Fusarium graminearum mycotoxins associated with grain mould of maize and sorghum in South Africa

by

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DECLARATION

'I, Mudzuli Mavhunga, declare that the dissertation hereby submitted by me for the degree of Magister Scientiae Agriculture at the University of the Free State is my own independent work and has not previously been submitted by me at another University/Faculty. I cede copyright of this dissertation to the University of the Free State.'

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PREFACE

This dissertation consists of four chapters. The main objective of this study was to investigate the presence of the *Fusarium graminearum* species complex (FGSC) and associated mycotoxins in maize and sorghum grain in South Africa.

The first chapter is a literature review of *F. graminearum* mycotoxins associated with ear rot of maize (Figure 1) and grain mold of sorghum (Figure 2) in South Africa. In this chapter, an overview of the genus *Fusarium* is provided, followed by an in-depth look at *F. graminearum*, with special reference to species concepts, host range, sources of inoculum and dispersal. Symptoms in maize and sorghum grain, the economic impact, mycotoxins and human and animal health perspectives are addressed. Methods for mycotoxin analysis are also presented and regulations for both the local and international arenas are discussed.

Chapter 2 deals with the morphological and molecular detection, identification and quantification of the FGSC. Microscopy, species-specific polymerase chain reaction (PCR) and sequencing were used to identify members of the FGSC and other *Fusarium* spp. isolated from maize and sorghum grains. Quantitative real-time PCR was used for simultaneous detection and quantification of FGSC deoxyribonucleic acid (DNA) in maize and sorghum grain samples.

In chapter 3, the mycotoxins and concentrations produced by the FGSC in maize and sorghum grain were evaluated. Enzyme linked immunosorbent assays (ELISA) was used to screen all the maize and sorghum grain samples for deoxynivalenol (DON) and zearalenone (ZEA) using commercial kits. Since this technique was questioned subsequent to the study being initiated, liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays for DON, nivalenol (NIV) and ZEA were carried out on selected grain samples collected over the three year period.

The occurrence of trichothecene chemotypes among the FGSC isolates are presented in Chapter 4. To assist in identification of isolates to species level, sequencing of the translation elongation factor 1-α (*TEF*1-α) and ammonia ligase (*URA*) genes was conducted. Isolates of the NIV-, DON- and 15-acetyldeoxynivalenol (15-ADON) chemotypes were identified using multiplex PCR targeting the *Tri*6 and *Tri*12 genes.

Figure 1. Maize ear rot (Photo by Prof. B.C. Flett)

Figure 2. Grain mold of sorghum (Photo by Prof. N.W. McLaren)

LIST OF ABBREVIATIONS AND SI UNITS

ARC	Agricultural Research Council
ARC-GCI	Agricultural Research Council-Grain Crops Institute
ARC-PPRI	Agricultural Research Council-Plant Protection Research Institute
ARC-OVI	Agricultural Research Council-Onderstepoort Veterinary Institute
a_w	water activity
bp	base pair
CAST	Council for Agricultural Science and Technology
CLA	carnation leaf agar
cm	centimetre
°C	degree celsius
CTAB	hexadecyltrimethyl ammonium bromide
DNA	deoxyribonucleic acid
DON	deoxynivalenol
EDTA	ethylene diamine tetraacetic acid
*TEF*α	translation elongation factor alpha
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FAO	Food and Agricultural Organisation of the United Nations
g	gram
HPLC	high performance liquid chromatography

ITS	internal transcribed spacers
kg	kilograms
L	litre
LC-MS-MS	liquid chromatography tandem mass spectrometry
M	molar
mg	milligram
ml	millilitre
mM	millimolar
MMB	minimal medium broth
NaOH	sodium hydroxide
ng	nanogram
NIV	nivalenol
nM	nanomoles
PCR	polymerase chain reaction
PDA	potato dextrose agar
PDB	potato dextrose broth
pg	picogram
PPA	modified nash snyder medium
ppb	parts per billion
ppm	parts per million
qPCR	quatitative real-time polymerase chain reaction
SAGIS	South African Grain Information Services
SNA	Spezieller Nährstoffarmer agar

TES	trace element solution
TAE	tris acetic acid ethylene diamine tetraacetic acid
µl	microlitre
µg	microgram
UV	ultra violet
WA	water agar
ZEA	zearalenone

CHAPTER 1
A REVIEW OF *FUSARIUM GRAMINEARUM* ASSOCIATED WITH EAR ROT OF MAIZE AND GRAIN MOULD OF SORGHUM

1.1. INTRODUCTION

1.1.1. Maize and sorghum production in South Africa

Maize (*Zea Mays* L.) and sorghum (*Sorghum bicolor* (L.) Moench) are amongst the world's top five most important cereal crops, including wheat (*Triticum* spp.), rice (*Oryzae sativa* L. or *O. Glaberrima* Steudel) oats (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) (Reischer *et al.,* 2004; Strange & Scott, 2005; Nicolaisen *et al.,* 2009). With the exception of Antarctica, maize is produced on all continents, given sufficient heat and water. The annual world crop production of cereals was reported to exceed 2 billion tons (Eskola, 2002). This figure is increasing due to the increased demand for food throughout the world. Production of cereal grain crops is greater in developed than in developing countries, despite the fact that the former are self-sustaining. It is estimated that 10-30% of the harvest from the millions of hectares cultivated annually, is lost due to fungal infections (Eskola, 2002; Munkvold, 2003).

Within the Southern African Development Community (SADC), South Africa is the primary maize producer, with over 9000 commercially recognised maize producers and millions of small-scale subsistence producers. According to Akpalu *et al.* (2008) nearly 50% of the required maize supply for the SADC region is sourced from South Africa. Furthermore, South Africa is listed among the top 20 maize producing countries worldwide by the Food and Agriculture Organisation (FAOSTAT, 2004).

The most important grain crop cultivated in South Africa is maize which also serves as the major feed grain and staple food for the majority of the South African population. Commercial production of maize focuses primarily on meeting the demands of both the feed and food industries (du Plessis, 2003) while thousands of subsistence farmers' health and wealth depend on the quality of the maize they produce (Fandohan *et al.,* 2003). Production

of maize is encouraged due to the high yields per hectare, ease of cultivation and adaptability to different agro-ecological zones (Fandohan *et al.,* 2003).

Annual maize yields vary considerably due to fluctuations in seasonal precipitation (du Toit *et al.,* 2002). This crop contributes up to 70% of total grain production in South Africa. Production is done on over 60% of South Africa's arable area. Maize production is divided into two primary areas, namely the dry western area (i.e. western Free State and North West Provinces) and the wet eastern area (i.e. eastern Free State, Gauteng, Mpumalanga and KwaZulu-Natal Provinces). In South Africa, both white (WM) and yellow maize (YM) are produced. White maize is produced primarily for human consumption while both white and yellow maize can be used for animal feed. Most of the maize produced in South Africa is consumed locally, with at least 50% being used for human consumption, 40% in the animal feed industry and the remainder being used for seed and industrial purposes (Maize Market Value Chain, 2010-2011).

Sorghum is the fifth most important cereal crop cultivated worldwide after maize, wheat, rice and barley (FAO, 2004). Worldwide, approximately 70 million tons of grain is produced from about 50 million ha of land (NDA, 2010). In Africa, approximately 20 million tons of sorghum is produced annually. Although many countries produce sorghum as both a feed and forage crop (Audilakshmi, 1999), Africa continues to retain this crop as an important food source. Surpluses are used to supplement the needs of the feed industry and some are sometimes exported (Sorghum Section 7 Committee, 2007).

After maize and wheat, sorghum is the third most important grain crop produced in South Africa (du Plesssis, 2008) but contributes only a small percentage of the total domestic grain crop. According to the Grain Sorghum, Market Value Chain Profile 2010-2011 report, approximately 182 000 tons of the total sorghum produced in South Africa is used for human consumption while 43 000 tons is used for animal feed production. In South Africa, sorghum is used in the manufacturing of a variety of foods and beverages, including bread, porridge, beer and other non-alcoholic beverages. However, this grain has to compete directly with maize based foods such as maize meal and other grains such as rice. In the 2008/09 production years, 62% of South Africa's total sorghum was produced in the Free State Province while in Mpumalanga and Limpopo Provinces, 24% and 8% respectively were produced. In the same season, only 5% and 1% of the total production occurred in the North

West and Gauteng Provinces, respectively (Grain Sorghum, Market Value Chain Profile 2010-2011).

Cultivation of sorghum generally takes place in drier areas, with shallow and heavy clay soils. These include the drier heavy clay areas of the North-West Province, the western Free State and the Limpopo Province, as well as the south-eastern production areas of Mpumalanga where heavier clay soil is found. Recently, commercial production of sorghum has begun to shift from the drier western production areas to the wetter eastern areas. This change is the result of the identification and development of cultivars which are more tolerant to lower temperatures (Pannar Seed, 2006). Sorghum production is suited to many of the arable areas on the Highveld as this crop has the ability to withstand high temperatures and drought (Onyike & Nelson, 1992). Comparisons of South African plantings, production and annual yields for maize, sorghum and wheat from 2006/07 to 2010/11 are presented in Table 1.1.

1.1.2. Maize and sorghum grading in South Africa

Grading of local maize is guided by specifications detailed in the Government Gazette No. 19131 dated 14 August 1998 (www.nda.agric.za). Adherence to these regulations determines the grade, price and subsequent use of the grains i.e. whether the maize is fit for human or animal consumption or is to be totally rejected. Grading is done on the basis of visual assessment of the percentage of mouldy, discoloured and broken kernels as well as the presence of foreign matter (Rheeder *et al.,* 1995). Three classes of maize exist in South Africa, namely Class White Maize (WM), Class Yellow Maize (YM) and Class Other Maize. Maize of the Class White Maize is graded as WM1, WM2 or WM3, Class Yellow Maize is be graded as YM1, YM2 or YM3 while no grades are determined for Class Other Maize. The best quality of maize is graded the highest, namely WM1 or YM1.

Grain sorghum is graded according to its malting and fodder qualities as well as its tannic acid content. There are currently three classes used, namely: GM, GL and GH (Government Gazette No. 31042, 2008) as indicated in Table 1.2.

1.2. THE GENUS *FUSARIUM*: OVERVIEW AND TAXONOMY

The genus *Fusarium* was introduced in 1809 by Link (Nelson, *et al.,* 1981) and contains species that are ubiquitous in nature (Nelson *et al.,* 1983; Logrieco *et al.,* 2003). It is a large group of filamentous fungi that is found in the air, soil, in association with plants and on occasion with humans. Some of the most important plant pathogenic fungal species known today are members of this genus. Worldwide, a substantial number (~81) of economically important plant species are believed to be susceptible to at least one or more *Fusarium* spp. (Leslie & Summerell, 2006). Fungi now included in the genus *Fusarium* were originally described and defined as *Fusisporium* based on the type *Fusisporium roseum* described by Link in 1809 (Summerell *et al.,* 2010). Wollenweber & Reinking (1935) reclassified the two *F. roseum* type specimens as *F. sambucinum* and *F. graminearum*, with *F. sambucinum* now being accepted as the type species for the genus. Although the taxonomy of *Fusarium* continues to undergo major changes, especially on the basis of molecular classifications, the Wollenweber and Reinking classification system continues to form the foundation on which species are described (Leslie & Summerell, 2006).

Members of the genus *Fusarium* are characterised by the production of septate, hyaline, delicately curved, elongate macroconidia (Moss & Thrane, 2004; Leslie & Summerell, 2006). Mycelia and spore masses are generally brightly coloured (Booth, 1971). In some species, smaller 0 to 1 septate microconidia and chlamydospores are common, while some authors recognize a third conidial type known as mesoconidium. In addition to their disease causing ability, many *Fusarium* spp. produce an array of mycotoxins (toxic secondary metabolites) that are associated with plant, animal and human diseases (CAST, 2003; Desjardins, 2006).

1.3. FUSARIUM AND ASSOCIATED MYCOTOXINS: RESEARCH IN SOUTH AFRICA

Fusarium spp. are important pathogens of cereal grain crops such as maize, wheat, oat, barley and sorghum (Lysøe *et al.,* 2006; Schollenberger *et al.,* 2006). These fungi are known as field fungi that require high moisture levels to colonise and infect grain (Placinta *et al.,* 1999; Gale *et al*., 2002). At any stage during plant development, *Fusarium* spp. can cause seedling, root and crown rot as well as stalk and ear rot (Marasas *et al.,* 1981; Rheeder *et al.,* 1992;

Cotten & Munkvold, 1998). Cereal crops infected with *Fusarium* spp. are often characterized by reduced grain quality and yield losses (Nelson *et al.,* 1981). Members of the genus are not only of agricultural importance as infected grain can also be contaminated with a variety of toxic secondary metabolites (mycotoxins). Some of these secondary metabolites can be toxic to man and/or animal and are therefore referred to as mycotoxins.

In South Africa, the natural occurrence of *Fusarium* spp. has been well documented over the past decades (Nelson *et al.,* 1981; Rheeder *et al.,* 1992; Rheeder & Marasas, 1998). The main *Fusarium* spp. associated with maize production worldwide are, *F. graminearum* Schwabe [teleomorph = *Gibberella zeae* Schwein. (Petch)] [hereafter refered to as *F. graminearum sensu lato* (*s.l.*)]; *F. verticillioides* (Saccardo) Nirenberg, [synonym = *F. moniliforme* J. Sheldon], teleomorph = *G. fujikuroi* (Sawada) Ito in Ito & K. Kimura]; *F. proliferatum* (T. Matsushima) Nirenberg ex Gerlach & Nirenberg and *F. subglutinans* (Wollenw. & Reinking) P.E. Nelson, T.A. Toussoun, & Marasas [(teleomorph = *G. subglutinans* (E. Edwards) P.E. Nelson, T.A. Toussoun, & Marasas)] (Rheeder *et al.,* 1995; Desjardins, 2006). The most common fungal species associated with sorghum grain worldwide include *F. pseudograminearum*, *F. chlamydosporum, F. equisetti, F. nygamai*, *F. verticillioides*, *F. thapsinum*, *F. graminearum s.l.* and *F. semitectum* (Onyike & Nelson, 1992; Lefyedi *et al.,* 2005).

South Africa has a long and reputable history of mycotoxin research. Over 100 mycotoxins have been identified, making South Africa a world leader in mycotoxins research (Gelderblom *et al.,* 1988; Dutton, 2003). The focus of the research has been primarily on detection and identification of different mycotoxins in food commodities. Another important focus area was the determination of the effects of these mycotoxins on animal and human health (Dutton, 2003). Earlier studies included work on such mycotoxins as the fumonisins and aflatoxins (Marasas *et al.,* 1979). These toxins are highly toxic and carcinogenic to farm and experimental animals and have been implicated in human oesophageal cancer and birth defects (Leslie & Summerell, 2006). Due to the prevalence of many chronic diseases of humans and animals in South Africa, research began to focus on the relationship between such diseases and the consumption of mycotoxin-contaminated foods and feed (Marasas *et al.,* 1979; Marasas *et al.,* 1981; Marasas, 2001; Dutton, 2003). This led to suggestions of a possible link between the consumption of *Fusarium*-contaminated maize and/or home-brewed beer made from highly infected grains (and in particular fumonisins contaminated

grain) and oesophageal cancer in rural Eastern Cape Province of South Africa. Following such findings, more publications were released as the fumonisins were constantly found in contaminated maize based meals and beverages consumed by residents of this area (Gqaleni *et al.,* 1997).

By the onset of the 1980's, the South African Maize Board had begun annual mycological surveys of commercial maize. Research was able to reveal that the mycotoxin challenge in South African cereal grain commodities was similar to that of other countries. Viljoen (2003) reported that there were good data for maize while that of other local grains was lacking.

1.4. THE PATHOGEN - FUSARIUM GRAMINEARUM

F. graminearum s.l. was first described as *Phaeria zeae* by Fries in 1822. It was later renamed by Schwabe in 1838 to its present name and linked to its teleomorphic state in 1936 by Petch (Booth, 1971; Nelson *et al.,* 1981). Based on the sexual stage, *F. graminearum s.l.* can be classified as follows: Superkingdom Eukaryota; Kingdom Fungi; Phylum Ascomycota; Subphylum *Pezizomycotina*; Class *Sordariomycetidae*; Subclass *Hypocreomycetidae*; Order *Hypocreales*; Family *Nectriaceae*; Genus *Gibberella* (Goswami & Kistler, 2004). *F. graminearum s.l.* is a member of the *Fusarium* section Discolor which contains some of the world's most important cereal crops pathogens (Booth, 1971). The section Discolor is morphologically distinguished from other *Fusarium* sections by the production of chlamydospores, absence of microconidia and may also be characterized by thick-walled, distinctly septated macroconidia (Leslie & Summerell, 2006). Fungi in the section Discolor are known to cause seedling blights, pre- and post-emergence damping off, crown rot, head blight (scab), grain mold and cob rot of cereal grains (Booth, 1971). Worldwide, *F. graminearum s.l.* is an important pathogen of maize, sorghum and other cereal grain crops. In an effort to generate strategies for the control and management of this pathogen in agriculture, research continues to focus on all aspects of its life cycle, particularly on infection, colonization and overwintering mechanisms (Goswami & Kistler, 2005).

1.4.1. Species concepts in *Fusarium graminearum*

Species concepts define criteria through which species can be recognized and create the basis upon which the species can be differentiated from one another. In *Fusarium*, three different species concepts are recognised, namely morphological, biological, and phylogenetic species concepts (Summerell *et al.,* 2003). The fungus *F. graminearum s.l.* was initially split into two taxa, namely *F. graminearum* Group 1 and *F. graminearum* Group 2. This split was done on the basis of fertility (heterothallic vs. homothallic), disease association, (Leslie & Summerell, 2006) and at a later stage on the basis of phylogenetic differences (Aoki & O'Donnell, 1999a). The heterothallic strains were classified as Group 1 and are commonly associated with crown rot of wheat, barley, triticale, oats and grasses while Group 2 strains are homothallic and associated with stalk and cob rot of maize, head blight or scab of wheat as well as stub dieback of carnations (*Dianthus caryophyllus* L.). These groups are morphologically identical and thus indistinguishable on the basis of macroconidium or conidiophores (Burgess *et al.,* 1988). However, Aoki & O'Donnell (1999a, b) used molecular techniques to assign the name *F. pseudograminearum* (teleomorph = *Gibberella coronicola*) to Group 1 strains while Group 2 strains retained the names *F. graminearum s.l.* (teleomorph = *Gibberella zeae*). Leslie & Summerell (2006) give comprehensive descriptions of both *F. pseudograminearum* and *F. graminearum s.l.*, providing sufficient morphological characters to distinguish the two.

1.4.1.1. Morphological species concept

Morphological species concept is based on the idea that the morphology of a "type" or individual represents the variation present within an entire species (Leslie *et al.,* 2001). The genus *Fusarium* contains species that are highly variable genetically and in the environments in which they grow, resulting in morphological changes (Nelson *et al.,* 1983). Physical and physiological characters have been used to distinguish *Fusarium* spp. (Leslie *et al.,* 2001), however, the number of readily detectable characters within the genus is far smaller than the number of species that need to be distinguished (Leslie & Summerell, 2006). The Gerlach & Nirenberg (1982) and Nelson *et al.* (1983) taxonomic systems are based on morphological characterisation of *Fusarium* spp. Both systems are the foundation on which the biological and phylogenetic species concepts in *Fusarium* are laid. These systems are used by many Fusarium specialists as the basis for identifying *Fusarium* spp. and describing new taxa

(Leslie *et al.,* 2001). The most important characteristics used in identification of *Fusarium* spp. are the shape of the macroconidia as well as the presence or absence of microconidia and chlamydospores (Booth, 1971; Leslie & Summerell, 2006). Since the shape and size of macroconidia can be influenced by environmental factors in which they are produced (Leslie *et al.,* 2001), possible confusion and misidentification of species can take place. Morphology alone is currently not sufficient for descriptions and definitions of species within the genus *Fusarium* (Leslie & Summerell 2006).

1.4.1.2. Biological species concept

The biological species concept considers species as groups of populations that actually or potentially interbreed with each other (Leslie & Summerell, 2006). *F. graminearum s.l.* is a haploid and homothallic ascomycetous fungus. All *F. graminearum s.l.* isolates possess two mating type idiomorphs, namely *MAT1-1* and *MAT1-2* (Miedaner *et al.,* 2008). Sexual outcrossing has also been observed in culture (Bowden & Leslie, 1999; Lee *et al.,* 2008) and genetic analysis of pathogen populations suggests that outcrossing does occur in nature, though at a much slower rate (Goswami & Kistler, 2004).

F. graminearum s.l. must constantly adapt to changes in the environment it inhabits in order to survive. Evolutionary changes within pathogen populations occur through several mechanisms such as mutations, mating systems, gene flow or migration, population size and selection (Cumagun *et al.,* 2004). These changes along with changes in the agricultural ecosystems (i.e. the use of resistant cultivars, fungicides, fertilizers as well as irrigation and crop rotation practices) impose a strong directional selection on all pathogen populations (McDonald, 1997) and as such contribute to changes in the population structure of a pathogen. According to Leslie & Bowden (2008), all individuals within the *F. graminearum s.l.* population are potential partners and therefore there is no mating restriction, allowing for recombination to occur.

1.4.1.3. Phylogenetic species concept

The genome size of *F. graminearum s.l.* is typical of filamentous fungi (36.1 MB) and contains genes encoding 13,937 predicted proteins which are distributed over four chromosomes (Trail, 2009). At least 2001 of these genes are not similar to those of any other sequenced organism (orphans) and 5812 have homology to proteins of unknown function. This genome contains fewer high identity duplicated sequences in comparison with the genome sequences of other filamentous ascomycetes (Cuomo *et al.,* 2007).

Until recently, *F. graminearum s.l.* was thought to represent a single cosmopolitan species based on morphological species recognition (Booth, 1971; Nelson *et al.,* 1983; Leslie & Summerell, 2006). According to phylogenetic species concept, *F. graminearum s.l.* now comprises at least 15 cryptic species that are biogeographically and phylogenetically distinct to form what is currently referred to as the *F. graminearum* species complex (FGSC) (O'Donnell *et al.,* 2000; 2004; 2008; Starkey *et al.,* 2007; Yli-Mattila *et al.,* 2009; Boutigny *et al.,* 2011; Davari *et al.,* 2012). These species (where available, the corresponding lineage numbers are given in brackets) are: *F. austroamericanum* T. Aoki, Kistler, Geiser et O'Donnell (lineage 1); *F. meridionale* Aoki, Kistler, Geiser et O'Donnell (lineage 2); *F. boothii* O'Donnell, T. Aoki, Kistler et Geiser (lineage 3); *F. mesoamericanum* T. Aoki, Kistler, Geiser et O'Donnell (lineage 4); *F. acaciae-mearnsii* O'Donnell, T. Aoki, Kistler et Geiser (lineage 5); *F. asiaticum* O'Donnell, T. Aoki, Kistler et Geiser (lineage 6); *F. graminearum s.s.* Schwabe-Flora Anhaltina (lineage 7); *F. cortaderiae* O'Donnell, T. Aoki, Kistler et Geiser, (lineage 8); *F. brasilicum* T. Aoki, Kistler, Geiser et O'Donnell (no lineage number); *F. aethiopicum* O'Donnell, Aberra, Kistler et T. Aoki (no lineage number); *F. gerlachii* T. Aoki, Starkey, Gale, Kistler, O'Donnell (no lineage number); *F. vorosii* B Toth, Varga, Starkey, O'Donnell, Suga et T. Aoki (no lineage number); *F. ussurianum* T. Aoki, Gagkaeva, Yli-Mattila, Kistler, O'Donnell, (no lineage number); *F. louisianense* Gale, Kistler, O'Donnell et T. Aoki (no lineage number) and *F. nepalense* T. Aoki, Carter, Nicholson, Kistler & O'Donnell (no lineage number). According to Desjardins & Proctor (2011), each of these species may differ significantly in aggressiveness and in mycotoxin production.

The splitting of *F. graminearum s.l.* into separate species is met with cynicism by some *Fusarium* researchers around the globe and remains a controversial classification system that

promotes the adoption of contradicting opinions amongst renowned *Fusarium* researchers (Trail, 2009). Leslie & Bowden (2008) for example, using strains developed by Lee *et al.* (2003), were able to successfully cross strains from ten lineages within the FGSC with *F. graminearum s.s.* As a result, they concluded that this outcrossing served as an indication of intra-species variation rather than a confirmation of differences in species. They suggested that these phylogenetic lineages need to be evaluated at both morphological and biological levels to ascertain their current elevation to species within the genus *Fusarium*. Thus, some *Fusarium* experts are not entirely convinced that the use of phylogenetic species concept alone for species definition provides sufficient information to elevate strains to species level.

1.4.2. Host range

Members of the FGSC are known to infect a wide range of plant hosts in both temperate and subtropical regions (Karugia *et al.,* 2009). They are found either as pathogens or secondary invaders (Lysøe *et al.,* 2006) of among other crops, maize, sorghum, wheat, barley, rice, rye (*Secale cereale* L.) and oats (Gilbert & Tekauz 1999; Desjardins *et al.,* 2004; Goswami & Kistler, 2004; Tesso *et al.,* 2004; Schmale *et al.,* 2005). These fungi are also commonly known as the primary aetiological agents of fusarium head blight (FHB) of wheat, barley and rye in for example Australia (Akinsanmi *et al*., 2006), Canada (Gilbert *et al.,* 2008), the Netherlands (Waalwijk *et al.,* 2004), South Africa (Boutigny *et al.,* 2011; Lamprecht *et al*., 2011) and the USA (Brennan *et al.,* 2005). Over the years, the host range of the FGSC has expanded from cereal to non-cereal crops such as dry bean, canola, potato and soybean (Goswami & Kistler, 2004; Burkaloti *et al.,* 2008). Members of the FGSC cause stalk rot and Gibberella ear rot (or pink mold) of maize, seedling blight and stalk rot of sorghum (Vigier *et al.,* 1997; Zeller *et al.,* 2003; Goswami & Kistler, 2004) as well as root rot of cereals (McMullen *et al.,* 1997). Up to 30-70% of crop yield can be lost due to FGSC linked diseases (Waalwijk *et al.,* 2003).

Cereal grain diseases caused by members of the FGSC are important in South Africa, both for commercial and subsistence farming systems (Marasas *et al.,* 1981). Previous reports seemed to suggest that *F. graminearum s.l.* was no threat to maize and sorghum production within South Africa (Viljoen, 2003) as the pathogen was found only in lower frequencies in maize producing areas of South Africa. However, a recent study (Boutigny *et al*, 2011) has shown *F. graminearum s.l.* to be on the increase in South African maize production areas.

Continued monitoring of the FGSC in cereal grains within South Africa is essential in developing management strategies since infected grains can be contaminated with mycotoxins such as deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA). Waniska (2000) reported that maize was more prone to contamination with mycotoxins than sorghum due to the differences in fungal species that colonise these crops. However, *F. graminearum s.l.* is a pathogen of cereal crops worldwide. The supply of high quality, healthy maize and sorghum grains is a national priority for South Africa since these commodities represent the staple food of many South Africans.

1.4.3. Sources of inoculum, dispersal and survival

F. graminearum s.l. produces two forms of inoculum, namely sexual spores (ascospores) and asexual spores (macroconidia) (Beyer *et al.,* 2004; Gilbert & Fernando, 2004). Both these forms are important in disease development in the field (Burlakoti *et al.,* 2008) and can cause significant infections under favourable conditions (Beyer *et al.,* 2004). Sexual development of *F. graminearum s.l.* is especially important in both dispersal and initiation of disease (Goswami & Kistler, 2004). Macroconidia can be spread by splash dispersal during rainy seasons or distributed by insect vectors (Beyer *et al.,* 2004). Ascospores are forcibly discharged into the air and dispersed primarily by wind currents (Xu, 2003). *G. zeae* ascospores have been found to cause disease epidemics over large distances (Bentley *et al.,* 2008). Figure 1.1 illustrates a simplified life cycle of *F. graminearum s.l.* on cereal grain crops.

Asymptomatic inflorescences of wild grasses that surround cultivated fields have been found to carry the pathogen (Gilbert & Fernando, 2004). Crop residue such as maize stalks, as well as wheat and sorghum straw/stubble serves as the primary source of inoculum for infections in field crops (Gale *et al.,* 2002; Munkvold, 2003). *F. graminearum s.l.* can enter the host tissue through insect and/or bird wounds (Reid *et al.,* 2002; Wagacha & Muthomi, 2008), however, the silk-channel is the most important pathway for maize ear rot infections (Reid *et al.,* 2002).

1.4.4. Disease symptoms in maize

Symptoms of Gibberella ear rot of maize are affected in most part by the presiding moisture levels during silk emergence and prevalence is increased with wet weather later in the season (Miller, 1995). Infection often starts at the tip of the ear, spreading to the base of the ear, resulting in subsequent kernel discolouration (du Toit & Pataky, 1999; Reid *et al.,* 2002). Young maize ears are more susceptible to infection than mature ears (Reid & Sinha, 1998). Infections of the stalk, leaves and the roots also occur (Mansfield *et al.,* 2005). Gibberella ear rot symptoms are characterised by pinkish red discolourations of infected kernels or grain that spread from the tip of the ear downward or outwards from an insect wound (du Toit & Pataky, 1999; Reid *et al.,* 2002). Stalk rot symptoms on the other hand also show the characteristic pink-to-red discolouration of the pith tissues as well as internal shredding of the lower internodes (Reid *et al.,* 2002). Moreover, stalk rots, coupled with severe leaf damage, insect and bird damage as well as lodging can aggravate the problems of cob and kernel rots caused by this fungus. The invasion of cobs often results in major yield and quality losses due to kernel discolouration and subsequent damage thereof. In severe cases, the crop cannot be used for feed or seed purposes (Williams & McDonald, 1983).

1.4.5. Disease symptoms on sorghum

Grain mold of sorghum is caused by one or more fungal species from different genera (Navi *et al*., 2005), thus the symptoms of infection are not as distinct as in maize as they are primarily dependent on the fungal species involved as well as the time and severity of infection (Thakur *et al.,* 2006). Grain mold symptoms often manifest as pink, grey, white or black discoloration of the grain surface and yield reduction due to reduced grain size, dry matter accumulation or complete destruction of the grain itself (Williams & McDonald, 1983). Stalk rot symptoms in sorghum are relatively similar to those of maize and are characterised by internal shredding of lower nodes with tan or pink-to-purple internal discoloration (Jardine, 2006).

According to Bandyopadhyay *et al.* (2000), grain mold symptoms between early infections and post-maturity colonization differ considerably (Figure 1.2). Early infection of grain is characterised by pigmentation of the lemma, palea, glumes and lodicules, which is highly cultivar dependent, and may be linked to mechanisms of resistance. Post-maturity

colonization is characterised by a "moldy appearance" of grain when maturing in humid environments (Bandyopadhyay *et al.,* 2000). Severely infected grain is fully covered with mold while partially infected grain may look normal or discoloured. According to Thakur *et al.* (2006), symptoms are more prominent on white grain than in brown or red grain sorghums.

Asymptomatic grain has also been found to produce grain mold fungi when plated on agar media or blotters after surface sterilization (Thakur *et al.,* 2006). Sorghum can also act as an asymptomatic host for the FGSC where perithecia of these fungi have been found in abundance on newly senescent sorghum stalks and on sorghum residues from previous crops (Quazi *et al.,* 2009).

1.4.6. Economic impact of FGSC related diseases

Members of the FGSC can infect many plant parts during the life cycle of the host, resulting in a wide range of diseases (Carter *et al.,* 2002). Infections may affect both the physical and physiological aspects of seed quality, including seed size, weight and composition (Argyris *et al.,* 2003). Worldwide, economic and crop losses are attributed to FGSC species. During the 1990's, losses in North America resulting from FHB on wheat and barley exceeded US $3 billion (Gale *et al.,* 2007) while between 1998 and 2000, at least nine US states lost close to US $870 million due to FHB (Voigt *et al.,* 2005). Through this period alone, direct and indirect economic losses assessed for all crops were estimated at US $2.7 billion (Goswami & Kistler, 2004). Moreover, increases in reports of *F. graminearum s.l.* outbreaks are an indication that the FGSC is becoming an increasing threat to grain production within Asia, Canada, Europe and South America (Yli-Mattila *et al.,* 2009).

Several fungal species in the genera *Alternaria, Bipolaris, Curvularia, Colletotrichum, Fusarium* and *Phoma* have been reported to be associated with grain mold of sorghum. Of these, *Fusarium* spp. are dominant within the sorghum grain mold complex (Sharma *et al.,* 2011). The unfavourable effects on yield and quality caused by these fungi in sorghum include complete destruction of the grain, severe grain discolouration, reductions in size and weight of grain, reduction in market value, reduction in nutritional value, the production of mycotoxins, vivipary, the loss of seed viability and subsequent seedling mortality (Williams & McDonald, 1983; Thakur *et al.,* 2006). The International Crops Research Institute for the

Semi-Arid Tropics (ICRISAT) estimates that at least US $130 million loss occurs due to grain mold of sorghum in Africa and the semi-arid tropical areas of Asia (Bandyopadhyay *et al.,* 2000). In highly susceptible cultivars, yield losses can reach 100% (Ibrahim *et al*., 1985).

1.4.7. Mycotoxins

Coined in 1962, the word mycotoxin was derived from the Greek word 'mykes', meaning mold, and "toxicum" meaning poison. Several fungal species (Table 1.3), including *Acremonium*, *Alternaria, Aspergillus*, *Claviceps, Fusarium* and *Phomopsis* are prominent producers of a wide range of toxigenic metabolites (Švábová & Lebeda, 2005). All mycotoxins are low molecular weight, non-enzymatic, secondary metabolites produced by filamentous fungi (Bennett & Klich, 2003). They can be enclosed in spores and mycelium, or may be excreted, as exotoxins, into the substrates (foodstuff) on which fungal growth occurs. The negative effects of mytoxins in cereal grains are reduction in grain weight and quality, reduced crop yields and subsequently major economic losses, (Ciegler & Bennett, 1980; Eskola, 2002; Zinedine *et al.,* 2006). Over the ages, mycotoxins have been shown to exhibit four basic kinds of toxicoses towards humans and animals, namely acute, chronic, carcinogenic and teratogenic (Pitt *et al*., 2000; CAST, 2003), leading to abnormalties in plant, animals and humans. These toxic metabolites have been associated with for example Turkey X disease in turkeys, leukoencephalomalacia in horses and rabbits, feed refusal in pigs and death of humans in Kenya (Bennett & Klich, 2003; Murphy *et al*., 2006).

1.4.7.1. Factors affecting mycotoxin production

Mycotoxins are produced under various environmental conditions that support the growth of fungi during production, harvest, storage and food and feed processing (CAST, 2003; Wagacha & Muthomi, 2008). Conditions that predispose grain crops to mycotoxin production include moisture/water activity (aw), substrate temperature, aeration and substrate availability. Furthermore, mycotoxins are generally produced by fungi under stress conditions and later in the life cycle of the fungus. Several other factors also play a role in mycotoxin accumulation in grains, including fungal inoculum, insect damage, mechanical injury, wind, storm and rain, hail damage to crops, crop physiology, nutritional content of the plant, susceptibility of the cultivar and poor storage conditions (Eskola, 2002; Munkvold, 2003). Moisture content and temperature remain the most critical factors affecting both

fungal growth and mycotoxins. Both moisture content and temperature determine and influence the types of fungi that will colonise crops in specific countries (Eskola, 2002). Optimal temperature and water activity required for the production of some mycotoxins by *Aspergillus*, *Fusarium* and *Penicillium* spp. are presented in Table 1.4.

Moderate temperatures and high rainfall during crop maturation are known to favour fungal infections, while heavy wet weather conditions prior to silking, encourage the invasion of developing cobs by *F. graminearum s.l.* (Martins & Martins, 2002). *F. graminearum s.l.* predominates in warmer areas with average temperatures between 24 and 28ºC and high humidity (~80%) (Booth, 1971). Disease development occurs within the range of 15-30°C, and at an optimum temperature of 25°C and a moist period of longer than 16 hours (Beyer *et al.,* 2004). These conditions are ideal for maize silk infection and the spread of *F. graminearum s.s.* into the maize cob (ear or rachis) (Mansfield *et al.,* 2005). When wetness or high moisture events are discontinuous, reductions in infection efficiency have been observed (De Wolf *et al.,* 2003). Frequent rainfall, high humidity, and/or heavy dews that coincide with the flowering and early kernel-fill period of the crop tend to favour infection and disease development (McMullen *et al.,* 1997). Overhead irrigation is highly advantageous to disease development and symptoms frequently occur under centre pivot irrigation. Disease severity is also optimal around the centre of the pivot (Strausbaugh & Maloy, 1986).

1.4.7.2. Trichothecene chemotypes within FGSC

Worldwide, epidemics due to FGSC not only result in yield and quality reduction (Gale *et al.,* 2002) but can lead to contamination of cereal grains with trichothecene mycotoxins, including DON, NIV and ZEA (CAST, 2003; Desjardins, 2006; O'Donnell *et al.,* 2008). Trichothecenes are a large group of more than 200 structurally related sesquiterpenoid metabolites (Reinehr & Furlong, 2003; Kumar *et al.,* 2008). They are produced by a number of fungal genera, including *Fusarium, Myrothecium, Trichothecium, Trichoderma, Stachybotrys, Phomopsis, Cylindrocarpon, Dendrodochium, Hypocrea, Peltaster, Verticimonosporium* and *Cryptomela* (Kumar *et al.,* 2008; Zhou *et al.,* 2008). These mycotoxins can be characterized into four structural groups, namely types A, B, C and D. Type A trichothecenes do not contain a carbonyl group at C-8 in contrast to type B which contains a carbonyl group at C-8. Type C carries a second epoxide group at C-7,8 or C-9,10

while type D trichothecenes carry a macrocyclic ring between C-4 and C-15, represented by roridins and verrucorins (Desjardins, 2006; Zhou *et al.,* 2008).

Fusarium trichothecenes are classified as type A or type B and are synthesized by a complex biosynthetic pathway (Audenaert *et al.,* 2009) that requires the coordinated expression of more than 14 trichothecene (*TRI*) genes (Desjardins *et al.,* 2004). Studies show that NIV is the ultimate product of the trichothecene biosynthesis pathway, while DON is seen as a pathway intermediate (Figure 1.3) (Desjardins, 2006; McCormic *et al.,* 2011). Furthermore, genes involved in the biosynthesis of these products also function in determining the type of chemotype produced by *Fusarium* spp. The division of trichothecene chemotypes as described by Ichinoe *et al.* (1983) resulted into only two chemotypes, namely DON and NIV, on the basis of 8-ketotrichothecene production. Currently, three strain-specific trichothecene profiles (chemotypes) have been identified in the FGSC (Ji *et al.,* 2007), namely DON and 3-acetyldeoxynivalenol (3ADON) chemotype, DON and 15-acetyldeoxynivalenol (15ADON) chemotype and NIV and acetylated derivatives (NIV chemotype). DON and NIV differ only in the C-4 position, where NIV has a hydroxyl group and DON does not. According to Kim *et al.* (2003) no single *F. graminearum s.l.* isolate has been found to produce both these trichothecenes and isolates are described as predominant producers of DON or NIV (Edwards *et al.,* 2002). These chemotypes have been found in different geographic locations (Lee *et al.,* 2002).

The most common representatives of the Type A trichothecenes include T-2 toxin (fusariotoxin), HT-2 toxin, T-2 triol, 15-monoacetoxyscirpenol, diacetoxyscirpenol, neosolaniol and scirpentriol. Type B trichothecenes include DON, NIV, 3-ADON, 15-ADON and fusarenon X (4-acetylnivalenol or 4-ANIV) (CAST, 2003; Logrieco *et al.,* 2003; Jurado *et al.,* 2005; Zhou *et al.,* 2008). The trichothecene gene cluster in FGSC is approximately 27 kb long and contains all the genes involved in the synthesis of trichothecene mycotoxins (Desjardins, 2006).

DON is the most widespread of the trichothecenes and is frequently detected worldwide (Sarlin *et al.,* 2006). More often than not, *F. graminearum s.l.* strains fail to hydroxylate the C-4 position and accumulate this toxin rather than NIV (Desjardins, 2006). The presence of DON in barley, wheat, maize, rye and mixed feeds is documented worldwide (Bennett & Klich 2003). Deoxynivalenol is a non-fluorescent, water-soluble mycotoxin that may be

translocated in the phloem, which may explain why DON produced in the stalk or ear of maize can be found in tissues not invaded by *F. graminearum s.l.* This mycotoxin is known to be produced during the early stages of the infection process in host plants.

Although restricted, the NIV chemotype, which usually produces both NIV and 4-ANIV has been reported in Africa, Asia and Europe but not in North America (Sydenham *et al.,* 1989; Desjardins, 2006; Ji *et al.,* 2007; Yoshida & Nakajima, 2010). NIV is produced by *F. graminearum s.l., F. culmorum, F. cerealis* and *F. poae* (Desjardins 2006).

Trichothecene-producing *Fusarium* spp. are described by Desjardins & Hohn (1997) as 'destructive pathogens' that infect a wide range of plant hosts. The occurrence of these mycotoxins in host tissues suggests that these mycotoxins play a role in the pathogenesis of *Fusarium* spp. on plants. Numerous studies, based on the generation of trichothecene non-producing mutants through the disruption of the *Tri*5 gene have been conducted in order to determine the role of these mycotoxins in disease severity (Desjardins & Hohn, 1997). Harris *et al.* (1999) found that although trichothecene non-producing strains were still capable of infecting host plants, they were less virulent on maize than the trichothecene-producing progenitor and revertant strains. Their observations clearly suggest that trichothecene production can act as a virulence factor in plant pathogenesis.

1.4.7.3. The zearalenones

In addition to the production of trichothecene mycotoxins, FGSC species are also considered the primary producers of ZEA (Marasas *et al.,* 1981; Krska & Josephs, 2001; Desjardins, 2006). Other species complexes that produce ZEA include *F. cerealis* (*F. crookwellense*), *F. culmorum*, *F. equiseti*, and *F. semitectum* (Martins & Martins, 2002; Jurado *et al.,* 2005; Desjardins, 2006). This mycotoxin has been detected in a wide variety of cereal crops, including sorghum, wheat and maize. The co-occurrence of ZEA, DON and NIV is common in cereal crops infected by FGSC (Kazanas, 1984; Placinta *et al.,* 1999; Kim *et al.,* 2005).

Chemically, ZEA is a nonsteroidal, phenolic compound derived by cyclization to form a resorcyclic acid lactone (Desjardins, 2006). Grains contaminated with ZEA normally also contain its associated metabolites α- and β-zearalenol as well as α- and β-zearalanol (Logrieco *et al.,* 2003; EFSA, 2004; Desjardins, 2006). ZEA has been detected in beers from

Lesotho, Swaziland and Zambia (Lovelace & Nyathi, 1977; Okoye, 1987). Bily *et al.* (2004) reported that ZEA is primarily produced at the end of the infection process, however, poor grain storage conditions as well as prolonged moist conditions have been shown to fuel mycotoxin production (Prelusky *et al.,* 1989; Abdulkadar *et al.,* 2004; Lysøe *et al.,* 2006).

The biological potency of ZEA and its metabolites is high, however, with oral LD_{50} values ranging between 2000 and 20,000 mg/kg (ppm) in experimental animals, the actual toxicity associated with consumption of food and feed sources contaminated with these compounds is low. The 50% lethal dose in female rats is reportedly higher than 10,000 mg/kg while in female guinea pigs it is 5,000 mg/kg. Swine are more sensitive to this mycotoxin, with as little as 1 µg/kg (ppb) known to cause detectable uterogenic responses in female swine (Lawlor & Lynch, 2001; Bennett & Klich, 2003). Concentrations between 50 and 100 ppm can interfere with conception, ovulation, implantation, fetal development, and the viability of newborn animals.

Following oral administration, ZEA is transformed into derivatives α- and β-zearalenol and α-and β-zearalanol. It has been suggested that toxicity of ZEA can also be increased through its derivatives, α- and β-zearalenol. Although this mycotoxin is most common in maize, very high levels (11–15 mg/kg) have been found in other cereals such as barley (Magan & Olsen, 2004). These cause hyperestrogenism and reproductive problems in experimental animals (Lysøe *et al.,* 2006). Vulval reddening and/or swelling which may progress to vaginal or rectal prolapse have been observed in pre-pubertal female swine following ingestion of 1-5 mg/kg zearalenone (Vincelli & Parker, 2002). Livestock fed contaminated cereals transmit this mycotoxin into meat and that the amount in meat can be used as an indication of the degree of contamination of the feed, duration of exposure, persistence in the animal as well as species difference in terms of metabolism (Magan & Olsen, 2004). Sáenz de Rodriguez *et al.* (1985) reported that ZEA and/or its metabolite zearalanol were potential causal agents for epidemic precocious pubertal changes in young children in Puerto Rico. The detection of ZEA in feed and food samples could also indicate the possible presence of other fusarial toxins (Bandyopadhyay *et al.,* 2000).

1.4.7.4. Mycotoxin management

In the food industry, strategies used in the control of mycotoxins include monitoring and control of water activity and pH, the two most important environmental factors that preclude mycotoxin accumulation. Contamination of maize with *Fusarium* mycotoxins depends on an interaction between host susceptibility, environmental conditions favourable for infection and in some cases vector activity. Changing the planting date of maize has been found to significantly reduce the risk of mycotoxin accumulation, especially in temperate areas (Munkvold, 2003). Because *F. graminearum s.l.* overwinters in crop residue, the risk of mycotoxins contamination is increased when maize is followed directly by wheat or related cereal crops during rotation practices (Mansfield *et al.,* 2005).

Physical and chemical methods have been used worldwide for decontaminating *Fusarium* mycotoxins in grain, and their degree of success varies greatly. Physical methods include density segregation of contaminated kernels from non-contaminated kernels (using water and saturated sodium chloride or sucrose solution), food-processing practices such as milling, cleaning, washing and baking as well as dilution of contaminated grain with clean grain. Although the latter is not entirely a method of decontaminating grains, this method has been used widely in animal production to reduce the toxicity of feed. It has also been observed that the use of density segregation, milling, cleaning, and baking strategies in decontamination do not completely remove DON and ZEA in flour fractions or whole wheat (CAST, 2003).

Fusarium spp. are known to proliferate and subsequently produce mycotoxins during malting processes (Sarlin *et al.,* 2006). Both ZEA and DON are extremely heat resistant, pH dependent and highly stable in storage and during processing (milling, cooking) (Bennett & Klich, 2003). In South Africa, ZEA has been associated with *F. graminearum s.l*, both in the field grown maize (Aucock, 1980) and in culture (Marasas *et al.,* 1979).

1.4.7.5. Regulations for mycotoxins in food and feed

A largely diverse group of mycotoxins is found as natural contaminants of food and feed sources worldwide. Many of these, including DON, NIV, ZEA, aflatoxins and the fumonisins have been known to cause serious health problems for both humans and animals

(CAST, 2003). The occurrence of mycotoxins in food and feed sources as well as their ability to induce disease has resulted not only in a surge of scientific research but also led to the establishment of control measures for prevention of such contaminants worldwide. Such steps are aimed at improving our understanding of the structures of these mycotoxins, elucidation of their mode of action as well as aspects related to human and animal safety.

Due to the toxicity and adverse effects of mycotoxins, many countries including the USA and Canada have developed guidelines for acceptable levels of contamination for the food and feed industries. At least 99 countries had mycotoxin regulations for food and/or feed in 2003, an increase of approximately 30% compared with 1995 (van Egmond *et al.,* 2007). These regulatory limits have been put in place for aflatoxins B1, total aflatoxins (aflatoxin B1, B2, G1 and G2), aflatoxin M1, some trichothecenes (i.e. DON, T-2, H-T2 and diacetoxyscirpenol (DAS), ZEA, the fumonisins B1, B2 and B3, ergot alkaloids, ochratoxin A, patulin, the phomopsins and sterigmatocystin.

Countries such as Australia, the European Union and New Zealand have several regulations that have been harmonised due to their need for economic trade (Table 1.5). Strict regulations however can cripple international trade if commodities fail to meet the regulatory limits. Millions of South Africans produce cereal crops such as maize and sorghum on a subsistence scale i.e. for household use. As a result, imposing mycotoxins regulations under those conditions, especially for human consumption, remains a major challenge for the law makers within the country. However, by 2003, at least 15 countries on the African continent were known to have regulations for one or more mycotoxins, while many more did not have any regulations in place (FA0, 2004). Mycotoxin contamination of cereals may differ, depending on the state of the grains, i.e raw or processed. According to the regulations for human consumption outlined in the Commision Regulation (EC) No 856/2005 (2005), the maximum acceptable level for DON is 1250 µg/kg in all unprocessed cereals with the exclusion of durum wheat, oats and maize. The EU will soon apply maximum levels for *Fusarium* toxins in unprocessed cereals and cereal products.

In South Africa, advisory regulations have been stipulated for animal feed (Table 1.6) by the Department of Agriculture, Forestry and Fisheries, although there are no limits set for local cereal-based foods. In addition, there is currently no monitoring of locally used maize and maize based products for mycotoxin contamination. There are currently no regulations by

international laws on NIV contents in food and feed products (Pasquali *et al.,* 2010). However, given the potential increased toxicity and synergistic effects of trichothecenes, considerations should be given to developing models for predicting the presence of more than one trichothecene mycotoxin at a given time.

1.4.7.6. Human and animal health perspectives

To date more than 200 trichothecene mycotoxins have been documented, with only a few known to pose a significant threat to both human and animal health (Murphy *et al.,* 2006). *Fusarium* trichothecenes DON, NIV, and T-2 toxin are considered to be the most important in food and feed. Trichothecene mycotoxins are extremely potent inhibitors of protein synthesis in eukaryotes and have been known to interfere with initiation, elongation and termination processes in DNA and RNA synthesis (Bennett & Klich, 2003). These mycotoxins can alter the functioning of the immune system, mitochondria, cell division and affect cell membranes (Zhou *et al.,* 2008). Table 1.7 outlines some of the reported diseases caused by exposure of humans and animals to mycotoxins. When ingested, these mycotoxins can cause acute and chronic diseases in humans and animals, including diarrhoea, weight loss, feed refusal, skin irritation, nausea, vomiting, abortions and neural disturbances. These mycotoxins are also reported to be immunosuppressive (CAST, 2003; Desjardins, 2006; Kumar *et al.,* 2008). In humans, *F. graminearum s.l.* is associated with alimentary toxic aleukia and Akakabi toxicosis which are characterized by nausea, vomiting, anorexia and convulsions (Goswami & Kistler, 2004).

The presence of DON in maize-based feeds such as silage increases the risk of health problems in livestock and is associated with poor performance in animal growth and production (Mansfield *et al.,* 2005). This problem can be further exacerbated by the co-occurrence of DON, NIV and ZEA in contaminated cereal crops (Kim *et al.,* 2005). Furthermore, NIV is believed to be ten times more toxic that DON (Ji *et al.,* 2007). In countries like the Netherlands, Japan and China, NIV occurs more frequently (Waalwijk *et al.,* 2003; Pasquali *et al.,* 2010). This is seen as a major concern in the food and feed industries since *Fusarium* contaminated cereals and cereal products may potentially be contaminated with these and other mycotoxins.

1.5. TECHNIQUES FOR IDENTIFYING *FUSARIUM* SPECIES

1.5.1. Culture techniques

The genus *Fusarium* contains a large group of species that are morphologically similar (or cryptic species) as is the case with the FGSC (Edwards *et al.,* 2002; O'Donnell *et al.,* 2008). This makes it more difficult to depend solely on the use morphological characteristics for identification of *Fusarium* spp. (Jurado *et al.,* 2005; Leslie & Summerell, 2006; Fredlund *et al.,* 2008). However, *Fusarium* spp. have traditionally been separated and described on the basis of morphology and cultural characteristics, such as pigment production, shape and size of macroconidia, the presence or absence of microconidia, conidiophores and chlamydospores (Moss & Thrane, 2004), mycotoxin profiles and host plant association (Mirhendi *et al.,* 2010) as well as pathogenicity (Edwards *et al.,* 2002). *Fusarium* spp. grow remarkably well under a broad range of conditions. Sporulation and pigmentation are favoured by light, including ultraviolet wavelengths and fluctuating temperatures (Leslie & Summerell, 2006). A flow chart for the identification protocol used for identifying *Fusarium* spp. (Leslie & Summerell, 2006) is given in Figure 1.4.

Although culture techniques are generally seen as laborious, time consuming and heavily reliant on the availability of living propagules, these techniques remain important in the characterization of fungi (Moss & Thrane, 2004). Morphological characteristics form the basis of species identification and taxonomic classification. New species are still described on the basis of morphological characteristics (Rheeder *et al.,* 1996).

1.5.2. Molecular techniques

A number of molecular techniques based on analysis of DNA or RNA have been used in resolving the complexity of identification of *F. graminearum s.l.* isolates to species level as well as in providing information on the structure of populations (Edwards *et al.,* 2002). Species identification using *F. graminearum s.l.* species specific primers (Schilling *et al.,* 1996a, b; Nicholson *et al.,* 1998) has included the use of quantitative detection of fungal DNA in grain samples (Waalwijk *et al.,* 2004; Nicolaisen *et al.,* 2009) as well as determination of chemotypes in isolates (Desjardins & Proctor, 2011).

Genetic variation in a population found within an area can be used as an indicator of pathogen reproduction as well as gene flow between the populations. Understanding genetic diversity and population biology is fundamental in the prediction of disease epidemics and in the choice and application of disease management strategies (Burlakoti *et al.,* 2008). In the history of *Fusarium* research, molecular markers, such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), inter-simple sequence repeat (ISSR), single strand conformational polymorphism (SSCP) and sequence related amplified polymorphism (SRAP) have all been used to investigate diversity among *F. graminearum s.l.* populations (Burlakoti *et al.,* 2008). In this study, the principles of real-time quantitative as well as conventional PCR will be discussed.

1.5.2.1. The polymerase chain reaction (PCR)

The PCR provides a rapid and specific tool that can be used for detection of target DNA molecules within a complex of molecules (Jurado *et al.,* 2005). Nicholson *et al.* (1998) and Waalwijk *et al.* (2004) applied PCR in studying *F. graminearum s.l.* populations, based on amplification of specific DNA fragments from a complex pool of DNA (Edwards *et al.,* 2002). Species specific primers have been designed for identification of *F. graminearum s.l.* (Nicholson *et al.,* 1998) as well as other toxigenic *Fusarium* spp. (Edwards *et al.,* 2002). Genes coding for mycotoxin production are being used frequently in identification of specific *Fusarium* spp. Primers developed for specificity to the *Tri5* gene encoding trichodiene synthase allows detection of trichothecene producing *Fusarium* spp. i.e. the trichothecene biosynthetic gene. The assays make it possible to determine the presence or absence of all trichothecene producing species, without differentiating between the chemotype produced.

Although classical PCR has been used for detection of plant pathogens in diseased plants and infected seed, this technique is not frequently used in plant disease diagnosis as it is a laborious and time-consuming process that also requires verification of the amplified product (Schaad & Frederick, 2002). Another disadvantage of PCR when used in the detection of *Fusarium* spp. and in particular *F. graminearum s.l.* is that the technique cannot be used to differentiate between lineages or species within this species (O’Donnel *et al.,* 2004; Jurado *et*

al., 2005). The DNA coding regions of the small (18S and 5.8S) and large (26 or 28S) subunits of eukaryotes are also too conserved to be used for species identification (Seifert & Lévesque, 2004). As a result, many PCR-based protocols used in identification of *Fusarium* spp. are based on portions of the genomic sequences encoding translation elongation factor 1-α (*TEF*1-α), β-tubulin, intergenic spacer (*IGS*) and internal transcribed spacer (*ITS*) regions of the rDNA unit (*ITS*1 and *ITS*2) (Jurado *et al.,* 2005). The *ITS* regions are variable and are therefore used frequently when distinguishing between species (Edwards *et al.,* 2002).

1.5.2.2. Quantitative real-time PCR (qPCR)

Quantitative real-time PCR (qPCR) is a technique that offers an alternative tool for both qualitative and quantitative analysis. An overview of the fundamentals of quantitative real-time PCR is illustrated in Figure 1.5. This technique is fast becoming the method of choice for direct quantification of target DNA in composite samples. It offers an alternative to traditional techniques of amplification and culturing, allowing scientists to circumvent the constraints of end-point analysis as is done with classical PCR (Sarlin *et al.,* 2006). Currently, there are at least two classes of fluorescence assay methods, namely sequence-independent detection assays and sequence-specific probe binding assays. The fluorescence methods available for detecting the production of PCR amplicons include Taqman®probes (Waalwijk *et al.,* 2004) and SYBR (Nicolaisen *et al.,* 2009). qPCR amplification is a convenient, rapid and safe method for detecting pathogens. However, real-time amplification must be able to differentiate between a true negative and a false negative result caused by amplification inhibitors. To overcome this, the use of internal control oligonucleotides are recommended (Burggraf & Olgemöller, 2004).

TaqMan PCR exploits the 5' nuclease activity of *Taq* DNA polymerase in conjunction with fluorescent probes. The TaqMan probes are dual labelled, carrying a fluoregenic reporter dye on the 5'- end and a quencher dye on the 3'-end. These probes are designed to hybridise with specific PCR products. When the probe is intact, fluorescent energy transfer occurs and the reporter dye fluorescent emission is absorbed by the quenching dye. During PCR amplification, the probed is digested by *Taq* DNA polymerase, which results in separation of the reporter and quencher dyes. This activates increased fluorescence of the reporter dye. With repeated PCR cycles, the fluorescence intensity increases as the PCR product is amplified exponentially (Hogg *et al.,* 2007). The reactions are characterised by the cycle

threshold (CT). In comparison with conventional PCR where products must be at least 200 bp long, TaqMan amplicons are generally between 60 and 100 bp long, which increases PCR efficiency. Despite being an extremely powerful technique, real-time has its shortfalls, one of the most important being the need to normalise runs by using either reference or housekeeping genes (Jain *et al.,* 2006).

Generally, qPCR data analysis can be classified as ‘absolute quantification’ or ‘relative quantification’ (Peirson *et al.,* 2003). For absolute quantification, a standard curve is constructed using a sample of know concentration or known copy numbers and is then used to determine the concentration (copies or ng/µl) of unknown samples (Kühne & Oschmann, 2002). In relative quantification, the change in expression level relative to another set of experimental samples is determined, typically the experimental control group. The requirement to generate stable and reliable standards for quantification purposes is both time-consuming and requires precise quantification, making determination of exact copy numbers slightly more challenging. Moreover, the use of readily available nucleic acids such as plasmids introduces considerable risks of contamination.

1.6. QUALITATIVE AND QUANTITATIVE ANALYSIS OF MYCOTOXINS

Several analytical methods for the determination of *Fusarium* mycotoxins have been proposed and used. These include thin-layer chromatography (TLC), gas chromatography (GC) methods based on electron-capture detection (ECD) or mass spectrometric detection (MS), supercritical fluid chromatography (SFC) and high-performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection (FLD) (Tanaka *et al.,* 2000; Edwards *et al.,* 2002) and enzyme immunoassays (Rahmani *et al.,* 2009).

1.6.1. Chromatographic methods

Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) techniques have both been used for the qualitative and quantitative detection of *Fusarium* and other mycotoxins (Schollenberger *et al.,* 1998). Chromatographic methods measure the compound (mycotoxin) after sample extraction and extract clean-up steps, by separating the compound using either gas or liquid chromatography. Polar solvents like water are used to dissolve and extract mycotoxins from ground cereal samples.

The coupling of HPLC with a variety of detectors allows for the separation and detection of practically all mycotoxins (Rahmani *et al.,* 2009). Liquid chromatography with tandem mass spectrometry (LC-MS/MS) offers a great opportunity for the simultaneous analysis of a wide variety of mycotoxins in an array of matrices. This method has been used to determine trichothecenes such as DON, NIV, fusarenon X, 3- and 15-ADON as well as macrocyclic lactones such ZEA, α- and β-zearalenol, and α- and β-zearalalanol in maize (Turner *et al.,* 2009). The use of LC-MS/MS technology eliminates the need to derivatize samples while tandem mass spectrometry provides a highly selective and sensitive detection of target molecules through the use of ionization techniques such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (Santini *et al.,* 2008). What makes LC-MS a method of choice and a valuable tool is the ability to detect multiple mycotoxins in a single run, following a single extraction. Several methods for type A and type B trichothecenes that use APCI or ESI have been described (Biselli *et al.,* 2005). Today, the simultaneous determination of type A and B trichothecenes can be achieved using GC-ECD, GC-MS, LC-MS or LC-MS/MS.

1.6.2. Enzyme-linked immunosorbent assay (ELISA)

The generic principle of competitive direct enzyme-linked immunosorbent assay (CD-ELISA) is based on the ability of free toxin in samples and in the positive controls (standards) to compete with enzyme-labelled conjugate for antibody binding sites in a microwell. This technique has been used for analysis of mycotoxins such as the fumonisins (Parsons & Munkvold, 2010), aflatoxins B1 in maize, wheat and peanut butter, aflatoxin B1 in milk and T-2 toxin in maize and wheat (Van Egmond & Paulsch, 1986). In the process, no clean-up procedures are necessary, allowing for direct detection and quantification of target mycotoxins and can be used for routine analysis of matrices that are extensively tested (Pascale, 2009). Results for naturally contaminated grain crops tend to give good correlation to values obtained by an HPLC method, although the former tends to overestimate toxin levels (Magan & Olsen, 2004).

1.7. SUMMARY

The FGSC is comprised of at least 15 species, each geographically and phylogenetically characterised. Of these, at least six members have been officially identified as pathogens of maize crowns, kernels and roots, barley as well as wheat in South Africa. Worldwide, these fungi are important pathogens of cereal grain crops that can lead to losses in grain quality, nutritional value and yields, which in turn translate to losses in revenue. Moreover, these fungi contaminate grain with a wide range of mycotoxins, including those within the trichothecene and estrogenic clusters. These mycotoxins can cause a variety of diseases in both humans and animals. Within the trichothecenes, the FGSC fungi produce three strain specific chemotypes, namely the DON and 3-ADON, DON and 15-ADON and NIV and acetylated forms, as well as ZEA. Of the trichothecenes, DON occurs more frequently worldwide and is one of the most studied. Worldwide, DON, NIV and ZEA have been found as contaminants of cereal grains at unacceptably high levels (CAST, 2003) while countries such as Poland, Japan, New Zealand and the Americas (Placinta *et al*., 1999) have also reported on the prevalence of these mycotoxins in food and feed cereal crops.

Disease symptoms associated with FGSC fungi remain an important tool in the identification of these fungi as causal agents of disease in the field. However, in grain sorghum, the symptoms are not easily recognisable due to the multiple numbers of species forming the grain mold complex. This is especially true for red pigmented sorghum cultivars. In maize however, Gibberella ear rot symptoms are easier to observe and are mainly characterised by pink to red discoloration of infected tissue. In view of the complexity of the disease complex, more techniques need to be applied to facilitate correct identification of the disease causing fungi within the complex. Currently, a wide range of molecular tools that target genes that aid in identifying these fungi, including the *TEF*-1α and *ITS* regions of filamentous fungi. Both the conventional and qPCR technologies can be used to provide information on FGSC species and the levels of contamination. In addition, chromatographic techniques such as LC-MS/MS have broad applications in detection and quantification of mycotoxins produced by FGSC pathogens in culture as well as in cereal grain samples.

In view of the global importance of the FGSC and their mycotoxins both in food and feed sources, this study aims to assess the current state of maize and sorghum grain produced

commercially in both the wetter and drier areas of South Africa, with special reference to incidence of the FGSC deoxyribonucleic acid (DNA) and the mycotoxins DON, NIV and ZEA.

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Table 1.1. Area, production and yields of commercial maize, sorghum and wheat from 2006/07 to 2010/11[*]

Season	Crop	Area (ha)	Production (t)	Yield (t/ha)
2006/2007	Maize	2 551 800	7 215 000	2.79
	Sorghum	69 000	176 000	2.55
	Wheat	632 000	1 905 000	3.01
2007/2008	Maize	2 799 000	12 700 000	4.54
	Sorghum	86 800	255 000	2.94
	Wheat	748 000	2 130 000	2.85
2008/2009	Maize	2 427 500	12 050 000	4.96
	Sorghum	85 500	276 500	3.23
	Wheat	642 500	1 958 000	3.05
2009/2010	Maize	2 742 400	12 815 000	4.67
	Sorghum	86 675	196 500	2.27
	Wheat	558 100	1 430 000	2.56
2010/2011	Maize	2 372 300	10 679 400	4.5
	Sorghum	69 200	159 700	2.31
	Wheat	606 700	1 861 880	3.07

[*]Source: www.nda.agric.za/docs/statsinfo/Trends2011.pdf

Table 1.2. Sorghum classes, characteristics and grades in South Africa*

Class	Characteristics	Grades
GM (feed and malt)	Low tannin	GM
	Suitable for malting and milling	
	Also known as sweet sorghum	
GL (feed)	Low tannin	GL1, GL2
	Suitable for milling and animal feed	
	Also known as sweet sorghum	
GH (high tannic acid)	High tannin content	GH1, GH2
	Bird resistant	
	Also known as bitter sorghum	

*Source: www.nda.agric.za/docs/Brochures/prodGuideSorghum.pdf

Table 1.3. Mycotoxigenic fungal species and the primary mycotoxins they produce in food and feed commodities*

Fungal species	Primary mycotoxin(s) produced
Acremonium coenophialum	Ergopeptine alkaloids
A. lolii	Lolitrem alkaloids
Aspergillus flavus; A. parasiticus	Aflatoxin cyclopiazonic acid
A. ochraceus; Penicillium viridicatum; P. cyclopium	Ochratoxin A
Fusarium culmorum; F. graminearum; F, sporotrichioides; F. poae	Zearalenone, deoxynivalenol, nivalenol, T-2 toxin, diacetoxyscirpenol
F. subglutinans	Moniliformin
F. verticillioides	Fumonisin
Phomopsis leptostromiformis	Phomopsin
Pithomyces chartarum	Sporidesmin
P. expansum	Patulin

* Adapted from CAST (2003) and Murphy *et al.* (2006)

Table 1.4. Optimum conditions[*] required for mycotoxin production in culture

Mycotoxin (s)	Pathogen (s)	Temperature (°C)	Water activity(Aw)
Aflatoxins	*Aspergillus flavus*	33	0.99
	A. parasiticus		
Deoxynivalenol; Nivalenol and their derivatives	*Fusarium proliferatum*	11	0.9
	F. Verticillioides		
Fumonisin	*F. proliferatum*	10 – 30	0.93
	F. Verticillioides		
Ochratoxin	*A. ochraceus, ,*	10 – 30	0.85 - 0.98
	A. carbonarius		
	Penicillium verrucosum		
Patulin	*P. expansum*	0 - 25	0.95 - 0.99
Zearalenone	FGSC pathogens	25 – 30	0.98

[*] Adapted from CAST (2003) and Murphy *et al.* (2006)

Table 1.5. European Union maximum levels and feed guidance levels for mycotoxins*

Mycotoxin	Maximum levels: Lowest - highest (µg/kg)	Feed guidance levels: Lowest - highest (µg/kg)	Formation of mycotoxins
Aflatoxins B1,B2, G1, G2	0.10 (infant food) - 20 (animal feed)	-	In the field and during storage
Aflatoxin M1	0.025 (infant food) - 0.050 (milk)	-	Metabolic formation from aflatoxin B1 in dairy cows
Deoxynivalenol (DON)	200 (infant food) - 1750 (unprocessed maize)	900 (pig feed) - 12 000 (maize by products)	In the field and during storage
Fumonisins B1, B1	200 (infant food) - 4000 (unprocessed maize)	5000 (feed for pigs, horses and pet aminals)	In the field and during storage
		60 000 (maize based feed)	
Ochratoxin A (OTA)	0.50 (infant food) - 80 (liquorice extract)	50 (pig feed) 250 (cereal based feed)	During storage (cereals)
		250 (cereal based feed)	
Patulin	10 (infact food) - 50 (fruit juices)	-	In the field (apples)
Zearalenone (ZEA)	20 (infant food) - 400 (refined maize oil)	100 (feed for piglets)	In the field and during storage
		3000 (maize by products)	In the field and during storage

*Source: http://www.www.efsa.europa.eu/de/topics/topic/mycotoxins.htm

Table 1.6. Maximum content of mycotoxins in South Africa in mg/kg (ppm) relative to farm feed with a moisture content of 120 g/kg

Mycotoxin	Farm feeds	Maximum content mg/kg (ppm)
Aflatoxin B1	Feed ingredients with the exception of:	0.02
	- groundnut, copra, palm kernel, cotton seed, maize and products derived from processing thereof	0.05
	Complete farm feeds for cattle, sheep and goats with the exception of:	0.05
	- dairy cattle	0.005
	- calves and lambs	0.01
	Complete feeds for pigs and poultry (except young animals)	0.02
	Other complete farm feeds	0.01
	Supplement/concentrates for cattle, sheep and goats (except for dairy animals, calves and lambs)	0.05
Deoxynivaleneol	Feeding stuffs on full ration basis for:	
	- pigs	1
	- cattle	5
	- calves up to 4 months	2
	- dairy cattle	3
	- poultry	4
Fumonisins B1	Horses	5
	Pigs	10
	Beef and poultry	50
Ochratoxin A	Feeding stuffs on full ration basis for:	
	- sows, pigs and piglets	0.05
	- poultry	0.2
Zearalenone	Feeding stuffs on full ration basis for:	
	- sows and pigs	0.25
	- young pigs	0.1
	- young cattle and dairy cattle	0.5

*Source: www.nda.agric.za (Government gazette # 19131).

Table 1.7. Major mycotoxins contaminating cereal crops and the diseases they cause in humans and animals*

Mycotoxin	Cereal crops	Some of the affected species	Pathological effects
Aflatoxins (B1, B2, G1,G2, M1, M2)	maize, wheat, rice	birds, fish, pigs, pregnant sows, cattle, sheep, humans	hepatotoxicity (liver damage), bile duct hyperplasia, hemorrhage of the intestinal tract and kidneys,
Citrine	wheat, barley, maize, rice	swine, laboratory animals	liver carcinogenesis
Cyclopiazonic acid	maize, millet	swine, chicken, rat, human	muscle necrosis, intestinal hemorrhage and edema, oral lesions
Ochratoxin A	wheat, barley, maize, oats	swine, chicken, rat, human	nephrotoxicity (tubular necrosis of kidney), Porcine nephropathy, mild liver damage, enteritis, teratogenesis, kidney carcinogenesis, urinary tumours
Patulin	wheat straw residue	chicken, cattle, human	brain and lung edema, hemorrhage, lungs, liver spleen and kidney capillary damage, paralysis of motor nerves, convulsions, carcinogenesis, antibiotic
Sterigmatocystin	moldy wheat, maize, barley, sorghum oats	mouse, rat	carcinogenesis, hepatotoxin
Trichothecenes	maize, wheat, barley, oats, barley	swine, cattle, horse, rat, human	digestive disorders (emesis, diarrhea, refusal to eat), hemorrhage (stomach, heart, intestines, lungs, bladder, kidney), edema, oral lesions, dermatitis, blood disorders (leucopenia)
Zearalenone	maize, wheat, barley, oats, barley	swine, dairy cattle, chicken, lamb, rat, human	estrogenic effects (edema of vulva, prolapse of vagina, enlargement of uterus), atrophy of testicles, atrophy of ovaries, enlargement of mammary glands, abortion
Fumonisins	maize, wheat, barley, oats, barley	swine, dairy cattle, chicken, lamb, rat, human, horses	leucoencephalomalacia in horses; pulmpnary edema in swine; esophageal cancer in humans; liver and kidney cancer in rats; neurqal tube defects

* Sources: CAST, 2003; Desjardins, 2006

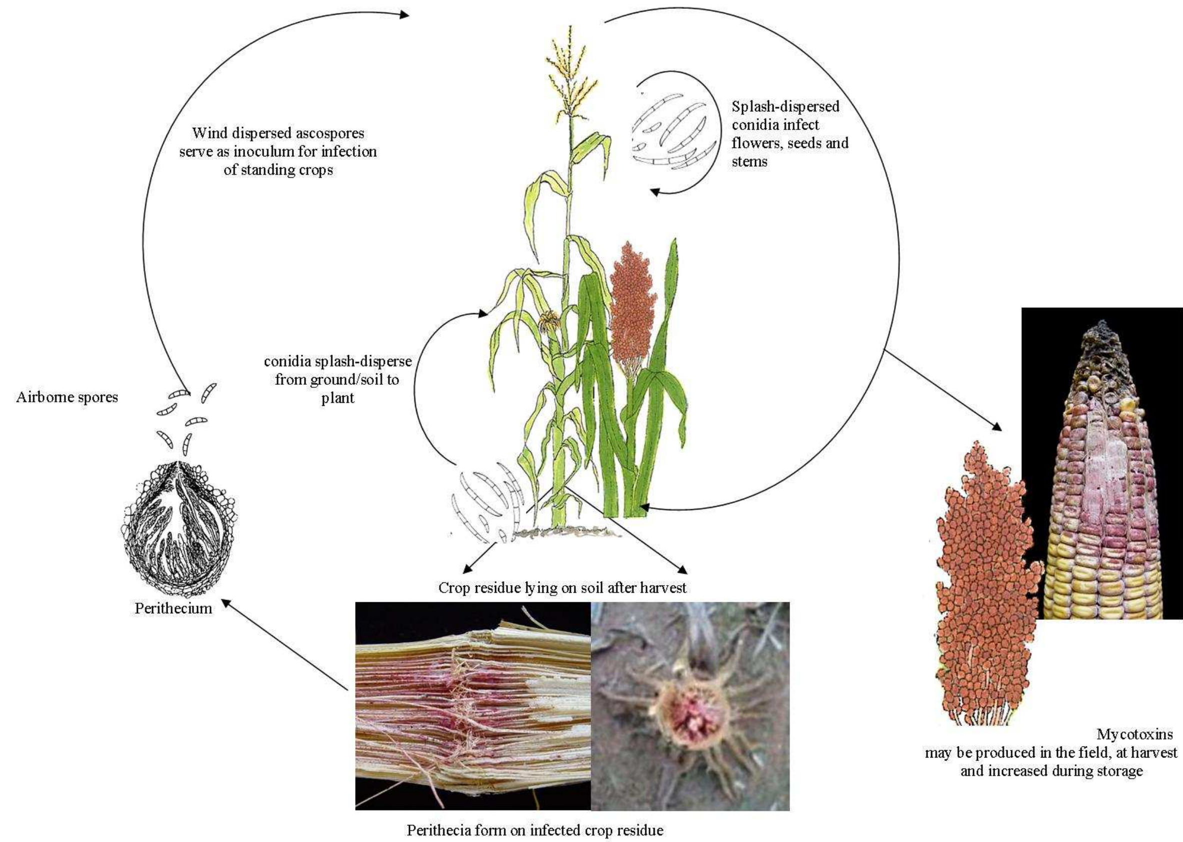

Figure.1.1. Simplified life cycle of *F. graminearum s.l.* on cereal grain crops, adapted from Trail (2009). (photos: Dr Rikus Kloppers; Drawings: Dr Susanna Hermina Koch)

Figure 1.2. Symptoms of grain mold on sorghum panicles - pink, grey or black discolourations of the grain and incomplete grain fill. (Thakur *et al.,* 2006)

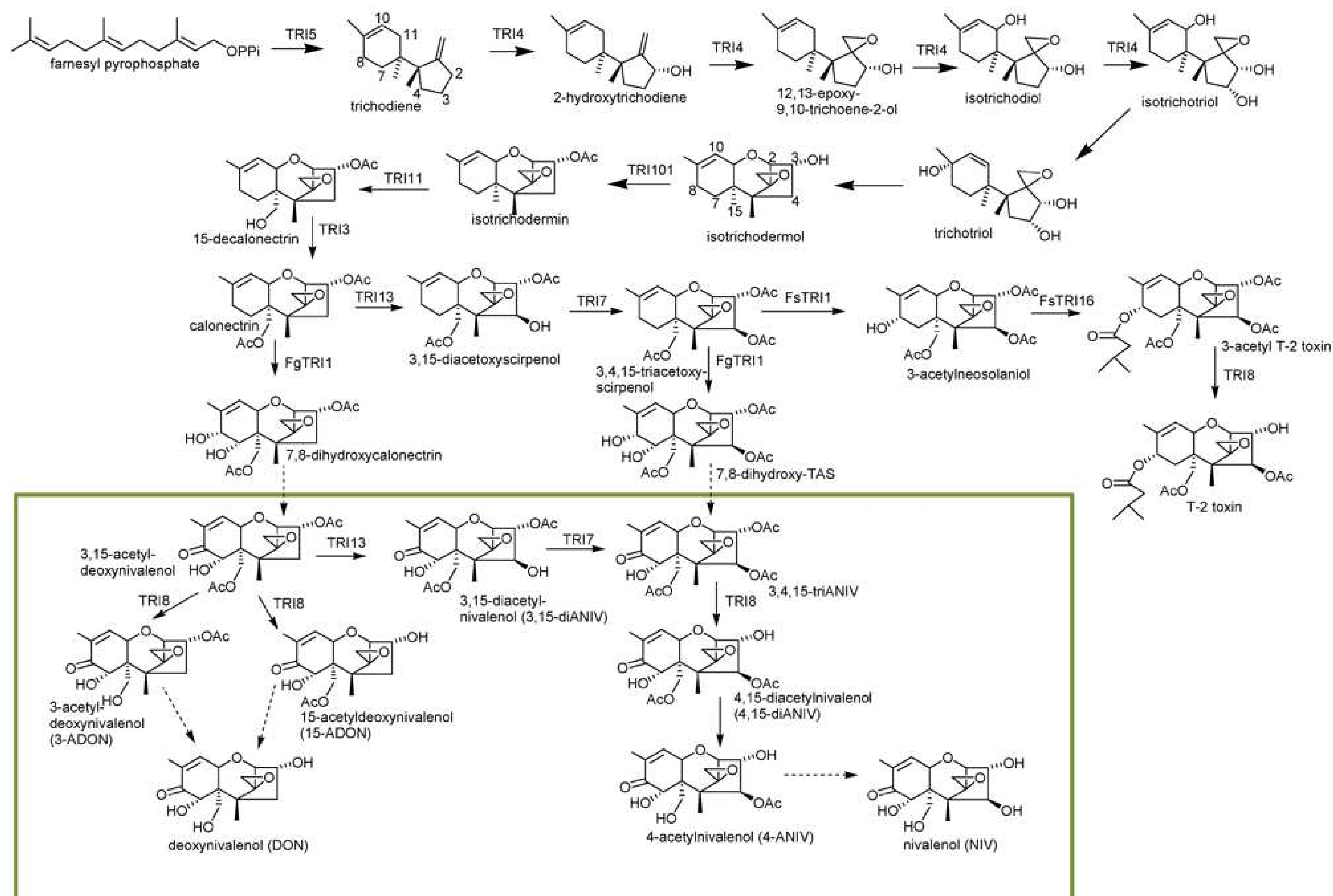

Figure 1.3. Trichothecene biosynthesis pathways to T-2 toxin, deoxynivalenol, and nivalenol. Genes encoding an enzymatic step are identified near the arrow indicating the step. The dashed arrows indicate steps for which a gene has not been assigned and the green box identifies Type B trichothecenes (McCormick *et al*., 2011)

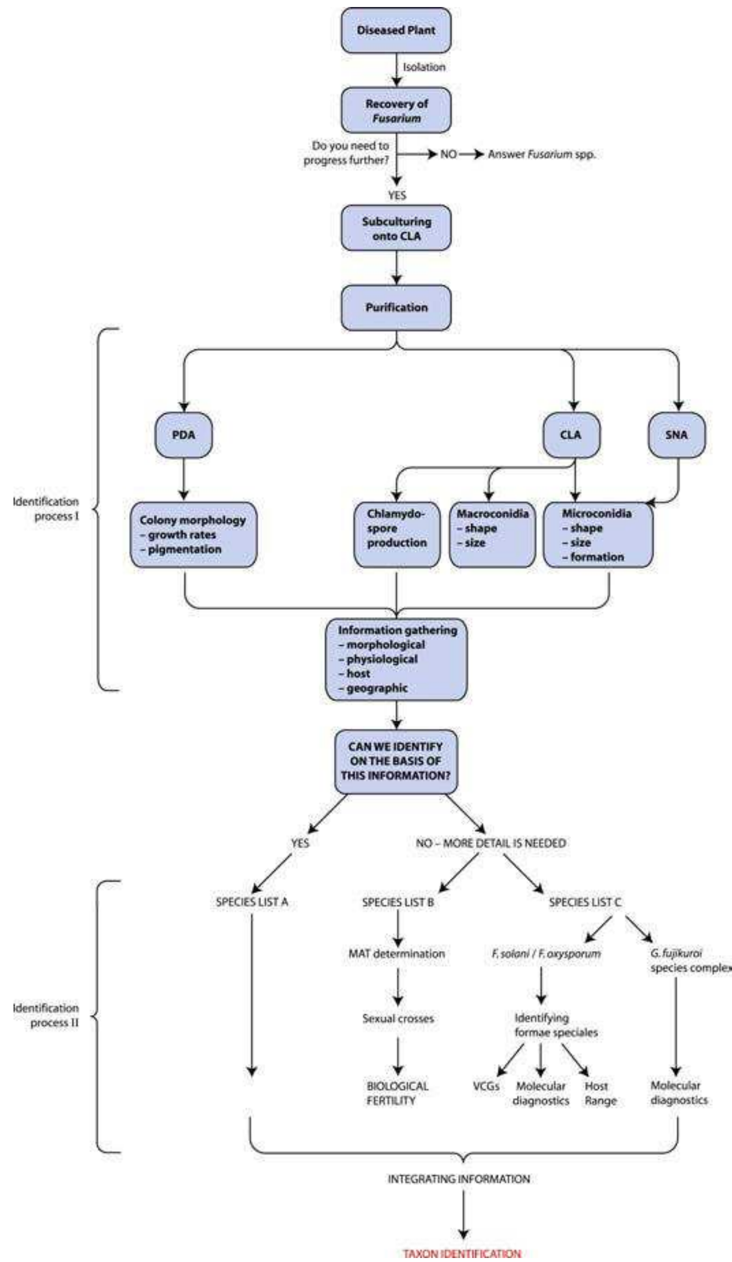

Figure 1.4. Flow chart of identification protocol used for identifying *Fusarium* spp. (Leslie & Summerell, 2006)

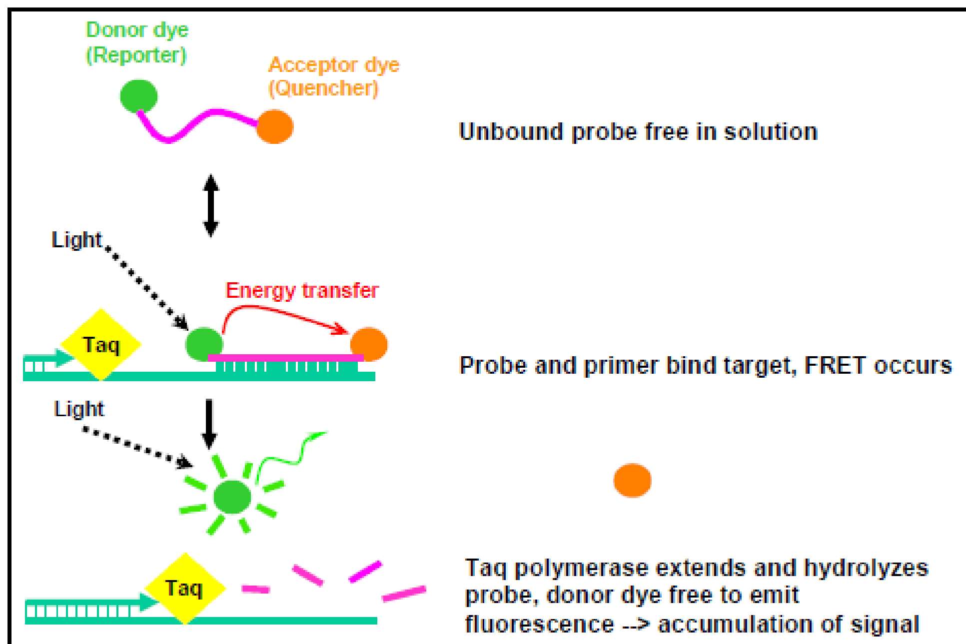

Figure 1.5. Overview of the fundamentals of quantitative real-time PCR. Source: http://www.cdc.gov/meningitis/lab-manual/chpt10-pcr.html

CHAPTER 2

QUANTITATIVE DETECTION OF *FUSARIUM GRAMINEARUM* DNA IN COMMERCIAL SOUTH AFRICAN MAIZE AND SORGHUM CULTIVARS

Abstract

Colonisation of cereal grain by fungi within the *Fusarium graminearum* species complex (FGSC) may result in losses due to reduction in grain yield, quality and accumulation of mycotoxins in the grains. Early detection and management of this pathogen complex is crucial to preventing toxins from entering the food and feed chain. In this study, quantitative real-time PCR assays were used to quantify the total FGSC DNA in milled grain samples. The distribution of FGSC DNA in 558 samples comprising of six maize and seven sorghum cultivars collected from 34 and 22 localities, respectively, between 2006 and 2009 was determined. Total DNA was extracted from ground grain samples using a commercial DNA extraction kit and analysed in a LightCycler® system using species specific primers and a fluorogenic TaqMan probe. Members of the FGSC occurred at variable concentrations as a natural contaminant of over 47% of local maize and sorghum grain, ranging from non-detectable to 3920 and non-detectable to 3790 pg/mg, respectively. Significant differences ($P \leq 0.05$) in the concentrations of FGSC DNA in maize kernel samples were observed between the seasons and localities, as well as among cultivars. Similarly, these variables also affected the prevalence of FGSC DNA in sorghum grain significantly. These results emphasize the need for genotype x environment interactions to be considered in the assessment of the risk of grain colonisation by members of the FGSC and the selection of low risk production areas.

2.1. INTRODUCTION

The *Fusarium graminearum* species complex (FGSC) is comprised of 15 biogeographically and phylogenetically distinct species that have been characterised and described by O'Donnell *et al.* (2000, 2004, 2008), Starkey *et al. (*2007), Yli-Mattila *et al.* (2009) and Davari *et al.* (2012). Some of these species are associated with a biogeographical region, particularly in Asia, Africa and South America (Summerell *et al*., 2010). These fungi are important pathogens of cereal crops worldwide. They are causal organisms of diseases of maize (*Zea Mays* L.), sorghum [*Sorghum bicolour* (L.) Moench], wheat (*Triticum* spp.), oats (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) (Reischer *et al.,* 2004; Nicolaisen *et al.,* 2009). Members of the FGSC are considered the primary causal agents of Gibberella ear and stalk rot of maize as well as grain mold of sorghum (Trail, 2009). Reports from all continents show that the FGSC have large economic and food safety impacts on grain production, particularly during epidemic seasons (Gale *et al.,* 2011). Economic losses result from a reduction in yield, poor seedling germination and contamination of grain with mycotoxins such as deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) (Trail, 2009; Gale *et al.,* 2011). These diseases have also reached epidemic proportions in North America, while outbreaks have also been reported in Asia, Australia Canada, Europe and South America where this complex poses a threat to global food production (Waalwijk *et al.,* 2003; Akinsanmi *et al*., 2008; Trail, 2009).

Studies during the 1970's and 1980's demonstrated that *F. graminearum sensu lato* (*s.l.*) [teleomorph = *Gibberella zeae* Schwein (Petch)] exists under field conditions in South Africa (Marasas *et al.,* 1979; 1981). Viljoen (2003) reported that, although present in cereal grain crops, this pathogen occurs at low frequencies in commercial crops. However, a recent survey by Boutigny *et al.* (2011a) revealed that the FGSC occurred at significant levels in a variety of South African cereal grains, including maize, wheat and barley. Furthermore (Kriel, 2010) reported that the *F. graminearum sensu stricto* (*s.s.*) is particularly prominent in wheat under irrigation and in high rainfall areas. Twenty five percent infected wheat seed can result in approximately 67% reduction in stand.

Yield losses due to members of the FGSC may reach 10-30% in wheat although losses of up to 70% have been reported under severe disease conditions. In susceptible cultivars, yield losses of 22-24% were recorded, while only 7-17% losses were reported in less susceptible

cultivars (Kriel & Pretorius, 2008). Regional differences, cropping systems as well as temperature and rainfall may influence the profiles of *Fusarium* spp. infecting grain, which in turn affect the mycotoxins that contaminate the grain (Waalwijk *et al.,* 2004).

Traditionally, the detection and identification of *Fusarium* spp. involved isolation and single sporing to obtain pure cultures for morphological identification and pathogenicity tests. The polymerase chain reaction (PCR), on the other hand offers a sensitive, specific, fast and high throughput method to detect, identify and quantify fungal species in grains (Sarlin *et al.,* 2006). More recently, real-time quantitative PCR assays have been developed for *Fusarium* spp. (Waalwijk *et al.,* 2004; Yli-Matilla *et al.,* 2006; Boutigny *et al*., 2011b). The aim of this study was to use TaqMan real-time PCR (qPCR) technology to determine the distribution of the FGSC in maize and sorghum samples collected from different localities in grain production areas of South Africa and to quantify grain colonisation levels.

2.2. MATERIAL AND METHODS

2.2.1. Field samples

Maize kernel and sorghum grain samples harvested from the National Cultivar Trials were provided by the Agricultural Research Council-Grain Crops Institute (ARC-GCI), Potchefstroom, South Africa. A total of 308 maize kernel samples from the 2006/07, 2007/08 and 2008/09 seasons and 250 sorghum grain samples from the 2007/08 and 2008/09 seasons were used in this study. The samples were collected from 47 grain production localities in South Africa and are presented in Table 2.1. All were composite grain samples from three replicates of approximately 0.2 to 2.0 kg from each locality per season. Sub-samples were milled in a Retsch cross beater mill with a 1 mm sieve and stored at 4°C in airtight containers until use. The maize cultivars were CRN3505, DKC78-15B, DKC80-10, DKC80-12B, LS8521B and PAN6611 and the sorghum cultivars were NS5511, PAN8247, PAN8420, PAN8609, PAN8625, PAN8648 and PAN8816.

2.2.2. Fungal isolation and identification

Approximately 200 kernels per sample were surface sterilized in commercial 1% aqueous solution of sodium hypochlorite (NaOCL) for 3 min. The samples were rinsed three times with sterile distilled water and air dried in a laminar flow cabinet for a minimum of 30

minutes (Leslie & Summerell, 2006). One hundred kernels per sample were plated on Nash-Snyder medium [peptone pentachlornitrobenzene (PCNB) agar/PPA] at a rate of five and ten grains per petri dish for maize and sorghum respectively. The plates were incubated at room temperature for seven days and *Fusarium* spp. were subcultured onto potato dextrose agar (PDA). Only isolates resembling *F. graminearum s.l.* were purified by single spore technique (Choi *et al.*, 1999) and cultured on potato dextrose agar (PDA), Spezieller Nährstoffarmer agar (SNA) and CLA plates for identification and incubated at 25ºC for 7-10 days before attempts were made to identify cultures. Identification was done according to Booth (1971), Nelson *et al*. (1983) and Leslie & Summerell (2006).

2.2.3. DNA extraction

Reference strains were purchased from the Plant Protection Reasearch Institute of the Agricultural Research Council (ARC-PPRI) as well as the PROMEC Unit of the Medical Research Council (MRC) culture collectections, South Africa. The species and their reference numbers are listed in Table 2.2. Each isolate was grown on PDA plates for 5-7 days and mycelia were harvested and stored in 2 ml Eppendorf tubes at -70°C until use. The ZymoResearch fungal/bacterial DNA kits™ (Zymo Research, Irvine, California, USA) were used for extraction of genomic DNA from milled grain samples as well as fungal mycelia. Total DNA was extracted from 200 mg milled grain samples and 100 mg fungal mycelia according to the manufacturer's instructions. The DNA concentrations and purity was determined using a NanoDrop® spectrophotometer (ND1000) (Thermo, Waltham, MA, USA) by absorbance at 260 nm (OD_{260}). All the DNA samples were diluted to 2 ng/µl and aliquoted into 20 µl sub-samples and stored at -20ºC.

2.2.4. TaqMan assays

The *F. graminearum s.l.* major groove binding (MGB) primers and probe, as developed by Waalwijk *et al.* (2004) were used. The probe was labeled at the 5' end with the fluorescent reporter dye 6-carboxylfluoresceine (FAM) and at the 3' end with the quencher dye 6-carboxy-tetramethyl rhodamine (TAMRA). The primers and probe were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa, USA). Quantitative real-time PCR assays were performed in a LightCycler® Carousel based 1.5 instrument (Roche Applied Science, Penzberg, Upper Bavaria, Germany).

The reactions were carried out in capillaries containing 20 µl reaction mix consisting of 4.0 µl of the 5 X LightCycler TaqMan® Master (Roche Diagnostics, Mannheim, Germany), 2 µl primer mix (10 µM), 0.5 µl MGB-TaqMan probe (5 µM), 8.5 µl molecular grade water, and 5 µl of sample DNA. To eliminate possible false positive results due to PCR contamination (Pennings *et al.,* 2001), 1 U uracil DNA *N*-glycosylase (UNG) was added to each PCR master mix. Each run included a negative control to which no DNA was added and a sample with a known concentration of *F. graminearum s.l.* DNA from the standard.

The following thermal cycling conditions were used: Single steps of 10 min at 40°C and 10 min at 95°C, followed by 40 cycles of 10 sec at 95°C, 30 sec at 52°C, 15 sec at 60°C and 30 sec at 40°C. Data were analysed using the LightCycler® software-4.0 (Roche Applied Science, Penzberg, Upper Bavaria, Germany). The fluorescence signal was measured during the last 15 sec of the extension phase. Quantification of DNA was done using an external standard curve. The cycle threshold (CT) values were used for further calculations of FGSC DNA concentrations in the grain samples using the LightCycler® software. Unspecific amplification may occur during the late PCR cycles, therefore, to minimise wrong positive results, a PCR reaction was considered positive if the CT value was less than 35. All the negative samples were re-analysed to verify that they were true negative samples.

2.2.5. Gel electrophoresis

Evaluation of the quality and integrity of DNA extracts from fungal isolates, grain samples, PCR products as well as sequencing reaction products was done by means of gel electrophoresis. The gel was prepared by dissolving 1 g of agarose in 100 ml tris acetic acid EDTA (TAE) buffer. Ten µl ethidium bromide (10 mg/ml) was added to the cooled agarose prior to casting into a gel tray. A comb was inserted into the tray and the poured agarose was allowed to set at room temperature before DNA templates were loaded into the wells. To assess the DNA purity, 4 µl extracts per sample were loaded into separate wells. In addition, 2.5 µl each of a 1 kb molecular marker was loaded into the first and the last wells. The cast gels were electrophoresed at 80 volts for 45 min and viewed on the UVP GelDoc-It™ imaging system (Upland, Carlifonia, USA).

2.2.6. Sensitivity and specificity of TaqMan primers and probe

To determine the sensitivity of the TaqMan assays, 10-fold serial dilutions of purified *F.*

graminearum s.l. genomic DNA (MRC4712) were prepared in PCR-grade water and tested using the *F. graminearum* real-time PCR assay. The absence of cross-reactivity in TaqMan reactions was tested on 18 reference isolates representing 11 *Fusarium* spp. These were *Fusarium* spp. (M260/45), *F. verticillioides* (PPRI 7259), *F. crookwellense* (PPRI 9106), *F. culmorum* (PPRI 10138), *F. equiseti* (PPRI 10141), *F. graminearum s.l.* (MRC4712, M637/36, M637/47), *F. pseudograminearum* (MRC4927),, *F. boothii* (M260/47, M260/51), *F. cortaderiae* (M431/17), *F. meridionale* (M431/18, M431/28, M431/60, M431/91) and *F. acaciae-mearnsii* (M431/35, M431/92).

2.2.7. Standard curve

A series of assays were conducted in order to generate a standard curve that would be used for quantitative detection of *F. graminearum s.l.* DNA in maize and sorghum grain samples. A negative control to which no DNA template was added was included with each PCR run. A standard curve was generated by analysis of 10-fold serial dilution of pure *F. graminearum s.l.* DNA (MRC 4712). The dilution range was 275 000, 27 500, 2 750, 275, 27.5, 2.75, 0.275, 0.0275 and 0.00275 pg. Three replicates per dilution were used to generate the standard curve.

2.2.8. Agrometeorological data

Total rainfall (mm), maximum temperature (°C) and maximum relative humidity (%) data for the 2006/07, 2007/08 and 2008/09 seasons of all the localities monitored in this study were supplied by the Agricultural Research Council-Institute of Soil, Climate and Water. Data of the three variables for the months of February and March.

2.2.9. Data Analysis

Grains from the two commodities were regarded as two separate surveys. Maize samples were planted over three seasons, 34 localities and six cultivars. Sorghum samples were planted over two seasons, 22 locations and seven cultivars. The data collected were FGSC DNA concentrations in the milled samples. The initial analysis was to test if the season variances of the log transformed and untransformed data from each survey are of comparable magnitude using Levene's test (Levene, 1960). There was not sufficient evidence against

homogeneity therefore a three factor analysis (season x locality x cultivar) was performed on the transformed data using the three factor interaction as error term. The standardised residuals were subjected to Shapiro-Wilk's test to test for deviation from normality (Shapiro & Wilk, 1965). In all cases, deviation from normality was due to Kurtosis and not skewness as a result of numerous zero values, therefore we accept the data as reliable (Glass *et al*., 1972). Means of significant effects were compared using Fisher's protected t-test and least significant differences (LSD) at a 5% significance level (Snedecor & Cochran, 1967). All the statistical procedures were performed using SAS/STAT version 9.2 statistical software (SAS, 1999). Additionally, the Jenks optimization method, also known as the Jenks natural breaks classification method (Jenks, 1967) was used to determine the best arrangement of data into different classes and these were plotted on maps (Figures 2.1 to 2.5).

2.3. RESULTS

2.3.1. Fungal isolation and identification

Fungal isolates representing five of the 15 FGSC pathogens isolated from maize and sorghum grain samples are presented in Table 2.2. These were *F. graminearum s.s* (M637/36, M637/47), *F. boothii* (M260/47, M260/51), *F. cortaderiae* (M431/17), *F. meridionale* (M431/18, M431/28, M431/60, M431/91) and *F. acaciae-mearnsii* (M431/35, M431/92). Non-FGSC fungi were not identified to species level as they were not of interest for this study.

2.3.2. Sensitivity and specificity of TaqMan primers and probe

The sensitivity and specificity of the primer-probe combination was evaluated in a single real-time PCR using total DNA extracts from 18 reference isolates (Table 2.2) representing 11 *Fusarium* spp, among them representatives of the five FGSC pathogens isolated from maize kernel and sorghum grain samples. The assays yielded positive results (CT values > 0) only from samples infected with FGSC pathogens. The average CT value for the five isolates representing members of the FGSC was 22.30. The amplification curves for each of the five FGSC tested are illustrated in Figure 2.6. No fluorescence could be measured with any of the non-FGSC spp. included in the sensitivity and specificity assays.

2.3.3. Standard curve

The quantitative detection of FGSC DNA in maize and sorghum grain samples was achieved by the generation of a high quality standard curve using known concentration of *F. graminearum* (MRC 4712) DNA. The standard curve had an efficacy of 1.995 and the primer/probe combination displayed a linear range of at least four orders of magnitudes. The primer-probe set detected up to 10^{-6} FGSC in the DNA samples (Figure 2.7a, b)

2.3.4. TaqMan assays

The number of maize and sorghum samples contaminated with FGSC DNA over the three seasons is provided in Table 2.3. In the absence of FGSC DNA, no fluorescence could be detected, as illustrated in Figure 2.8 and only the fluorescence of a positive control could be detected. Overall, 53% maize and 41% sorghum samples were contaminated with FGSC DNA (Table 2.3).

2.3.4.1. Maize kernel samples

In the 2006/07 season, only 9% of the samples were contaminated with FGSC DNA. The highest concentration recorded in a sample was 950.00 pg/mg (CRN3505, Delareyville) while the lowest concentration recorded was 13.30 pg/mg (LS8521B, Wesselsbron). The average FGSC DNA concentrations at the different localities ranged from non-detectable to 194.17 pg/mg while Delareyville, Hartebeesfontein and Bethlehem (Table 2.4) had the highest mean FGSC concentrations of 194.17, 133.17 and 40.67 pg/mg respectively. Cultivar means during 2006/07 ranged from non-detectable to 118.93 pg/mg while cultivar CRN3505 was the most susceptible to colonisation by FGSC fungi. Statistical differences at a 95% confidence level were found between localities and cultivars. During this season, only three mean groupings (based on LSD), were observed for the localities, namely a, ab and b (Table 2.4).

During the 2007/08 season, 71% of the maize samples contained FGSC DNA. The highest concentration recorded in a sample was 3920.00 pg/mg (DKC78-15B, Vrede) while the lowest concentration detected was 1.04 pg/mg (LS8521B, Tweebuffelsfontein) (Table 2.4) with significant differences between between cultivars. The average FGSC DNA

concentrations at the different localities ranged from non-detectable to 1478.20 pg/mg. Vrede had the highest mean concentration of 1478.20 pg/mg and did not differ significantly from Danielsrus, Delmas, Bloekomspruit (1st planting) and Tweeling (Table 2.4). During this season, these localities, along with Leeudoringstad were more conducive for the growth of FGSC fungi in the grain. Eight mean groupings were observed, with group (de) having 13 out of the 28 localities (Table 2.4) and all the localities within this group were less conducive for the growth of FGSC fungi. Cultivar means ranged from 170.40 to 494.10 pg/mg for cultivars LS8521B and CRN3505 respectively. Similar to the 2006/07 season, cultivar CRN3505 (Table 2.4) was the most susceptible of the six cultivars. Cultivar LS8521B was least susceptible to colonisation by FGSC fungi while the remaining cultivars formed the intermediate group (Table 2.4).

In the 2008/09 season, 65% of the maize kernel samples were contaminated with FGSC DNA. The highest concentration recorded in a sample was 1280.00 pg/mg (DKC78-15B, Cedara) while the lowest concentration recorded was 29.80 pg/mg (DKC80-10, Cedara) (Table 2.4). The average concentrations at the different localities ranged from non-detectable to 469 pg/mg and Cedara (2nd planting) produced the highest mean concentration (Table 2.4). Only five mean groupings were observed, with group (c), (which comprised localities that were less conducive for the growth of the FGSC fungi) representing four of the ten localities. Both Cedara (2nd planting) and Bethlehem (2nd planting) were the most conducive localities for the growth of FGSC fungi. Cultivar means ranged from 54.00 to 218.20 pg/mg for cultivars LS8521B and DKC80-12B respectively. There were no significant differences among the cultivars for infection by FGSC fungi during 2008/09.

A combined analysis of the variance (ANOVA) (Table 2.5) for the three seasons indicated a significant difference ($P \leq 0.05$) between seasons, localities and cultivars. The ANOVA for locality by cultivar indicated that there were significant interactions between these two factors. No significant interactions between season and locality and season and cultivar were recorded

2.3.4.2. Sorghum grain samples

In the 2007/08 season, only 24% of the samples contained FGSC DNA. The highest concentration detected in a sample was 2700.00 pg/mg (PAN8609, Cedara) while the lowest concentration detected was 4.49 pg/mg (PAN8420, Klerksdorp). The average FGSC concentrations at the different localities ranged from non-detectable to 1235.29 pg/mg (Table 2.6). Cedara (1st planting) contained the highest mean concentration of FGSC DNA (1235.29 pg/mg) followed by Cedara (2nd planting) with a mean concentration of 624.43 pg/mg FGSC DNA. This locality was more conducive for the growth of FGSC fungi and FGSC DNA concentrations differed significantly from remaining localities. There were no significant differences between the remaining localities during 2007/08 which were less conducive. There were also no significant differences among cultivars for colonisation by FGSC fungi (Table 2.6).

During the 2008/09 season, 56% of the samples were contaminated with FGSC DNA. The highest concentration detected in a sample was 3790 pg/mg (PAN8609, Cedara) while the lowerst concentration detected was 12.10 pg/mg (NS551, Dover). The average concentrations for localities ranged from non-detectable to 2450 pg/mg. Similar to the 2007/08 season, Cedara had the highest mean FGSC concentration (2450.20 pg/mg) and was significantly different ($P \leq 0.05$) from the rest of the localities which formed the less conducive group for FGSC growth and did not differ from one another. The planting of sorghum on three different dates at Potchefstroom yielded mean FGSC DNA concentrations of 245.00, 58.16 and 0.00 pg/mg for the 1st, 2nd and 3rd plantings respectively, with no statistical difference between these recordings. There were also no significant differences in planting sorghum grain at this locality under irrigation and dry conditions as both these conditions did not yield any FGSC DNA. Cultivar NS5511 was the most susceptible while cultivars PAN 8648 and PAN 8816 were the most resistant to FGSC colonisation (Table 2.6).

A combined analysis of variance (ANOVA) for the two seasons indicated significant differences ($P \leq 0.05$) between seasons, localities and cultivars. The ANOVA for locality by cultivar; season by locality and season by cultivar also indicated that there were significant interactions between these factors (Table 2.7). Overall, seasons differed significantly, with 2007/08 being the more conducive of the two.

2.3.5. Agrometeorological data

Total rainfall (Table 2.8), maximum temperature (Table 2.9) and maximum relative humidity (Table 2.10) data for the months of February and March at the localities where maize and sorghum samples were monitored during the 2006/07, 2007/08 and 2008/09 seasons are presented in the afore mentioned tables respectively. It is evident from these data that the climatic conditions during the critical stages of silking and grain maturation were probably major contributors to the increase in the prevalence of FGSC fungi, especially during the 2007/08 seasoon as most of the localities received rainfall >60mm for each month during this period. At Cedara, the weather conditions during the 2007/08 and 2008/09 seasons may have been the cause of the increase in FGSC DNA in sorghum grain. During the 2007/08 season, mean rainfall of 67.5 mm was recorded while the mean temperature was 25°C and in the 2008/09 season, the mean rain recorded was 36.5 mm and the mean temperature was 25.9°C. These conditions favour the growth of FGSC fungi. At Vrede and Warden, total rain for the months of February and March was 181.50 mm and the average maximum temperature was 33.5°C, and it would appear that these conditions also supported the growth of FGSC fungi.

2.4. DISCUSSION

Diagnostic methods for plant pathogens are important in the identification of fungal contaminants in cereal grain crops. The PCR offers a sensitive and potentially specific means to detect, identify and quantify the presence of species within grain and other plant tissues (Nicholson *et al*., 2003). As not all members of the FGSC are distinguishable using conidial morphology, the targeting of specific sequences in the genomes of these fungi can improve detection in grain samples without the need for culturing (Suga *et al*., 2008). This study made use of TaqMan based quantitative real-time PCR for the simultaneous detection and quantification of FGSC DNA in maize kernel and sorghum grain samples collected in grain producing localities within South Africa.

The qPCR assays revealed noteworthy differences in the concentrations of FGSC DNA and its distribution in maize kernel and sorghum grain samples over the three seasons. These differences may be crop (cultivar) related but also the results of factors such as the absence or presence and amount of inoculum or the predominance of environmental conditions that promote or prevent the growth of the FGSC fungi. Cultivar data clearly show that differences

in genetic susceptibility to FGSC pathogens exist in South African maize and sorghum cultivars. It does not appear as though these characteristics are stable at the various localities over the seasons, making cultivar selection and evaluation for the different maize and sorghum producing areas less feasible. It is also evident from this study that none of the cultivars from both crops exhibited complete resistance to FGSC pathogens, although some variation in their degree of susceptibility or tolerance towards these fungi was observed. In this study, qPCR was a reliable tool to detect and quantify FGSC pathogens in maize and sorghum samples throughout the seasons when compared to plating out of kernels on agar media.

Temperature, humidity and rainfall are some of the environmental factors that have a great importance for the incidence and severity of *Fusarium* species (Vigier *et al.* 1997). The optimum temperature for *F. graminearum s.l.* has been estimated at 24-26ºC (Reid & Sinha, 1998) while the fungus grows better under wet or high humidity conditions (Stewart *et al.*, 2002). In the 2007/08 season, most of the localities recorded rainfall exceeding 60 mm as well as relative humidities above 90% which is reported as optimal for the growth of *F. graminearum s.l.* (Reid & Sihna, 1998). The period of greatest susceptibility to ear rot and grain mold is silking or flowering and early kernel development (Reid & Sinha, 1998) and it is during this period that environmetal conditions can greatly influence incidence and severity of disease. This was evident in Cedara, where average maximum temperatures of 26.7, 25.0 and 25.9 and maximum RH>90% were recorded during the 2006/07; 2007/08 and 2008/09 seasons respectively in Febraury and March and could explain the high concentrations of FGSC DNA recorded in this study. Furthermore, Cedara falls within the KwaZulu-Natal mist belt which is characterised by high humidity and erratic rainfall, hence the high concentrations of FGSC DNA recovered from the area. According to Xu (2003), relative humidity (>90%) coinciding with warm conditions promotes spore germination and is critical for disease development while the incidence of infection increases with the duration of wet periods. Overall, the maximum RH at most of the localities was higher than 90%. Year-to-year variations in the amounts of disease are highly dependent on variable weather conditions within localities. The prevailing wet weather conditions coupled with RH >90% during the 2007/08 season created a more favourable environment for the growth of FGSC pathogens in both maize and sorghum grain samples, resulting in the increased levels of FGSC DNA concentrations.

Generally, the eastern production localities, which include the eastern Gauteng, eastern Free State, Mpumalanga and KwaZulu-Natal, had a higher prevalence of FGSC contamination in grain than the western localities. The eastern regions are classified as temperate and are generally cooler than the western localities (Figure 2.9) (ARC-GCI, 2012), therefore, the prevailing environmental conditions in these regions were more conducive for the growth of FGSC fungi over the seasons. However, this study also reveals the importance of some areas of the western producing localites where members of the FGSC were detected, albeit in lower numbers.

Planting early in the season generally exposes seed to low soil temperatures and more moisture which increases the time required for germination of the seed and favours *F. graminearum s.l.* (Broders *et al*., 2007). In this study however, no evidence of statistical differences between early and late planting of maize and sorghum grain were reported, although a reduction in FGSC concentrations was observed in localities such as Potchefstroom, Cedara and Bloekomspruit. According to ARC-GCI (2002) and Du Toit *et al*. (2002), production in the cooler eastern production areas starts at the beginning of October and continues into the first week of November, in the central regions, production begins during the last week of October and ends towards beginning of March. Broders *et al*. (2007) also reports that temperature and moisture at the time of planting are important factors in the ability of *F. graminearum s.l.* to infect seed or seedlings. While production periods and weather conditions could be useful in predicting the most suitable times to cultivate crops and in the reducing grain colonisation by FGSC pathogens, results of this study indicate that further investigation into the effects of planting date and environmental conditions on FGSC pathogens in maize and sorghum production localities of South Africa can contribute towards generating management strategies for local conditions.

Reid *et al.* (1999) observed that in mixed field inoculations, *F. verticillioides* interfered with the growth of *F. graminearum*. Likewise, Stewart *et al.* (2002) found that in the presence of *F. verticillioides,* the growth rates of *F. graminearum* were much lower. In this survey, low numbers of FGSC fungi were recovered from grain samples while other *Fusarium* spp. were more prevalent, especially on maize kernels. It is possible that the prevalence of FGSC DNA in grain samples was affected by interactions between these pathogens and other *Fusarium* spp. within the grain. Thus in the future, studying all the major pathogens associated with maize and sorghum production in South Africa will broaden our understanding of the

interactions that take place under field conditions and the impact of these on total fungal populations within grain.

Previous reports within South Africa have maintained that *F. graminearum s.l.* occurrence was insignificant and was the lesser contributor to the maize ear rot complex in commercial grain production (Rheeder *et al.,* 1994; Viljoen, 2003). Contrary to these reports, this survey shows a widespread distribution of the FGSC pathogens throughout grain producing localities in South Africa, especially when weather and environtmental conditions are favourable for pathogen prevalence and disease. These were the same observations made by Boutigny *et al.* (2011a, b) and Lamprecht *et al*. (2008, 2011) who also reported an increase in the prevalence of FGSC pathogens as pathogens of cereal hosts within South Africa. Finally, a combination of factors such as season, temperature region (locality), previous crop, cultivation practices, and cultivar could potentially be contributing to the FGSC populations colonising maize and cereal crops.

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Table 2.1. Localities, provinces and seasons in which maize kernel and sorghum grain samples were collected

Locality	Province	Season		
		2006/07	2007/08	2008/09
Amersfoort	Mpumalanga	*	*	sorghum
Bainsvlei	Free State	maize	*	*
Bethlehem	Free State	maize	maize & sorghum	maize & sorghum
Bloekomspruit	Gauteng	maize	maize	*
Bothaville	Free State	maize	maize	*
Cedara	KwaZulu Natal	*	maize & sorghum	maize & sorghum
Coligny	North West	*	maize	*
Danielsrus	Free State	*	maize	*
Delareyville	North West	maize	*	*
Delmas	Mpumalanga	*	maize	*
Dover	Free State	*	maize & sorghum	sorghum
Frankfort	Free State	*	maize	*
Goedgedacht	Mpumalanga	*	maize & sorghum	sorghum
Gottenburg	Free State	*	*	sorghum
Greenlands	Free State	*	maize & sorghum	sorghum
Hartebeesfontein	North West	maize	maize	*
Holmdene	Mpumalanga	*	sorghum	sorghum
Hoogekraal	North West	*	*	maize
Jim Fouche	Free State	*	maize	*
Kafferskraal	North West	*	*	sorghum
Klerksdorp	North West	*	sorghum	sorghum
Klipdrift	North West	*	sorghum	sorghum
Koster	North West	maize	*	*
Leeudoringstad	North West	maize	maize	maize
Leeuwkraal	Mpumalanga	*	*	sorghum
Marquard	Free State	maize	*	*
Nampo	Free State	*	maize	*
Nooitgedacht	Mpumalanga	*	maize	*
Ottosdal	North West	maize	*	maize
Parys	Free State	*	*	sorghum
Perdekop	Mpumalanga	*	*	sorghum
Platrand	Mpumalanga	*	sorghum	*
Platrand northeast	Mpumalanga	*	*	sorghum
Potchefstroom	North West	maize	maize & sorghum	maize & sorghum
Rietfontein	Mpumalanga	*	*	sorghum
Robertsdrift	Mpumalanga	*	maize	*
Rushof	Free State	maize	maize	*
Skaapplaas	Free State	*	*	sorghum
Tweebuffelsfontein	North West	maize	maize	*
Tweeling	Free State	*	maize	*
Vaalharts	North West	*	maize & sorghum	maize & sorghum
Val	Mpumalanga	*	sorghum	sorghum
Ventersdorp	North West	maize	*	*
Viljoenskroon	Free State	*	maize	*
Vrede	Free State	*	maize	*
Warden	Free State	*	maize	*
Weiveld	Free State	*	sorghum	sorghum
Wesselsbron	Free State	maize	*	maize
Wonderfontein	Mpumalanga	*	maize	*

*= no samples

Table 2.2. *Fusarium* spp. used to test the sensitivity and specificity of the TaqMan real-time PCR for detection of FGSC DNA in milled maize kernel and sorghum grain samples

Species name	Strain / isolate No.	Plant part	TaqMan Real-time PCR	
			Target calls	Average Crossing Point#
FGSC				
1 *Fusarium acaciae-mearnsii*	M431/35	Sorghum grain	+	21.82
2 *F. acaciae-mearnsii*	M431/92	Sorghum grain	+	21.70
3 *F. boothii*	M260/47	Maize kernels	+	21.85
4 *F. boothii*	M260/51	Maize kernels	+	21.24
5 *F. cortaderiae*	M431/17	Sorghum grain	+	21.95
6 *F. graminearum*	MRC4712	Maize kernels	+	15.83
7 *F. graminearum*	M637/36	Maize kernels	+	22.75
8 *F. graminearum*	M637/47	Maize kernels	+	22.40
9 *F. meridionale*	M431/18	Sorghum grain	+	22.63
10 *F. meridionale*	M431/28	Sorghum grain	+	22.97
11 *F. meridionale*	M431/60	Sorghum grain	+	22.77
12 *F. meridionale*	M431/91	Sorghum grain	+	23.20
Other *Fusarium* spp.				
13 *F. crookwellense*	PPRI9106	Maize roots	-	-
14 *F. culmorum*	PPRI10138	Maize kernels	-	-
15 *F. equiseti*	PPRI10141	Maize kernels	-	-
16 *F. pseudograminearum*	MRC4927	Maize kernels	+	13.84
17 *Fusarium*	M260/45	Maize kernels	-	-
18 *F. verticillioides*	PPRI7259	Maize kernels	-	-

mean of three replicates

Table 2.3. Number of maize kernel and sorghum grain samples contaminated with FGSC DNA during the 2006/07, 2007/08 and 2008/09 seasons as determined by TaqMan qPCR**

Season	Crop	
	Maize	Sorghum
2006/07	8 (86)	*
2007/08	118 (167)	28 (117)
2008/09	36 (55)	74 (133)

* = no grain samples collected

** = number in brackets is the total number of samples collected and tested per crop per season

Table 2.4. FGSC DNA (pg/mg**) in maize grain samples representing six cultivars collected during the 2006/07, 2007/08 and 2008/09 seasons in grain producing localities of South Africa

	Locality	Cultivars							
		DKC78-15B	PAN 6611	DKC80-10	DKC80-12B	CRN 3505	LS 8521B	Mean	t-Grouping#
2006/2007								**LSD =157.29**	
	Delareyville	nd	nd	215.00	nd	950.00	nd	194.17	a
	Hartebeesfontein	nd	nd	nd	nd	799.00	nd	133.17	ab
	Bethlehem	227.00	nd	nd	nd	16.60	nd	40.67	ab
	Koster	nd	nd	nd	nd	nd	68.80	11.50	b
	Bloekomspruit	nd	nd	nd	nd	17.80	nd	3.00	b
	Ottosdal	nd	nd	nd	nd	nd	13.30	2.17	b
	Bainsvlei	*	*	nd	nd	nd	*	0	b
	Bothaville	nd	nd	nd	nd	nd	*	0	b
	Leeudoringstad	nd	nd	nd	nd	nd	nd	0	b
	Marquard	nd	nd	nd	nd	nd	nd	0	b
	Potchefstroom	nd	nd	nd	nd	nd	nd	0	b
	Rushof	nd	nd	nd	nd	nd	nd	0	b
	Tweebuffelsfontein	nd	nd	nd	nd	nd	nd	0	b
	Ventersdorp	nd	nd	nd	nd	nd	nd	0	b
	Wesselsbron	nd	nd	nd	nd	nd	nd	0	b
	Mean (LSD =98.06)	16.21	0	14.33	0	118.93	6.31		
	t-Grouping	y	y	y	y	x	y		
2007/2008								**LSD =639.31**	
	Vrede	3920.00	258.00	955.00	629.00	2530.00	577.00	1478.20	a
	Danielsrus	143.00	238.00	265.00	2240.00	3340.00	115.00	1056.80	ab
	Delmas	273.00	223.00	1030.00	21.10	2940.00	1220.00	951.18	ab
	Bloekomspruit 1st planting	789.00	931.00	nd	718.00	2040.00	1110.00	931.30	abc
	Tweeling	2100.00	476.00	245.00	1450.00	875.00	115.00	876.80	abc
	Leeudoringstad	*	131.00	nd	2310.00	556.00	282.00	655.80	bcd
	Frankfort	931.00	1280.00	211.00	105.00	89.90	21.00	439.70	bcde
	Bothaville	5.75	3.48	2480.00	6.27	17.20	4.24	419.30	bcde
	Viljoenskroon	449.00	566.00	nd	684.00	122.00	nd	303.50	cde
	Nampo	65.30	nd	nd	1140.00	161.00	nd	227.70	de
	Cedara 2nd planting	6.11	494.00	13.50	294.00	258.00	nd	177.70	de
	Warden	126.00	97.30	183.00	96.40	123.00	292.00	152.80	de
	Robertsdrift	58.20	128.00	108.00	18.40	58.30	473.00	140.50	de
	Hartebeesfontein	46.70	132.00	nd	225.00	375.00	47.00	137.70	de
	Jim Fouche	97.70	61.30	287.00	148.00	25.50	19.80	106.70	de
	Bloekomspruit 2nd planting	5.43	208.00	3.40	239.00	134.00	25.10	102.30	de
	Wonderfontein	80.80	109.00	92.80	nd	nd	315.00	97.80	de
	Nooitgedacht	193.00	62.20	106.00	71.40	41.70	55.70	88.30	de
	Cedara 1st planting	328.00	78.90	7.64	nd	29.70	53.10	83.00	de
	Rushof	5.76	4.33	nd	32.30	104.00	38.70	30.80	de
	Potchefstroom 2nd planting	15.70	nd	89.00	nd	nd	nd	17.50	de
	Tweebuffelsfontein	4.00	nd	nd	82.60	11.90	1.04	16.70	de
	Potchefstroom 1st planting	nd	nd	80.00	nd	nd	6.39	14.30	e
	Bethlehem	nd	nd	68.90	nd	nd	nd	11.50	e
	Vaalharts 2nd planting	nd	nd	56.00	nd	nd	nd	9.30	e
	Vaalharts 1st planting	nd	nd	36.20	nd	1.28	nd	6.20	e
	Coligny	nd	nd	nd	nd	nd	nd	0.00	e
	Potchefstroom	nd	nd	nd	nd	nd	nd	0.00	e
	Mean (LSD = 295.96)	357.20	198.90	225.60	375.30	494.10	170.40		
	t-grouping	xy	xy	xy	xy	x	y		
2008/2009								**LSD = 354.14**	
	Cedara 2nd planting	1280.00	987.00	83.00	71.80	330.00	61.80	469.00	a
	Bethlehem 2nd planting	76.40	301.00	83.20	868.00	295.00	1020.00	440.50	ab
	Potchefstroom 3rd planting	110.00	73.50	88.60	236.00	574.00	206.00	214.80	abc
	Potchefstroom 2nd planting	98.20	317.00	nd	297.00	152.00	92.70	159.50	abc
	Cedara 1st planting	185.00	229.00	29.80	64.50	112.00	255.00	146.00	abc
	Potchefstroom 1st planting	59.00	nd	152.00	318.00	nd	nd	88.20	bc
	Leeudoringstad	nd	55.70	49.00	nd	93.30	nd	33.00	c
	Vaalharts 2nd planting	39.90	nd	nd	nd	nd	nd	6.70	c
	Vaalharts 1st planting	nd	nd	nd	nd	nd	nd	0.00	c
	Wesselsbron	*	*	nd	*	*	*	0.00	c
	Mean (LSD = 234.12)	210.70	200.90	163.60	218.20	172.90	54.00		
	t-grouping	x	x	x	x	x	x		

*no samples collected

**pg/mg = pg FGSC DNA per mg milled grain sample

nd = not detected

#Means followed by the same letters do not differ significantly at P≤0.05

Table 2.5. Analysis of variance of combined maize FGSC DNA data for the seasons 2006/07, 2007/08 and 2008/09 and six cultivars

Source of Variation	*df*	Sum of Squares	Mean Square	F Value	Pr > F
Season	2	4418801.66	2209400.83	25.51	<0.0001
Locality	37	25578682.02	691315.73	7.98	<0.0001
Cultivar	5	1800683.63	360136.73	4.16	0.0027
Locality*Cultivar	181	40022802.13	221120.45	2.55	<0.0001
Season*Locality	13	2041917.22	157070.56	1.81	0.0624
Season*Cultivar	10	426551.69	42655.17	0.49	0.8881

P values underlined are significant at 95% confidence level

Table 2.6. FGSC DNA (pg/mg[**]) in sorghum grain samples representing seven cultivars collected during the 2007/08 and 2008/09 seasons in grain producing localities of South Africa

	Locality	Cultivars								
		PAN 8420	PAN 8625	PAN 8247	PAN 8609	PAN 8648	PAN 8816	NS 5511	Mean	t-grouping[#]
2007/2008									**LSD=255.27**	
	Cedara 1st planting	0.00	1370.00	1400.00	2700.00	1030.00	1450.00	697.00	1235.29	a
	Cedara 2nd planting	953.00	198.00	608.00	369.00	1470.00	773.00	0.00	624.43	b
	Val	12.80	4.58	4.70	88.10	52.40	0.00	0.00	23.23	c
	Klerksdorp	4.49	0.00	99.30	0.00	0.00	0.00	0.00	14.70	c
	Potchefstroom Dryland	nd	40.80	nd	nd	nd	51.30	0.00	13.10	c
	Dover	0.00	61.80	0.00	0.00	*	16.40	0.00	13.00	c
	Goedgedacht	0.00	0.00	0.00	0.00	62.30	28.10	0.00	12.91	c
	Platrand	0.00	0.00	68.00	0.00	0.00	0.00	*	11.30	c
	Vaalharts 1st planting	0.00	0.00	62.80	0.00	0.00	0.00	0.00	10.50	c
	Holmdene	nd	nd	nd	15.90	nd	nd	0.00	2.30	c
	Greenlands	nd	nd	nd	nd	nd	nd	0.00	0	c
	Klipdrift	nd	nd	nd	nd	nd	nd	0.00	0	c
	Potchefstroom 1st planting	nd	nd	nd	nd	nd	nd	0.00	0	c
	Potchefstroom Irrigation	nd	nd	nd	nd	nd	nd	0.00	0	c
	Vaalharts 2nd planting	nd	nd	nd	nd	nd	nd	0.00	0	c
	Bethlehem	nd	nd	nd	nd	nd	nd	0.00	0	c
	Weiveld	nd	nd	nd	nd	nd	nd	0.00	0	c
	Mean (LSD= 163.47)	57.06	98.59	131.94	186.65	163.38	136.35	43.56		
	t-grouping	x	x	x	x	x	x			
2008/2009									**LSD=310.12**	
	Cedara	*	1900.00	3370.00	3790.00	*	2560.00	631.00	2450.20	a
	Potchefstroom 1st planting	*	136.00	319.00	369.00	*	198.00	203.00	245.00	b
	Vaalharts 1st planting	*	nd	224.00	174.00	*	115.00	151.00	132.80	b
	Val	*	60.80	42.60	308.00	*	19.20	28.80	92.00	b
	Potchefstroom 2nd planting	*	nd	142.00	126.00	*	nd	22.80	58.20	b
	Kafferskraal	*	nd	126.00	156.00	*	nd	nd	56.40	b
	Goedgedacht	*	59.30	67.40	77.80	*	31.20	nd	47.00	b
	Leeuwkraal	*	22.70	92.70	82.00	*	25.20	nd	44.60	b
	Klerksdorp	*	63.30	100.00	nd	*	35.50	nd	39.80	b
	Weiveld	*	20.90	19.60	75.80	*	6.20	42.20	33.00	b
	Weiveld 2nd Planting	*	nd	101.00	nd	*	55.60	nd	31.40	b
	Bethlebem 2nd planting	*	nd	102.00	13.60	*	nd	39.60	31.20	b
	Skaapplaas	*	47.70	21.90	*	*	20.90	*	30.30	b
	Potchestroom Dryland	*	89.10	55.70	nd	*	nd	nd	29.00	b
	Gottenburg	*	24.40	52.50	58.00	*	nd	nd	27.00	b
	Dover	*	37.50	12.30	20.40	*	50.10	12.10	26.40	b
	Holmdene	*	nd	nd	102.00	*	nd	nd	20.40	b
	Vaalharts 2nd planting	*	nd	52.70	45.70	nd	12.80	nd	18.70	b
	Platrand NorthEast	*	24.00	31.20	33.90	*	nd	nd	17.80	b
	Perdekop	*	26.70	34.80	25.60	*	nd	nd	17.60	b
	Rietfontein	*	nd	42.90	20.10	*	nd	*	15.80	b
	Parys	*	30.70	13.40	13.00	*	nd	nd	11.40	b
	Greenlands	*	nd	nd	nd	*	nd	34.90	7.00	b
	Amersfoort	*	nd	nd	nd	*	nd	nd	0.00	b
	Klipdrift	*	nd	nd	nd	*	nd	nd	0.00	b
	Potchefstroom 3rd planting	*	nd	nd	nd	*	nd	nd	0.00	b
	Potchestroom Irrigation	*	nd	nd	nd	*	nd	nd	0.00	b
	Mean (LSD=135.72)	*	40.78	38.15	83.00	*	33.33	170.04		
	t-Grouping	*	xy	xy	xy	y	y	x		

* = no samples

[**]pg/mg = pg FGSC DNA per mg milled grain sample

nd = not detected

[#]Means followed by the same letters do not differ significantly at P≤0.05

Table 2.7. ANOVA for combined FGSC DNA data in sorghum grain samples over the 2007/2008 and 2008/2009 seasons and seven cultivars

Source of Variation	*df*	Sum of Squares	Mean Square	F Value	Pr > F
Season	2	4418801.66	2209400.83	25.51	<0.0001
Locality	37	25578682.02	691315.73	7.98	<0.0001
Cultivar	5	1800683.63	360136.73	4.16	0.0027
Locality*Cultivar	181	40022802.13	221120.45	2.55	<0.0001
Season*Locality	13	2041917.22	157070.56	1.81	0.0624
Season*Cultivar	10	426551.69	42655.17	0.49	0.8881

P values underlined are significant at 95% confidence level.

Table 2.8. Total rainfall** (mm) and means for the months of February and March during the 2006/07, 2007/08 and 2008/09 seasons

Locality	2006/07			2007/08			2008/09		
	February	March	Mean	February	March	Mean	February	March	Mean
Amersfoort	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Bainsvlei	12.0	48.6	30.3	115.0	94.6	104.8	113.6	66.6	90.1
Bethlehem	25.1	33.9	29.5	*	*	*	67.9	20.2	44.1
Bloekomspruit	*	*	*	72.1	78.6	75.4	532.2	157.7	344.9
Bothaville	25.4	37.3	31.4	36.2	28.1	32.2	45.7	39.4	42.5
Cedara	18.9	107.9	63.4	76.0	58.9	67.5	33.2	39.8	36.5
Coligny	17.4	34.3	25.9	30.6	2.8	16.7	80.0	60.2	70.1
Danielsrus	25.1	33.9	29.5	72.1	78.6	75.4	171.45	38.86	105.2
Delareyville	29.5	59.9	44.7	63.3	164.8	114.1	34.0	72.1	53.1
Delmas	5.5	25.3	15.4	38.5	132.5	85.5	200.0	73.5	136.8
Dover	31.7	30.5	31.1	25.3	119.9	72.6	86.2	78.2	82.2
Frankfort	33.0	31.5	32.3	45.9	81.0	63.5	115.9	35.1	75.5
Goedgedacht	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Gottenburg	*	*	*	*	*	*	96.3	22.4	59.3
Greenlands	31.7	30.5	31.1	25.3	119.9	72.6	86.2	78.2	82.2
Hartebeesfontein	1.2	12.9	7.1	34.2	91.4	62.8	107.2	48.5	77.9
Holmdene	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Jim Fouche	33.0	31.5	32.3	45.9	81.0	63.5	115.9	35.1	75.5
Kafferskraal	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Klerksdorp	*	*	*	*	*	*	*	*	*
Klipdrift	73.4	50.3	61.9	108.7	138.9	123.8	20.8	73.9	47.4
Koster	25.9	37.1	31.5	73.6	108.7	91.2	33.5	107.4	70.5
Leeudoringstad	24.4	33.8	29.1	113.0	132.8	122.9	0.0	0.0	0.0
Leeuwkraal	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Marquard	38.7	17.4	28.1	56.8	103.0	79.9	92.7	99.1	95.9
Nampo	*	*	*	47.8	144.0	95.9	46.7	235.0	140.9
Nooitgedacht	*	*	*	*	*	*	*	*	*
Ottosdal	16.9	24.4	20.7	57.3	113.0	85.2	0.0	0.0	0.0
Parys	*	*	*	*	*	*	96.3	22.4	59.3
Perdekop	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Platrand	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Platrand NorthEast	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Potchestroom	73.4	50.3	61.9	108.7	138.9	123.8	20.8	73.9	47.4
Rietfontein	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Robertsdrift	33.0	31.5	32.3	45.9	81.0	63.5	115.9	35.1	75.5
Rushof	55.5	43.5	49.5	18.2	0.0	9.1	42.2	6.1	24.1
Skaapplaas	*	*	*	*	*	*	96.3	22.4	59.3
Tweebuffelsfontein	40.0	47.5	43.8	57.3	113.7	85.5	43.7	128.5	86.1
Tweeling	40.9	19.2	30.1	69.8	111.7	90.8	156.5	17.0	86.7
Vaalharts	9.4	40.4	24.9	61.1	49.2	55.2	166.9	36.1	101.5
Val	0.0	5.8	2.9	*	*	*	42.3	13.4	27.9
Ventersdorp	1.2	12.9	7.1	34.2	91.4	62.8	107.2	48.5	77.9
Viljoenskroon	55.5	43.5	49.5	18.2	0.0	9.1	42.2	6.1	24.1
Vrede	40.9	19.2	30.1	69.8	111.7	90.8	156.5	17.0	86.7
Warden	40.9	19.2	30.1	69.8	111.7	90.8	156.5	17.0	86.7
Weiveld	*	*	*	*	*	*	96.3	22.4	59.3
Wesselsbron	3.9	11.6	7.8	15.3	62.3	38.8	44.5	13.0	28.7
Wonderfontein	2.4	18.2	10.3	73.6	90.2	81.9	90.2	67.6	78.9

*= no metereological data

**= metereological data supplied by Agricultural Research Council-Institute of Soil Climate and Water

Table 2.9. Maximum temperatures** (°C) and means for the months of February and March during the 2006/07, 2007/08 and 2008/09 seasons.

Locality	2006/07			2007/08			2008/09		
	February	March	Mean	February	March	Mean	February	March	Mean
Amersfoort	27.7	*	27.7	*	*		25.7	25.0	25.4
Bainsvlei	32.7	28.9	30.8	*	*	*	27.4	28.3	27.9
Bethlehem	29.3	27.2	28.2	26.3	23.3	24.8	24.5	24.7	24.6
Bloekomspruit	*	*	*	28.9	25.2	27.0	26.8	26.5	26.7
Bothaville	32.5	29.8	31.1	30.8	30.0	30.4	27.9	27.8	27.8
Cedara	28.2	25.2	26.7	25.8	24.3	25.0	26.1	25.6	25.9
Coligny	31.4	29.1	30.2	27.9	27.5	27.7	25.9	25.5	25.7
Danielsrus	29.3	27.2	28.2	26.3	23.3	24.8	24.5	24.7	24.6
Delareyville	32.0	29.3	30.7	29.2	25.1	27.1	27.1	26.5	26.8
Delmas	30.3	28.8	29.5	27.5	24.7	26.1	26.0	25.5	25.8
Dover	31.7	29.6	30.7	29.7	25.4	27.5	26.6	26.3	26.4
Frankfort	30.4	28.8	29.6	28.4	23.6	26.0	26.4	26.1	26.3
Goedgedacht	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Gottenburg	*	*	*	*	*	*	34.5	34.7	34.6
Greenlands	31.7	29.6	30.7	29.7	25.4	27.5	26.6	26.3	26.4
Hartebeesfontein	31.4	29.3	30.4	28.9	25.4	27.2	26.9	26.4	26.6
Holmdene	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Jim Fouche	30.4	28.8	29.6	28.4	23.6	26.0	26.4	26.1	26.3
Kafferskraal	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Klerksdorp	*	*	*	*	*	*	*	*	*
Klipdrift	31.2	29.5	30.4	29.3	25.2	27.3	27.4	26.8	27.1
Koster	30.7	29.1	29.9	27.1	24.5	25.8	26.2	25.3	25.8
Leeudoringstad	31.6	29.4	30.5	30.3	26.9	28.6	28.4	29.1	28.8
Leeuwkraal	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Marquard	32.0	29.3	30.7	28.8	25.2	27.0	26.3	26.3	26.3
Nampo	*	*	*	32.1	27.4	29.8	29.3	29.7	29.5
Nooitgedacht	*	*	*	*	*	*	*	*	*
Ottosdal	32.6	29.9	31.2	29.9	25.3	27.6	26.8	27.6	27.2
Parys	*	*	*	*	*	*	34.5	34.7	34.6
Perdekop	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Platrand	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Platrand NorthEast	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Potchestroom	31.2	29.5	30.4	29.3	25.2	27.3	27.4	26.8	27.1
Rietfontein	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Robertsdrift	30.4	28.8	29.6	28.4	23.6	26.0	26.4	26.1	26.3
Rushof	32.1	30.6	31.3	30.6	31.2	30.9	27.5	27.3	27.4
Skaapplaas	*	*	*	*	*	*	34.5	34.7	34.6
Tweebuffelsfontein	29.5	28.0	28.8	26.7	23.9	25.3	25.4	24.8	25.1
Tweeling	29.4	27.6	28.5	28.1	38.9	33.5	25.8	26.0	25.9
Vaalharts	33.7	30.9	32.3	33.1	29.7	31.4	31.5	31.5	31.5
Val	27.7	*	27.7	*	*	*	25.7	25.0	25.4
Ventersdorp	31.4	29.3	30.4	28.9	25.4	27.2	26.9	26.4	26.6
Viljoenskroon	32.1	30.6	31.3	30.6	31.2	30.9	27.5	27.3	27.4
Vrede	29.4	27.6	28.5	28.1	38.9	33.5	25.8	26.0	25.9
Warden	29.4	27.6	28.5	28.1	38.9	33.5	25.8	26.0	25.9
Weiveld	*	*	*	*	*	*	34.5	34.7	34.6
Wesselsbron	32.6	30.4	31.5	31.3	27.0	29.1	27.7	28.5	28.1
Wonderfontein	29.9	28.9	29.4	28.3	25.5	26.9	27.8	27.7	27.8

*= no metereological data

**= metereological data supplied by Agricultural Research Council-Institute of Soil Climate and Water

Table 2.10. Maximum relative humidity[**] and means for the months of February and March over three seasons, 2006/07, 2007/08 and 2008/09.

Locality	2006/07			2007/08			2008/09		
	February	March	Mean	February	March	Mean	February	March	Mean
Amersfoort	98.0	*	98.0	*	*	*	100.0	100.0	100.0
Bainsvlei	69.5	75.5	72.5	*	*	*	86.5	80.4	83.4
Bethlehem	89.5	90.8	90.1	91.6	93.6	92.6	93.9	93.0	93.4
Bloekomspruit	*	*	*	87.2	90.6	88.9	88.8	89.3	89.0
Bothaville	92.2	89.3	90.7	97.3	98.2	97.7	99.9	99.8	99.9
Cedara	94.6	93.3	93.9	93.6	93.8	93.7	95.9	96.1	96.0
Coligny	71.3	71.9	71.6	87.4	86.8	87.1	89.3	89.1	89.2
Danielsrus	89.5	90.8	90.1	91.6	93.6	92.6	93.9	93.0	93.4
Delareyville	79.2	84.0	81.6	89.2	93.8	91.5	92.6	91.5	92.0
Delmas	93.7	91.0	92.3	97.4	98.1	97.7	97.5	97.9	97.7
Dover	88.3	85.3	86.8	93.2	96.9	95.0	98.8	99.4	99.1
Frankfort	92.3	89.4	90.8	92.9	94.7	93.8	93.9	94.2	94.0
Goedgedacht	98.0	*	98.0	*	*	*	100.0	100.0	100.0
Gottenburg	*	*	*	*	*	*	85.6	80.2	82.9
Greenlands	88.3	85.3	86.8	93.2	96.9	95.0	98.8	99.4	99.1
Hartebeesfontein	87.9	85.4	86.6	92.9	95.7	94.3	95.4	96.1	95.7
Holmdene	98.0	*	98.0	*	*		100.0	100.0	100.0
Jim Fouche	92.3	89.4	90.8	92.9	94.7	93.8	93.9	94.2	94.0
Kafferskraal	98.0	*	98.0	*	*	*	100.0	100.0	100.0
Klerksdorp	*	*	*	*	*	*	*	*	*
Klipdrift	82.3	78.6	80.4	86.0	91.9	89.0	89.5	89.8	89.7
Koster	84.4	85.5	84.9	93.3	94.6	94.0	95.0	95.1	95.0
Leeudoringstad	85.9	85.4	85.7	93.3	95.5	94.4	95.6	94.9	95.2
Leeuwkraal	98.0	*	98.0	*	*		100.0	100.0	100.0
Marquard	83.7	85.3	84.5	90.6	93.4	92.0	93.5	92.7	93.1
Nampo	*	*	*	92.1	94.8	93.4	94.8	94.8	94.8
Nooitgedacht	*	*	*	*	*	*	*	*	*
Ottosdal	87.0	91.8	89.4	99.5	100.0	99.8	100.0	99.7	99.8
Parys	*	*	*	*	*	*	85.6	80.2	82.9
Perdekop	98.0	*	98.0	*	*	*	100.0	100.0	100.0
Platrand	98.0	*	98.0	*	*	*	100.0	100.0	100.0
Platrand NorthEast	98.0	*	98.0	*	*	*	100.0	100.0	100.0
Potchestroom	82.3	78.6	80.4	86.0	91.9	89.0	89.5	89.8	89.7
Rietfontein	98.0	*	98.0	*	*		100.0	100.0	100.0
Robertsdrift	92.3	89.4	90.8	92.9	94.7	93.8	93.9	94.2	94.0
Rushof	90.0	86.6	88.3	89.9	84.6	87.2	91.7	92.7	92.2
Skaapplaas	*	*	*	*	*	*	85.6	80.2	82.9
Tweebuffelsfontein	82.4	82.5	82.4	90.1	91.9	91.0	92.2	92.0	92.1
Tweeling	88.7	88.1	88.4	92.4	95.4	93.9	94.5	93.4	93.9
Vaalharts	81.7	87.3	84.5	86.8	89.1	88.0	91.9	87.2	89.6
Val	98.0	*	98.0	*	*	*	100.0	100.0	100.0
Ventersdorp	87.9	85.4	86.6	92.9	95.7	94.3	95.4	96.1	95.7
Viljoenskroon	90.0	86.6	88.3	89.9	84.6	87.2	91.7	92.7	92.2
Vrede	88.7	88.1	88.4	92.4	95.4	93.9	94.5	93.4	93.9
Warden	88.7	88.1	88.4	92.4	95.4	93.9	94.5	93.4	93.9
Weiveld	*	*	*	*	*	*	85.6	80.2	82.9
Wesselsbron	83.9	84.8	84.3	89.4	94.7	92.0	96.5	95.7	96.1
Wonderfontein	89.7	85.2	87.4	91.7	91.9	91.8	95.8	96.2	96.0

[*]= no metereological data

[**]= metereological data supplied Agricultural Research Council-Institute of Soil Climate and Water

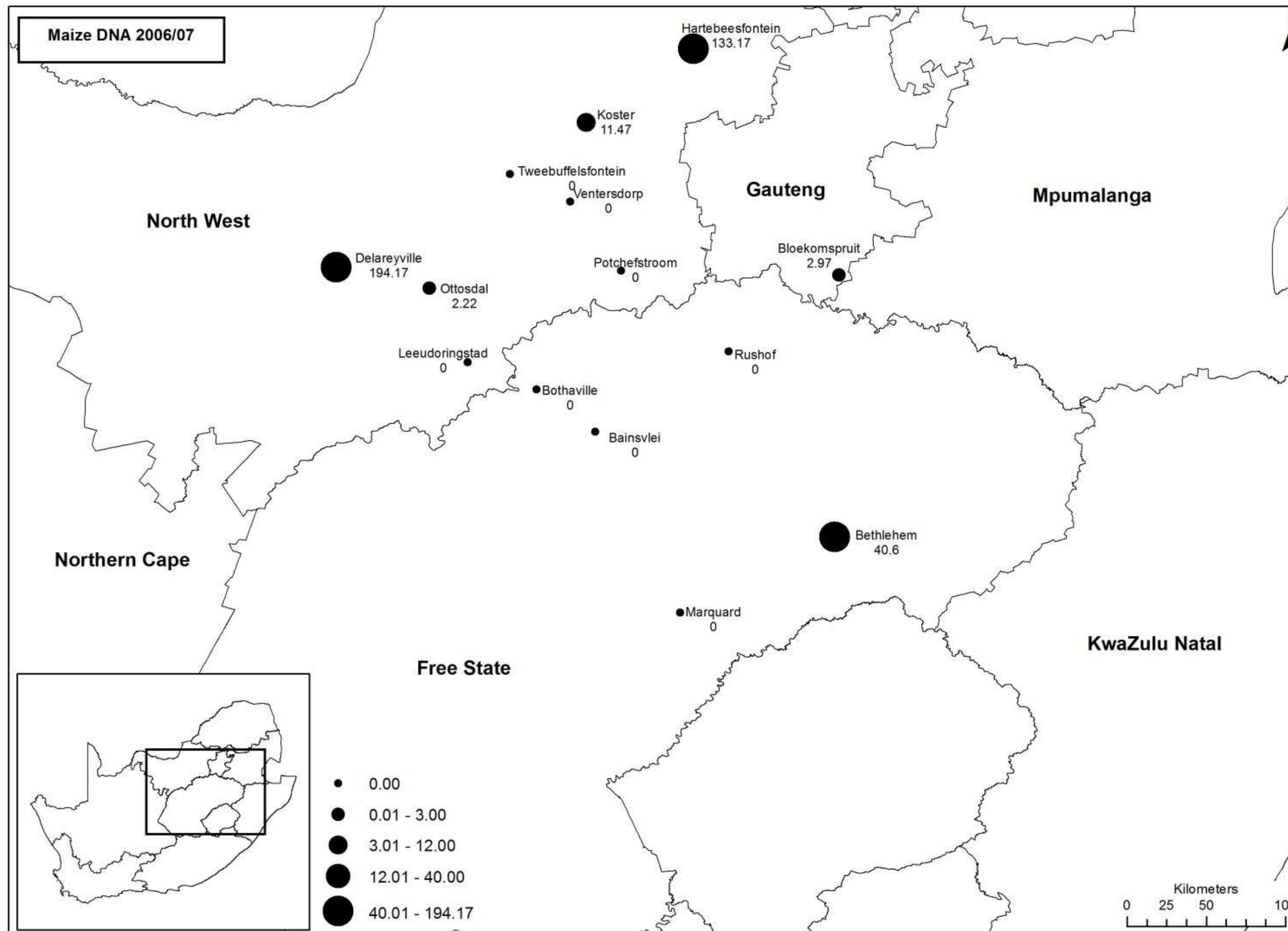

Figure 2.1. Distribution of FGSC DNA (pg/mg) in maize kernel samples collected during the 2006/07 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes

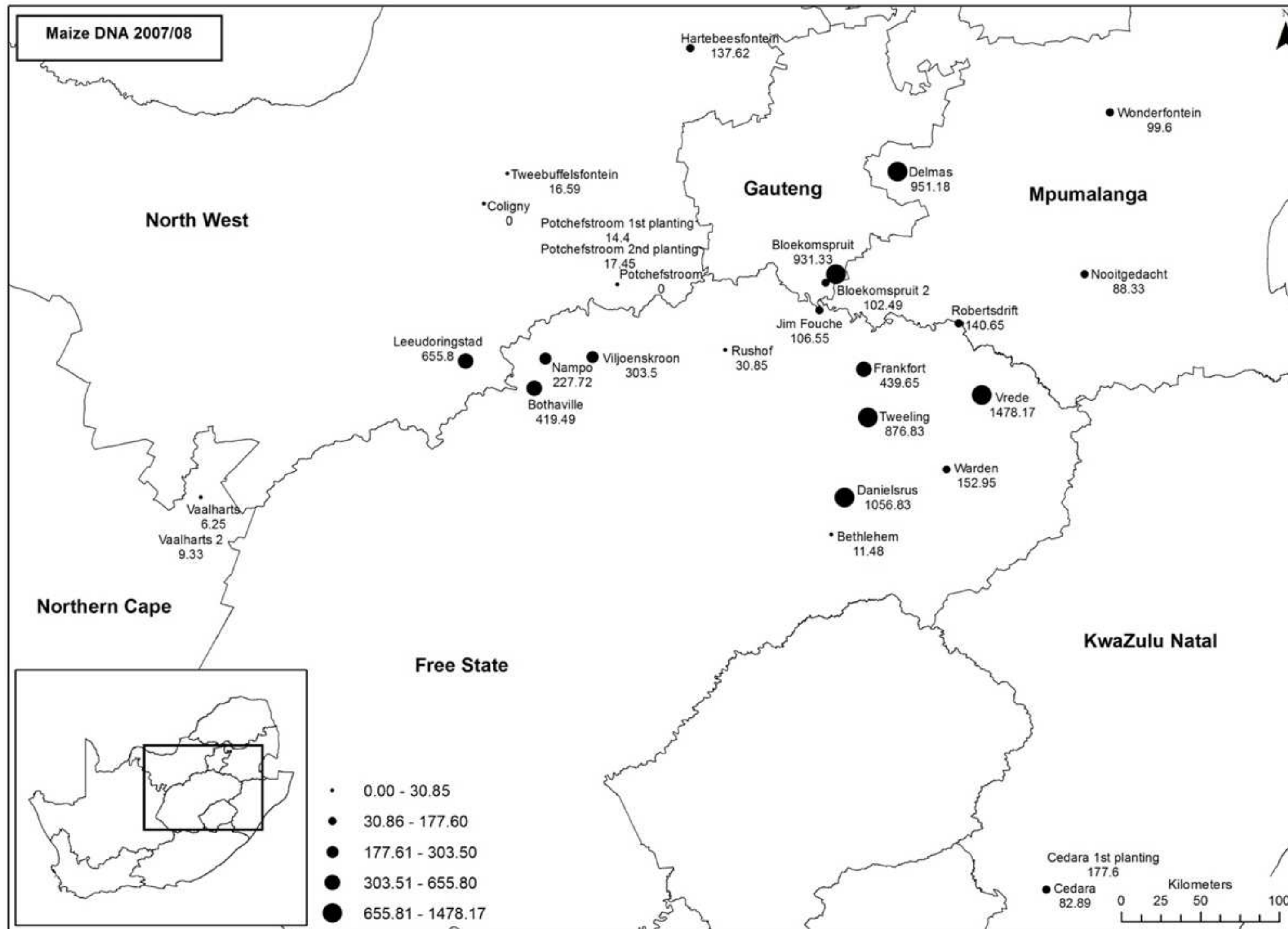

Figure 2.2. Distribution of FGSC DNA (pg/mg) in maize kernel samples collected during the 2007/08 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes

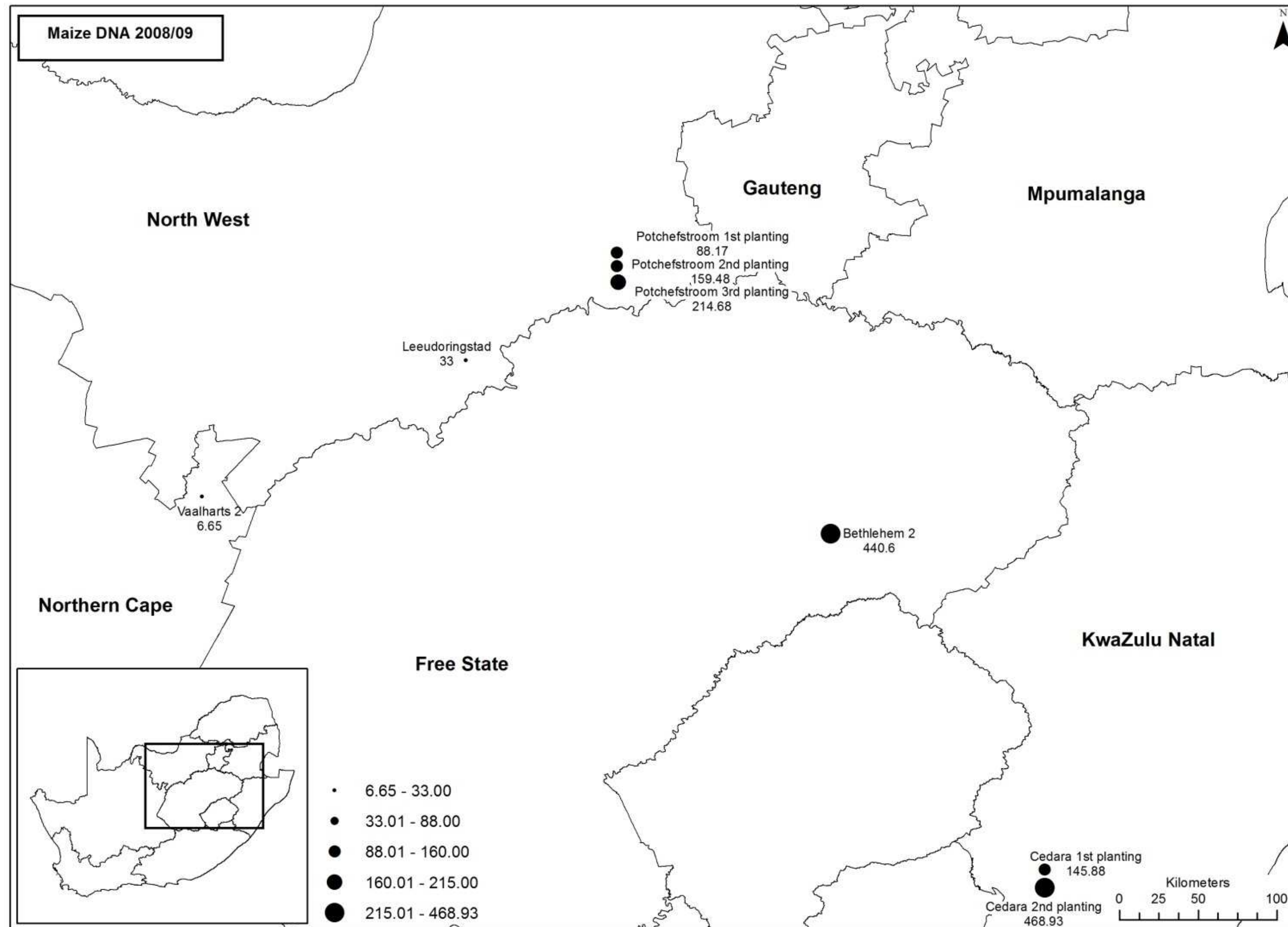

Figure 2.3. Distribution of FGSC DNA (pg/mg) in kernel samples collected during the 2008/09 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes

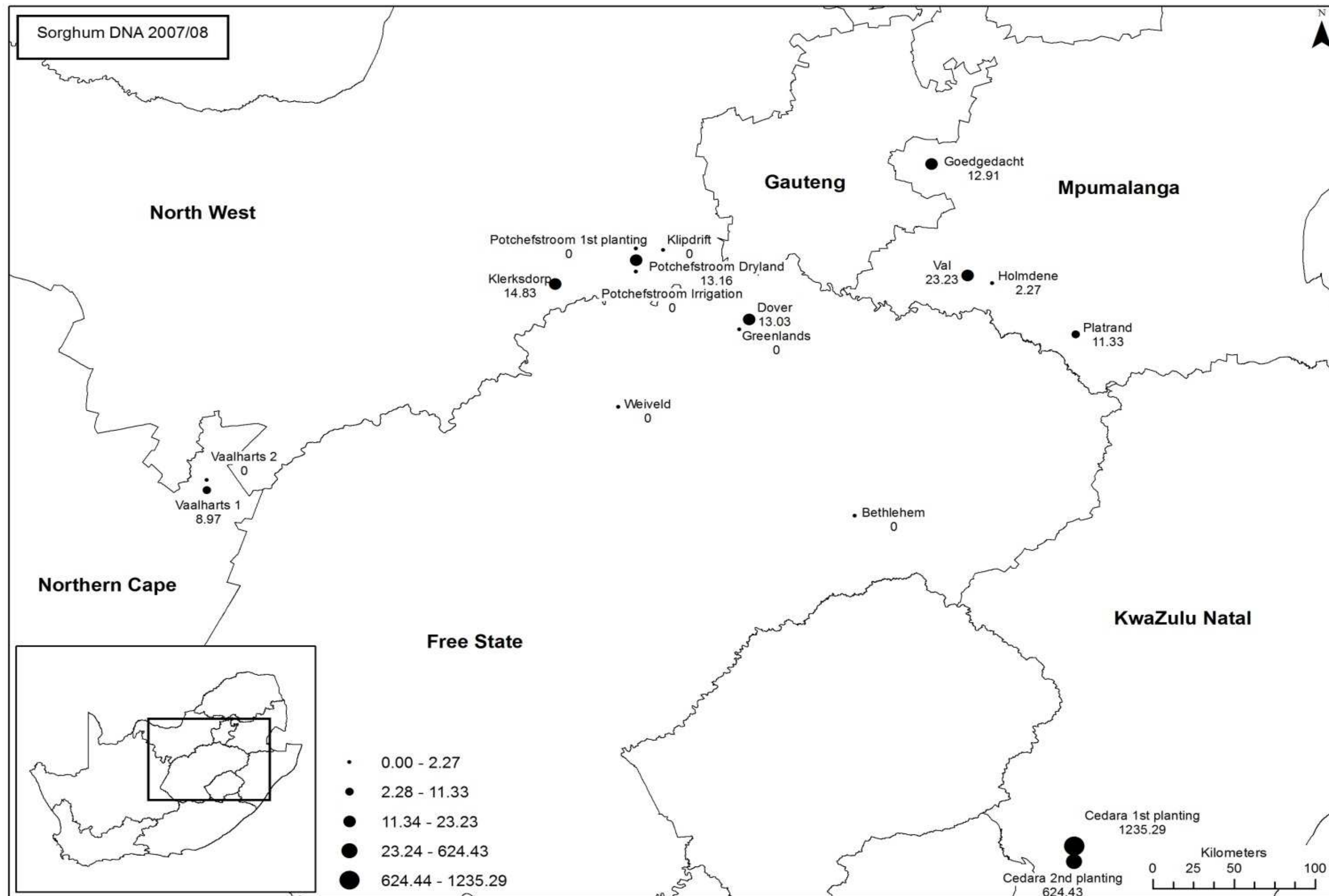

Figure 2.4. Distribution of FGSC DNA (pg/mg) in sorghum grain samples collected during the 2007/08 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes

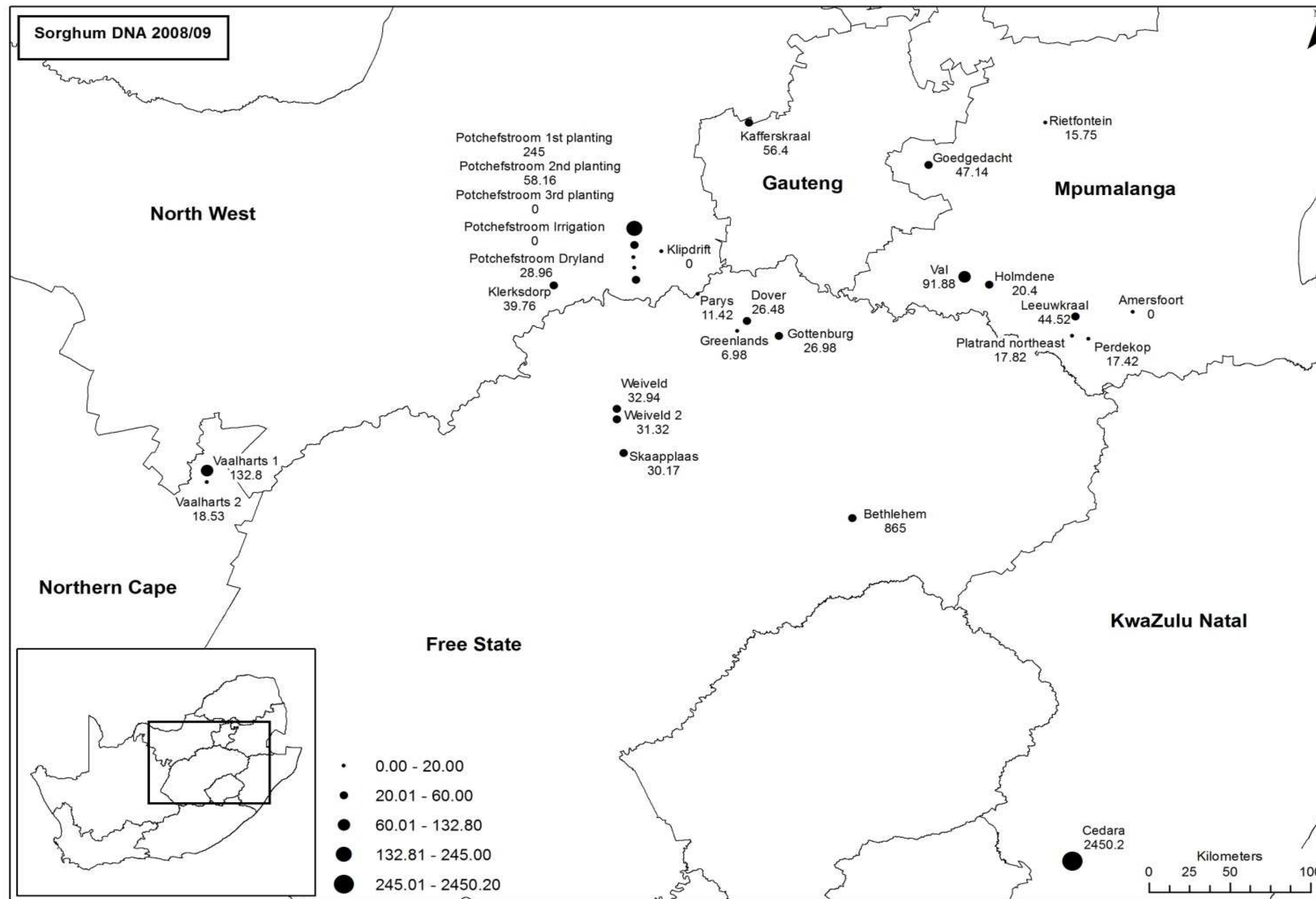

Figure 2.5. Distribution of *F. graminearum* DNA (pg/mg) in sorghum grain sam ples collected during the 2008/09 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes

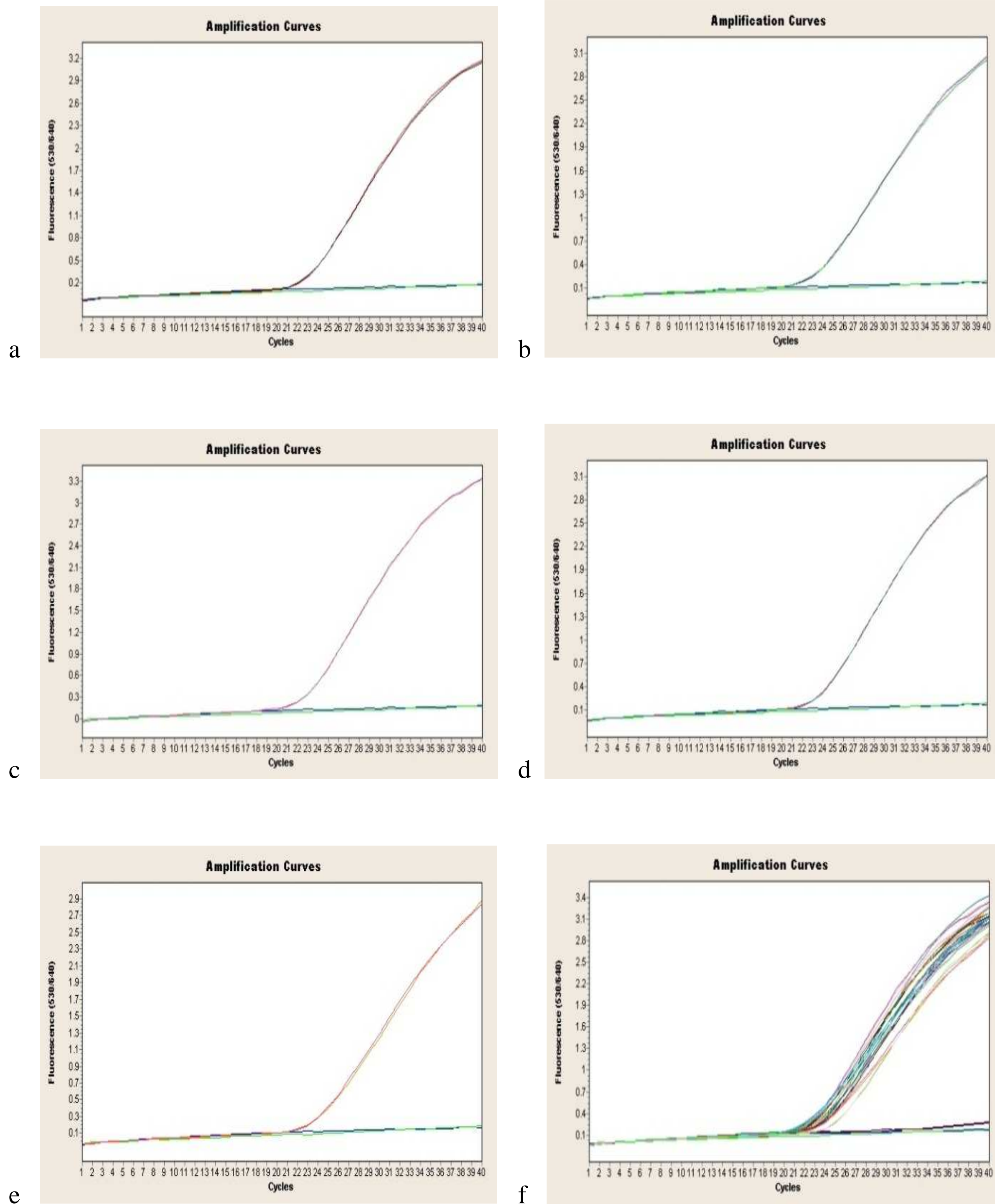

Figure 2.6. Amplification curves obtained for the *Fusarium graminearum* species complex (FGSC) isolates tested using TaqMan real-time PCR. a = *F. boothii*; b = *F. cortaderiae;* c = *F. acacia mearnsii*, d = *F. meridionale;* e = *F. graminearum* and f = a-e combined

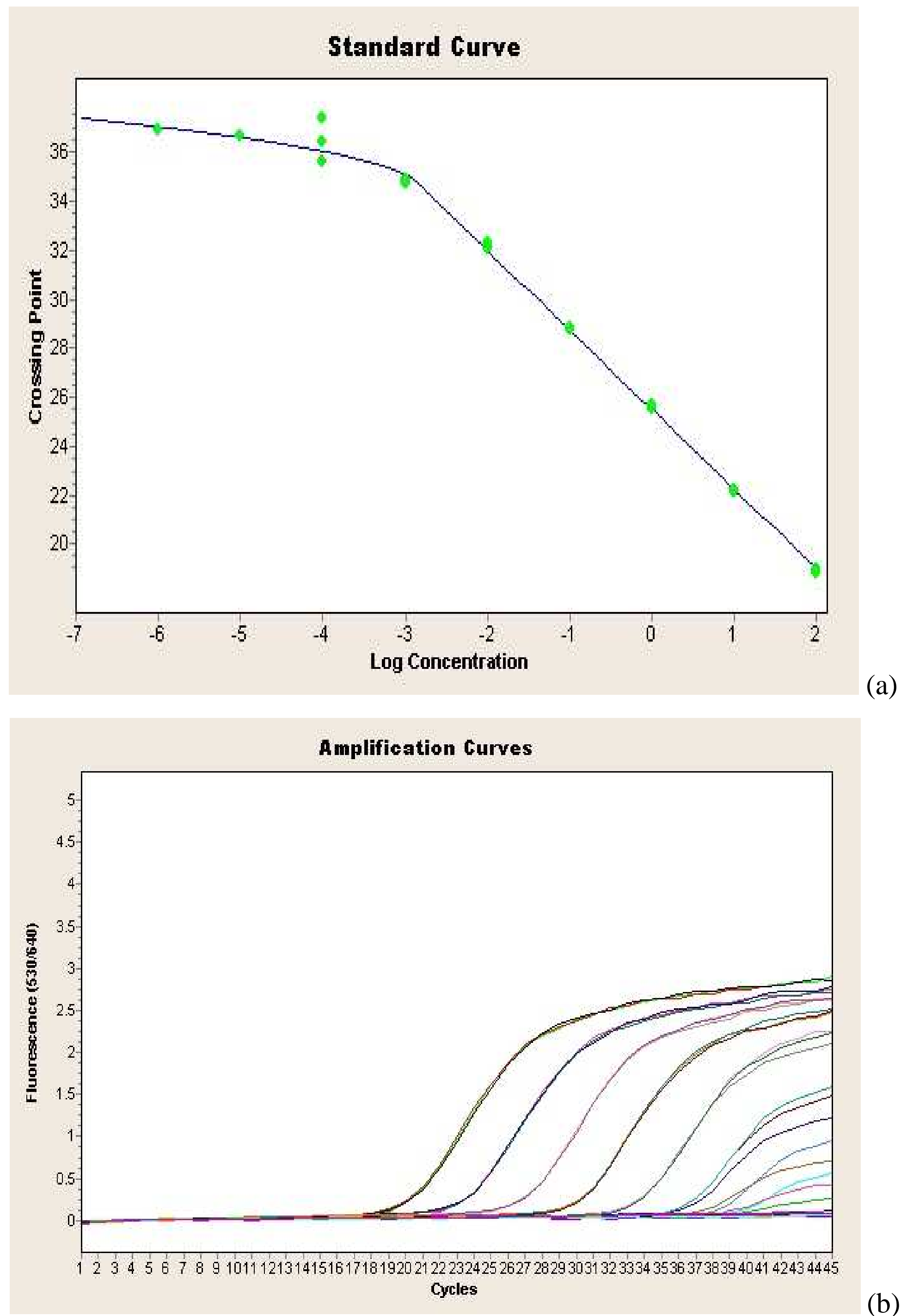

Figure 2.7. A standard curve (a) and amplification curves (b) demonstrating the quantification of *Fusarium graminearum* DNA using TaqMan real-time PCR. The crossing points (CP) or cycle thresholds (CT) were plotted against the logarithm of known standard concentration of *F. graminearum* DNA. The efficiency of this curve was 1.995 and the error was 0.0143

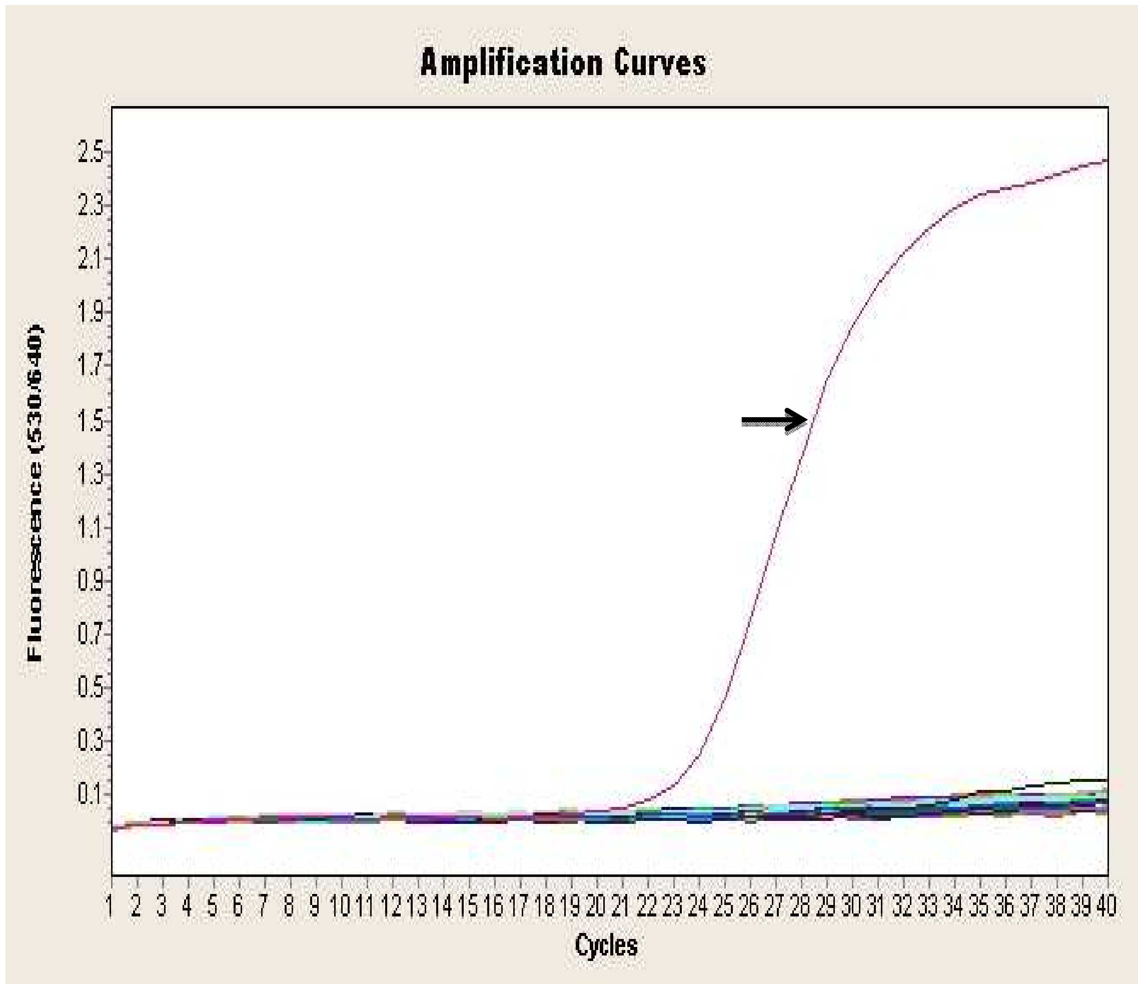

Figure 2.8. A fluorescence profile showing no *Fusarium graminearum* species complex (FGSC) detection in grain samples. The indicated pink curve is the positive control containing known concentration of *F. graminearum* DNA.

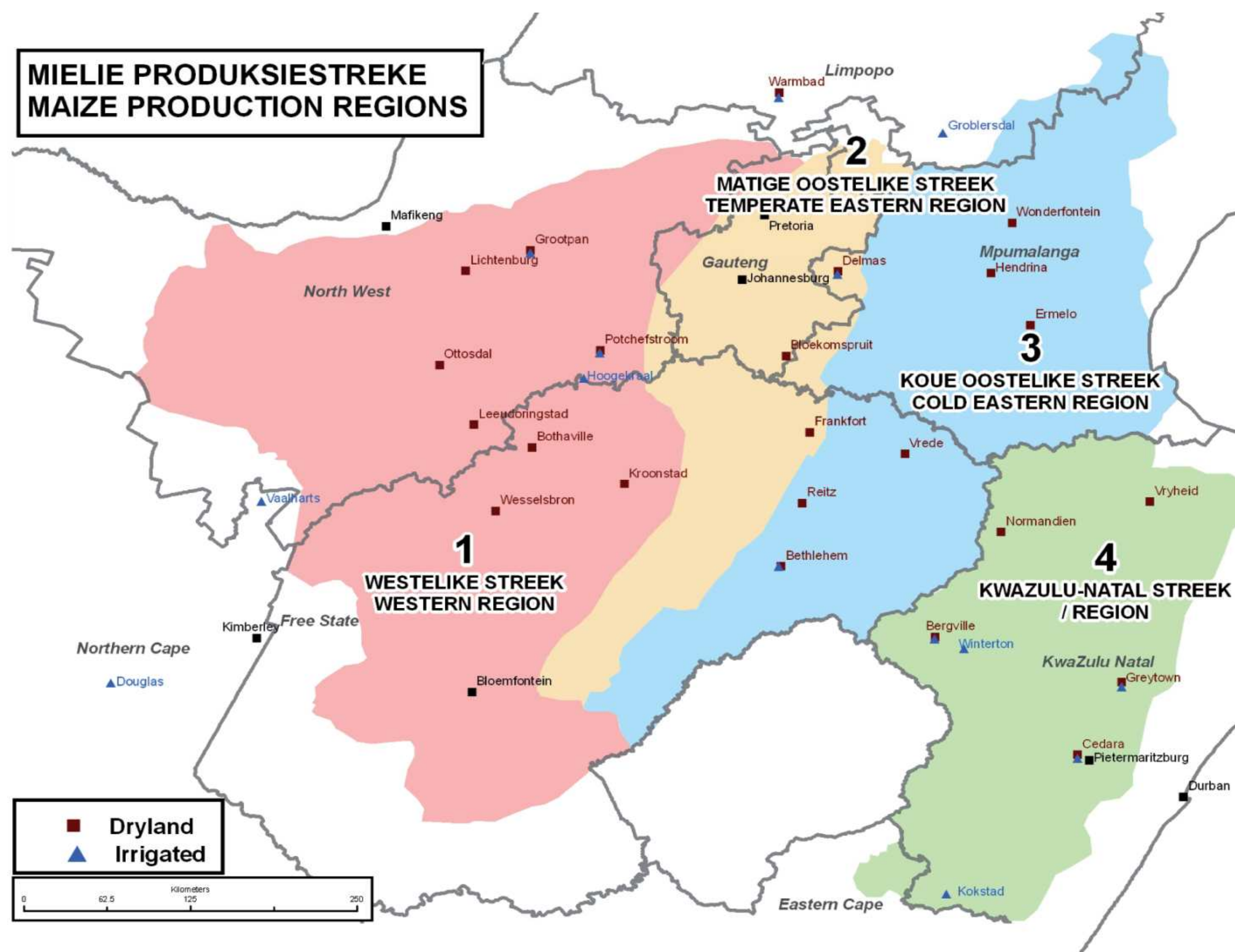

Figure 2.9. Map showing the subdivision of maize production of areas South Africa into four regions. Source: Maize information guide, 2012 (http://www.arc.agric.za)

CHAPTER 3

FUSARIUM GRAMINEARUM SPECIES COMPLEX MYCOTOXINS ASSOCIATED WITH GIBBERELLA EAR ROT AND GRAIN MOLD IN SOUTH AFRICA

Abstract

The aim of the present study was to investigate the occurrence of *Fusarium graminearum s.l.* mycotoxins deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) in South African maize and sorghum cultivars, in order to estimate the risk of contamination. A total of 308 maize samples were collected over three seasons from 2006/07, 2007/08 and 2008/09 while 250 sorghum samples were collected during 2007/08 and 2008/09. Deoxynivalenol and ZEA were determined using enzyme-linked immuno-sorbent assay (ELISA) test kits. Results show that maize and sorghum grain produced in South Africa are frequently contaminated with these mycotoxins at high concentrations. Over the respective seasons, ZEA was frequently found in both maize (67% positives; median 1105 µg/kg, maximum 11 804 µg/kg) and sorghum (86% positives, median 1360 µg/kg, maximum 4350). Confirmatory analyses for DON, ZEA, and NIV were conducted on 56 maize and 66 sorghum samples using liquid chromatography-mass spectrometry (LC-MS/MS). The LC-MS/MS results showed that DON, NIV and ZEA co-occurred in both maize and sorghum grain and that NIV was a contaminant of all the samples tested. Concentrations at some localites exceeded EU and USA legal limits in both crops which could impact on human and animal health as well as having impications for international marketing of grain.

3.1. INTRODUCTION

Fusarium spp. are frequently found as pathogens of cereal crops including maize and sorghum production worldwide. Among these are members of the *Fusarium graminearum* species complex (FGSC). This group of fungi comprises 15 biogeographically structured and phylogenetically distinct species (O'Donnell *et al.,* 2000, 2004, 2008; Starkey *et al.,* 2007; Davari *et al.,* 2012) that contaminate grains with trichothecene and estrogenic mycotoxins (Lysøe *et al.,* 2006; Wang *et al*., 2008). Type B trichothecene mycotoxins, including nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON) and 15-acetydeoxynivalenol (15-ADON); fusarenon X (4-acetylnivalenol, Fus X) and the estrogenic zearalenones (ZEA, α-and β-zearalenol and α- and β-zearalanol) are the principal FGSC mycotoxins reported as contaminants of cereal grains worldwide (Marasas *et al.,* 1981; Perkowski *et al.,* 1997; Eriksen *et al.,* 2004; Desjardins, 2006; Pasquali *et al.,* 2010)

The trichothecenes are a large group of structurally related sesquiterpenoid metabolites (Kumar *et al*., 2008). They are extremely potent inhibitors of protein synthesis in eukaryotes and interfere with initiation, elongation and termination processes (Bennett & Klich, 2003). When ingested at high doses, these mycotoxins induce nausea, vomiting and diarrhoea in humans and farm animals (Kumar *et al.,* 2008). Pigs are the most sensitive farm animals to trichothecenes, and these mycotoxins are associated with feed refusal and weight loss, leading to economic losses for the pig farmers (Eriksen *et al.,* 2004). Zearalenone can produce estrogenic syndromes in both man and animals. Children are more likely to be affected as a result of consuming ZEA-contaminated foods such as cereals and cereal-based food products (Bhat *et al.,* 2010).

The presence of trichothecene and estrogenic mycotoxins in food and animal feed has made testing of agricultural commodities such as maize, sorghum, wheat, oil seeds, soybean, forage, compound animal feeds and fishmeal a priority worldwide. The aim of the present study was to investigate the incidence and distribution of DON, NIV and ZEA in South African maize and sorghum. Enzyme-linked immunosorbent assay (ELISA) was initially used to quantify DON and ZEA. However, due to questions regarding the accuracy of this method, further studies were conducted using liquid chromatography-mass spectrometry coupled with mass spectrometry (LC-MS/MS).

3.2. MATERIAL AND METHODS

3.2.1. Field samples

Maize kernel and sorghum grain samples collected from National Cultivar Trials were supplied by the Agricultural Research Institute-Grain Crops Institute, Potchefstroom, South Africa. The maize samples were collected over three seasons (2006/07; 2007/08 and 2008/09) while sorghum grain samples were collected during two seasons (2007/08 and 2008/09) (Table 3.1). During this period, 308 maize samples from six cultivars namely, CRN3505, DKC78-15B, DKC80-10, DKC80-12B, LS8521B and PAN6611 and 250 sorghum samples from seven cultivars namely NS5511, PAN8247, PAN8420, PAN8609, PAN8625, PAN8648 and PAN8816 were collected. The distribution of localities from which the maize samples were collected over the three seasons is illustrated in Figures 3.1, 3.2 and 3.3 while Figures 3.4 and 3.5 illustrate respectively, the localities from which sorghum samples were collected in 2007/08 and 2008/09. Sub-samples were milled in a Retsch cross beater mill with a 1 mm sieve and stored at 4°C in airtight containers until use.

3.2.2. Enzyme-linked immunosorbent assays for DON and ZEA

The Veratox® 5/5 quantitative DON test and Veratox® for Zearalenone test kits (Neogen Corporation, Lansing, Michigan, USA) were respectively used for the analysis of DON and ZEA. The tests were done according to the manufacturer's instructions. Both test kits are competitive direct enzyme-linked immunosorbent assays (CD-ELISA) wherein free toxin in the samples and in the positive controls (standards) competes with enzyme-labelled conjugate (toxin) for antibody binding sites in a microwell. After washing the wells with distilled water, a substrate which reacts with the bound conjugate to produce a blue colour is added into each well. The intensity of the blue colour is used to calculate toxin concentrations in the samples, i.e. the darker the blue colour, the lower the toxin is in a sample. Optical densities obtained for the standards are used to generate a standard curve against which sample densities were compared to calculate the exact concentrations of the mycotoxins in the samples. For DON, the limit of detection was 250 µg/kg, the range of quantification was 250 to 2000 µg/kg while for ZEA, the limit of detection was 25 µg/kg and the range of quantitation was 25 to 500 µg/kg.

Due to the unavailability of commercial ELISA kits, NIV was not evqaluated using this methodology.

3.2.2.1. Extraction of DON and ZEA in maize kernel and sorghum grain samples

DON was extracted from 10 g sub-samples of finely ground maize and sorghum by homogenising in 100 ml Millipore water for 3 min using the Ika UltraTurrax homogenisor (model TP18/10). Approximately 10 ml of the extract was filtered through Whatman$^{(R)}$ no. 1 (Maidstone, England) filter paper.

Extraction of ZEA was done in 25 g sub-samples of finely ground maize and sorghum samples. Each sample was homogenised in 125 ml of 70 % methanol for 3 min using an Ika UltraTurrax homogenisor (model TP18/10). At least 15 ml of the extract was filtered through Whatman$^{(R)}$ No. 1 filter paper. One millilitre of the collected filtrate was diluted in a 1:5 ration using Millipore water (Merck Millipore, Billerica, Massachusetts, USA).

3.2.2.2. Quantification of DON and ZEA in maize kernel and sorghum grain extracts

Sample analysis for both DON and ZEA was conducted using 100 µl of the extract for each individual sample and controls according to the manufacturer's instructions. The following standards were respectively included by the manufacturer of the CD-ELISA kits: DON: 0, 0.25, 0.5, 1, 2 ppm; ZEA: 0, 25, 75, 150, and 500 ppb. The concentrations of both mycotoxins were quantified using a Thermo Scientific Multiskan Ex (Thermo Electron Corporation, Vantaa, Finland) microwell reader with a 650 nm filter. Neogen's Veratox for Windows software (Neogen Corporation, Lansing, Michigan, USA) was used to determine the concentration of the mycotoxins. All samples with concentrations that exceeded the 2 ppm and 500 ppb DON and ZEA respectively were diluted and retested.

3.2.3. Multitoxin extraction and detection of DON, NIV and ZEA in maize and sorghum using LC-MS/MS

3.2.3.1. Reagents

Mycotoxin standards (DON, NIV and ZEA) were purchased from Sigma Aldrich (Milan, Italy) and stored at 4°C in the dark. The standards were individually dissolved in 99% methanol, mixed and conditioned to room temperature before use. Working standard solutions were prepared by serially diluting each individual standard stock solution with a suitable solvent mixture (methanol/water, 70:30; v/v). These solutions were also kept at 4°C. Water for LC mobile phase and organic solvents (methanol and acetonitrile) were high performance liquid chromatography (HPLC) grade from Merck (Darmstadt, Germany) and ammonium acetate (MS grade) was bought from Sigma-Aldrich, South Africa. The extracts were filtered using Whatman No. 1 filter papers (Maidstone, England). SGE C18-GP125 columns were purchased from SGE Analytical Science (Melbourne, Australia). Filtek syringe filters (0.22 µm; 25 mm) were purchased from Chemtek Analytica (Bologna, Italy).

3.2.3.2. Sample preparation

A total of 56 maize kernel and 66 sorghum grain samples were selected to represent high, medium and low levels of ZEA contamination as determined by ELISA. Since these samples were postitive for ZEA, the assumption made was that other two toxins would be present. Ten grams of finely ground maize kernels and sorghum grain samples were mixed with 50 ml of acetonitrile/water (84:16; v/v) in Erlenmeyer flasks. The flasks were stirred on a rotary shaker for 1 h. The extracts were filtered through Whatman No. 1 (Maidstone, England) filter paper and 5 ml of the extract was recovered.

The filtered extracts were evaporated to dryness using a gentle stream of nitrogen. The dry extracts were reconstituted using 400 µL of mobile phase. The vials were vigorously vortexed and the samples were filtered through a 0.22 µl cellulose filter prior to analysis.

3.2.3.3. LC parameters

Liquid chromatography (LC) analysis was performed using a system consisting of two micropumps (Series 200, PerkinElmer, Waltham, Massachusetts, USA). Chromatographic separation was performed using a Phenomenex® RP-C_{18} (150mm, 2.0 mm, 5.0 µm) column (Torrance, California, USA). The flow rate was 250 µl/min, while the injection volume was 20 µl.

Mobile phase A consisted of water containing 5 mM ammonium acetate, while mobile phase B was made of 100% methanol containing 5 mM ammonium acetate. An isocratic mobile phase was used, consisting of 20% solvent A and 80% solvent B.

3.2.3.4. MS/MS parameters

Tandem mass spectrometry (MS/MS) data were obtained using the an Agilent 1200 system interfaced to an API 2000 triple-quadruple mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada), coupled with an electrospray ionization (ESI) source. The declustering potential (DP) and collision energy (CE) were optimized for each compound by direct infusion of standard solutions (10 µg/ml) into the mass spectrometer at a flow rate of 8 µl/min, using a Model 11 syringe pump (Harvard Apparatus, Holliston, Massachusetts, USA). Biosystems/MDS SCIEX Analyst software version 1.4.2 was used for data acquisition. The spray voltage was kept at 4000 V in the negative mode and acquisition was carried out by selected reaction monitoring (SRM). DON, NIV and ZEA were identified. Additional mass spectrometer settings are given in Table 3.2.

3.2.4. Data Analysis - ELISA

Grains from two commodities were regarded as two separate surveys. Maize samples were planted over three seasons, 34 localities and six cultivars. Sorghum samples were planted over two seasons, 22 localities and seven cultivars. The data collected were concentrations of DON and ZEA in the milled samples. The initial analysis was to test if the season variances of the log transformed and untransformed data from each survey are of comparable magnitude using Levene's test (Levene, 1960). There was not sufficient evidence against homogeneity, therefore a three factor analysis (season x locality x cultivar) was performed on

the transformed data using the three factor interaction as error term. The standardised residuals were subjected to Shapiro-Wilk's test to test for deviation from normality (Shapiro & Wilk, 1965). In all cases, deviation from normality was due to Kurtosis and not skewness as a result of numerous zero values, therefore we accept the data as reliable (Glass *et al*., 1972). Means of significant effects were compared using Fisher's protected t-test and least significant differences (LSD) at a 5% confidence level (Snedecor & Cochran, 1967). All the statistical procedures were performed using SAS/STAT version 9.2 statistical software (SAS, 1999). Additionally, the Jenks optimization method, also known as the Jenks natural breaks classification method (Jenks, 1967) was used to determine the best arrangement of data into different classes and these were plotted on maps (Figures 3.1 to 3.10).

3.3. RESULTS

3.3.1. Enzyme linked immunosorbent assays

The number of maize and sorghum samples contaminated with DON and ZEA over the three seasons is provided in Table 3.9. The mean concentrations and geographic distributions of DON in maize kernel samples during the 2006/07, 2007/08 and 2008/09 seasons are presented in Figures 3.1, 3.2 and 3.3, respectively, while the means and distributions of ZEA are presented in Figures 3.4, 3.5 and 3.6 for the three seasons, respectively. Figures 3.7 and 3.8 illustrate the DON concentrations and geographic distributions in sorghum grain samples during the 2007/08 and 2008/09 seasons, while the means and distributions of ZEA during the same seasons are presented in Figures 3.9 and 3.10, respectively.

3.3.1.1. Distribution of DON in maize kernel samples

The mean groupings for the DON concentrations in maize samples during the 2006/07, 2007/08 and 2008/09 seasons are presented in Table 3.3. In the 2006/07 season DON was not detected in the 86 maize samples collected at 15 individual localities. Overall, 12% maize samples were contaminated with DON over the three seasons (Table 3.3).

In the 2007/2008 season, 18% maize samples were contaminated with DON and this mycotoxin was detected in all the cultivars but not at all localities (Table 3.4). The highest concentration of DON detected in a sample was 5520.00 µg/kg (DKC-78-15B, Delmas)

while the lowest concentration of the same mycotoxin detected was 282 µg/kg (DKC-78-15B, Delmas). Cultivar means ranged from 104.40 µg/kg (LS8521B) to 448.00 µg/kg (CRN3505). Of the six cultivars, LS8521B was the most resistant to contamination with DON. Delmas had the highest mean concentration of 2061.30 µg/kg and differed significantly from the Danielsrus and the remaining localities (Table 3.4).

DON was detected in 15% (Table 3.3) of the maize samples collected during the 2008/09 season and similar to 2007/08, this toxin was detected in all the cultivars but not all localities The highest concentration detected in a sample was 1440.00 µg/kg (PAN6611, Cedara) and the lowest concentration detected was 353.00 µg/kg (DKC-78-15B, Potchefstroom). Bethlehem (2nd planting) had the highest DON means of 448.00 µg/kg and differed significantly from the remaining localities, with the exception of Cedara 2nd planting and Potchefstroom 3rd planting. Similarly to 2006/07 and 2007/08, DON was not detected in Leeudoringstad (Table 3.4).

A combined analysis of variance (ANOVA) for the 2006/07, 2007/08 and 2008/09 data indicated significant differences ($P \leq 0.05$) between seasons, localities and cultivars . The ANOVA for localitiy by cultivar indicated that there were significant interactions between these two factors. No significant interactions for season by locality and season by cultivar were recorded (Table 3.5).

3.3.1.2. Distribution of ZEA in maize kernel samples

In the 2006/07 season, 59% (Table 3.3) maize samples were contaminated with ZEA. The cultivar and locality means for ZEA in maize during the 2006/07, 2007/08 and 2008/09 seasons are presented in Table 3.6. The highest concentration detected in a sample was 362.32 µg/kg (DKC78-15B, Bethlehem) and the lowest concentration detected was 25.23 µg/kg (LS8521B, Bethlehem). Cultivar means ranged from 14.85 µg/kg to 49.13 µg/kg. LS8521B has the lowest mean concentration but did not differ significantly from the other cultivars (Table 3.6). Only two mean groupings were observed for locality responses to ZEA contamination with group (b) having 14 of the 15 localities (Table 3.6). With the highest mean ZEA concentration of 179.00 µg/kg, Bethlehem differed significantly ($P \leq 0.05$) from

the remaining localities and thus appeared to be favourable for grain contamination with this mycotoxin.

During the 2007/2008 season, 83% maize samples (Table 3.3) were contaminated with ZEA. The highest concentration recorded in a sample was 3516.99 µg/kg (DKC80-10, Bothaville) and the lowest concentration recorded in a sample was 25.39 (CRN3505, Robertsdrift). Cultivar means ranged from 165.21 µg/kg to 461.14 µg/kg. Zearalenone concentrations were generally higher in this season than during the 2006/07 and 2008/09 seasons. Twelve mean groupings were observed for locality responses to ZEA contamination, with group (cdefg) containing seven of the 12 groupings (Table 3.6). Bothaville was the locality with the highest mean ZEA concentration (1507.28 µg/kg) and differed significantly from the rest of the localities.

In the third season, 2008/2009, 42% (Table 3.3) maize samples were contaminated with ZEA. The highest concentration detected in a sample was 1529.15 µg/kg (PAN6611, Cedara) while the lowest concentration in a sample was 25.71 µg/kg (DKC-80-10, Potchefstroom). Cultivar means ranged from 28.80 µg/kg to 217.90 µg/kg (Table 3.6) and no significant differences between the means were recorded. Leeudoringstad yielded the highest mean ZEA cpmcemtration of 309.50 µg/kg, although localites did not differ from one another (Table 3.6). Similarly to DON, ZEA concentrations were higher in the 2007/08 season than in the 2006/07 and 2008/09 seasons.

A combined analysis of variance (ANOVA) (Table 3.7) for the three seasons indicated significant differences ($P \leq 0.05$) between seasons, localities and season by locality. No significant differences between cultivars, locality by cultivar and season by cultivar were recorded.

3.3.1.3. Distribution of DON in sorghum grain samples

During the 2007/2008 season, 13% sorghum samples were contaminated with DON (Table 3.3). The highest and lowest concentrations recorded in a sample were 1520.00 µg/kg (NS5511, Cedara) and 273 µg/kg (NS5511, Vaalharts), respectively. Cultivar means ranged from 28.12 to 331.00 µg/kg. Cultivar NS5511 yielded the highest mean and differed

significantly from the remaining six localites. Only four mean groupings were observed for localities, with group (c) having 14 of the 18 localities. Cedara 1st and 2nd plantings differed significantly from each other and from the remaining localities, with mean concentrations of 605.9 and 330.4 µg/kg respectively (Table 3.8).

In the 2008/09 season, 14% (Table 3.3) sorghum samples were contaminated with DON. The highest concentration recorded in a sample was 1330.00 µg/kg (PAN8609, Cedara) and the lowest concentration was 260 µg/kg (PAN8625, Perdekop). Cultivar means ranged from non-detectable to 170.04 µg/kg and differed significantly from one another (Table 3.8). There were only four mean groupings for localities with group (c) containing 14 of the 27 localities. Cedara had the highest mean DON concentration (929.60 µg/kg) and differed significantly from the rest of the localities (Table 3.8). Overall, DON concentrations were higher in the 2008/09 than in 2007/08 season.

A combined analysis of variance (ANOVA) for the two seasons indicated significant differences ($P \leq 0.05$) between localities and cultivars. No significant interaction between seasons, locality by cultivar, season by locality and season by cultivar were recorded (Table 3.9).

3.3.1.4. Distribution of ZEA in sorghum grain samples

In the 2007/2008 season, 100% sorghum samples (Table 3.3) were contaminated with ZEA. The highest concentration recorded in a sample was 2174.89 µg/kg (PAN8609, Cedara) while the lowest concentration recorded was 169.65 µg/kg (PAN8816, Dover). Cultivar means ranged from 490.79 µg/kg to 798.50 µg/kg (Table 3.10). Cultivar NS5511 ranked as the most susceptible of all the cultivars while PAN8420, PAN8648 and PAN8816 ranked as the least susceptible. Average ZEA concentrations at the various localities ranged from 207.41 to 1404.76 µg/kg. Cedara 2nd plantings yielded the highest locality mean and did not differ significantly from Cedara 1st planting, Vaalharts 2nd planting and Potchefstroom 1st planting. Eight of the localities had mean groupings within group (d) (Table 3.10) which formed the least conducive localities in the season.

Seventy nine percent of the samples collected in 2008/09 (Table 3.3) were contaminated with ZEA. The highest concentration recorded in a sample was 2602.95 µg/kg (PAN8816, Dover) while the lowest concentration recorded was 26.35 µg/kg (PAN8625, Potchefstroom). Cultivar means ranged from 390.52 µg/kg to 565.76 µg/kg (Table 3.10). No significant differences between cultivars were recorded. Average ZEA concentrations at the various localities ranged from 8.40 to 1586.12 µg/kg . Dover yielded the highest locality mean and differed significantly from other localities, with the exception of Skaapplaas (Table 3.10). Overall, grain contamination with ZEA was greater in the 2008/09 season than in the 2007/08.

A combined analysis of variance (ANOVA) (Table 3.11) over the two seasons showed significant differences ($P \leq 0.05$) between the seasons, localities and season by locality. No significant differences were recorded between cultivars, locality by cultivar and season by cultivar.

3.3.2. LC-MS/MS detection and quantification of DON, NIV and ZEA in maize kernel and sorghum grain samples.

The chromatograms for the three mycotoxins are illustrated in Figures 3.11. The calibration curves for DON, NIV and ZEA had R^2 values of 0.9985, 0.9943 and 0.9916 respectively (Figure 3.12). These three mycotoxins were detected in both crops at varying concentrations.

3.3.2.1. DON, NIV and ZEA in maize kernel samples

DON was detected in 77% maize samples, with mean concentrations ranging from from 60.69 to 237.46 µg/kg. When the same samples were analysed using ELISA, 39% were contaminated with DON and the mean concentrations ranged from 256.22 to 9315.33 µg/kg (Table 3.12). Only 20% of the maize samples were contaminated with ZEA, with mean concentrations ranging from 4415.75 to 25 678.45 µg/kg. The ELISA assays for ZEA showed that 79% of the samples were contaminated although the concentrations were less than those determined using LC-MS/MS and ranged from 361.45 to 3935.19 µg/kg. NIV was detected in 100% of the samples, with mean concentrations ranging from 3275.68 to 8983.38 µg/kg (Table 3.12). Regression analysis for the relationships between ELISA determinations

and LC-MS/MS values yield R^2 = 0.56 for DON while those for ZEA were R^2= 0.12 (not significant). A regression coefficient of 6.37 indicates that ELISA values were generally six times greater than those recorded with LC-MS/MS.

3.3.2.1. DON, NIV and ZEA in sorghum grain samples

Data for sorghum contamination with DON, NIV and ZEA are presented in Table 3.13. DON was detected in 58% of the samples, and the concentrations ranged from 10.62 to 61.62 µg/kg. The same samples when tested using ELISA, yielded higher contamination levels (41%) and the mean concentrations ranged from non-detectable to 411.17 µg/kg. NIV was detected in 100% of the samples and the mean concentrations ranged from 6597.39 to 9144.00 µg/kg. Eighteen % of the samples contained ZEA, and the mean concentrations ranged from non-detectable to 42 608.32 µg/kg. Regression analysis for the relationships between ELISA determinations and LC-MS/MS values yield R^2 = 0.18 and R^2=0.007 (not significant) for DON and ZEA respectively, indicating no relationship between detection levels using the two assessment methods.

3.4. DISCUSSION

The occurrence and concentrations of DON, ZEA and NIV were determined in maize and sorghum grain samples collected from various production areas within South Africa over three seasons between 2006 and 2009. According to the Food and Agricultural Organization (FAO), over 25% of the world's agricultural production is contaminated with mycotoxins (Fink-Gremmels & Leistner, 1990). South Africa is no exception, as previous studies conducted locally have indicated the co-occurrence of DON, NIV and ZEA in locally grown maize and wheat grain (Sydenham *et al.,* 1989; Viljoen, 2003). This study has confirmed this and highlights the importance of FGSC mycotoxins in South African cereal grain, adding sorghum to the list of locally affected grains.

DON is one of the most common *Fusarium* mycotoxins found in cereal grains worldwide (CAST, 2003). The detection of this mycotoxin in maize kernel and sorghum grain samples from localities within South Africa indicates that local grain is continually predisposed to contamination with this mycotoxin. The ELISA results showed that sorghum samples had DON concentrations between 260 and 1520 µg/kg. The concentrations detected were lower

when LC-MS/MS technique was used, ranging from 26.9 to 183.38 µg/kg. Worldwide regulations, guidelines or reference levels for DON in cereal grain ranges from 200 to 2000 µg/kg depending on the country and regulating authority (Joint FAO/WHO, 2012). Many samples fell within this range while a few exceeded the 2000 µg/kg level when ELISA was used. Although the effects of DON on human health are not completely understood, the EU has set the maximum level allowed in raw cereals at 200 and 1250 µg/kg for baby foods and cereal products respectively (Bhat *et al.,* 2010). Previous reports by Rheeder *et al.* (1995) showed that DON was a contaminant of maize in South Africa, with concentrations up to 1830 µg/kg being recorded. The authors concluded that this mycotoxin did not pose a threat to animal health in South Africa. Despite this, the continual occurrence of DON as a natural contaminant of cereal grain calls for regular monitoring as implications for human and animal health can be huge.

Worldwide, there are currently no advisory levels or regulations set for NIV in food and feed as this mycotoxin is often overshadowed by the widely distributed and well-studied DON. However, NIV remains one of the most important *Fusarium* trichothecenes as it is believed to be at least ten times more toxic than DON (Yoshida & Nakajima, 2010). Detection of NIV in all the samples tested using LC-MS/MS is of concern for the safety of food and feed products in South Africa due to the toxicity of this mycotoxin. Moreover, NIV is known to co-exist with DON which could augment the toxicity of either toxin. Thus, control measures effective for both NIV and DON in cereal crops need to be established within the South African cereal grain industry.

ZEA is a *Fusarium* mycotoxin with potent estrogenic activity. This toxin has been implicated in several incidents of precocious pubertal changes and children are most sensitive. In domestic animals, heperestrogenic or feminizing syndromes have been recorded, with swine being the most sensitive. The EU has set the maximum tolerated levels for ZEA in food commodities between 20 and 100 µg/kg as from 1 July 2006 (Santini *et al.,* 2008) while some reports have this level set at 200 µg/kg. The Joint Expert Committee on Food Additives (JECFA) on the other hand has established a provisional maximum tolerable daily intake of ZEA to be 0.5 µg/kg (JECFA, 2000). The ELISA results in this study showed that at least 78% of the samples had concentrations above 20 µg/kg. This could signal the importance of this mycotoxin within South Africa as the majority of the South African population consume these cereal grains as major sources of energy on a daily basis. The

maximum tolerable levels for ZEA in animal feeds in South Africa are 100 and 250 µg/kg for swine and cattle respectively (Animal Feed Manufacturers Association, 2001). At least 43% of the total number of samples processed with in this study had concentrations above 200 µg/kg. Due to its heat stability, ZEA may be transmitted from contaminated grains into beer at various stages of the brewing process.

The high ZEA concentrations have implications for both human and animal health as these concentrations can induce mycotoxicoses in farm animals. Furthermore, high concentrations of ZEA have been previously reported in African beers in Nigeria (< 2 000 µg/L); Swaziland (< 53 000 µg/L); Zambia (< 4 600 µg/L); (Okoye, 1987; Scott, 1996; Sibanada *et al.,* 1997). Although the exact mechanism of ZEA toxicity is not completely established, this mycotoxin has been shown to be hepatotoxic, haematotoxic, immunotoxic and genotoxic, making this mycotoxin an important one for the South African consumer.

Although detected in fewer samples when LC-MS/MS technique was used, ZEA concentrations were over 200 times more than the EU level and much higher than those reported in South African commodities by Dutton *et al.* (2001). The most important feature of ZEA distribution in cereal grain and animal feed is its co-occurrence with other *Fusarium* mycotoxins, including DON and NIV. We also noted that samples contaminated with ZEA were also contaminated with DON and/or NIV, which could enhance the synergistic and or additive effects of these Fusarium mycotoxins in both humans and domestic animals.

The ELISA technique is useful as a screening tool for mycotoxins in a wide range of matrices. However, this technique has certain disadvantages, including the possibility of false positives due to cross-reactivity and the possibility of false negatives. We found some of the samples that were selected as positive turned out to be negative, highlighting the need for confirmatory assays in routine analysis of mycotoxins. It is also apparent from these findings that using more precise quatification methods (i.e. using LC-MS/MS instead of ELISA or TLC) will reduce variability associated with mycotoxin testing.

Mycotoxins DON, NIV and ZEA were detected in field samples that were contaminated while the crop was still standing in the field. Thus, the presence of one or more *Fusarium* spp. on the maize ears and grain sorghum as well as the environmental conditions that favour fungal proliferation could have contributed to the production of DON, NIV and ZEA in the

grain. DON was not detected in the maize samples collected from the 2006/07 season, in contrast to samples contaminated during the 2007/08 and 2008/09 seasons. This could be the result of factors such as favourable prevailing weather conditions while the crop is still in the fields as well as the presence of inoculum. There was more rain in 2007/08 than in 2006/07 and 2008/09, and this could be one of the factors that contributed the contamination of large numbers of samples with mycotoxins DON, NIV and ZEA. *Fusarium graminearum* grows optimally at 24-28°C and at a minimum of 0.90 a_w, while toxin production largely mirrors growth conditions (Pitt *et al.,* 2000). The average maximum temperature throughout the grain producing localities over the three seasons were between this range as discussed in chapter two, creating environmental conditions for grain colonisation and subsequent contamination with mycotoxins which influenced mycotoxin production.

Although symptomatic and asymptomatic grain is often blended post-harvest, undoubtedly lowering the concentration of mycotoxins, we estimate that these mycotoxins occur in amounts that are sufficient to cause mycotoxicoses, including hyperestrogenism and infertility in domestic animals and may have health implications for small children who are primarily fed cereal-based foods. The major concern with the contamination of cereal grains with DON, NIV and ZEA is the stability of these toxins during food processing and milling (CAST, 2003). Consequently, even low levels of these mycotoxins in raw grain should be seen as a potential threat to human and animal health, given the dependence of the majority of the South African population on maize as a staple food. The long term exposure of consumers to these mycotoxins is also cause for concern as there are no regulatory or advisory guidelines for any of these toxins set for local producers and for industry as a whole, with particular reference to the food and feed industry. Thus, the continual occurrence and co-occurrence of these mycotoxins in South African cereal grains is cause for concern, especially because there are currently no studies evaluating the amounts consumed daily by the majority of the South African population that is dependent on maize and sorghum as staple sources of energy.

From the preceding discussion, it can be concluded that in the samples evaluated, both maize and sorghum had significant levels of DON, NIV and ZEA. The results of the current study highlights co-occurrence of DON, NIV and ZEA in both maize and sorghum grain samples. It is likely that the intake of mycotoxin contaminated cereal grain products is greater in South Africa as the majority of the population may have less well controlled grain storage systems.

It is therefore recommended that these and other mycotoxins in cereal grains are monitored regularly as they could have adverse effects on animal production as well as human health.

3.5. REFERENCES

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Table 3.1. Localities, Provinces and seasons in which maize kernel and sorghum grain samples were collected.

Locality	Province	Season		
		2006/07	2007/08	2008/09
Amersfoort	Mpumalanga	*	*	sorghum
Bainsvlei	Free State	maize	*	*
Bethlehem	Free State	maize	maize & sorghum	maize & sorghum
Bloekomspruit	Gauteng	maize	maize	*
Bothaville	Free State	maize	maize	*
Cedara	KwaZulu Natal	*	maize & sorghum	maize & sorghum
Coligny	North West	*	maize	*
Danielsrus	Free State	*	maize	*
Delareyville	North West	maize	*	*
Delmas	Mpumalanga	*	maize	*
Dover	Free State	*	maize & sorghum	sorghum
Frankfort	Free State	*	maize	*
Goedgedacht	Mpumalanga	*	maize & sorghum	sorghum
Gottenburg	Free State	*	*	sorghum
Greenlands	Free State	*	maize & sorghum	sorghum
Hartebeesfontein	North West	maize	maize	*
Holmdene	Mpumalanga	*	sorghum	sorghum
Hoogekraal	North West	*	*	maize
Jim Fouche	Free State	*	maize	*
Kafferskraal	North West	*	*	sorghum
Klerksdorp	North West	*	sorghum	sorghum
Klipdrift	North West	*	sorghum	sorghum
Koster	North West	maize	*	*
Leeudoringstad	North West	maize	maize	maize
Leeuwkraal	Mpumalanga	*	*	sorghum
Marquard	Free State	maize	*	*
Nampo	Free State	*	maize	*
Nooitgedacht	Mpumalanga	*	maize	*
Ottosdal	North West	maize	*	maize
Parys	Free State	*	*	sorghum
Perdekop	Mpumalanga	*	*	sorghum
Platrand	Mpumalanga	*	sorghum	*
Platrand northeast	Mpumalanga	*	*	sorghum
Potchefstroom	North West	maize	maize & sorghum	maize & sorghum
Rietfontein	Mpumalanga	*	*	sorghum
Robertsdrift	Mpumalanga	*	maize	*
Rushof	Free State	maize	maize	*
Skaapplaas	Free State	*	*	sorghum
Tweebuffelsfontein	North West	maize	maize	*
Tweeling	Free State	*	maize	*
Vaalharts	North West	*	maize & sorghum	maize & sorghum
Val	Mpumalanga	*	sorghum	sorghum
Ventersdorp	North West	maize	*	*
Viljoenskroon	Free State	*	maize	*
Vrede	Free State	*	maize	*
Warden	Free State	*	maize	*
Weiveld	Free State	*	sorghum	sorghum
Wesselsbron	Free State	maize	*	maize
Wonderfontein	Mpumalanga	*	maize	*

*= no samples

Table 3.2. Mass spectrometer settings for the detection of NIV, DON and ZEA using LC-MS/MS

Mass spectrometer settings	NIV	DON	ZEA
Precursor ion [M + H]+ (m/z)	371	355	317
Product ion (m/z)	281	265	175
Declustering potential (V)	-1	-1	-16
Collision energy (eV)	-17	-14	-28
Nebulizer gas	30	30	30
Auxiliary gas	75	75	75
Curtain gas	20	20	20
Collision gas, N2	5	5	5
Source temperature (0C)	450	450	450
Ionization voltage (V)	-4500	-4500	-4500

Table 3.3. Number of maize and sorghum grain samples contaminated with DON and ZEA during the 2006/07, 2007/08 and 2008/09 seasons in South Africa as determined by ELISA** technique

Season	Maize		Sorghum	
	DON	ZEA	DON	ZEA
2006/07	0 (86)	50 (86)	*	*
2007/08	30 (167)	138 (167)	15 (117)	117 (117)
2008/09	8 (55)	23 (55)	19 (133)	105 (133)

* =no samples

** =number in brackets is the total number of samples collected and tested per crop per season

Table 3.4. Mean groupings of DON concentrations (µg/kg**) obtained using ELISA in maize kernel samples representing six cultivars collected during the 2006/07, 2007/08 and 2008/09 seasons in grain producing localities of South Africa

	Locality	Cultivars							
		DKC78-15B	PAN 6611	DKC80-10	DKC80-12B	CRN 3505	LS 8521B	Mean	t-Grouping
2006/2007								LSD =0.00	
	Delareyville	nd	nd	nd	nd	nd	nd	nd	a
	Hartebeesfontein	nd	nd	nd	nd	nd	nd	nd	a
	Bethlehem	nd	nd	nd	nd	nd	nd	nd	a
	Koster	nd	nd	nd	nd	nd	nd	nd	a
	Bloekomspruit	nd	nd	nd	nd	nd	nd	nd	a
	Ottosdal	nd	nd	nd	nd	nd	nd	nd	a
	Bainsvlei	*	*	nd	nd	nd	*	nd	a
	Bothaville	nd	nd	nd	nd	nd	*	nd	a
	Leeudoringstad	nd	nd	nd	nd	nd	nd	nd	a
	Marquard	nd	nd	nd	nd	nd	nd	nd	a
	Potchefstroom	nd	nd	nd	nd	nd	nd	nd	a
	Rushof	nd	nd	nd	nd	nd	nd	nd	a
	Tweebuffelsfontein	nd	nd	*	nd	nd	nd	nd	a
	Ventersdorp	nd	nd	nd	nd	nd	nd	nd	a
	Wesselsbron	nd	nd	nd	nd	nd	nd	nd	a
	Mean (LSD =0.00)	0.00	0.00	0.00	0.00	0.00	0.00		
	t-Grouping	x	x	x	x	x	x		
2007/2008								LSD =740.47	
	Delmas	5520.00	565.00	1580.00	nd	3850.00	853.00	2061.30	a
	Danielsrus		284.00	nd	nd	4000.00	1030.00	885.70	b
	Bloekomspruit 1	3590.00	nd	nd	1070.00	nd	nd	776.70	bc
	Frankfort	315.00	1500.00	1860.00	nd	nd	nd	612.50	bcd
	Vrede	0.00	nd	nd	nd	1860.00	371.00	371.80	bcd
	Cedara 2nd planting	nd	nd	620.00	nd	1490.00	nd	351.70	bcd
	Wonderfontein	nd	nd	1020.00	nd	590.00	nd	322.00	bcd
	Tweeling		305.00	nd	420.00	753.00		246.30	bcd
	Bothaville	1140.00	nd	nd	nd	nd	nd	190.00	bcd
	Robertsdrift	nd	279.00	nd	nd	nd	668.00	157.80	bcd
	Vaalharts 2nd planting	nd	nd	nd	866.00	nd	nd	144.33	cd
	Viljoenskroon	nd	nd	nd	816.00	nd	nd	136.00	cd
	Nooitgedacht	487.00	nd	nd	nd	nd	nd	81.17	cd
	Jim Fouche	282.00	nd	nd	nd	nd	nd	47.00	cd
	Leeudoringstad	*	nd	nd	nd	nd	nd	0.00	d
	Nampo	nd	nd	nd	nd	nd	nd	0.00	d
	Warden	nd	nd	nd	nd	nd	nd	0.00	d
	Hartebeesfontein	nd	nd	nd	nd	nd	nd	0.00	d
	Bloekomspruit 2	nd	nd	nd	nd	nd	nd	0.00	d
	Cedara 1st planting	nd	nd	nd	nd	nd	nd	0.00	d
	Rushof	nd	nd	nd	nd	nd	nd	0.00	d
	Potchefstroom 2nd planting	nd	nd	nd	nd	nd	nd	0.00	d
	Tweebuffelsfontein	nd	nd	nd	nd	nd	nd	0.00	d
	Potchefstroom 1st planting	nd	nd	nd	nd	nd	nd	0.00	d
	Bethlehem	nd	nd	nd	nd	nd	nd	0.00	d
	Vaalharts 1st planting	nd	nd	nd	nd	nd	nd	0.00	d
	Coligny	nd	nd	nd	nd	nd	nd	0.00	d
	Potchefstroom	nd	nd	nd	nd	nd	nd	0.00	d
	Mean (LSD =342.44)	419.80	108.60	181.40	113.30	448.00	104.40		
	t-grouping	xy	xy	xy	xy	x	y		
2008/2009								LSD = 370.71	
	Bethlehem 2nd planting	669.00	nd	666.00	492.00	861.00	nd	537.60	a
	Cedara 2nd planting	nd	1440.00	nd	728.00	nd	nd	361.33	ab
	Potchefstroom 3rd planting	nd	nd	nd	nd	566.00	nd	94.33	ab
	Potchefstroom 1st planting	353.00	nd	nd	nd	nd	nd	58.83	b
	Potchefstroom 2nd planting	nd	nd	nd	nd	nd	nd	0.00	b
	Cedara 1st planting	nd	nd	nd	nd	nd	nd	0.00	b
	Leeudoringstad	nd	nd	nd	nd	nd	nd	0.00	b
	Vaalharts 2nd planting	nd	nd	nd	nd	nd	nd	0.00	b
	Vaalharts 1st planting	nd	nd	nd	nd	nd	nd	0.00	b
	Wesselsbron	*	*	nd	*	*	*	0.00	b
	Mean (LSD = 245.07)	113.60	160.00	66.60	135.60	158.60	0.00		
	t-grouping	x	x	x	x	x	x		

* = no samples

** µg/kg = µg DON per kg milled grain sample

nd = not detected

#Means followed by the same letters do not differ significantly at P≤0.05

Table 3.5. Combined analysis of variance of DON concentration in maize kernel samples for 2006/07, 2007/08 and 20008/09 growing seasons

Source	*df*	Sum of Squares	Mean Square	F Value	Pr > F
Season	2	3060549.74	1530274.87	28.7	<0.0001
Locality	37	31788273.23	859142.52	16.11	<0.0001
Cultivar	5	2084476.15	416895.23	7.82	<0.0001
Locality*Cultivar	181	55882582.92	308743.55	5.79	<0.0001
Season*Locality	13	184228.53	14171.43	0.27	0.9942
Season*Cultivar	10	395443.75	39544.37	0.74	0.6826

P values underlined are significant at 95% confidence level

Table 3.6. Mean groupings of ZEA concentrations (µg/kg) obtained using ELISA in maize kernel samples representing six cultivars collected during the 2006/07, 2007/08 and 2008/09 seasons in grain producing localities of South Africa

	Locality	Cultivars							
		DKC78-15B	PAN 6611	DKC80-10	DKC80-12B	CRN 3505	LS 8521B	Mean	t-Grouping
2006/2007								LSD =76.70	
	Bethlehem	362.32	398.30	196.75	45.85	45.85	25.23	179.05	a
	Wesselsbron	nd	nd	38.08	29.14	351.31	nd	69.76	b
	Marquard	nd	30.00	97.40	93.27	80.40	nd	50.00	b
	Delareyville	50.61	56.21	39.35	41.78	40.49	36.76	44.17	b
	Bainsvlei	*	*	31.60	40.28	43.95	*	38.67	b
	Koster	0.00	43.83	29.87	50.95	nd	51.61	29.50	b
	Leeudoringstad	0.00	25.47	122.70	nd	27.71	nd	29.33	b
	Rushof	0.00	25.38	40.81	30.72	28.19	40.26	27.50	b
	Ottosdal	31.69	26.76	*	33.77	25.35	nd	27.00	b
	Ventersdorp	40.28	nd	nd	120.26	nd	nd	26.67	b
	Tweebuffelsfontein	37.79	nd	26.97	27.08	25.41	38.96	26.00	b
	Bloekomspruit	nd	nd	69.01	52.70	28.44	nd	25.00	b
	Bothaville	nd	27.00	nd	nd	nd	*	5.40	b
	Hartebeesfontein	nd	nd	nd	27.71	nd	nd	4.67	b
	Potchefstroom	nd	nd	nd	nd	nd	nd	0.00	b
	Mean (LSD =47.82)	37.36	45.14	49.13	39.60	46.33	14.85		
	t-Grouping	x	x	x	x	x	x		
								LSD =370.04	
2007/2008	Bothaville	799.48	2817.12	3516.99	938.27	383.81	588.00	1507.28	a
	Bloekomspruit 1	459.00	513.37	2295.00	236.58	371.81	281.24	692.83	b
	Delmas	259.72	925.55	728.67	109.48	348.23	656.57	504.70	bc
	Warden	178.00	440.40	1818.06	243.05	68.19	221.46	494.86	bcd
	Wonderfontein	80.76	*	326.75	785.64	400.03	340.26	386.69	bcde
	Leeudoringstad	*	296.93	439.37	306.04	297.23	539.48	375.81	bcde
	Potchefstroom 2nd planting	224.76	372.35	391.43	391.43	440.63	414.98	372.60	bcdef
	Potchefstroom 1st planting	411.93	322.41	440.63	307.85	349.70	359.52	365.34	bcdefg
	Vrede	286.57	219.92	284.87	251.12	410.25	491.64	324.06	bcdefg
	Jim Fouche	210.97	210.68	464.29	88.91	105.15	720.10	300.02	cdefg
	Cedara 1st planting	159.90	289.28	263.08	223.44	282.40	202.88	236.83	cdefg
	Bloekomspruit 2	198.66	227.14	206.12	187.65	261.56	212.82	215.66	cdefg
	Bethlehem	187.48	159.67	333.41	246.31	80.29	249.03	209.37	cdefg
	Robertsdrift	187.47	291.53	134.51	243.05	25.39	145.75	171.28	cdefg
	Vaalharts 2nd planting	nd	197.35	257.46	155.16	132.40	205.60	157.80	cdefg
	Nooitgedacht	210.97	257.36	182.17	79.99	nd	134.51	144.20	cdefg
	Vaalharts 1st planting	147.31	175.06	198.36	112.01	120.34	50.24	133.70	defg
	Tweeling	259.72	52.18	72.60	162.96	107.73	142.31	133.00	defg
	Cedara 2nd planting	112.51	73.01	84.95	152.33	196.60	165.13	130.76	defg
	Tweebuffelsfontein	157.58	150.87	157.58	174.79	nd	120.62	127.20	defg
	Frankfort	175.47	60.48	253.78	50.22	49.01	64.51	108.80	efg
	Danielsrus	102.99	81.98	nd	47.32	140.75	66.06	73.20	efg
	Hartebeesfontein	28.48	27.18	30.69	33.85	54.78	26.55	33.70	efg
	Nampo	31.18	32.09	30.98	27.18	nd	nd	20.20	efg
	Viljoenskroon	nd	nd	nd	32.19	nd	nd	5.30	fg
	Rushof	nd	nd	nd	nd	nd	nd	nd	g
	Coligny	nd	nd	nd	nd	nd	nd	nd	g
	Potchefstroom	nd	nd	nd	nd	nd	nd	nd	g
	Mean (LSD =171.13)	180.41	303.41	461.14	199.46	165.21	228.61		
	t-grouping	y	xy	x	x	x	x		
								LSD = 331.91	
2008/2009	Leeudoringstad	401.66	nd	165.13	469.19	376.74	443.89	309.50	a
	Cedara 2nd planting	58.10	1529.15	nd	105.15	nd	32.20	287.30	a
	Vaalharts 2nd planting	nd	406.93	nd	nd	nd	415.04	137.00	a
	Potchefstroom 3rd planting	337.53	nd	30.84	33.05	nd	nd	67.00	a
	Cedara 1st planting	30.53	25.30	66.18	66.19	33.21	nd	36.80	a
	Bethlehem 2nd planting	nd	nd	nd	36.91	26.25	nd	10.50	a
	Potchefstroom 1st planting	nd	nd	nd	nd	nd	26.12	4.35	a
	Potchefstroom 2nd planting	nd	nd	25.71	nd	nd	nd	4.29	a
	Vaalharts 1st planting	nd	nd	nd	nd	nd	nd	0.00	a
	Wesselsbron	*	*	nd	*	*	*	0.00	a
	Mean (LSD = 219.42)	92.11	217.90	28.80	78.90	48.40	101.90		
	t-grouping	x	x	x	x	x	x		

* = no samples

** µg/kg = µg ZEA per kg milled grain sample

nd = not detected

#Means followed by the same letters do not differ significantly at $P \leq 0.05$

Table 3.7. Combined analysis of variance of ZEA concentration in maize kernel samples for 2006/07, 2007/08 and 20008/09 growing seasons

Source	*df*	Sum of Squares	Mean Square	F Value	Pr > F
Season	2	3032295.98	1516147.99	19.15	<0.0001
Locality	37	9806731.74	265046.8	3.35	<0.0001
Cultivar	5	931822.42	186364.48	2.35	0.0516
Locality*Cultivar	181	11132613.91	61506.15	0.78	0.8926
Season*Locality	13	6625001.08	509615.47	6.44	<0.0001
Season*Cultivar	10	629703.41	62970.34	0.8	0.6332

P values underlined are significant at 95% confidence level

Table 3.8. Mean groupings of DON concentrations (µg/kg) obtained using ELISA in sorghum grain samples representing six cultivars collected during the 2007/08 and 2008/09 seasons in grain producing localities of South Africa

	Locality	Cultivars								
		PAN 8420	PAN 8625	PAN 8247	PAN 8609	PAN 8648	PAN 8816	NS 5511	Mean	t-grouping#
2007/2008									LSD=198.89	
	Cedara 1st planting	332.00	730.00	729.00	478.00	426.00	446.00	1100.00	605.86	a
	Cedara 2nd planting	nd	312.00	nd	nd	481.00	nd	1520.00	330.40	b
	Dover	nd	nd	nd	nd	*	nd	949.00	158.20	bc
	Goedgedacht	nd	nd	nd	nd	nd	nd	895.00	127.90	c
	Holmdene	nd	nd	nd	nd	nd	585.00	0.00	83.60	c
	Val	nd	nd	nd	nd	nd	nd	559.00	79.86	c
	Vaalharts 2nd planting	nd	nd	nd	nd	nd	nd	273.00	39.00	c
	Klerksdorp	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Potchefstroom Dryland	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Platrand	nd	nd	nd	nd	nd	nd	*	0.00	c
	Vaalharts 1st planting	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Greenlands	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Klipdrift	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Potchefstroom 1st planting	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Potchefstroom Irrigation	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Bethlehem	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Weiveld	nd	nd	nd	nd	nd	nd	nd	0.00	c
	Mean (LSD= 127.51)	19.53	61.29	42.88	28.12	56.69	60.65	331.00		
	t-grouping	y	y	y	y	y	y	x		
2008/2009									LSD=138.19	
	Cedara	*	568.00	1030.00	1330.00	*	900.00	820.00	929.60	a
	Val	*	nd	nd	449.00	*	nd	398.00	169.40	b
	Amersfoort	*	nd	nd	379.00	*	nd	377.00	151.20	b
	Skaapplaas	*	273.00	nd	*	*	nd	*	91.00	bc
	Kafferskraal	*	nd	nd	nd	*	nd	432.00	86.40	bc
	Gottenburg	*	nd	nd	nd	*	nd	376.00	75.20	bc
	Klipdrift	*	nd	nd	nd	*	nd	369.00	73.80	bc
	Leeuwkraal	*	nd	nd	nd	*	nd	326.00	65.20	bc
	Bethlebem 2nd planting	*	nd	nd	nd	*	nd	314.00	62.80	bc
	Vaalharts 1st planting	*	nd	nd	nd	*	nd	305.00	61.00	bc
	Platrand NorthEast	*	nd	nd	nd	*	nd	269.00	53.80	bc
	Perdekop	*	nd	nd	nd	*	nd	265.00	53.00	bc
	Potchestroom Dryland	*	260.00	nd	nd	*	nd	nd	52.00	bc
	Potchefstroom 1st planting	*	nd	nd	nd	*	nd	nd	0.00	c
	Potchefstroom 2nd planting	*	nd	nd	nd	*	nd	nd	0.00	c
	Goedgedacht	*	nd	nd	nd	*	nd	nd	0.00	c
	Klerksdorp	*	nd	nd	nd	*	nd	nd	0.00	c
	Weiveld	*	nd	nd	nd	*	nd	nd	0.00	c
	Weiveld 2nd Planting	*	nd	nd	nd	*	nd	nd	0.00	c
	Dover	*	nd	nd	nd	*	nd	nd	0.00	c
	Holmdene	*	nd	nd	nd	*	nd	nd	0.00	c
	Vaalharts 2nd planting	*	nd	nd	nd	*	nd	nd	0.00	c
	Rietfontein	*	nd	nd	nd	nd	nd	*	0.00	c
	Parys	*	nd	nd	nd	*	nd	nd	0.00	c
	Greenlands	*	nd	nd	nd	*	nd	nd	0.00	c
	Potchefstroom 3rd planting	*	nd	nd	nd	*	nd	nd	0.00	c
	Potchestroom Irrigation	*	nd	nd	nd	*	nd	nd	0.00	c
	Mean (LSD=135.72)		40.78	38.15	83.00	0.00	33.33	170.04		
	t-Grouping	*	xy	xy	xy	y	y	x		

* = no samples

**µg/kg = µg DON per kg milled grain sample

nd = not detected

#Means followed by the same letters do not differ significantly at P≤0.05

Table 3.9. Combined analysis of variance of DON concentration* in sorghum grain samples during the 2007/2008 and 20008/2009 seasons and six cultivars from 31 localities.

Source	*df*	Sum of Squares	Mean Square	F Value	Pr > F
Season	1	10376.412	10376.412	0.52	0.4762
Locality	30	6811253.168	227041.772	11.28	<0.0001
Cultivar	6	1220178.389	203363.065	10.11	<0.0001
Locality*Cultivar	147	3561200.871	24225.856	1.2	0.2313
Season*Locality	12	231974.911	19331.243	0.96	0.4979
Season*Cultivar	5	106863.765	21372.753	1.06	0.3930

P values underlined are significant at 95% confidence level

Table 3.10. Mean groupings of ZEA concentrations (µg/kg) obtained using ELISA in sorghum grain samples representing six cultivars collected during the 2007/08 and 2008/09 seasons in grain producing localities of South Africa

	Locality	Cultivars								
		PAN 8420	PAN 8625	PAN 8247	PAN 8609	PAN 8648	PAN 8816	NS 5511	Mean	t-grouping#
2007/2008									**LSD=417.62**	
	Cedara 2nd planting	1296.07	1293.37	1361.12	1433.58	1907.73	1280.35	1261.13	1404.76	a
	Cedara 1st planting	579.30	1048.81	1911.28	2174.89	278.14	818.25	983.76	1113.49	ab
	Vaalharts 2nd planting	1579.50	1388.75	1564.05	340.47	745.10	1502.68	395.38	1073.70	ab
	Potchefstroom 1st planting	1105.48	1152.75	302.08	1635.35	423.19	738.75	1579.50	991.01	abc
	Vaalharts 1st planting	839.65	1434.80	1382.35	1490.20	357.41	245.22	426.46	882.30	bc
	Bethlehem	338.15	450.56	1274.10	929.31	1338.70	488.89	1235.30	865.00	bc
	Weiveld	184.86	407.38	1018.45	1236.08	469.10	497.18	253.53	580.94	cd
	Greenlands	247.12	211.87	319.10	369.16	399.98	382.73	2107.87	576.83	cd
	Dover	281.24	266.32	315.69	335.69	*	169.65	1250.78	436.56	cd
	Holmdene	178.14	175.11	221.94	180.19	423.25	367.83	1008.96	365.06	d
	Klipdrift	235.95	192.54	208.12	178.42	206.70	238.49	1048.45	329.81	d
	Potchefstroom Dryland	181.98	312.55	277.22	282.53	253.94	402.14	305.24	287.94	d
	Klerksdorp	227.50	286.33	322.79	243.12	285.87	269.87	189.04	260.65	d
	Val	266.49	209.52	241.64	241.64	265.17	345.19	227.32	256.71	d
	Potchefstroom Irrigation	413.59	189.99	216.14	184.40	230.98	209.54	315.05	251.38	d
	Platrand	263.80	198.85	340.37	287.71	286.77	139.56	*	253.00	d
	Goedgedacht	264.46	104.58	229.94	195.66	221.95	247.12	188.17	207.41	d
	Mean (LSD= 267.43)	499.02	548.48	676.85	690.49	505.87	490.79	798.50		
	t-grouping	y	xy	xy	xy	y	y	x		
2008/2009									**LSD=522.67**	
	Dover	*	1982.16	1120.37	83.58	*	2602.95	2141.56	1586.12	a
	Skaapplaas	*	1034.16	1297.90	*	*	940.20	*	1090.75	ab
	Platrand NorthEast	*	1192.72	1444.26	611.10	*	1597.62	188.31	1006.80	bc
	Amersfoort	*	243.34	321.00	945.78	*	1862.10	871.44	848.73	bcd
	Val	*	27.56	77.30	1723.69	*	63.98	1859.96	750.50	bcde
	Goedgedacht	*	442.57	1198.26	425.17	*	304.54	1015.00	677.11	bcdef
	Leeuwkraal	*	214.17	1512.40	1335.23	*	67.19	27.31	631.26	bcdefg
	Gottenburg	*	1565.64	318.17	329.70	*	374.93	318.17	581.32	bcdefg
	Weiveld	*	846.40	235.08	88.20	*	1415.88	287.03	574.52	bcdefgh
	Perdekop	*	461.01	496.08	866.94	*	62.88	751.00	527.58	cdefghi
	Rietfontein	*	743.25	76.33	774.35	*	62.38	*	413.80	defghi
	Weiveld 2nd Planting	*	365.25	251.25	280.84	*	338.72	225.58	292.33	efghi
	Klipdrift	*	153.27	257.21	176.82	*	173.19	338.31	219.76	fghi
	Cedara	*	110.18	377.58	325.68	*	210.60	55.80	216.20	fghi
	Kafferskraal	*	138.79	172.01	243.59	*	nd	181.82	147.40	ghi
	Vaalharts 2nd planting	*	nd	nd	325.05	nd	nd	nd	54.20	hi
	Potchefstroom 1st planting	*	nd	77.72	78.78	*	45.58	51.34	50.80	i
	Potchestroom Irrigation	*	26.35	61.40	71.35	*	26.59	52.44	47.40	i
	Parys	*	nd	53.58	60.95	*	48.70	46.61	42.20	i
	Bethlebem 2nd planting	*	nd	31.91	48.13	*	36.79	29.68	29.40	i
	Klerksdorp	*	nd	26.35	34.79	*	54.28	25.12	28.00	i
	Potchestroom Dryland	*	71.18	27.84	nd	*	38.65	nd	27.60	i
	Potchefstroom 2nd planting	*	nd	60.36	75.81	*	nd	nd	27.20	i
	Vaalharts 1st planting	*	nd	36.02	nd	*	nd	47	16.60	i
	Greenlands	*	nd	31.26	nd	*	nd	50	16.20	i
	Potchefstroom 3rd planting	*	nd	nd	34.52	*	43.15	nd	15.60	i
	Holmdene	*	nd	nd	41.93	*	nd	nd	8.40	i
	Mean (LSD=513.31)	*	565.76	398.40	390.52	*	493.85	428.18		
	t-Grouping		x	x	x	*	x	x	x	

* = no samples

** µg/kg = µg ZEA per kg milled grain sample

nd = not detected

Means followed by the same letters do not differ significantly at P≤0.05

Table 3.11. Combined analysis of variance of ZEA concentration in sorghum grain samples during the 2007/2008 and 20008/2009 seasons and seven cultivars from 31 localities

Source	*df*	Sum of Squares	Mean Square	F Value	Pr > F
Season	1	3780560.3	3780560.3	18.86	<0.0001
Locality	30	24498595.61	816619.85	4.07	<0.0001
Cultivar	6	789895.15	131649.19	0.66	0.6845
Locality*Cultivar	147	22989073.69	156388.26	0.78	0.8673
Season*Locality	12	12233480.55	1019456.71	5.09	<0.0001
Season*Cultivar	5	235484.76	47096.95	0.23	0.9452

P values underlined are significant at 95% confidence level

Table 3.12. Number of positive samples and the mean concentrations[#] of ZEA, NIV and DON (µg/kg) in a selection of maize kernel samples as assessed using ELISA and LC-MS/MS

Cultivar	Number of samples	ELISA				LC-MS/MS					
		DON		ZEA		DON		NIV		ZEA	
CRN 3505	11	4	(4033.55)	8	(489.70)	10	(237.46)	11	(6333.60)	1	(22863.41)
DKC 78-15B	11	6	(300.45)	9	(361.45)	9	(139.25)	11	(6933.83)	1	(4415.75)
DKC80-10	7	2	(314.29)	7	(1490.10)	7	(110.25)	7	(3275.68)	3	(20359.52)
DKC80-12B	9	3	(256.22)	7	(881.75)	8	(60.69)	9	(8983.38)	2	(10081.86)
LS 8521B	6	3	(9315.33)	5	(1920.70)	1	(66.08)	6	(5268.27)	1	(18380.28)
PAN 6611	12	4	(622.88)	8	(3935.19)	8	(220.22)	12	(5936.41)	3	(25678.45)

[#]Mean values are in brackets.

Table 3.13. Number of positive samples and the mean concentrations# of ZEA, NIV and DON (µg/kg) in a selection of sorghum grain samples as assessed using ELISA and LC-MS/MS

Cultivar	Number of samples	ELISA				LC-MS/MS					
		DON		ZEA		DON		NIV		ZEA	
NS 5511	18	14	(411.17)	18	(900.05)	9	(26.33)	18	(6597.39)	1	(2096.54)
PAN 8247	7	2	(251.29)	7	(673.88)	4	(53.09)	7	(7767.19)	2	(42608.32)
PAN 8420	4	0	(0)	4	(1352.63)	4	(61.62)	4	(9144.00)	0	(0)
PAN 8609	16	3	(141.06)	10	(639.35)	11	(49.51)	16	(7506.57)	3	(37988.69)
PAN 8625	12	4	(156.92)	10	(9709.94)	3	(10.62)	12	(7441.21)	2	(10905.62)
PAN 8648	4	2	(226.75)	4	(1409.16)	4	(57.55)	4	(8114.30)	2	(26021.84)
PAN 8816	5	2	(247.98)	5	(1203.99)	3	(58.48)	5	(7911.58)	2	(14409.88)

#Mean values are in brackets.

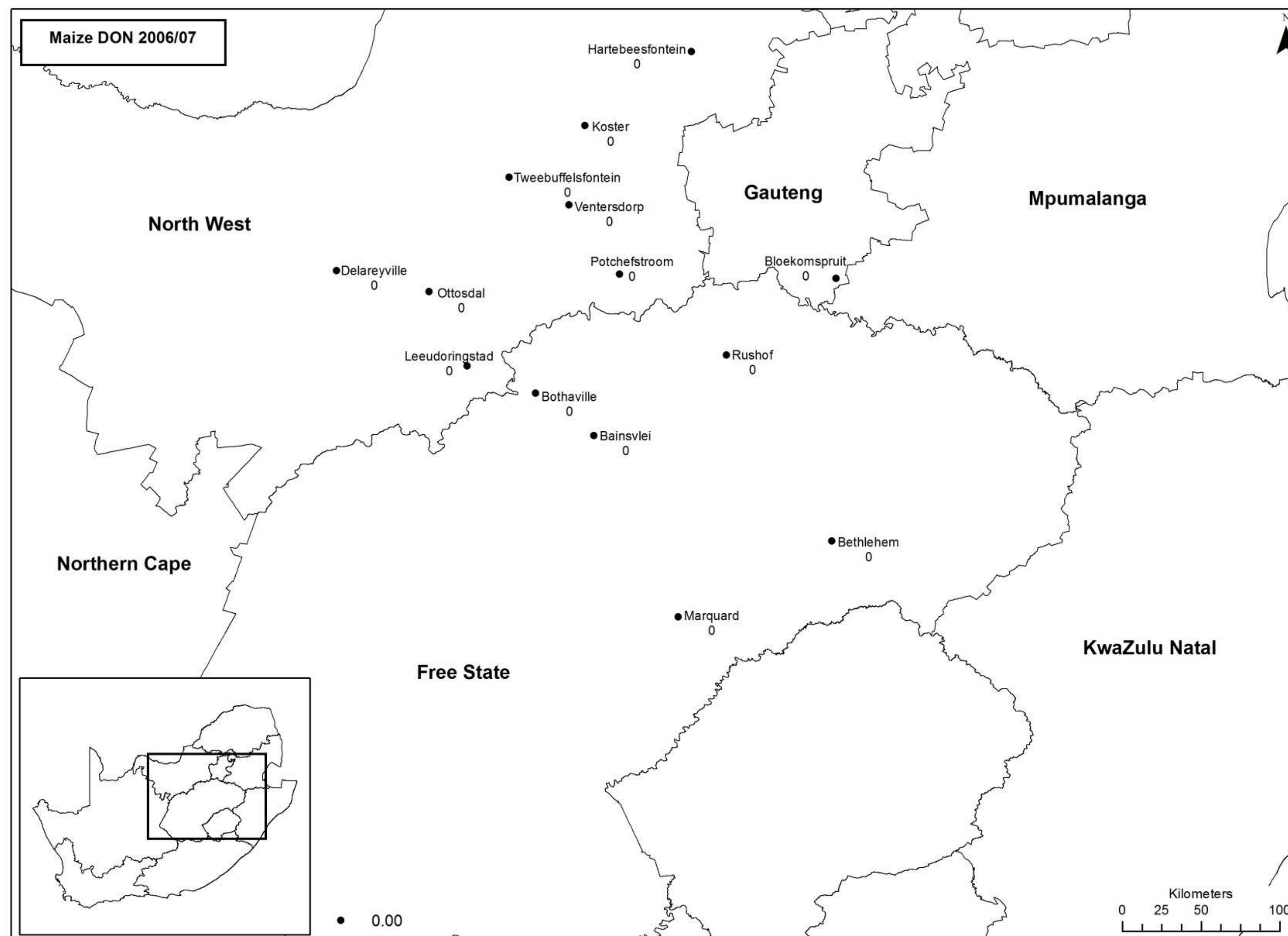

Figure 3.1 Distribution of DON (µg/kg) in maize kernel samples collected during the 2006/07 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

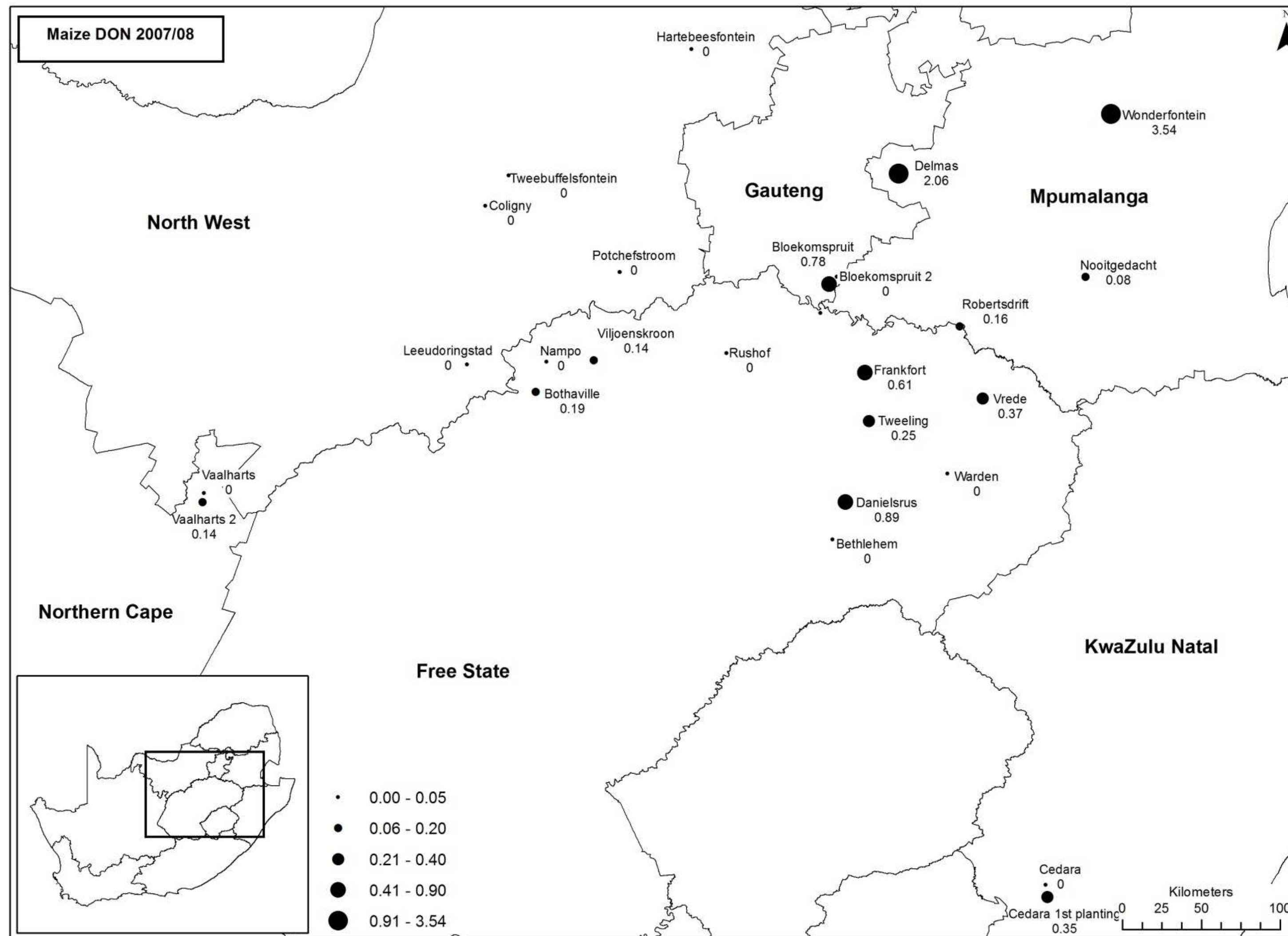

Figure 3.2 Distribution of DON (µg/kg) in maize kernel samples collected during the 2007/08 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

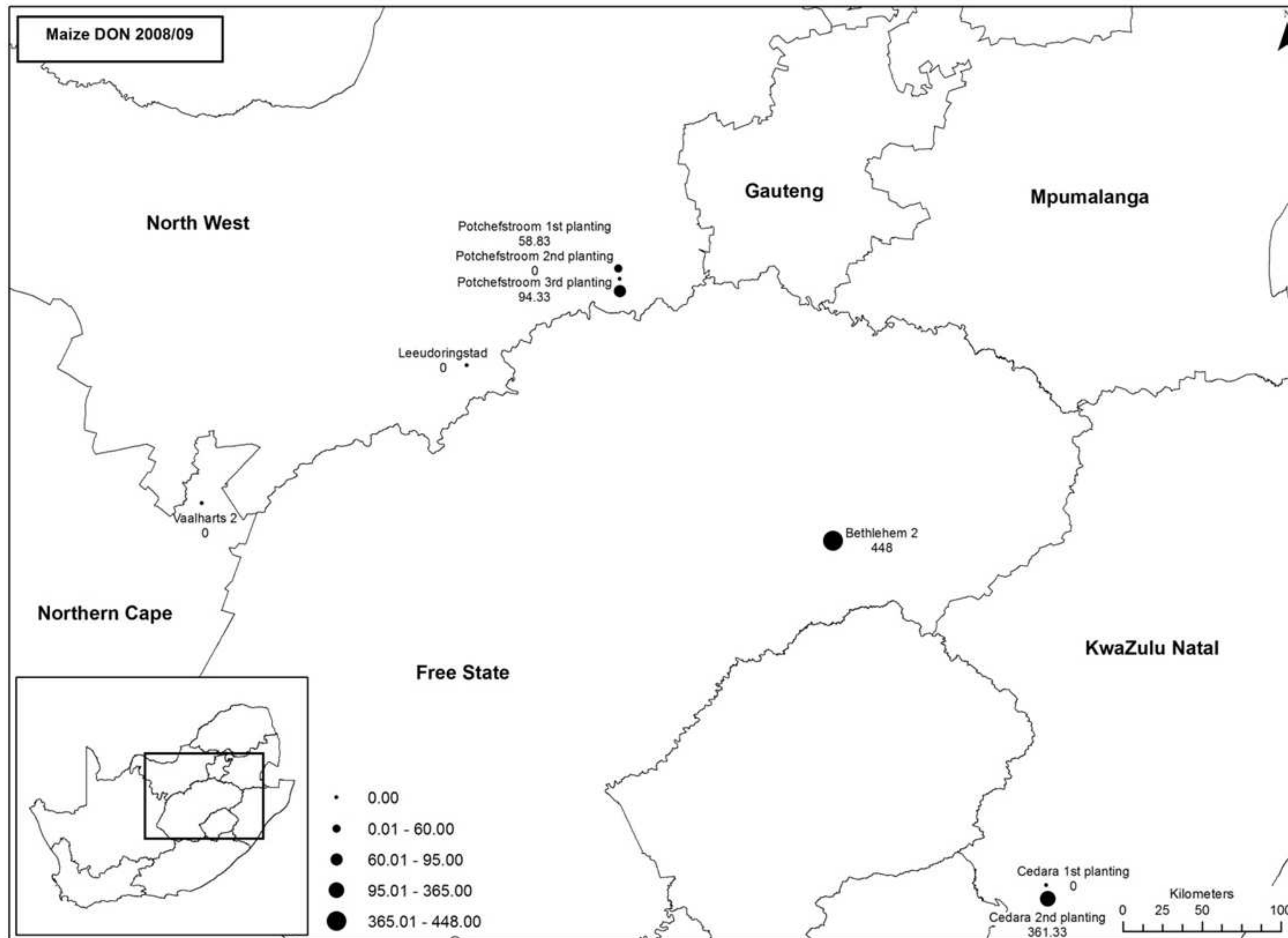

Figure 3.3. Distribution of DON (µg/kg) in maize kernel samples collected during the 2008/09 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

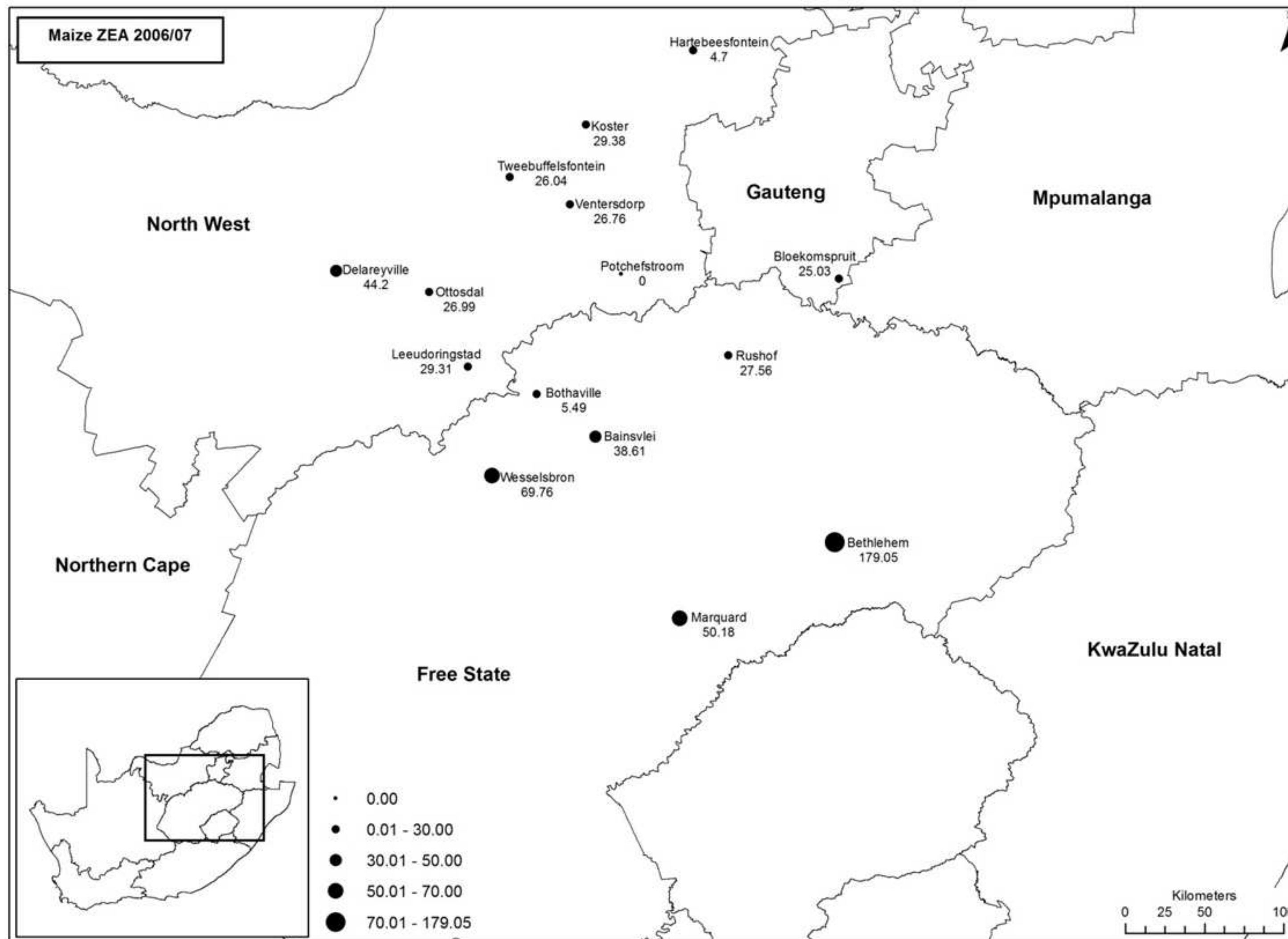

Figure 3.4. Distribution of ZEA (µg/kg) in maize kernel samples collected during the 2006/07 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

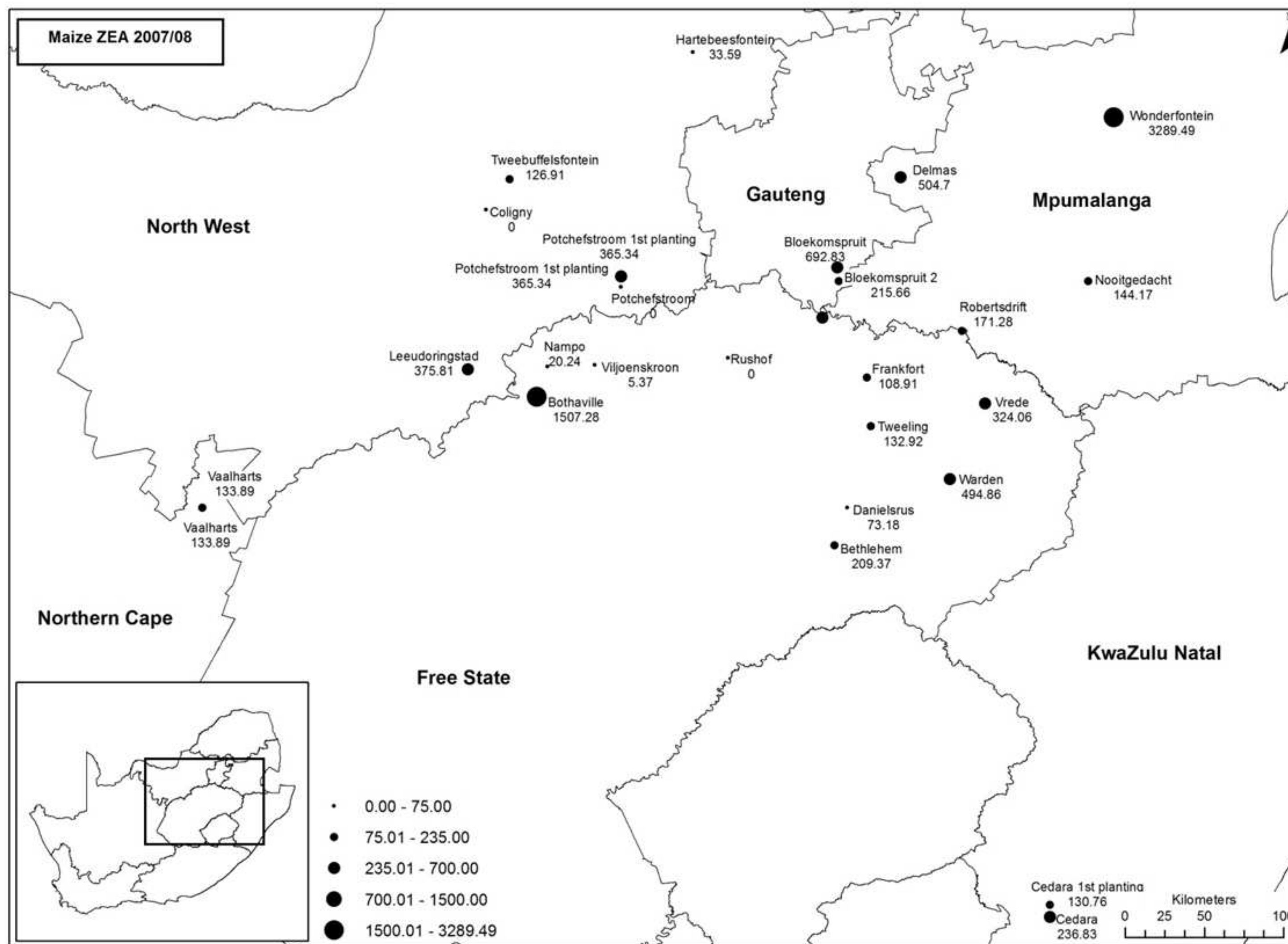

Figure 3.5. Distribution of ZEA (µg/kg) in maize kernel samples collected during the 2007/08 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

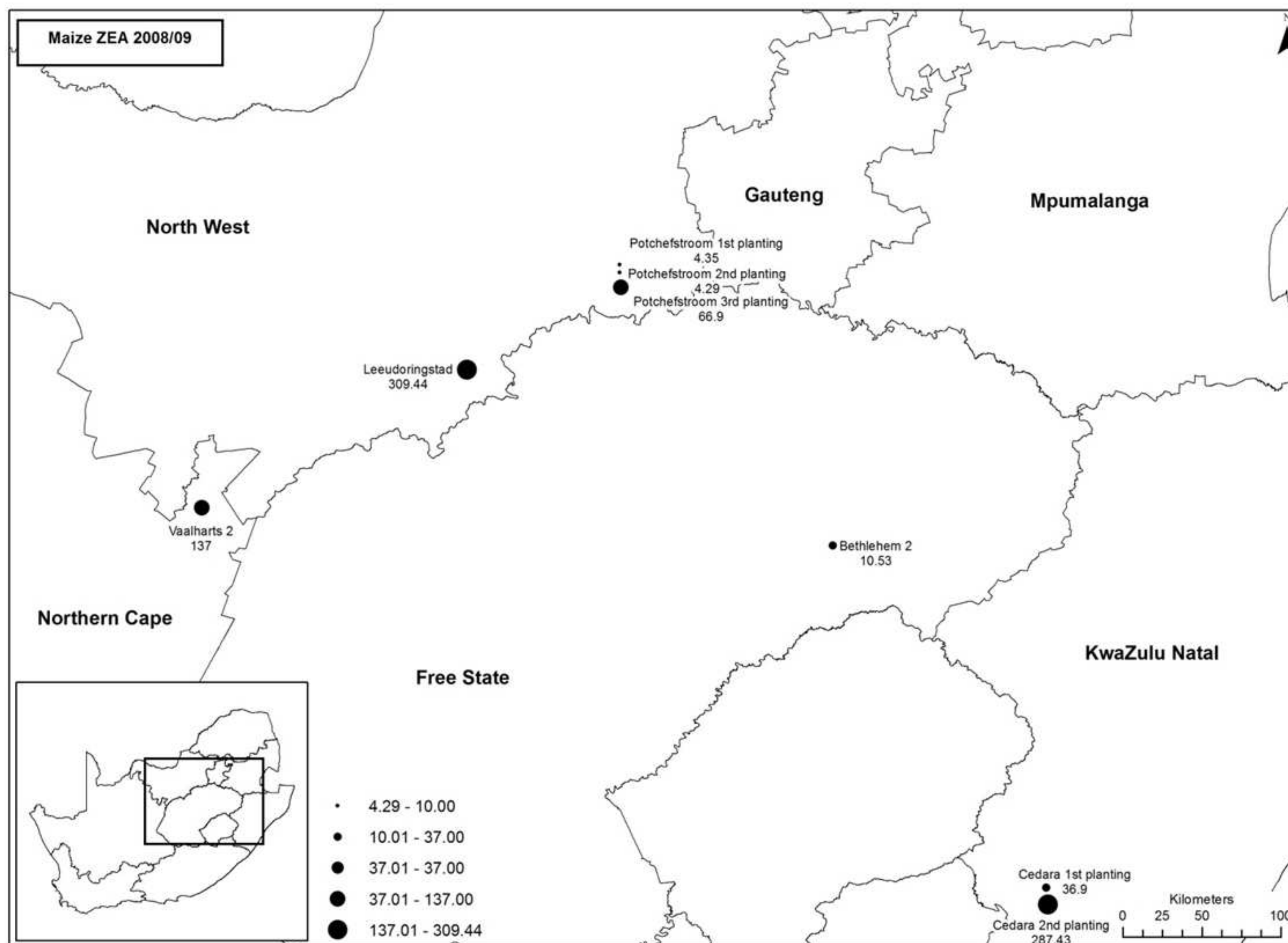

Figure 3.6. Distribution of ZEA (µg/kg) in maize kernel samples collected during the 2006/07 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

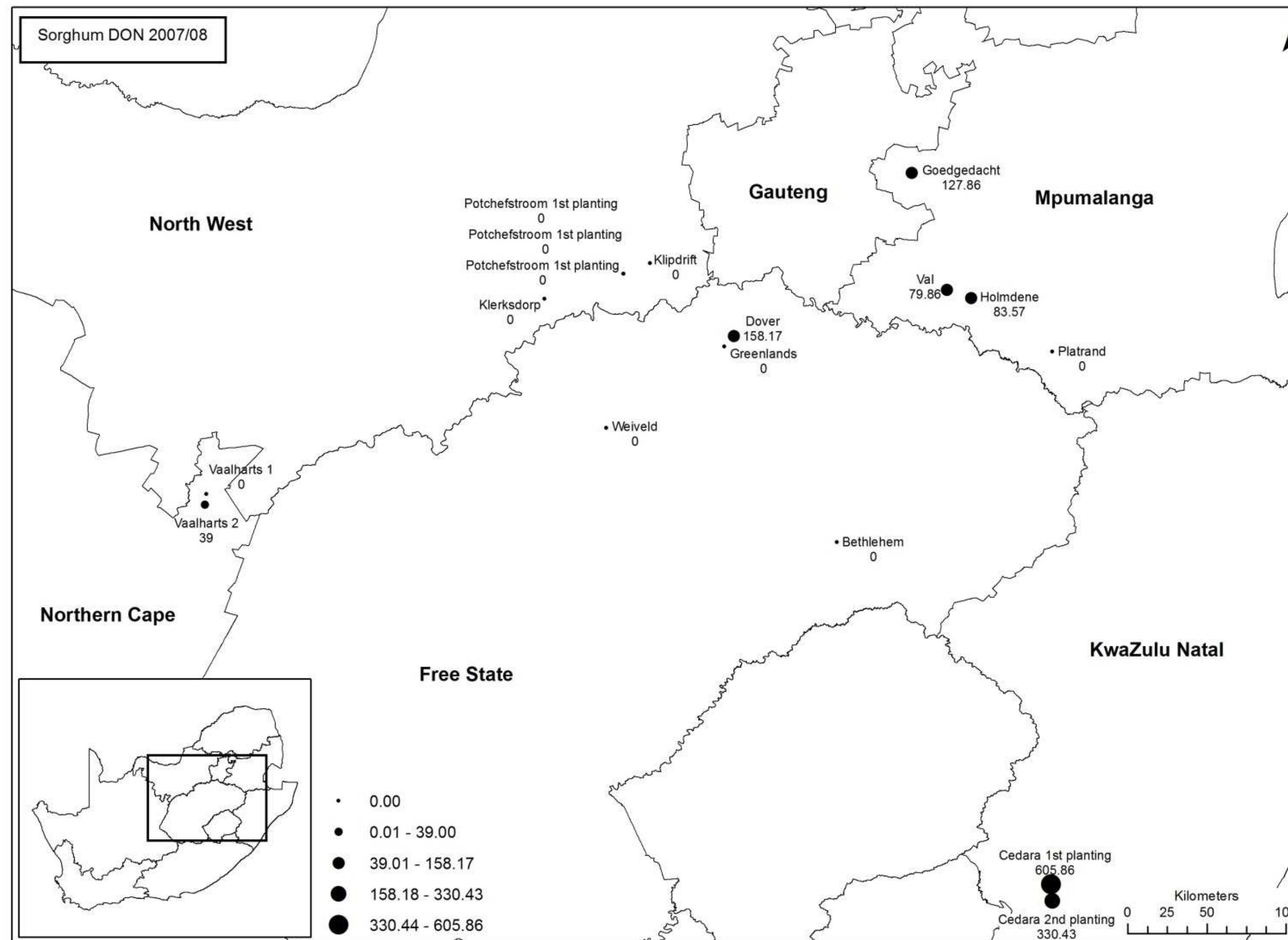

Figure 3.7. Distribution of DON (µg/kg) in sorghum grain samples collected during the 2007/08 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

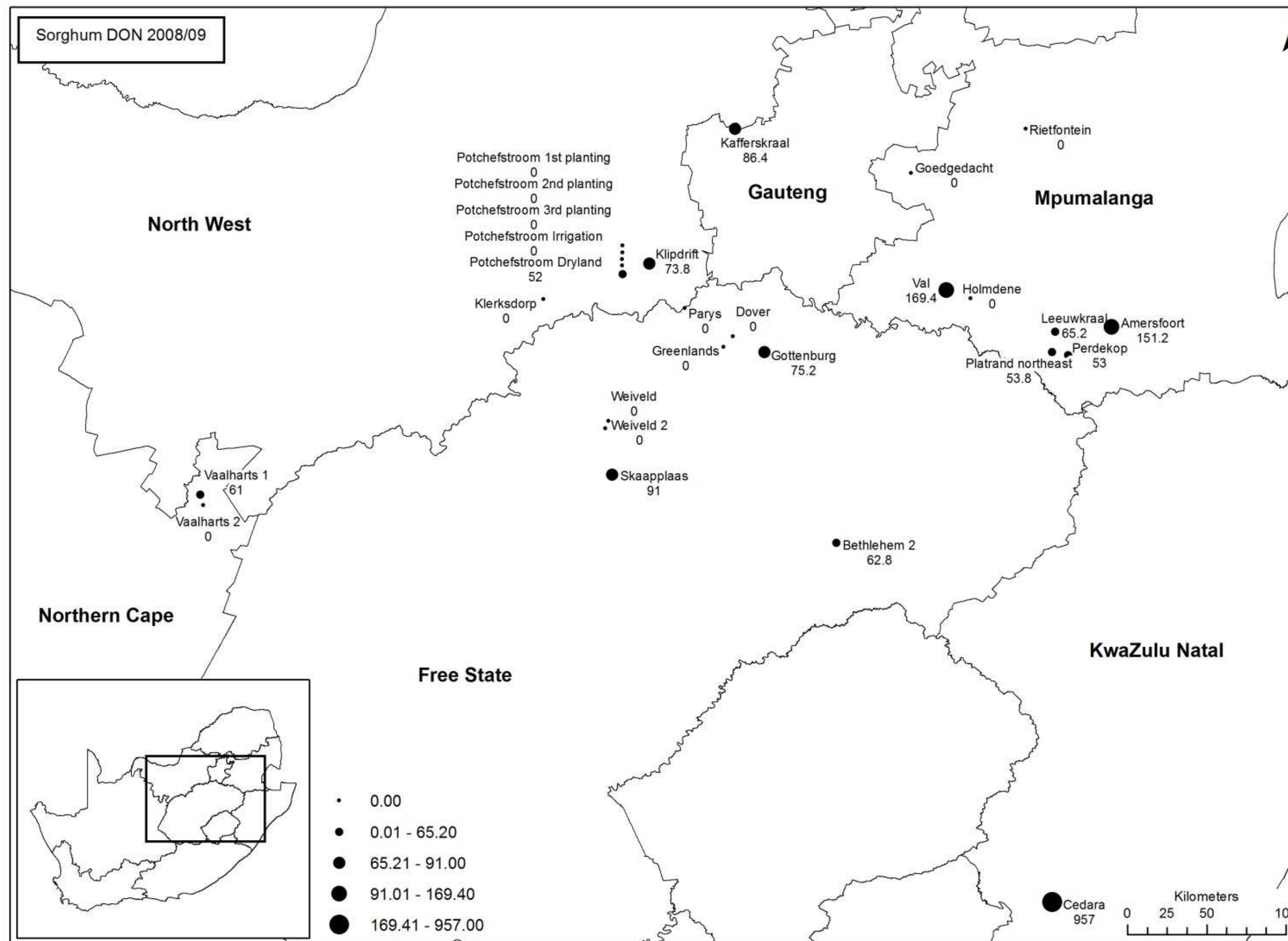

Figure 3.8. Distribution of DON (µg/kg) in sorghum grain samples collected during the 2008/09 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

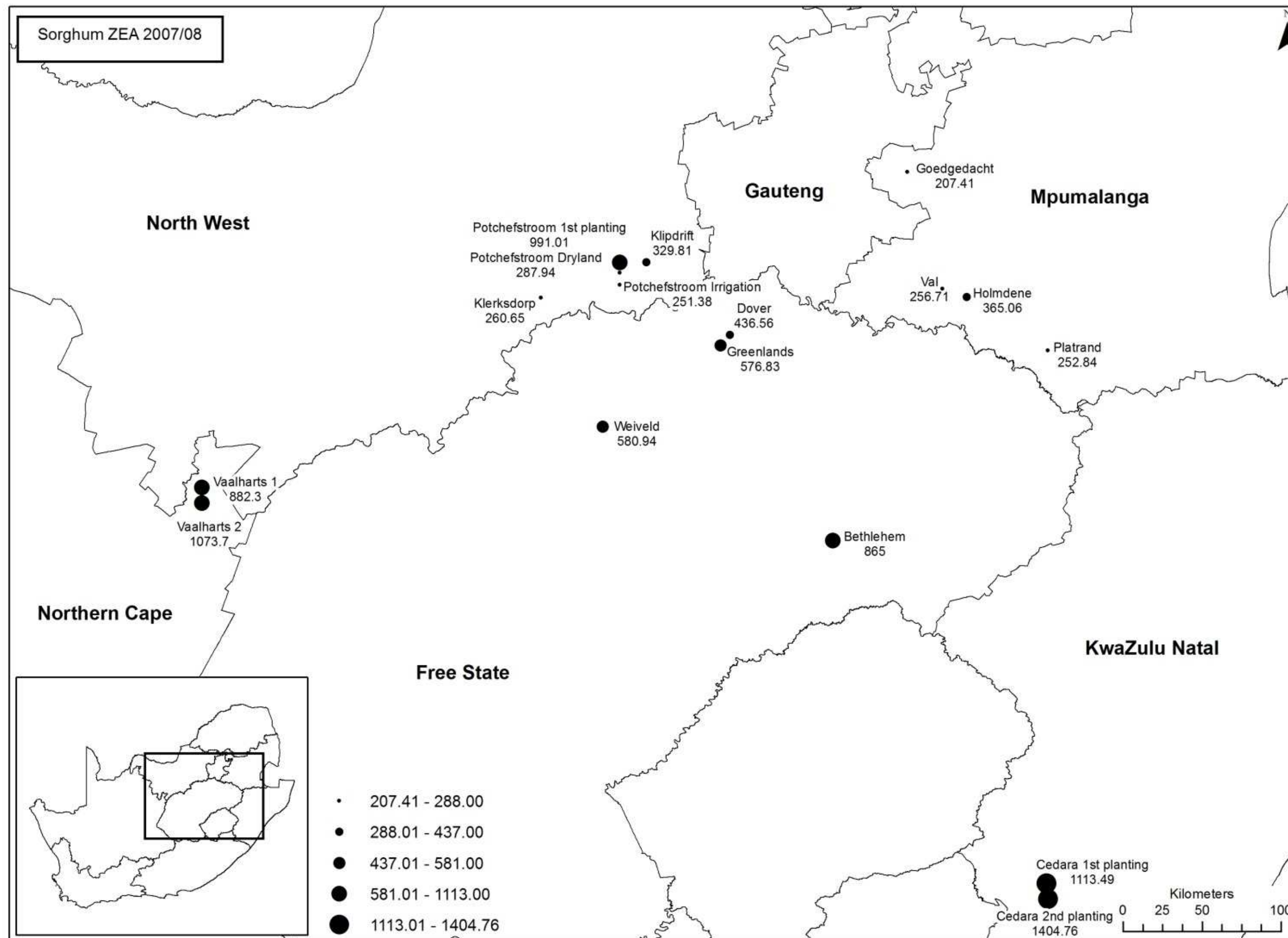

Figure 3.9. Distribution of ZEA (μg/kg) in sorghum grain samples collected during the 2007/08 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

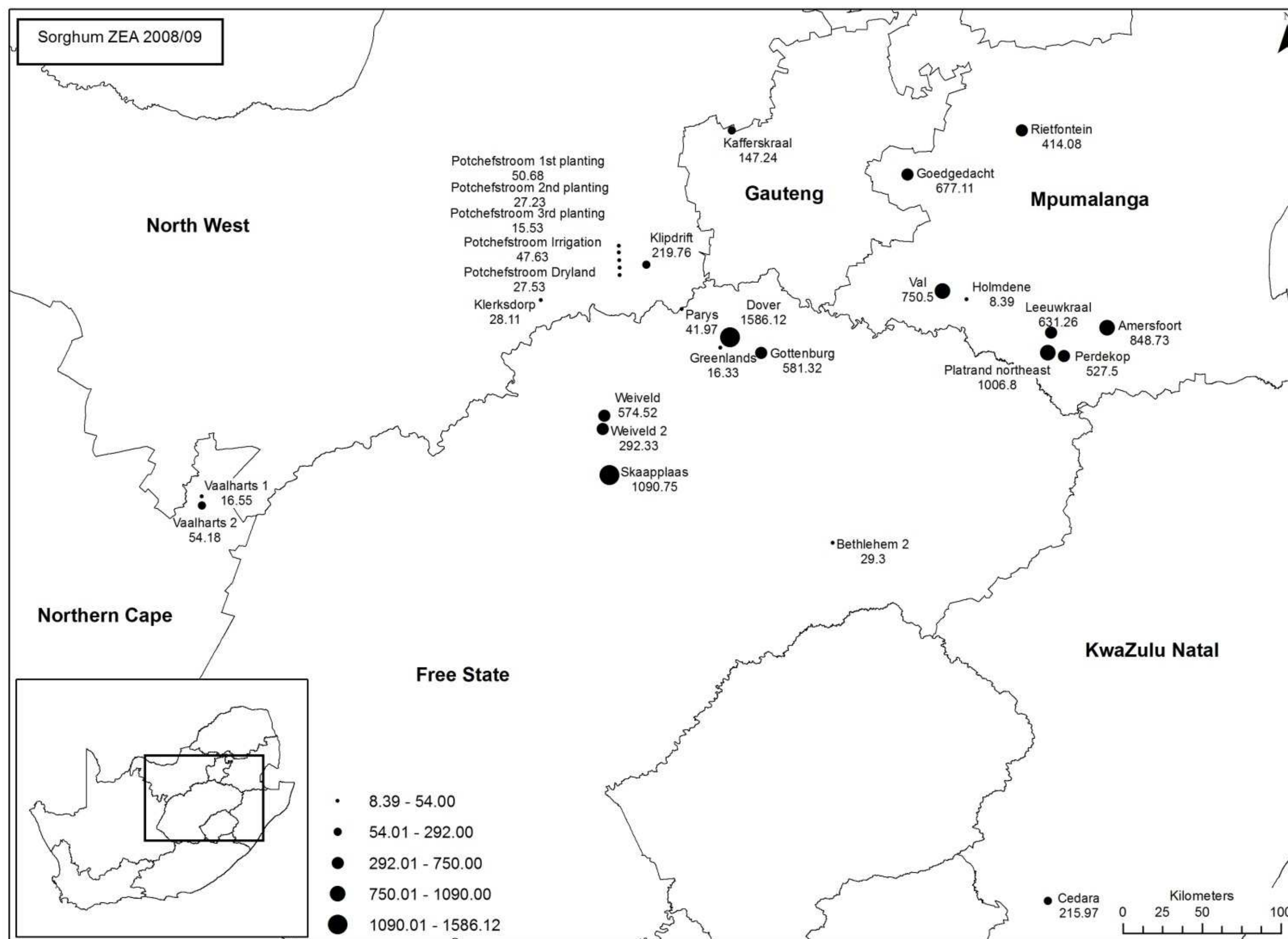

Figure 3.10. Distribution of ZEA (µg/kg) in sorghum grain samples collected during the 2008/09 season from different grain production areas of South Africa. The Jenks natural breaks classification method was used to arrange values into different classes.

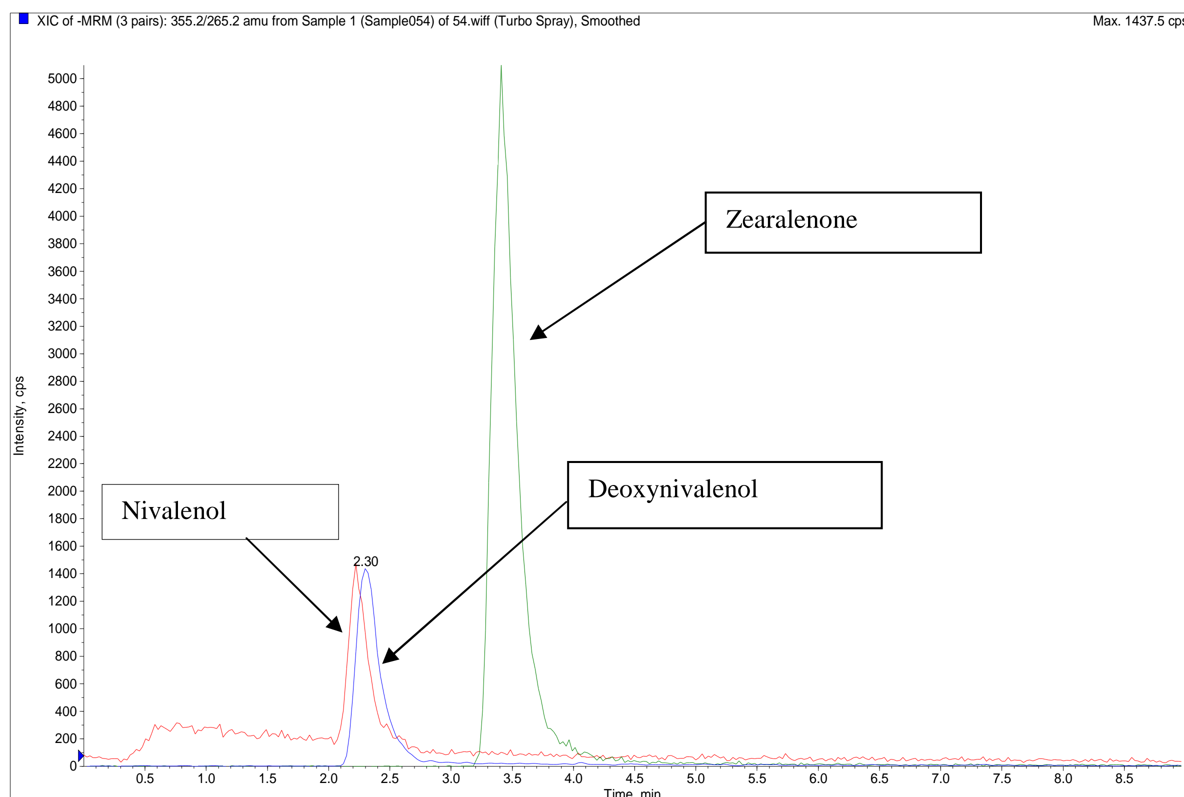

Figure 3.11. Normalised chromatograms of NIV, DON and ZEA mycotoxin standards. The retention times were 2.22, 2.30 and 3.41 min for INV, DON and ZEA respectively

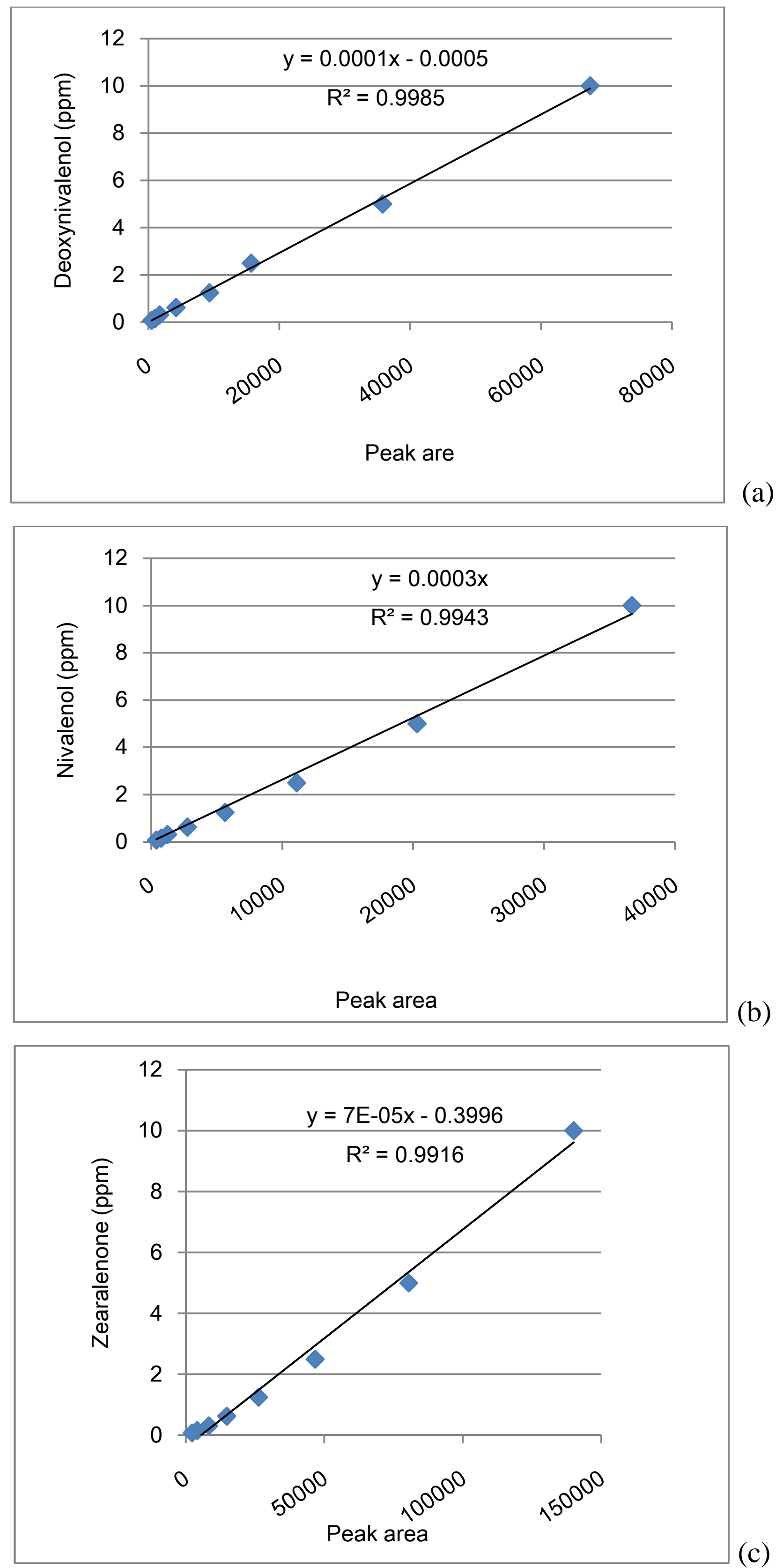

Figure 3.12. Calibration curves for DON (a), NIV (b), and ZEA (c)

CHAPTER 4

TRICHOTHECENE CHEMOTYPE PROFILES OF *FUSARIUM GRAMINEARUM* SPECIES COMPLEX MEMBERS ISOLATED FROM FROM MAIZE AND SORGHUM

Abstract

Species within the *Fusarium graminearum* species complex (FGSC) are capable of producing deoxynivalenol (DON), nivalenol (NIV) and their derivatives. Sixty-four maize and nine sorghum isolates were selected and screened for their trichothecene chemotypes using the polymerase chain reaction (PCR) based on the *Tri*12 and *Tri*6 portion of the trichothecene gene. Phylogenetic analyses using the ammonia ligase (*URA*) and translation elongation factor (*TEF1*-α) gene region sequences revealed the presence of *F. graminearum sensus stricto* (two isolates) and *F. boothii* (48 isolates) in maize kernel samples as well as *F. meridionale* (four isolates), *F. acacia-mearnsii* (two isolates) and *F. cortaderiae* (one isolate) in sorghum grain samples. The MRC strain 4712 clustered with *F. boothii* while MRC strain 4927 clustered with *F. graminearum sensu stricto*. All the maize isolates were of the DON/15-ADON while all the sorghum isolates were of the NIV chemotype. No isolates of the 3-ADON chemotype were found in the maize and sorghum. This is the first report of *F. meridionale, F. acacia-mearnsii* and *F. cortaderiae* in sorghum grain samples in South Africa.

4.1. INTRODUCTION

Members of the *Fusarium graminearum* species complex (FGSC) are some of the main causal agents of fusarium head blight (FHB) of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), sorghum [*Sorghum bicolor* (L.) Moench] and other small grains. They also infect maize (*Zea mays* L.), causing seedling blight, root-, crown-, stem- and Gibberella ear rot (Booth, 1971; Carter *et al.,* 2002). These fungi not only reduce grain quality and yield (Lysøe *et al.,* 2006), but can subsequently contaminate infected grain with trichothecene and estrogenic mycotoxins which are harmful to humans and animals (CAST, 2003; Pasquali *et al.,* 2010).

Trichothecene mycotoxins are a large family of structurally related fungal secondary metabolites produced, by among other fungi, *Fusarium* spp. (Zhou *et al*., 2008). Although structurally diverse, *Fusarium* trichothecenes can be divided into two major groups, i.e. type A and type B trichothecenes (Desjardins, 2006). Type B trichothecenes, deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivatives are of great concern for cereal growing regions worldwide (Jurado *et al*., 2005). According to O'Donnell *et al.* (2008), every species within the FGSC is capable of producing type B trichothecenes *in planta*. Trichothecene mycotoxins are potent inhibitors of eukaryotic protein synthesis (CAST, 2003), interferering with initiation, elongation and termination stages (Bennett & Klich, 2003). Some of the diseases in humans and animals associated with these toxins include feed refusal, nausea, vomiting, abortions, weight loss, inflammation of the skin, haemorrhaging of internal organs, blood disorders, immunosuppression and disturbance of the nervous system (Bennett & Klich, 2003; Logrieco *et al.,* 2003; Desjardins, 2006; Kumar *et al.,* 2008).

Three chemotypes have been identified in the FGSC, namely DON and 3-acetyldeoxynivalenol (3-ADON), DON and 15-acetylatedeoxynivalenol (15-ADON) and NIV and 4 acetylnivalenol (4-ANIV) (Wang *et al*., 2011). Chemotype characterisation has been used extensively to characterise the FGSC for their toxigenic potential. The chemotyping methods developed over the past decade are all based on the polymorphism in coding genes and introns of the *TRI* gene cluster, such as *tri*5, *tri*6*, tri*12 and *tri*13 (Desjardins, 2006; Pasquali *et al.,* 2010). PCR assays for

the diagnosis of trichothecene chemotypes in the FGSC have been developed based on genes involved in trichothecene synthesis, including the *tri*5 and tri6 (Li *et al.,* 2005), *tri*3 and *tri*12 (Ward *et al.,* 2002; Suga *et al.,* 2008) and the *tri*7 and *tri*13 (Waalwijk *et al.,* 2003). The intergenic spaces between *tri*5 and *tri*6 are chemotype specific and as such, have been used to design primers for differentiation of DON and NIV production based on PCR fragment sizes (Li *et al.,* 2005).

The FGSC chemotypes appear to differ in geographic distribution, i.e. the NIV chemotype has been reported in Africa, Asia and Europe while the DON chemotypes are more common worldwide (Ji *et al*., 2007). Until recently, little was known about the diversity of the FGSC and their trichothecene chemotypes in South African cereal grains. Boutigny *et al.* (2011) revealed that at least six of the 15 members of the FGSC occur within South Africa and that these produced NIV and 15-ADON. Due to significant differences in the toxicity of these trichothecenes, it is important to understand the nature and diversity of the FGSC infecting maize and sorghum in South Africa as these toxins may pose a health risk for humans and animals. Based on this, it was hypothesised that DON/3-ADON; DON/15-ADON and NIV chemotypes of the members of the FGSC are present in maize and sorghum fields in South Africa. The hypotheses was tested in the current study on a collection of isolates from maize kernel as well as sorghum grain, PCR assays were used to characterise and compare FGSC populations and to determine trichothecene chemotype profiles of the populations.

4.2. MATERIAL AND METHODS

4.2.1. Fungal isolates

Seventy three single-spore isolates representing members of the FGSC were selected for this investigation. These included 64 maize and nine sorghum isolates. The maize kernel samples were collected at Bethlehem, Frankfort and Wonderfontein while the sorghum grain samples were collected at Cedara during the 2007/08 and 2008/09 seasons respectively. For identification purposes, isolates were cultured on Spezieller Nährstoffarmer agar (SNA), potato dextrose agar (PDA) and carnation leaf agar (CLA) plates (Leslie & Summerell, 2006). Two reference strains, namely *F.*

graminearum strain (MRC4712) and *F. pseudograminearum* (MRC4927) were also included and used as positive controls. The isolates were preserved at -70°C as spore suspensions in 15% glycerol.

4.2.2. DNA extraction

The isolates were grown on sterile cellophane membranes placed on the surface of PDA plates for five days at 23°C ±2 under 12 h photoperiod. Mycelia were harvested, frozen in liquid nitrogen and ground to a fine powder. DNA was extracted from 100 mg samples using the ZymoResearch ZR Fungal/Bacterial DNA kit™ (Zymo Research, Irvine, California, USA) according to the manufacturer's instructions. The quality and quantity of the extracted DNA was measured using NanoDrop® ND 1000 (Thermo, Waltham, Massachusettes, USA). Samples were diluted to 2 ng/µl for PCR optimisation. Diluted DNA samples were stored at -20°C.

4.2.3. Species specific PCR

F. graminearum species-specific PCR was performed using primer pair Fg16F and Fg16R (Nicholson *et al*., 1998). These primers produce a monomorphic product of 400-500 bp DNA fragment specific to *F. graminearum*. Amplification reactions were done in 20 µl PCR reaction mixtures containing 14-20 ng fungal DNA, 0.2 µM of each primer and 1X Kapa Taq readymix. A negative control containing all the reagents but no DNA was used in every set of reactions. *F. graminearum* (MRC4712) and *F. pseudograminearum* (MRC4927) were also included as positive controls. The following cycling conditions consisted of initial denaturation at 95°C for 5 min, 30 cycles at 94°C for 30s, 57°C for 30s, 72°C for 60s and final extension at 72°C for 5 min. The samples were cooled to 4°C until recovery. PCR products were separated by electrophoresis on 2% agarose gels stained with 10 mg/µl EtBr and visualised under UV light.

4.2.4. Sequence-assisted species identification

To assist in the identification of isolates, portions of the translation elongation factor 1α (*EF*-1α) and the ammonia ligase (*URA*) genes (O'Donnell *et al*, 2000) were

amplified and bi-directionally sequenced using primers *EF*1 and *EF*2 for the *TEF*-1α gene and primers *URA*1 and *URA*6 for the ammonia ligase gene (Table 4.1). The primers were synthesised by Integrated DNA Technologies (Coralville, Iowa, USA). The *EF*1 and *EF*2 primers produced a 725 bp fragment while *URA*1 and *URA*6 primers produced a 1388 bp fragment (O'Donnell *et al.,* 2008).

PCR amplification of the *TEF*-1α and *URA* genes was done separately in a total volume of 20 µl, containing 10 µl 1 x KAPA Taq PCR ReadyMix (KapaBiosystems, Lasec, South Africa), 0.1 µM primer mix, and 14 ng of DNA. The following cycling conditions were applied: initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 15 sec, 55°C for 20 sec and 72°C for 1 min and a final step of 5 min at 72°C. Following PCR amplification, the PCR products were electrophoresed to verify amplification of the target fragment.

The amplification products for both genes were purified using the Wizard® SV gel and PCR clean-up system (Promega, Madison, USA) or the FavorPrep Gel/PCR purification Kit (Favorgen Biotech Corporation, Taiwan) according to the manufacturer's instructions.

Sequencing of purified PCR products for the two genes was done using the ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlifonia, USA). The same primers as used in the amplification reactions were used. Each reaction contained 1 µl Sequencing Ready Reaction mix, 3.2 pmol of the forward or reverse primer and a 1 X sequencing buffer (1 X final concentration). The PCR conditions were as follows: 96°C for 1 min; 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

The sequenced products were purified by adding 5 µl of 125 mM EDTA and 60 µl 100% ethanol. The solution was mixed and incubated at room temperature for 15 min Samples were centrifuged at 12 000 *g* for 15 min at 4°C, the supernatant removed and the pellet washed with 60 µl 70% (v/v) ethanol. Tubes were centrifuged for 5 min at 12 000 *g* at 4°C and the pellet was air-dried overnight in the dark. The nucleotide composition of each sample was analysed by using the Applied Biosystems 3130 *x* l Genetic Analyser.

Raw chromatograms (forward and reverse sequences) were edited on Chromas Lite (www.technelysium.com.au) while alignment and consensus sequences were done on BioEdit version 7.0.9.0 (Hall, 1999). The consensus sequences from BioEdit were used for BLAST identification of isolates on the *Fusarium* Id website (http://isolate.fusariumdb.org/index.php) as well as on the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/).

To assess evolutionary relationships, sequences from 10 reference strains representing five of the 15 members of the FGSC were included in the phylogenetic analysis. Sequences of the FGSC isolates and the reference strains were aligned automatically using the online MAFFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/software/). Maximum likelihood (ML) analysis was performed using MEGA version 5 (Tamura *et al*., 2011). *F. pseudogramiearum* and *F. cerealis* sequences were used as outgroups.

4.2.5. PCR assays for trichothecene mycotoxin profiles

Trichothecene chemotypes of FGSC isolates were identified using a suite of primers (Table 4.1) directed at portions of the genes that are predictive of isolates producing DON, NIV, 3-ADON and 15-ADON. Two PCR assays were used to determine the chemotype of these isolates, one targeting the *Tri*5-*Tri*6 intergenic spaces and the other targeting the *Tri*12 gene. The ToxP1 and ToxP2 primers which amplify a 300 bp fragment for the DON-chemotype and a 360 bp for the NIV-chemotypes were designed from the *Tri5-Tri6* intergenic sequences and evaluated for specificity by Li *et al.* (2005). In addition, the *Tri*12 primer set described by Ward *et al.* (2002) were used to amplify PCR products of 410, 670 and 840 bp for the 3-ADON, 15-ADON and NIV chemotypes respectively.

The cycling conditions for primers ToxP1/ToxP2 were as follows: initial denaturation at 95°C for 5 min, 30 cycles at 94°C for 1 min, 55 °C for 1min, 72°C for 50 s and a final extension at 72°C for 6 min. The samples were cooled to 4°C until recovery.

The cycling conditions for the *Tri*12 consisted of an initial step at 94°C for 10 min, followed by two cycles at 94°C for 30 s, 59°C for 30 s and 72°C for 30s. The

annealing temperature was stepped down every two cycles to 58, 56, 54, 53, 52 and 51°C, then kept at 50°C for 21 cycles, with a final step at 72°C for 10 min.

In both PCR assays, the resulting PCR products were separated by gel electrophoresis, stained with EtBr at a final concentration of 10mg/ml and visualised under UV light using the Gel-Doc It system or the the Bio-Rad gel documentation system (3.4.2.1).

4.3. RESULTS

4.3.1. Fungal Isolates

All the isolates recovered were initially identified as *F. graminearum s.l.* using morphological species descriptions. Due to their cryptic nature, members of FGSC cannot be differentiated by morphology. To further classify these isolates into the various species, sequencing of two loci was conducted as described in section 4.3.3.

4.3.2. Species specific PCR

PCR amplification of the isolates using *F. graminearum* species-specific primers Fg16F and Fg16R yielded DNA fragments for all sorghum isolates, whilst no amplification was obtained with all the maize isolates. Preliminary data from the PCR assays using these primers indicated that the sorghum population contained species producing two different fragments of 400- and 500-bp. Furthermore, amplification of a 400-bp DNA fragment was achieved for MRC4927 whilst no amplification was obtained for MRC4712.

4.3.3. Sequence-assisted species identification

Amplification of the *TEF*1-α and *URA* genes yielded the desired fragments of 725 bp and 1388 bp respectively. BlastN identification of concensus sequences of both the *TEF1-α* and *URA* gene sequences allowed for identification of all the isolates to species level (Table 4.2). Five of the 15 previously described species within the FGSC were recovered in this survey. The majority of isolates collected from maize kernels (*n*=64) were identified as *Fusarium boothii* (97 %) while only 3% were *F.*

graminearum s.s. Three FGSC pathogens were recovered from sorghum grain (*n*=9), and were identified as *F. cortaderiae*, *F. acacia-mearnsii* and *F. meridionale*.

4.3.4. PCR assays for trichothecene mycotoxin profiles

The results of the chemotyping assays using the ToxP1/ToxP2 and *Tri*12 primer sets are presented in Table 4.2. PCR amplification of chemotype-specific fragments using ToxP1 and ToxP2 primers demonstrated that the DON chemotypes yielded a 300 bp fragment, while the NIV chemotypes yielded a 360 bp fragment. All of the maize isolates were of the DON chemotype while all the sorghum isolates were of the NIV chemotype. Further characterisation of these isolates using the *Tri*12 primers revealed that both the 15-ADON and the NIV chemotypes which yielded DNA fragments of 670 and 840 bp respectively were present among the FGSC isolates used in this study. Figure 4.1 illustrates the different fragment sizes obtained using the ToxP1/ToxP2 and *Tri*12 primer sets. The 3-ADON chemotype was not detected in any of the FGSC isolates tested in this study.

Maximum likelihood (ML) analysis of *URA* (Figure 4.2) and *TEF*1-α (Figure 4.3) genes strongly indicated that the FGSC isolates obtained from maize kernels and sorghum grain samples clustered with *F. boothii*, *F. meridionale*, *F. acacia-mearnsii*, *F. cortaderiae* and *F. graminearum.* Bootstraps for *URA* ranged from 63 to 99% while for *TEF*1-α, the boot strap values ranged from 64 to 99%. *F. boothii* was the predominant fungus recovered from maize kernels while *F. cortaderiae, F. meridionale* and *F. acacia-mearnsii* are the only species currently know to be associated with sorghum grains in South Africa.

4.4. DISCUSSION

The primary objective of this study was to use molecular phylogenetics to identify species of the FGSC isolated from maize kernels and sorghum grain within South Africa as well as determine the trichothecene chemotypes produced by these fungi using PCR assays based on the *Tri*5-*Tri*6 intergenic spaces and the *Tri*12 gene region involved in the biosynthesis of trichothecenes. In this study, maize and sorghum samples had very few *F. graminearum s.l.* infection levels. The low recovery rate of

fungal isolates versus the high concentrations of FGSC DNA (Chapter 2) and mycotoxins (Chapter 3) can be the result of host, pathogen and environment interactions. Rheeder *et al*. (1995) reported the presence of high levels of moniliformin (MON), DON and NIV in South African maize samples that had low levels of *F. graminearum s.l.* infection during the 1990 harvest. They concluded that environmental and physiological stress could have been the cause of such a discrepancy.

DNA sequencing has identified high levels of variability among the morphologically similar strains of the FGSC worldwide (Wang *et al*., 2011). According to the genealogical concordance with 13 independent loci totalling 16.3 kb, 15 species were identified within the FGSC (Davari *et al.,* 2012). Phylogenetic analysis of DNA sequences from portions of the *EF1*-α and *URA* gene region sequences revealed the presence of five species within the FGSC from maize kernels and sorghum grain in South Africa. This study shows that the Gibberella ear rot of maize in South Africa is associated with *F. boothii* and *F. graminearum*, with *F. boothii* being the predominant species. This is the first report of *F. meridionale*, *F. acacia-mearnsii* and *F. cortaderiae* in sorghum grain in South Africa, although these fungi have been found in other South African cereals such as wheat (Boutigny *et al*., 2011). *F. boothii* has been previously isolated from maize in South Africa (O'Donnell *et al*., 2000, 2004, 2008) and in recent surveys this pathogen has been found in association with locally diseased maize crowns and roots (Lamprecht *et al*., 2011) as well as wheat, barley, maize ears and roots (Boutigny *et al*., 2011). Furthermore, Lamprecht *et al*. (2011) also found that *F. graminearum s.s.* is primarily associated with maize crowns and roots. Although only two isolates of *F. graminearum s.s.* were recovered from maize kernels, this is consistent with worldwide reports that show this pathogen as a member of the maize complex (O'Donnell *et al*., 2000; Desjardins & Proctor, 2010). Cultivation of maize in rotation with wheat in some of the localities within South Africa could be contributing towards the spread of this pathogen from wheat to the maize crop. *F. graminearum s.s.* remains a contributor to maize diseases in other parts of the world, including the USA and Iran (O'Donnell *et al*., 2000).

This study also provides data on the trichothecene chemotype profiles of the FGSC isolates recovered from maize kernels and sorghum grain from four localities within

South Africa, namely Wonderfontein, Bethlehem, Frankfort and Cedara. According to O'Donnell *et al*. (2008), every species within the FGSC is capable of producing type B trichothecenes *in planta.* Using the ToxP1/P2 primers to chemotype the FGSC isolates allowed a clear differentiation between the DON and NIV chemotypes while further characterisation of the DON-producing isolates based on the *Tri*12 primer set indicated that the 15-ADON chemotype was present in all the maize isolates. These findings are consistent with previous studies that have shown that populations of the 15-ADON chemotype predominate in South African maize (Boutigny *et al*., 2011). An important issue that has recently gained interest is the relative toxicity of 3-ADON and 15-ADON which is of concern because these DON precursors are sometimes also detectable along with DON in cereal grains (Pestka, 2007). Studies have shown that the 3-ADON chemotypes produced more DON than 15-ADON chemotypes (Guo *et al*., 2008), which is a cause for increasing concern to the cereal industries. While the presence of the 15-ADON chemotype among the FGSC isolates poses a serious threat to both human and animal health, the absence of the 3-ADON chemotype in this survey is reassuring for local grain production since both these acetylated derivatives are believed to be more toxic than DON (Asam & Rychlik, 2007). The present results contribute to a deeper knowledge of the trichothecene mycotoxin profiles of members of the FGSC in South Africa.

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Table 4.1. Primers used for FGSC species identification and chemotyping

Primer name	Primer sequence (5'-3')	Target gene	Amplicon size (bp)	Reference
12CON	CATGAGCATGGTGATGTC	*Tri* 12	410	Ward *et al*., 2002
12NF	TCTCCTCGTTGTATCTGG		670	
12-15F	TACAGCGGTCGCAACTTC		840	
12-3F	CTTTGGCAAGCCCGTGCA			
EF1	AAGGCTGTTGCTTTCGTCC	Translation elongation factor-1α	725	O'Donnell *et al*., 2000
EF2	GCAAGCAATGTGGGCAGTG			
URA1	ATGAAGGTTGTTCTTGTGAGCGGCGG	Ammonia ligase	1388	O'Donnell *et al*., 2000
URA6	AATCCAATCCTGAATGGCGTC			
ToxP1	GCCGTGGGGRTAAAAGTCAAA	*Tri* 6	DON-300 & NIV-360	Li *et al*., 2005
ToxP2	TGACAAGTCCGGCGCACTAGCA			
Fg16F	CTCCGGATATGTTGCGTCAA		400-500	Ji *et al*., 2007
Fg16R	GGTAGGTATCCGACATGGCAA			

Table 4.2. Isolate number, species, locality, crop, season, *Fusarium graminearum* species specific PCR, amplicon size and NIV, DON, 3-ADON and 15-ADON chemotypes of FGSC from maize and sorghum grain

Species name	Isolate or accession number	Plant part	Locality	Season	*F. graminearum* species specific PCR	Amplicon size (bp)	Species Identification based on TEF1-α and URA gene sequences		FGSC chemotypes				
					Fg16F/Fg16R primers		EF1/EF2 primers	URA1/URA6	ToxP1/ToxP2 primers		Tri12 primers		
							TEF1-α	URA	DON	NIV	3-ADON	15-ADON	NIV
F. boothii	M260/01	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/02	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/04	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/07	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/08	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/13	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/20	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/23	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/37	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/38	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/39	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/40	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/47	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/51	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/57b	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/58	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/61	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/62	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/63	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/64	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/65	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/67	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/72	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/75	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/76	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/77	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/82	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/83	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/84	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/85	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/89	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/93	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/94	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/95	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M260/97	Maize kernels	Wonderfontein	2007/2008	-	-	+	+	+	-	-	+	-

Table 4.2. Continued.

Species name	Isolate or accession number	Plant part	Locality	Season	*F. graminearum* species specific PCR	Amplicon size (bp)	Species Identification based on TEF1-α and URA gene sequences		FGSC chemotypes				
					Fg16F/Fg16R primers		EF1/EF2 primers	URA1/URA6	ToxP1/ToxP2 primers		Tri12 primers		
							TEF1-α	URA	DON	NIV	3-ADON	15-ADON	NIV
F. boothii	M284/205	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/206	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/207	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/210	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/218	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/219	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/228	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/232	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M284/259	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/140	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/148	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/162	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/164	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/165	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/166	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/168	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/171	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/172	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/176	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/177	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M286/61	Maize kernels	Frankfort	2007/2008	-	-	+	+	+	-	-	+	-
F. boothii	M637/32	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. boothii	M637/37	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. boothii	M637/42	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. boothii	M637/43	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. boothii	M637/45	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. boothii	M637/48	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. cortaderiae	M431/17	Sorghum grain	Cedara	2007/2008	+	500	+	+	-	+	-	-	+
F. graminearum s.s	M637/36	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. graminearum s.s	M637/47	Maize kernels	Bethlehem	2008/2009	-	-	+	+	+	-	-	+	-
F. graminearum s.s	MRC4712				-	-	+	+	+	-	-	+	-
F. acaciae-mearnsii	M431/35	Sorghum grain	Cedara	2007/2008	+	400	+	+	-	+	-	-	+
F. acaciae-mearnsii	M431/92	Sorghum grain	Cedara	2007/2008	+	400	+	+	-	+	-	-	+
F. meridionale	M431/02	Sorghum grain	Cedara	2007/2008	+	500	+	+	-	+	-	-	+
F. meridionale	M431/11	Sorghum grain	Cedara	2007/2008	+	500	+	+	-	+	-	-	+
F. meridionale	M431/18	Sorghum grain	Cedara	2007/2008	+	500	+	+	-	+	-	-	+
F. meridionale	M431/28	Sorghum grain	Cedara	2007/2008	+	500	+	+	-	+	-	-	+
F. meridionale	M431/60	Sorghum grain	Cedara	2007/2008	+	500	+	+	-	+	-	-	+
F. meridionale	M431/91	Sorghum grain	Cedara	2007/2008	+	500	+	+	-	+	-	-	+
F. pseudograminearum	MRC4927				+	400	+	+	+	-	-	+	-

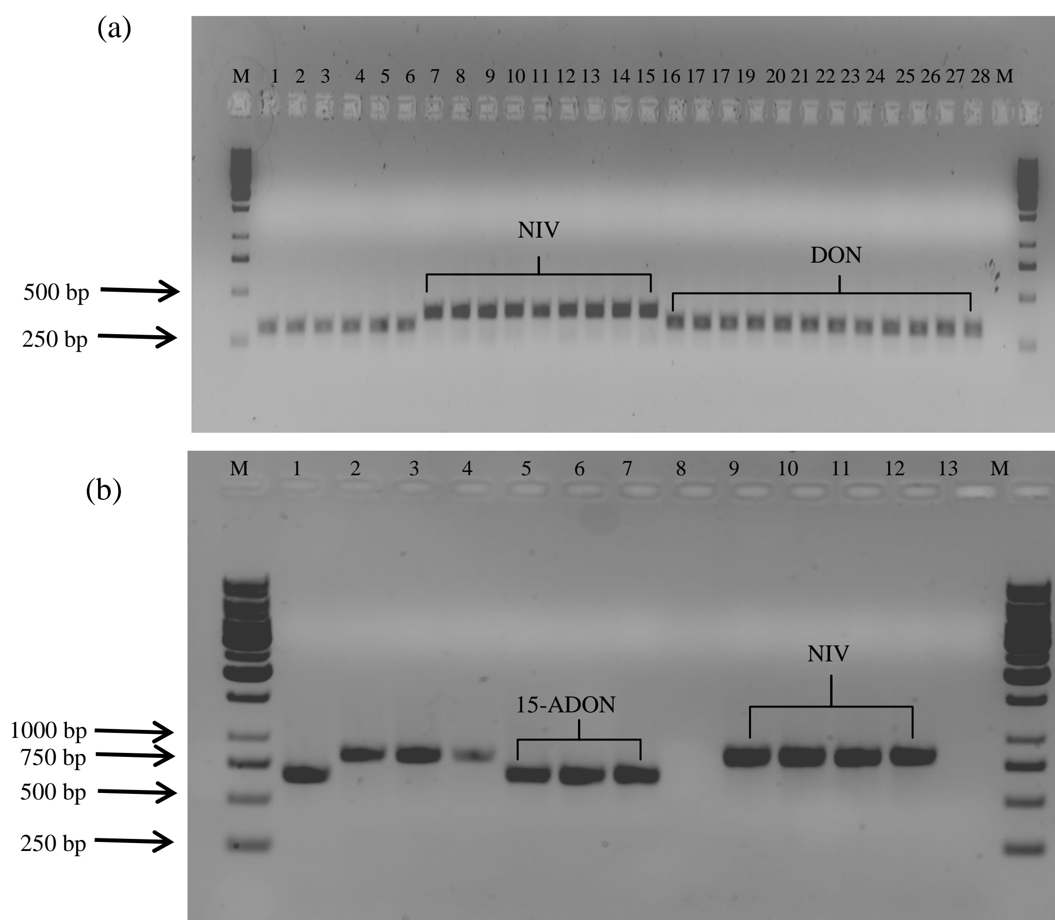

Figure 4.1. PCR products for FGSC trichothecene mycotoxin chemotypes using (a) ToxP1/ToxP2 primers to amplify a 300 and 360 bp fragments for DON and NIV respectively and (b) using *Tri*12 primers which amplify 15-ADON (670 bp; lanes 1,5-7); lane 8 *F. chlamydosporum* and NIV (840bp; lanes 2-4 and 9-12) fragments.

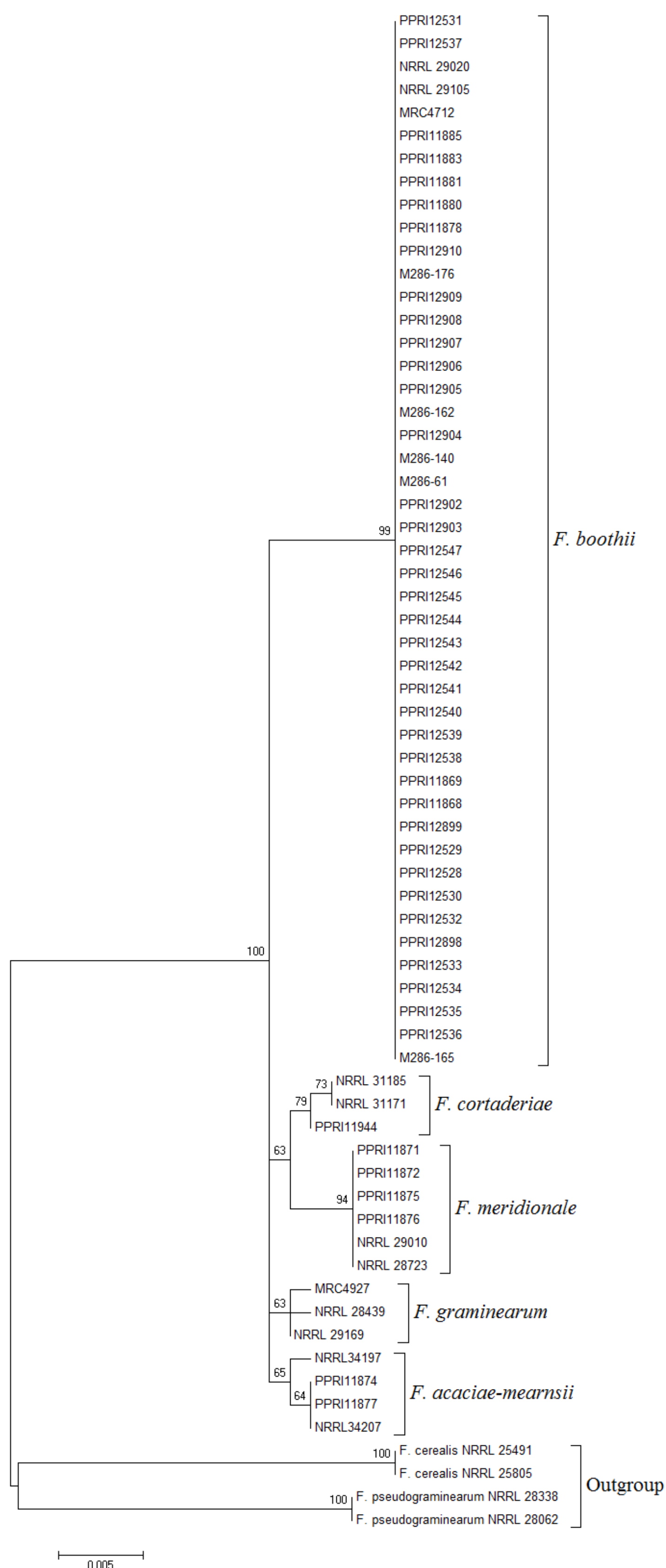

Figure 4.2. A maximum likelihood (ML) species phylogeny for FGSC based on the ammonia ligase gene sequence data set. Bootstrap values based on 1000 pseudoreplicates are indicated above the brances with those lower than 60% not shown. The tree was rooted with *F. cerealis* and *F. pseudograminearum*.

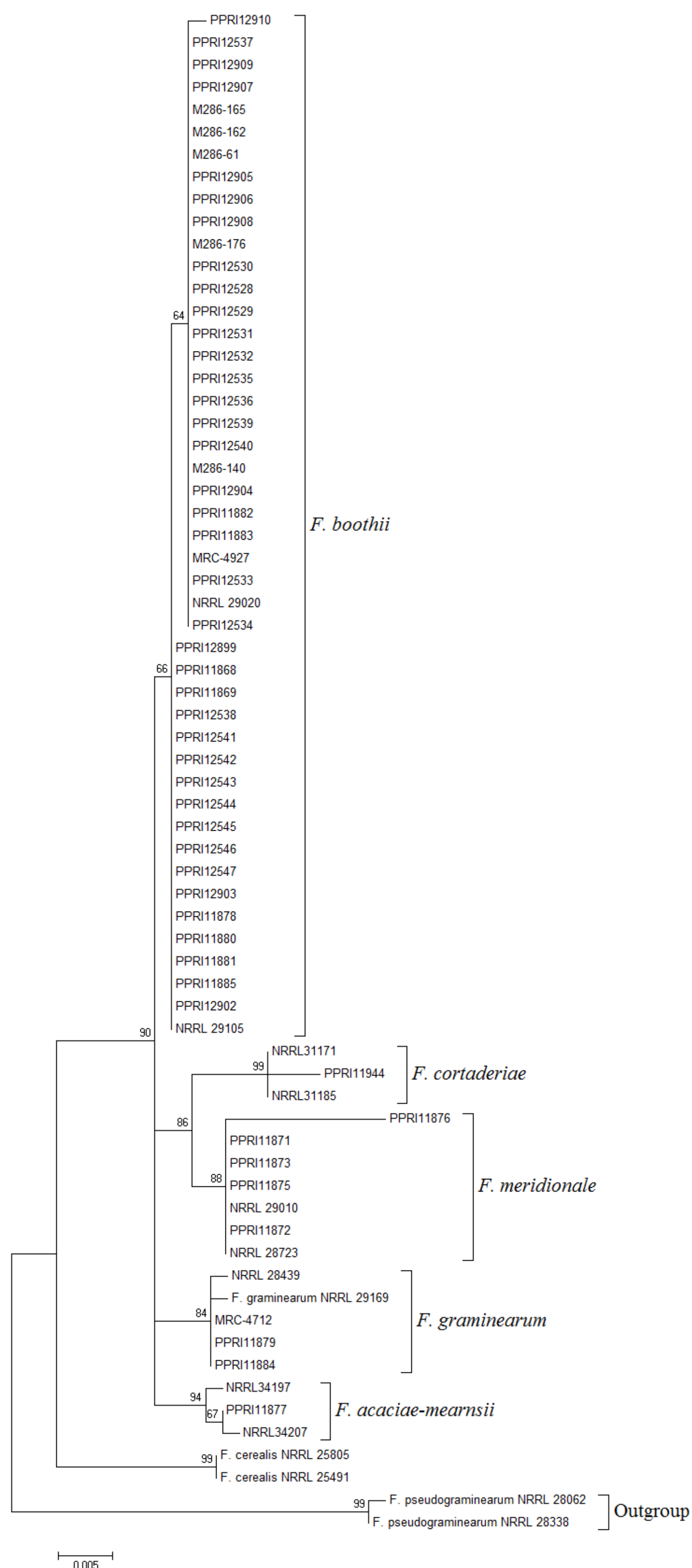

Figure 4.3. A maximum likelihood (ML) species phylogeny for FGSC based on the translation elongation factor 1-alpha gene sequence data set. Bootstrap values based on 1000 pseudoreplicates are incicated above the brances with those lower than 60% not shown. The tree was rooted with *F. cerealis* and *F. pseusograminearum*.

SUMMARY

Maize and sorghum are important crops in South Africa, meeting the dietary needs of millions of people as well as the needs of the feed industry. Fungi within the *Fusarium graminearum* species complex (FGSC) have recently become the subject of importance locally.

Maize kernels and sorghum grain were collected from the National Cultivar Trial over three and two seasons respectively. A total of 558 samples comprising of six maize and seven sorghum cultivars were collected from 34 and 22 localities, respectively. Although different conventional detection, isolation and identification methods were utilized, very low numbers of FGSC isolates could be obtained from the grain. Molecular identification was based on the translation elongation factor-1α (*TEF*1-*α*) and the ammonia ligase (*URA*) genes. *F. boothii* and *F. graminearum s.s.* were identified from maize kernels while *F. acacia-mearnsii, F. cortaderiae* and *F. meridionale* where associated with sorghum. Results indicated host-specificity between members of the FGSC.

Total genomic DNA was extracted from all milled kernel and grain samples and analysed for the presence or absence of FGSC DNA using TaqMan real-time PCR. *F. graminearum s.l.* DNA was detected and quantified in over 47% of the samples. These findings contradict previous reports that stated that this group of fungi is of minor importance. Reasons may be improved detection techniques as well as changes in agronomic practices, such as rotation of maize with wheat in South Africa, or to other shifts in environmental factors.

DON and ZEA concentrations were quantified using CD-ELISA. In the absence of an ELISA test for NIV, a selected number of samples were used in LC-MS/MS based detection and DON, NIV and ZEA were quantified. Concentrations varied considerably and no correlation between the two techniques was found. Based on CD-ELISA, maize and sorghum contamination with DON and ZEA differed significantly between seasons, localities and in some instances cultivars. An analysis of variance across all three seasons showed significant crop by locality interactions. In maize, high levels of DON were detected in the 2007/08 season, in particular at Delmas, while during the same season ZEA contamination of maize was significantly higher at Bothaville. In sorghum, DON and ZEA contamination was highest during the 2008/09 season, although both toxins were detected in the preceding

season. In both the 2007/08 and 2008/09 seasons, Cedara was the most conducive locality for grain contamination with both toxins.

Trichothecene chemotyping was conducted using both simplex and multiplex PCR. All the *F. boothii* and *F. graminearum s.s.* isolates from maize were DON/15-acetyldeoxynivalenol (15-ADON) producers and *F. acacia-mearnsii*, *F. cortaderiae* and *F. meridionale* isolates from sorghum grain produced NIV. DON can act as a virulence factor in plant disease and is usually associated with greater pathogenicity on plants than NIV producers. On the other hand, NIV is believed to be more toxigenic to both humans and animals. Isolates of the 15-ADON chemotype are reportedly less toxic that those of the 3-ADON chemotype. The 3-ADON chemotype was not recorded in this study. However, more FGSC isolates need to be collected from South African cereal grains and evaluated for their mycotoxin potential to establish safety guidelines for end users of these products.

The results of this study showed that levels of field infection of maize and sorghum grain by the FGSC are of significance. Future studies need to quantify the relationships between members of the FGSC and their tricthothecene and ZEA production in South African maize and sorghum production systems including the role of cultivar choice, weather, rotation, tillage and other practices with the aim of establishing intervention technologies.

OPSOMMING

Mielies en sorghum is belangrike gewasse in Suid Afrika en die stapel voedsel van miljoene mense asook dierevoeding. Fungi binne die *Fusarium graminearum* spesies kompleks (FGSK) het in die afgelope jare, plaaslik, toenemend belangriker geword.

Mielie pitte en sorghum graan is vanaf die Nasionale Kultivarproewe oor drie en twee seisoene onderskeidelik versamel. 'n Totaal van 558 monsters van ses mielie en sewe sorghum kultivars is vanaf 30 en 21 lokalitiete onderskeidelik versamel. Alhowevel verskillende konvensionele waarnemings, isolasie en identifikasie metodes toegepas is, is baie lae getalle van die FGSK ge-isololeer. Molekulêre identifikasie was op die translering verlenging facktor (*TEF*1-*α*) en die ammonium ligasie (*URA*) gene baseer. *F. boothii* en *F. graminearum s.s.* is op mieliepitte waargeneem terwyl *F. acacia-mearnsii, F. cortaderiae* en *F. meridionale* met sorghum geassosieer was. Resultate het gasheer spesifisiteit tussen lede van die FGSK aangedui.

Totalle genomiese DNA is van alle gemaalde monsters geëkstraeer en vir die teenwoordigheid van FGSK DNA geanaliseer deur gebruik te maak van werkstyd PKR. *F. graminearum s.l.* DNA is waargeneem en gekwantifiseer in 47% van die monsters. Die bevindending is teenstrydig met voorige verslae wat die fungusgroep as van minimale belangrikheid beskou. Redes hiervoor kan wees a.g.v. die verbetering van analitiese metodes en verandering in agronomiese praktyke soos wisselbou met mielies en koring in Siud Afrika of verskywings in omgewings faktore.

DON en ZEA konsentrasies is d.m.v. van CD-ELISA bepaal. In die afwesigheid van 'n ELISA toets vir NIV is 'n bepaalde aantal monsters ge-evalueer deur gebruik te maak van VC-MS/MS om die teenwoordigheid van DON, NIV en ZEA te bepaal en te kwantifiseer. Konsentrasies het aansienlik gevarieer en daar was geen korrelasie tussen die twee tegnieke nie. Op basis van CD-ELISA het kontaminasie van mielies en sorghum met DON en ZEA betekenis vol verskil tussen seisoen, lokalitieite en kultivars. Variansie analise oor die drie seisoene het betekenisvolle gewas X lokaliteit interaksies getoon. In mielies is hoë vlakke van DON in die 2007/08 season waargeneem, veral in Delmas, terwyl gedurende die selfde seisoen, ZEA vlakke in mielies betekenisvol hoër was op Bothaville. By sorghum was DON en ZEA vlakke die hooste gedurende die 2008/09 seisoen, alhowel albei toksiene in die

vorige seisoen voorgekom het. In beide die 2007/08 en 2008/09 seisoene was Cedara die mees voordelige lokalitiet vir graan kontaminasie deur albei toksiene.

Trichothesene chemotipering is uitgevoer deur van simpleks en multipleks PKR gebruik te maak. Al die *F. boothii* en *F. graminearum* s.s. isolate van mielies het DON/15-acetyldeoxynivalenol (15-ADON) geproduseer en *F. acacia-mearnsii*, *F. cortaderiae* en *F. meridionale* isolate van sorghum graan het NIV geproduseer. DON is ‘n virulensie faktor by plansiektes en is meer as NIV met plant patogenisiteit geassosieer. In teenstelling, word NIV beskou as meer toksigenies vir mens en diere. Isolate van die 15-ADON chemotipe is minder toksies as die van die 3-ADON chemotipe. Die 3-ADON chemotipe is nie in die huidige studie waargeneem nie. Meer FGSK isolate word benodig vanaf Suid Afrikanse grane vir evaluering van mikotoksiese potensial om veiligheids aanbvelings vir eingebruikers vas te stel.

Die bevindinge van die heidige studie het getoon dat vlakke van veld besmetting van mielies en sorghum grane deur lede van die FGSK betekenisvol is. Toekomstige studies behoort die verhouding tussen lede van die FGSK en tricthothecene en ZEA voorkoms in Suid Afrikanse mielies en sorghum produksiesisteme aan te spreek. Die rol van kultivarkeuse, weer, wisselbou, grondbwerking en ander praktyke met die doel on beheerstratigië vas te stel moet ingesluit word.