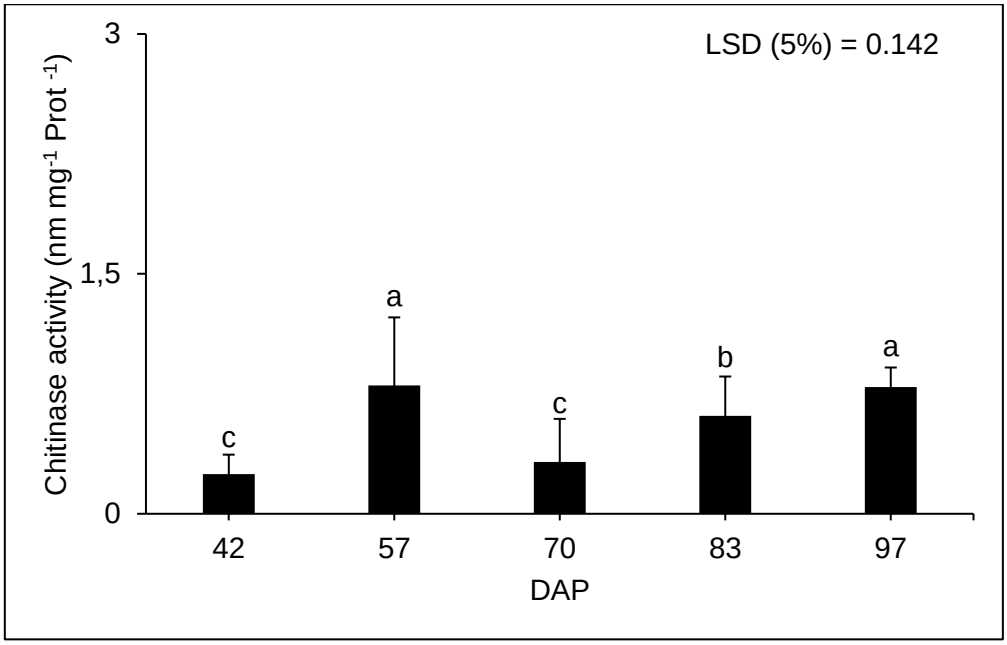

Figure 14: The effect of days after plant (DAP) on chitinase activity from maize leaf samples in the 2013 season.

3.5.3.3 *Peroxidase activity*

DAP, plant density and cultivar had no effect on peroxidase activity levels during the 2012 (Table 21) and 2013 seasons (Table 22).

The only correlation (moderate) observed was between fumonisin levels and peroxidase activity at 83 DAP (P=0.04, r=0.40) in the 2013 season.

Table 21: Analysis of variance of the effects of plant density (PD), cultivar and DAP on peroxidase activity during 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	2,04E+17	1.02E+17	1.18	0.3160
CULT	1	7.45E+16	7.45E+16	0.86	0.3579
PD	4	3.93E+17	9.83E+16	1.14	0.3507
PD*CULT	4	2.91E+17	7.27E+16	0.84	0.5055
REP(PD*CULT)	10	7.45E+17	7.45E+16	0.86	0.5736
DAP	3	5.98E+17	1.99E+17	2.31	0.0893
DAP*CULT	3	3.41E+17	1.14E+17	1.32	0.2809
PD*DAP	12	1.99E+18	1.66E+17	1.92	0.0668
PD*DAP*CULT	10	9.54E+17	9.54E+16	1.11	0.3792

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

Table 22: Analysis of variance of the effects of PD, cultivar and DAP on peroxidase activity during 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	2.55E+12	1.27E+12	0.82	0.4462
CULT	1	4.1E+11	4.1E+11	0.26	0.6123
PD	4	1.29E+13	3.24E+12	2.07	0.0927
PD*CULT	4	3.13E+12	7.8E+11	0.50	0.7359
REP(PD*CULT)	10	4.44E+12	4.4E+11	0.28	0.9832
DAP	4	1.44E+13	3.59E+12	2.29	0.0667
DAP*CULT	4	3.63E+12	9.1E+11	0.58	0.6783
PD*DAP	16	1.50E+13	9.4E+11	0.60	0.8746
PD*DAP*CULT	16	3.00E+13	1.87E+12	1.20	0.2883

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

3.5.3.4 *β-1, 3-glucanase activity*

Plant density and DAP as main variables significantly influenced β-1, 3-glucanase activity in maize leaves in the 2012 season ($P<0.0001$, Table 23). β-1, 3-glucanase activity was significantly lower at 123 DAP (0.54 mg mol^{-1} prot) when compared to the other DAP (Figure 15). Significantly lower levels of β-1, 3-glucanase were detected at 10 000 and

40 000 plants ha^{-1} (0.99 and 0.1 mg mol^{-1} prot) whilst significantly higher levels were noticed at 20 000, 30 000 and 50 000 plants ha^{-1} (1.14, 1.25 and 1.30 mg mol^{-1} prot) data not shown. The interaction between plant density and cultivar significantly influenced β-1, 3-glucanase activity (P=0.007, Table 23). β-1, 3-glucanase activity was significantly lower in PAN6P-110 and CRN3505 at 123 DAP compared to the other DAP (both cultivars, Table 24).

In the 2013 season, ANOVA results showed that only DAP had a significant effect on the activity of β-1, 3-glucanase (P<0.0001, Table 25). β-1, 3-glucanase activity was significantly higher at 42 DAP and significantly lower at 57 and 83 DAP (Figure 16).

There was a moderate correlation at 70 DAP between target DNA, fumonisins and β-1, 3-glucanase activity (P=0.02, r=0.42 and P=0.03, r= 0.41 respectively) in the 2012 season. A weak correlation was observed between fumonisin levels and β-1, 3-glucanase activity at 95 DAP (P=0.05, r=0.38).

An inverse correlation was observed at 97 DAP between β-1, 3-glucanase activity and fumonisin levels respectively during 2013 season (P=0.04, r=-0.38).

Table 23: Analysis of variance of the effects of plant density (PD), cultivar and DAP on β-1, 3-glucanase activity during 2012 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	0.726	0.363	9.01	0.0005
CULT	1	0.000	0.000	0.01	0.9247
PD	4	1.632	0.408	10.13	<.0001
PD*CULT	4	0.645	0.161	4.00	0.0073
REP(PD*CULT)	10	3.909	0.391	9.70	<.0001
DAP	3	4.957	1.652	41.00	<.0001
DAP*CULT	3	0.167	0.056	1.38	0.2597
PD*DAP	12	0.507	0.042	1.05	0.4243
PD*DAP*CULT	10	0.771	0.077	1.91	0.0680

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

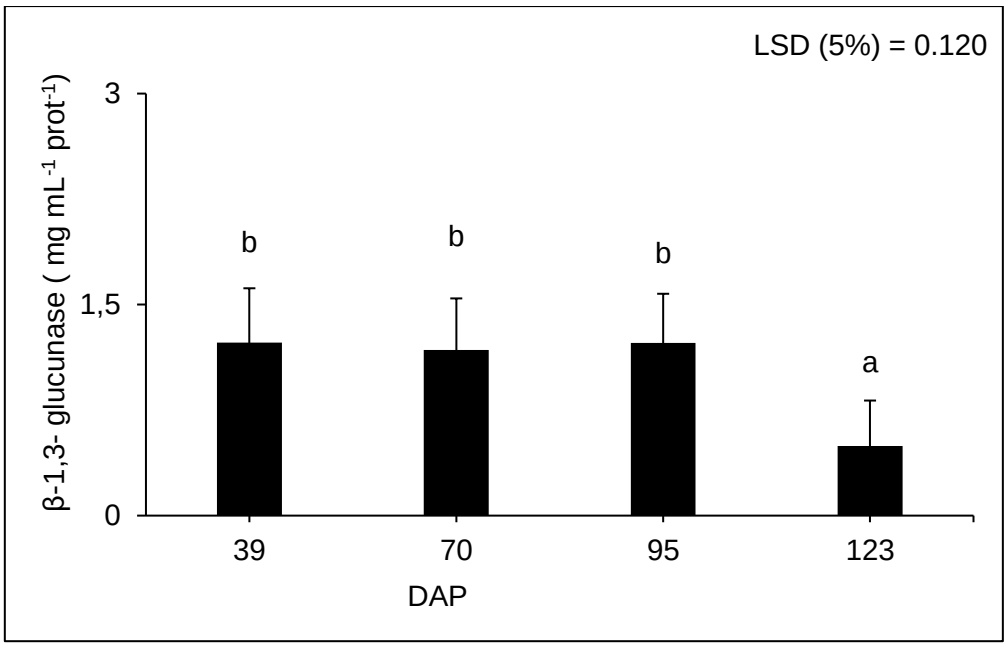

Figure 15: The effect of days after plant (DAP) on β-1,3-glucanase activity in maize leaves during the 2012 season.

Table 24: The effect of cultivar x DAP interaction on β-1,3-glucanase activity during the 2012 season.

DAP	PAN6P-110	CRN3505
39	1.23 [a]	1.23 [a]
70	1.13 [a]	1.23 [a]
95	1.21 [a]	1.25 [a]
123	0.60 [b]	0.49 [b]

Different letters shown indicate significant differences (P=<0.05) between mean β-1,3- glucanase activity at different DAP x cultivar based on Fisher's protected LSD.

Table 25: Analysis of variance of the effects of plant density (PD), cultivar and DAP on β-1, 3-glucanase activity during 2013 season.

Source	d.f.	s.s.	m.s.	v.r.	F pr.
REP	2	0.217	0.109	1.94	0.1507
CULT	1	0.001	0.001	0.03	0.8744
PD	4	0.431	0.108	1.92	0.1150
PD*CULT	4	0.241	0.060	1.07	0.3760
REP(PD*CULT)	10	0.471	0.0471	0.85	0.5863
DAP	4	7.834	1.959	34.91	<.0001
DAP*CULT	4	0.076	0.019	0.34	0.8525
PD*DAP	16	0.852	0.053	0.95	0.5184
PD*DAP*CULT	16	0.961	0.060	1.07	0.3959

d.f= Degree of Freedom, s.s =Sum of Squares, m.s= Mean Square, v.r= F value, F pr= P value

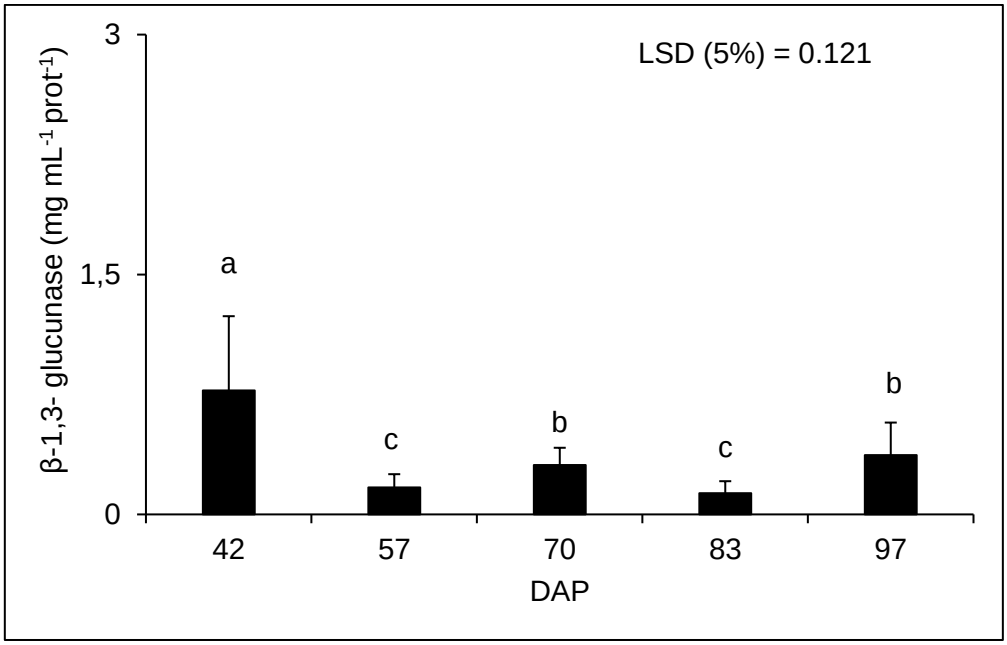

Figure 16: The effect of days after plant (DAP) on β-1,3-glucunase activity in maize leaves during the 2013 season.

3.6 DISCUSSION AND CONCLUSIONS

Chlorophyll *a* fluorescence

Chlorophyll *a* fluorescence monitors the activity of photosystem II, and the JIP-test permits the quantification of this activity at the levels of photosystem II photon absorption,

excitation trapping and electron transport. Photosystem II (PSII) activity plays a key role in the response of photosynthesis to environmental stresses (Martìnez-Ferri *et al.,* 2000). The photosynthetic efficiency of a plant reduces under adverse conditions (Santos *et al*., 2006); therefore, in this study measurements taken from maize leaves were used to quantify stress caused by plant density. The objective of the study aimed to determine whether chlorophyll fluorescence measurements could be used as an indicator of plant vitality under different plant densities.

In this study, chlorophyll fluorescence was analyzed in order to measure the photosynthetic efficiency of photosystem II on maize plants of cultivars PAN6P-110 and CRN3505 at different plant densities in the field. The performance index (PI) parameters provides a useful quantitative information about the state of plants and their vitality and therefore it can be used for the analysis of plant stress response (Oukarroum *et al.,* 2007; Kalaji *et al.,* 2012). Plant density had a highly significant effect on the photosynthetic performance (PI_{abs}) for seasons 2012 and 2013 with a decrease at increasing plant densities. Competition between plants for resources increase with an increase in plant density (Franić *et al.,* 2015) and furthermore, maize plants display photosynthetic adjustment to fluctuating light intensity and therefore have a reduced photosynthetic capacity in shaded environments created by dense maize populations.

Fluctuations could be observed in photosynthetic performance at different DAP which can be explained by the different growth processes of maize plants. At 39 DAP (8 leaf stage) rapid vegetative growth takes place and leaves are not fully enlarged, therefore photosynthesis is limited when compared to the 12 leaf stage when leaf enlargement will be complete (Du Toit, 1999). This finding emphasize careful selection of the DAP when measuring chlorophyll fluorescence as this can have a significant influence on the photosynthetic efficiency. In the 2012 season an interaction between DAP and cultivar showed the highest photosynthetic performance to be in cultivar CRN3505 at 123 (DAP) which corresponds to the soft dough stage. According to Dwyer *et al.* (1991), the photosynthetic performances of maize plants decreased at increasing plant densities from 20 000 to 130 000 plants ha^{-1}, however, the decrease was dependent on the genotype. Similar results were observed in die 2013 season for photosynthetic performance with an added interaction of DAP. The lowest photosynthetic performance could be observed in both cultivars at 97 DAP (milk stage) and the best photosynthetic

performance could be observed at 30 000 plants ha^{-1} (used as plant density control) irrespective of cultivar or DAP, which re-iterate the importance of utilizing recommended plant densities that are suitable to the environment.

Plant density did not have an effect on the photosynthetic performance ($PI_{abs, total}$) for seasons 2012 and 2013. $PI_{abs, total}$ was significantly affected by cultivar and DAP during the 2012 season and only DAP in the 2013 season. During the 2012 season, $PI_{abs, total}$ was significantly higher in PAN6P-110 at 39 DAP which is in contradiction with Gepstein (1988) that reported an increase in photosynthetic activity in maize plants during early leaf development that gradually reaches a maximum in fully expanded mature leaves.

Similar to PI_{abs} excitation during the 2013 season, $PI_{abs, total}$ was the lowest at 97 DAP (milk stage). This could be that photosynthesis in the leaves begins to decrease during later developmental stages of the maize plant. Nitrogen was not added to the soil before planting in the 2013 season and a reduction in available leaf nutrients was observed from the 2012 to 2013 season. The nitrogen percentage was 3.41 at 39 DAP and declined to 2.28 % at 123 DAP in the 2012 season. A reduction in leaf nitrogen percentage levels was recorded in the 2013 season with 1.36 % at 42 DAP and 0.32 % at 97 DAP. The decline in available leaf nutrients from the 2012 to 2013 season could explain the low $PI_{abs, total}$.

This study indicated that photosynthetic performance (PI_{abs}) can be used to measure plant stress due to plant density but that $PI_{abs, total}$ was not effective in measuring plant stress due to plant density. The importance of sampling at different physiological growth stages of the plants is emphasized, as this could significantly influence the photosynthetic performance. It was demonstrated that the best photosynthetic performance could be observed at 30 000 plants ha^{-1}. This plant density is used as a control in this study, as it is the recommended plant density for PAN6P-110 and CRN3505 in Potchefstroom. Although photosynthetic performance (PI_{abs}) can be used to measure plant stress due to plant density, added stressors such as nitrogen depletion can obscure the effects of photosynthetic performance.

Determination of plant nutrients

Plants, like other living organisms, require food for their growth and development. They require essential elements, which are supplied from either soil minerals, soil organic matter or fertilizers (Uchida, 2000). Nutrients in the soil are taken up by the roots of the plant after which they are translocated within the plant through the stems and leaves. However, for plants to utilize these nutrients resourcefully, they need adequate supply of light, heat and water. It is important to find a correct balance between macronutrients and micronutrients for maize crops. Plant tissue tests have been developed to assess the nutrient content in plants. By analysing this information, plant scientists have been able to determine the nutrient need of maize plants in the soil. According to Jones (1998) leaf analysis plays an important role in understanding the nutrient content and diagnoses nutritional problems under stress conditions. Fertilizers are generally applied during planting to increase crop yield, however they can also be used to modify the plant to resist abiotic and biotic stresses (Veresoglou *et al.,* 2013).

Maize plants exposed to fertility stress are also more susceptible to infection by microorganisms than plants not under stress. Elevated aflatoxin levels have been associated with fertility-related stresses, particularly N deficiency (Lisker & Lillehoj, 1991). Anderson *et al*. (1975) and Jones & Duncan (1981) reported that a higher rate of N fertilizer application consistently resulted in reduced aflatoxin rates. Blandino *et al.* (2008) related higher fumonisin contamination with N deficiencies. It is necessary to understand and quantify the effect of N levels on *F. verticillioides* and resultant fumonisin production under local conditions, therefore no fertilizers was applied at successive plantings to ensure a gradual depletion of N, C and S in the soil.

In the 2012 season, plant density significantly reduced the amount of nitrogen in the leaves at higher densities. This was expected due to the fact that high plant density ensures severe competition between plants for nutrients below the ground and as a result, nitrogen availability and nitrogen uptake are severely affected. These results are similar to the findings of Maddoni *et al.* (2001) who reported higher nitrogen levels at lower plant densities compared to lower nitrogen levels at higher plant populations. In line with this finding, both PAN6P-110 and CRN3505 had higher available leaf N % at 10 000 plants ha^{-1} when compared to the other plant densities.

In contrast to the 2012 season, plant density in the 2013 season did not influence available nitrogen percentage in the maize leaves. The rapid decline in nitrogen from the 2012 to 2013 season could be the reason why plant density did not have an effect on the available nitrogen in maize leaves. With a low nitrogen starting concentration in the soil, only DAP had a significant effect on available nitrogen percentage content in maize leaves. The available leaf nitrogen percentage declined as maize plants matured. Maize plants utilize nitrogen on a continuous basis from the soil and as plants develop the demand for nitrogen increase. Sufficient nitrogen is needed during plant development to optimize vitality and yield (Hague *et al.,* 2001). Furthermore, various genotypes is available to producers that can uptake and utilize nitrogen more efficiently (Hirel *et al.,* 2001). Correlation studies show an increase in fumonisin levels as available nitrogen percentage in maize leaves decreased in the 2012 season. This is in line with findings of Blandino *et al.* (2008) that reported higher fumonisin contamination with soil low nitrogen.

Maize plant take in carbon dioxide as their source of carbon and convert it into energy for growth and development. Therefore, the role of carbon in the maize plant is to promote healthier and more productive growth of the plant. In similarity with the nitrogen results, Carbon content decreased as plant densities increased and as plants matured (DAP). Correlation studies show an increase in fumonisin levels as available carbon content in maize leaves decreased in the 2012 season. Environmental factors such as maximum temperature may have played a role in fumonisin expression in the 2012 and 2013 season. It is known that temperature is one of the main factors influencing fumonisin synthesis and this could have prevailed over the effect that the available leaf nitrogen and carbon content in the 2013 season.

Sulphur is important in plant protein synthesis and aids in the formation of enzymes and in chlorophyll molecules (Jamal *et al.,* 2010). Sulphur uptake occurs over the entire growth season and is relatively constant from planting to maturity. The maize plant takes up a small percentage of sulphur from silking to physiological maturity (Haun, 2015). Since sulphur is immobile within the maize plant, and the plant is unable to move sulphur from older to newer growth stages to compensate for low levels that may occur late in the season, the crop depends on the continuous supply of available sulphur in the soil throughout the growing season. However, the uptake of sulphur is dependent on the cultivar characteristics and the climatic conditions in and outside the plant (Haun, 2015).

The results from this study show that sulphur content in the maize leaves was significantly influenced by the cultivar, plant density and DAP in both seasons. The effect of plant density was greater than that of DAP in the 2012 season. Although S % declined as plants matured it was significantly higher at 50 000 plants ha^{-1}. The same trend was observed in the 2013 season and PAN6P-110 had a lower available leaf S % when compared to CRN3505. Again, an increase in fumonisin levels were noted as available S % in maize leaves decreased in the 2012 season.

Although published literature is available regarding the effect of soil N on fumonisins, no literature could be found regarding the effects of available leaf nutrients such as N, C and S on fumonisin production in maize grain. This is therefore a first report of increasing fumonisin levels with a decrease in N, C and S available leaf nutrients. This study was conducted over a two year period and should be investigated further. By developing a management strategy that optimize available leaf nutrients such as N, C and S, producers can manage the risk of fumonisin production in maize grain.

PR proteins

Plant growth and development processes involve environmental and endogenous signals that contribute to determine the physiology and molecular features of the plant. Within these endogenous signals, pathogenesis related proteins (PR) have been recognized in maize plants as important participants in the biological processes. PR proteins have been implicated in the active defense and plays a role in restricting pathogen development and spread in the plant (van Loon *et al.,* 2006). PR proteins have been suspected to have a direct effect in defence mechanisms against fungi by hydrolyzing fungal cell walls, which consequently causes the lysis of fungal cells. In this study, the activity levels of β-1,3-glucanases, chitinases and peroxidase in response to natural infection by the *F. verticillioides* were examined at different plant density stages. The results of this study showed that total leaf protein content decreased as plants matured during the 2012 season. Data were more variable in the 2013 season with plant density x DAP that had a significant effect on total leaf protein content. Because total leaf protein content is used to quantify the activity levels of β-1,3-glucanases, chitinases and peroxidase, only the activity of the latter 3 PR proteins will be discussed.

Chitinases are digestive enzymes that hydrolyze glycosidic bonds in chitin that composes the cell walls of fungi. They are normally expressed at low levels in plants, but are dramatically increased when exposed to biotic and abiotic stresses (Punja & Zhang, 1993). Several studies have highlighted the overexpression of PR genes β-1,3-glucanase and chitinases to improve the plants resistance to *F.graminearum* (Chen *et al*., 2010; Makandar *et al*., 2006). Chitinase activity was significantly higher during 95 DAP (2012) and 57 and 97 DAP (2013). Fifty seven DAP corresponds to the 12 leaf stage and 95 as well as 97 DAP corresponds to the milk stage. Elevated chitinase activity during the 12 leaf stage could have been due to insect damage or fungal infection of the maize stalk or leaves, although these observations were not made. The milk stage is an important stage for fungal infection and furthermore fumonisin production in maize kernels (Janse van Rensburg, 2012). Induced chitinase activity at milk stage is an indication of a possible hypersensitive response. The hypersensitive response (HR) is a mechanism used by plants to prevent the spread of infection by pathogens. HR is characterized by the rapid death of cells in the local region surrounding an infection. It serves to restrict the growth and spread of pathogens to other parts of the plant. Gomez-Ariza *et al*. (2007) found that PR proteins in maize displayed broad-spectrum resistance to infection by bacterial and fungal pathogens such as *F.verticillioides* and appeared to respond quicker and stronger to fungal pathogens. These findings can be confirmed by a weak inverse correlation between chitinase activity and fumonisin levels in the 2013 season, where an increase in chitinase activity coincided with decrease in fumonisin levels.

Plant peroxidases have been implicated in a wide range of physiological processes, such as lignin formation, respiration and light-mediated processes to mention a few. In addition, peroxidase activity have also been correlated with plant defense against various pathogens and play a key role in the cell wall building process (Pegg, 1988; Sela-Buurlage *et al.,* 1993; Yeh *et al.,* 2000). Peroxidase has been proposed to enhance resistance through construction of a cell wall barrier that may slow down pathogen invasion and possible spread (Velazhahan *et al.,* 2010). However, peroxidase involvement as defense mechanism in this study was not significant.

Several studies have reported that the expression levels of β-1,3-glucanase increased after infection with pathogens, such as barley infected by powdery mildew (Ignatius *et al*., 1994) and maize infected with *Aspergillus flavus* (Lozovaya *et al*., 1998) to mention a few.

β-1,3-glucanase are abundant in maize and have been characterized from a wide range of species. In an invitro study, Liang *et al*. (2005) reported that β-1,3-glucanase activity increased after the inoculation of seeds with *A. flavus.* In this study, β-1,3-glucanase activity was induced at early stages of plant development and declined as plants reached maturity. Similar to that of chitinase activity, β-1,3-glucanase activity was significantly higher during the 70 and 95 DAP in the 2012 season. These DAP correspond to silk and milk stages respectively and is an indication of a plant defence response due to fungal infection and/or fumonisin production. It is not clear at this stages why β-1,3-glucanase expression varied amongst plant densities and this should be investigated further. The interaction between DAP and cultivar in the 2012 season, showed that β-1,3-glucanase expression was the lowest at 123 DAP for both PAN6P-110 and CRN3505. This could be co-incidental, as a decline in β-1,3-glucanase expression could be observed as maize plants mature. At the soft dough stage, grain mass continue to increase and sugars are converted to starch, therefore the plant is not as reliant on obtaining nutrients from leaves. At this stage, leaves start to senescence and therefore metabolic processes slow down. In future studies, maize ears can be inoculated from silk to milk stage and PR proteins can be sampled directly from maize ears. This will give insight into the expression of PR proteins during plant growth stages and optimize sampling procedures to obtain more accurate information.

β-1,3-glucanase involvement as defense mechanism in this study was not significant in the 2013 season and this may be due to the gradual depletion of soil nutrients and the additive effect of low levels of available leaf nutrients. Similar to chitinase expression, there was a weak inverse correlation between β-1,3-glucanase activity and fumonisin levels in the 2013 season, where an increase in β-1,3-glucanase activity lead to a decrease in fumonisin levels.

Significant findings in this study confirmed that photosynthetic performance (PI_{abs}) can be used to measure plant stress due to plant density with careful consideration to the sampling stage of plant maturity. The increase of PR proteins during critical infection stages of the maize plant (silk and milk) is a significant finding, as maize ears are susceptible to fungal infection and fumonisin production can occur as soon as fungal infection commenced. It was unexpected though, that available leaf nutrients as well as PR proteins did not impact fungal infection, but fumonisin levels. It was demonstrated

from the correlations, that an increase in the available leaf nutrients (N, S and C) as well as PR proteins (chitinase and β-1,3-glucanase) decreased fumonisin levels. Janse van Rensburg, *et al.* (2015) reported high target DNA (*F. verticillioides*) with low fumonisin content and low target DNA with high fumonisin content at certain localities/seasons/cultivars. *F. verticillioides* isolates differ in their ability to produce fumonisins, and fumonisin synthesis is often influenced by substrate (Schoeman *et al*., 2016). It was shown that different maize cultivars in South Africa have varying degrees of resistance to either fungal infection or fumonisin accumulation (Janse van Rensburg *et al*., 2015). Currently, an integrated approach is taken to manage fungal infection and subsequent fumonisin production in maize grain. Even though fungal infection can be managed, fumonisin production can be unpredictable due to genotype, environment and substrate. Chitinase and β-1,3-glucanase response to fumonisins in this study can be used in breeding programmes to improve resistance to specifically fumonisin production in maize grain.

Furthermore, available leaf nutrients can play a vital role in plant health and the ability of maize plants to activate defence responses to fungal infection and in this study, fumonisin production. Results from this chapter included a two year study with a decrease in soil nutrients. It is imperative that research continues using trials that represent commercial and developing farmer's fields. The gradual decline in soil nutrients obscured some effects and therefore responses of available leaf nutrients and PR proteins regarding fumonisins should be investigated in maize plants cultivated with adequate soil nitrogen.

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