Modelling the colonisation of sorghum grain by the *Fusarium graminearum* species complex and concomitant mycotoxin production

by

Lisa Ann Coetzee

A dissertation submitted in accordance with the requirements for the degree of *Magister Scientiae Agriculturae*

Faculty of Natural and Agricultural Sciences

Department of Plant Sciences

University of the Free State

Bloemfontein, South Africa

Supervisor: Prof. N. W. McLaren

Co-supervisors: Prof. B. Flett

June 2015

Declaration

I, Lisa Ann Coetzee, 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.

Lisa Ann Coetzee

.....

Date:

..... June 2015 .....

Contents

Acknowledgments

This study was supported financially by the Howard G. Buffett Foundation through the Norman E. Borlaug Institute for International Agriculture of Texas A&M AgriLife Research and the Sorghum Trust. Thank you to both parties involved in making this study possible.

Much appreciation goes to Ms. Gugu Khali from the Agricultural Research Council-Grain Crops Institute (Sub-Centre: Cedara), Mr. John Bow from Texas A&M AgriLife Research**,** Dr. Maryke Craven at the Agricultural Research Council-Grain Crops Institute in Potchefstroom, for the technical assistance with regard to the hard work that went into planting and maintaining all the field work.

Thank you to Dr. Botma Visser from the Plant Sciences department at the University of the Free State for his guidance with the molecular aspects of this study as well as the use of laboratory facilities. Appreciation must be extended to the students in his laboratory for assistance. A further thank you must be extended to the Plant Pathology division, particularly to Danelle van Rooyen, for being a great epidemiology team member.

I would also like to show gratitude towards Dr. Aneen Schoeman and the Agricultural Research Council-Grain Crops Institute in Potchefstroom for her patience and guidance with the molecular work which I did at their facilities.

I extend a special word of appreciation to Dr. Gabre Kemp in the Department of Microbial, Biochemical and Food-Biotechnology at the University of the Free State for the analyses on the LC-MS-MS and to Prof. John Taylor from the University of Pretoria's, Department of Food Science, for the use of the facilities and tangential dehulling device.

I would also like to thank Mrs. Maureen Fritz at the Agricultural Research Council - Grain Crops Institute and the South African Weather Services (SAWS) for providing the weather data for this study.

I am thankful to Christopher Rothmann for the extra hours that he assisted me with, in and out of the laboratory, during this study. Thank you to my parents, Linda and Petrus Coetzee, for the privilege to be a post-graduate student, endlessly motivating and loving me.

I thank my co-supervisor Prof. Bradley Flett for his guidance and patience.

My biggest academic and personal acknowledgements go to my supervisor, Prof. Neal McLaren, for his eternal inspiration, the opportunity to be his student and a student of epidemiology. Thank you for teaching me the wise words of Lord Kelvin, “to measure is to know and if you cannot measure it, you cannot improve it.” I hope to keep being one of your most difficult students and perhaps in the future to be as great a mentor and supervisor as you are to me.

Preface

This dissertation includes four chapters. The overall aim of this study was to explore the effect of genotype and environment on the colonisation of sorghum grain by the members of the *Fusarium graminearum* species complex (*Fg*SC) and mycotoxin contamination. Furthermore certain post-harvest processing activities, in particular, tangential abrasive dehulling, on removal of *Fg*SC and concomitant mycotoxin production from contaminated grain.

The first chapter is a literature review on modelling the colonisation of sorghum grain by *F. graminearum sensu lato*, which will be referred to *Fg*SC, and concomitant mycotoxin production, deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA).

In Chapter 2, the effect of cultivars, flowering date and associated weather conditions at two localities on the colonisation of sorghum grain by *Fg*SC, severity and mycotoxin accumulation in sorghum was explored. It was hypothesised that weather is a primary driving variable in host predisposition to grain mold pathogens and subsequent disease severity and the study was aimed at identifying and explaining the genotype and environment interactions associated with *FgSC* colonisation and concomitant mycotoxin contamination of sorghum.

In Chapter 3, the relationship between weather and the associated *Fg*SC development and mycotoxins production by *Fg*SC on sorghum grain in various production regions of South Africa was determined. This was aimed at providing a basis from which to develop a risk analysis model which would provide an indication of sorghum grain quality and safety for human and animal consumption.

Chapter 4 was based on the hypothesis that grain mold fungi colonise different layers of the endosperm and that mycotoxins therefore occur at different depths in grain. The effect of processing on mycotoxins associated with *Fg*SC in sorghum grain products, specifically associated with decortication of grains prior to milling or other processing methods was therefore investigated.

The work presented in this dissertation will contribute to a better understanding of sorghum grain mold and associated mycotoxin production and accumulation, caused by *Fg*SC. Understanding the climatic factors which drive the development of *Fg*SC and concomitant mycotoxin production could assist in the creation of *Fg*SC

colonisation and mycotoxin risk models specifically for South Africa. Furthermore, understanding the depth of *Fg*SC infections, DON, NIV and ZEA in processing could assist in reducing the health implications to consumers.

Chapter 1

Review of modelling the colonisation of sorghum grain by the *Fusarium graminearum* species complex and concomitant mycotoxin production

1. Introduction

Sorghum (Figure 1; *Sorghum bicolor* (L.) Moench) originated from Africa but is produced globally. Sorghum is the fifth most grown cereal worldwide, and is a staple food in 30 countries that sustains 500 million people in the semi-arid tropics (Rampho, 2005; Reddy *et al.,* 2010). Globally sorghum is grown in almost 100 countries and covers 42 million hectares. Drought tolerance associated with sorghum largely determines the production areas, and with current trends in climate change production of sorghum is expected to increase by approximately 9% globally (Reddy *et al.,* 2010).

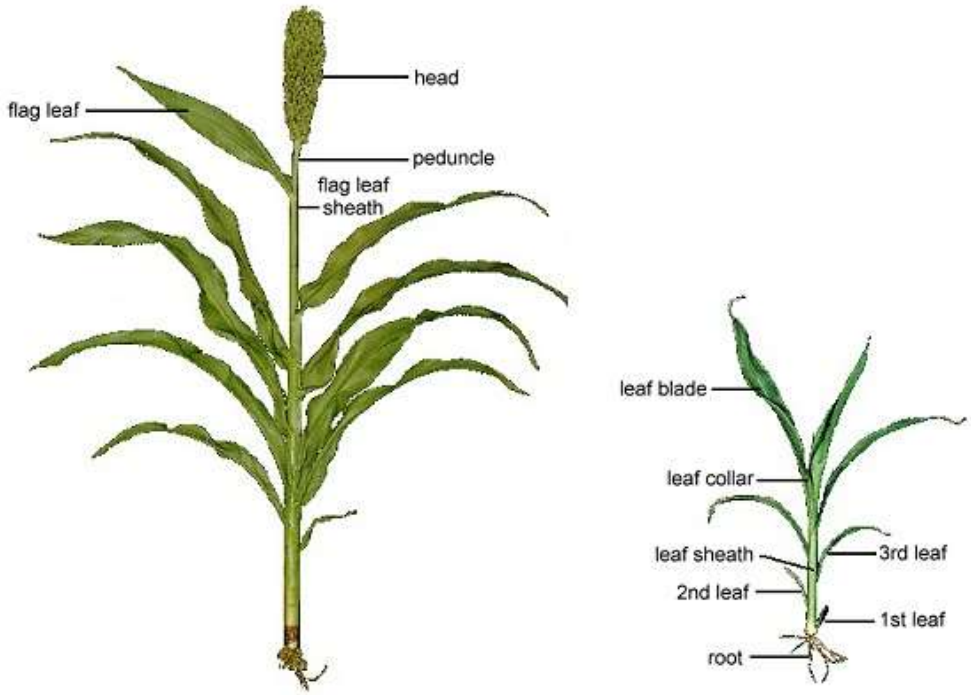


Figure 1. Grain sorghum plant morphology (Warrick, 2000).

Sorghum belongs to the grass family, *Graminea.* This crop has a deep profusely laterally branching root system and unique leaf characteristics. Motor cells are located along the midrib of the upper surface of the leaf, allowing the leaves to curl up rapidly under stress conditions such as drought. Sorghum grain is located on the

panicle, the characteristics and colour of this inflorescence varies between cultivars. The glumes which enclose the grains open during the early morning or at night and the opening of the entire inflorescence can take from six to nine days. Each panicle can contain between 800 to 3000 kernels, if optimum production conditions are achieved (Department of Agriculture, Forestry and Fishery (DAFF), 2010). The colour and characteristics of the grain varies between cultivars. Each grain consists of the seed coat (pericarp and testa), embryo and endosperm, as a fraction of total mass of 7.3–9.3%; 7.8–12.1% and 81.1–84.6% respectively. Polyphenolic compounds, tannins, anthocyanins, anthocyanidins, flavonoids and other compounds are present in the sorghum grain pericarp and testa. The advantage of these compounds is that they provide protection from biotic attacks on the grain and are associated with antioxidants. However, the digestibility of sorghum is reduced in the presence of tannins as they have the ability to bind with proteins and digestive enzymes associated with the grain (Figure 2; Taylor, 2003).

In drought prone areas of Africa, sorghum is the second most cultivated grain apart from maize (Taylor, 2003). Approximately 35% of sorghum grain produced is consumed directly by humans (Reddy *et al.,* 2010). Sorghum provides low income populations with dietary energy and micronutrient requirements (Sharma *et al*., 2011). Sorghum grain constituents include 73.8% starch, 12.3% protein and a source of vitamin B complex, which are important components required for human and livestock health (WHO/FAO, 2012). Sorghum grain is used in the production of sorghum meal (*mabele* as it is known in South Africa), sorghum rice, couscous, *injera* (gluten free, pancake-like, staple food of Ethiopia), leavened breads, *togwa* (porridge for toddlers under age of five in Tanzania), malt, non-alcoholic fermented beverages and beer. Sorghum grain is used for approximately 33% of livestock feed and will play a major role in the future production of ethanol. Not only is the grain used, but the stem can also be used for building materials, firewood, waxes, dyes and vegetable oil (Sheorain *et al.,* 2000; Belton & Taylor, 2004; Rampho, 2005; Taylor *et al.,* 2006; Du Plessis, 2008, Reddy *et al.,* 2010).

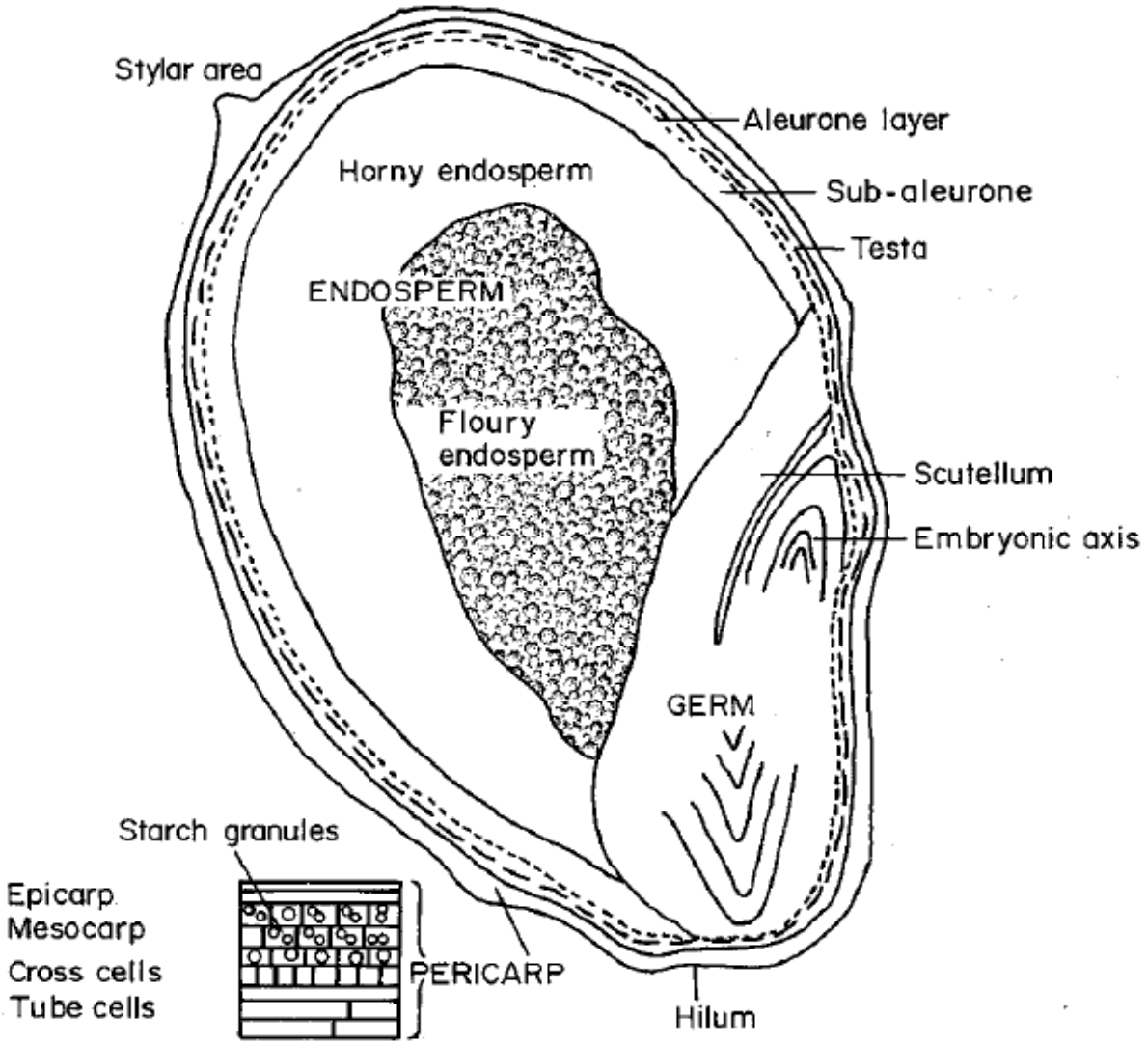


Figure 2. Diagrammatic cross section through sorghum grain (Taylor, 2003).

1.1 Sorghum Production in South Africa

Provinces in South Africa where the most sorghum is produced are Free State and Mpumalanga, as they are drier areas of the country with shallower clay soils and a lower yield potential. However, there has been a shift in the areas of sorghum production in South Africa due to the development of cultivars that can withstand lower temperatures and wetter periods (Du Plessis, 2008).

Smallholder farmers in the Southern African Development Community (SADC) region on average produce 0.8 $t.ha^{-1}$. This grain is mostly used for human and livestock consumption “on farm.” In South Africa the commercial yield in 2001 was 2.34 $t.ha^{-1}$ which was comparable with maize yields of approximately 2.49 $t.ha^{-1}$

during the same period (Belton & Taylor, 2004). However, current sorghum and maize yields are 3.4 $t.ha^{-1}$ and 5.3 $t.ha^{-1}$, respectively (SAGIS, 2014).

A knowledge base of sorghum production and production constraints is paramount to successfully increase yields of sorghum. Approximately 10 cm below the soil the minimum temperature requirement of sorghum germination is 15°C. The optimum temperature for growth and development is between 27 and 30°C, however no extreme damage has been observed at temperatures as low as 21°C. Sorghum requires short days and longer nights to stimulate reproductive growth. Water requirements which are ideal for the production of sorghum range from 400 mm to 800 mm, however sorghum is known to be a hardy, drought tolerant crop (Du Plessis, 2008). The Pan African Agribusiness and Agro Industry Consortium (PANAAC, 2012) state that sorghum requires 30% of the water requirements of maize to grow successfully. The plant remains in a vegetative state if moisture stress occurs (DAFF, 2010). Sorghum planting dates are dependent on various factors, however in South Africa mid-October to mid-December are seen as appropriate planting times. The base temperature of sorghum is 10°C and sorghum seed and seedlings are sensitive to low temperatures, i.e. 5 to 7°C and frost. Deciding on a specific planting date is critical, because there should be adequate moisture for panicle initiation (Du Plessis, 2008).

2. Biotic Constraints in Sorghum Production

Diseases which cause significant losses to sorghum production include ergot, anthracnose, leaf spots and blights, sooty stripe, downy mildew, rusts, smuts, seedling diseases and grain molds. The importance of these diseases varies across the globe, due to economic losses caused and the threat that the disease poses on food security. Superficial or internal damage can be inflicted by sorghum grain mold (SGM) (Figure 3A and 3B; Mtisi & McLaren, 2002). SGM is the one of the most important biotic constraints in sorghum production prior to harvest (Rao *et al*., 2012). It must not be confused with grain weathering, discolouration and grain damage, which is the result of superficial fungal colonisation that occurs during high moisture levels post-physiological maturity (Thakur *et al.,* 2006).

SGM is caused by a complex of fungi which colonises the sorghum grain, either saprophytically or parasitically. Infection is possible from anthesis, when flowers are most susceptible to infection and colonisation, until grain filling, if weather conditions permit (Figure 4). Two infection pathways followed by SGM fungal complex to infect the host plant have been proposed. The first infection pathway is known as natural unaided infection and the second as assistance which includes the role of biotic factors, such as insects (Marley & Ajayi, 1999).

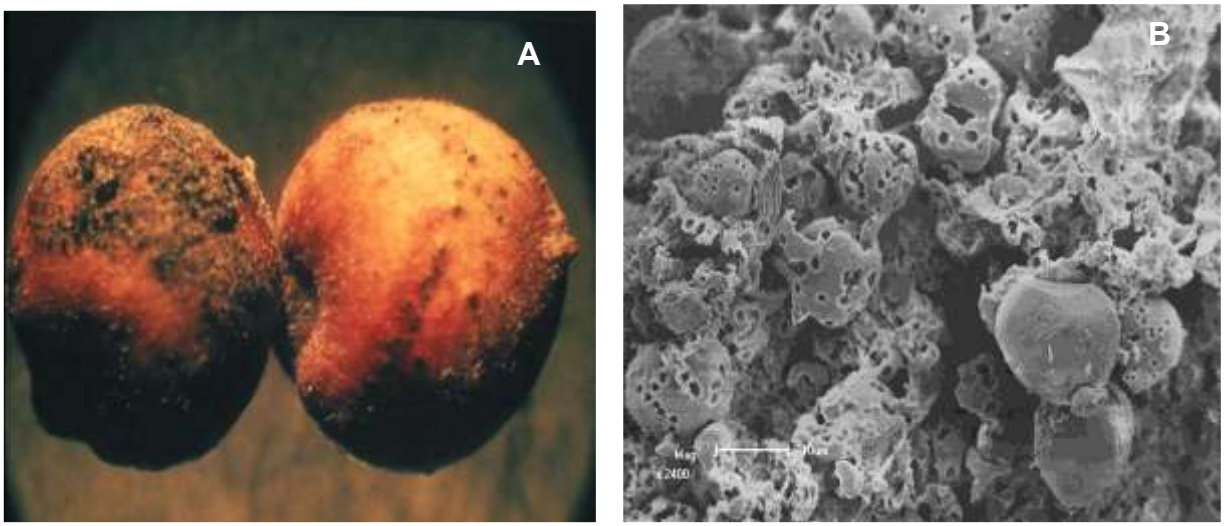


Figure 3. A. Superficial external disease presence. B. Starch density reduction, due to internal pathogen presence (Photo: Prof. N.W. McLaren).

2.1 Sorghum Grain Mold Pathogens

Over 40 genera of pathogenic fungi occur on sorghum grain and cause SGM. These fungi vary with geographic location, climatic conditions associated with the region and agronomic practices. Pathogens that are generally associated with SGM globally, include; *Alternaria* spp., *Aspergillus* spp. (*A*. *fumigatus* and *A*. *niger*), *Bipolaris* spp., *Cladosporium* spp., *Colletotrichum* spp., *Curvularia* spp. (*C. lunata*), *Fusarium* spp. (*F. graminearum (sensu lato)*, *F. moniliforme (sensu lato)*, *F. thapsinum* and *F. verticilliodes*) and *Phoma* spp. (*P. sorghina = Epicoccum sorghi*). The most important pathogens associated with SGM can be divided into two groups; i.e. fungi responsible for grain mold (i.e. discolouration and physical deterioration of the grain) and those that produce mycotoxins (i.e. toxic secondary metabolites in fungi contaminating grains).

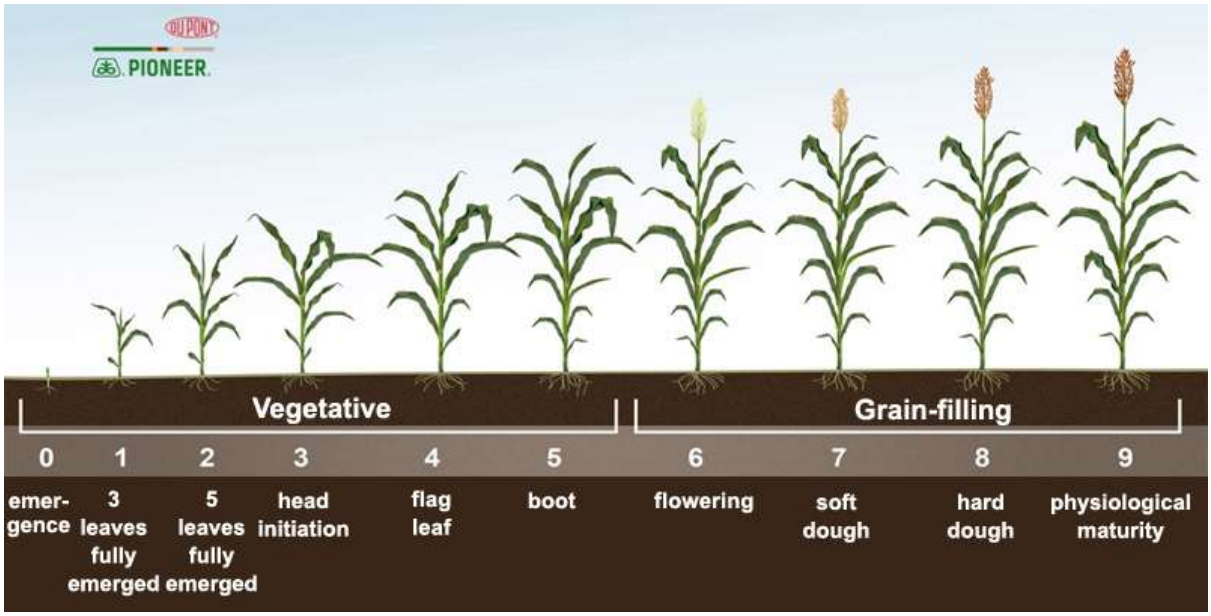


Figure 4. Sorghum growth stages from planting to physiological maturity (0 to 90 days after planting; Pioneer, 2014).

Fungi primarily responsible for the colonisation and molding of grain include *Alternaria* spp., *Cladosporium* spp., *Curvularia* spp. and *Phoma* spp. Fungi associated with SGM which are regarded as the most prominent mycotoxin producers globally include *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp. and *Phoma* spp. The metabolites may occur in both the spores and hyphae of the respective organisms (Marley & Ajayi, 1999; Little, 2000; Chandrashekar & Satyanarayana, 2006; Rahmani *et al.,* 2009; Köppen *et al*., 2010; Balota, 2012; Rao *et al*., 2012).

Various pathogens are associated exclusively with specific parts of the sorghum grain. The *Phoma* spp. are restricted to the pericarp whereas *Curvularia* spp. and *Fusarium* spp. begin at the hilar region of the pericarp and subsequently penetrate the endosperm. Furthermore, *Curvularia* spp. are mostly associated with grain infections, while the *Fusarium* spp. are known to attack the grains, stems and leaves of the sorghum plant and thus have a wider distribution throughout the plant (Chandrashekar & Satyanarayana, 2006).

SGM is favoured by environmental conditions associated with the semi-arid tropics, such as Africa and India (Rao *et al*., 2012). These areas are thus under the greatest threat by SGM due to their favourable environments and high levels of food insecurity (Taylor, 2003). Weather conditions which favour the development of the

disease complex are high humidity (75-100%) and moderate temperatures (21-27°C) from anthesis until harvest. However, it must be noted that SGM is a fungal complex and therefore, different weather conditions may favour development of different members of the fungal complex causing different fungal spectra to predominate at any one time (Menkir *et al.,* 1996).

Symptoms of SGM are divided into two distinct phases, i.e. those associated with initial infections and those with post-colonisation infections. Initial infections begin with discolouration of the apical regions of the floral tissues (Bandyopadhyay *et al*., 2000). After the infection of the floral tissues, the mycelial growth spreads to the grains from the base, near the pedicel. Fungal colonisation is responsible for small grains, grain abortions and premature formation of the black layer, which is associated with physiological maturity of the plant (Figure 5; Marley & Ajayi, 1999, Little, 2000). Superficial growth of fungi can be observed at the hilar end of the grain, extending to the pericarp surface. However, the response to SGM varies due to the interactions of cultivar, fungi and locality (Bandyopadhyay *et al.,* 2000).

Figure 5. Grain mold of sorghum (Photo by L.A. Coetzee)

2.2 Economic Importance

Annually Africa and Asia record an economic loss of over US$ 130 million due to the sorghum grain mold disease complex. However it must be noted that economic losses are directly correlated to the incidence and severity of the disease under various environmental conditions and plant host growth stages (Thakur *et al.,* 2006).

There is a vast diversity of economic impacts that mycotoxins can have on society including food recalls, import and export restrictions, detrimental impact on the livestock industry and the monetary impact on human and animal health care (Marroquin-Cardona *et al.,* 2014). Annually 25% of the world food crops are contaminated by mycotoxins and of this, 378 000 tonnes of sorghum and millet are wasted due to mycotoxin contamination. The majority of this waste is from developing countries in Africa and Asia (WHO/FAO, 2012). Deoxynivalenol (DON) annually results in loses of approximately US$ 637 million due to contaminated wheat and maize being rejected. While a loss of US$ 18 million due to contaminated feedlots has been reported (Bhat *et al.,* 2010). In order to meet the strict regulatory requirements of the European Union on aflatoxins, African countries exporting produce to Europe lose US$ 670 million due to the rejection of grain or loss of export markets (WHO/FAO, 2012). The use of biotechnologically engineered resistant plant varieties (predominantly *Bacillus thuringiensis* in maize (Bt maize)), contributes an additional US$ 23 million to the USA's agricultural economy through reducing contamination of grain by mycotoxins such as fumonisins and aflatoxins. The economic importance of grain mold is evident as the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) focuses a large portion of their research on breeding for resistance against SGM (Reddy *et al.,* 2010).

***3. Fusarium graminearum* Species Complex**

3.1 Etiology, Species Complex and Taxonomy

Fusarium graminearum is one of the most studied *Fusarium* species in recent years. This is attributed to the complexity of the *Fusarium* species life cycle, taxonomy, genetics and associated research with mycotoxins. Although the species complex has been widely researched owing the complexity of the species interaction with wide host ranges, geographic and climate variability, control of this pathogen

continues to be challenging (Summerell & Leslie, 2011). To date there are 16 phylogenetic species that have been identified within *Fusarium graminearum sensu lato* which contribute to the species complex associated with SGM infections (Wang *et al*., 2011; Van der Lee *et al.*, 2015). Therefore, in future SGM will be referred to as the *Fusarium graminearum* species complex (*Fg*SC). The *FgSC* is responsible for the majority of important mycotoxins associated with SGM and therefore, will form the focus of this study.

The taxonomy of the *Fg*SC has been continuously controversial. *Fusarium graminearum* is a homothallic fungus, that it has been subdivided into 16 different phylogenetic species, known as lineages, of which 15 are listed in Table 1 (Van der Lee *et al.*, 2015). These lineages are associated with biogeography and distribution of the species, there are eight species that have been designated specific lineages and seven that do not belong to specific lineages. *Fusarium graminearum sensu strictu* (lineage 7) is the most commonly distributed, associated with Fusarium head blight (FHB) and known to produce DON. However *F. meridionale* (lineage 2) is more commonly known to produce NIV. Novel *Fg*SC species, which include *F. louisianense* and *F. nepalense,* have not been designated specific lineages (Sarver *et al.,* 2011; Wang *et al.,* 2011). The constant scientific disagreement surrounding the *Fg*SC affects the taxonomic understanding of this pathogen complex (Summerell *et al.,* 2010).

Table 1. Lineages of *Fusarium graminearum* species complex, their distribution, trichothecenes, derivatives produced and authors as listed by O'Donnell *et al.* (2004), Leslie & Summerell, (2006), Sarver *et al.* (2011) and Wang *et al.* (2011).

Lineage	*Fusarium graminearum* clade species	Distribution	Trichothecene Derivatives	Authors
1	*F. austroamericanum*	Brazil and Venezuela	DON[1], 3-AcDON[2] & NIV[3]	T. Aoki, Kistler, Geiser & O'Donnell
2	*F. meridionale*	Australia, Brazil, Guatemala, Korea, Nepal, New Caledonia and South Africa	NIV	
3	*F. boothii*	Guatemala, Korea, Mexico, Nepal and South Africa	DON and 15-AcDON[4]	O'Donnell, T. Aoki, Kistler & Geiser
4	*F. mesoamericanum*	Honduras and Pennsylvania	DON, 3-AcDON & NIV	T. Aoki, Kistler, Geiser & O'Donnell
5	*F. acaciae-mearnsii*	Australia, South Africa	DON, 3-AcDON & NIV	O'Donnell, T. Aoki, Kistler & Geiser
6	*F. asiaticum*	Brazil, China, Japan, Korea and Nepal	DON, 3-AcDON, 15-AcDON & NIV	
7	*F. graminearum sensu stricto*	Globally	DON, 3-AcDON, 15-AcDON & NIV	Schwabe-Flora Anhaltina
8	*F. cortaderiae*	Argentina, Brazil and Australia and New Zealand	DON, 3-AcDON & NIV	O'Donnell, T. Aoki, Kistler & Geiser
No designated number	*F. brasilicum*	Brazil	DON, 3-AcDON & NIV	T. Aoki, Kistler, Geiser & O'Donnell
	F. aethiopicum	Africa	DON, 15-AcDON	O'Donnell, Aberra, Kistler & T. Aoki
	F. gerlachii	USA	NIV	T. Aoki, Starkey, Gale, Kistler & O'Donnell
	F. vorosii	Asia	DON and 15-AcDON	B Toth, Varga, Starkey, O'Donnell, Suga & T. Aoki
	F. ussurianum	Asia	DON and 3-AcDON	T. Aoki, Gagkaeva, Yli-Mattila, Kistler & O'Donnell
	F. louisianense	North America	DON, 15-AcDON & NIV	Gale, Kistler, O'Donnell & T. Aoki
	F. nepalense	Nepal	DON, 15-AcDON & NIV	T. Aoki, Carter, Nicholson, Kistler & O'Donnel

[1]Deoxynivalenol; [2]3-acetyldeoxy-nivalenol; [3]Nivalenol and [4]15-acetyldeoxynivalenol

3.2 Detrimental Effects of FgSC

Host Plant

The detrimental effects which *Fg*SC has on a sorghum crop and production thereof include reduced kernel germination, mass and density, reduced nutritional quality, market value, storage quality and unfavourable processing characteristics (Menkir, 1996; Marley & Ajayi, 1999; Navi *et al.*, 2005; Balota, 2012). Early symptoms associated with *Fg*SC are a white powdery mycelium which over time develops into a pinkish fluffy mycelium (Thakur *et al.,* 2006). The above detrimental effects are due to both superficial and internal colonisation of the grain or a combination of both (Balota, 2012). More specifically, certain pathogens are responsible for particular host-plant reactions. *Fusarium thapsinum* and *Curvularia lunata* are both known to be responsible for a great deal of seed germination failure and the latter pathogen is also known to infect seed without producing visible symptoms while reducing seed viability (Prom *et al.*, 2003).

Human and Livestock Health

The erroneous understanding of mycotoxins has been present throughout history, as early as the Babylonians (~1700 BC) and the controversial Salem witch trials in 1600's (Woolf, 2000; Köppen *et al*., 2010). However, in 1959 following the shipment of groundnut meal into Great Britain from Brazil for turkey feed that caused extensive disease in the turkeys, known now as Turkey "X" Disease, in 1961 the term mycotoxin was coined (Cole, 1986; Chukwukam *et al.,* 2010). Ancient languages describe mycotoxins with two derivations, "mukes" (Greek) meaning "fungi" and "toxicum" (Latin) referring to "poison" (Bhat *et al.*, 2010). The production of secondary metabolites is not crucial to survival of fungi, however in certain environments and circumstances they have potential to be highly beneficial to survival (McCormick *et al.*, 2011). There are over 500 known toxic fungal secondary metabolites with molecular weights lower than 700 and it is these that are classified as mycotoxins (Köppen *et al.*, 2010).

Fungi are able to produce mycotoxins in the field (pre-harvest) or during storage, transport and processing (post-harvest). It is thought that mycotoxins were an evolutionary trait which fungi developed as defence mechanisms against insects or

rodents. The presence of mycotoxins is not limited by hyphal or mycelial growth which occurs on or in spores and substrate where the fungal colony grows (Bhat *et al.,* 2010). Thus, translocation of DON produced in the base of the stem to other plant organs can occur (Obanor & Chakraborty, 2014).

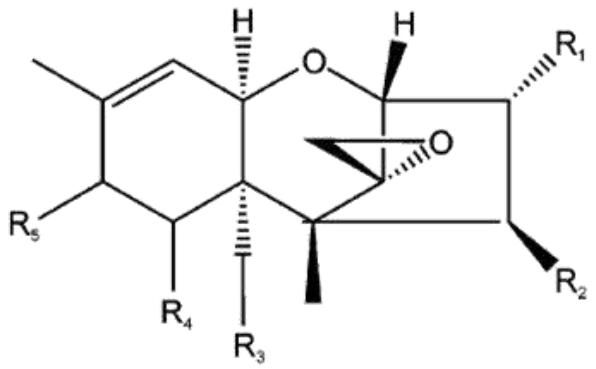
Fusarium spp. are responsible for a majority of mycotoxins including fumonisins, moniliformin, fusaproliferin, fusaric acid, fusarins, beauvercin, gibberellic acid and T-2 toxin. *Fusarium* spp. are also known to produce zearalenone (ZEA) and trichothecenes, nivalenol (NIV), DON and derivatives. The latter three mycotoxins are closely associated with *Fg*SC and globally distributed (Table 1). *Fusarium graminearum, F. culmorum and F. crookwellense* are the most commonly known pathogens to produce trichothecenes and ZEA (Lauren & Smith, 2001; Audenaert *et al.,* 2013). Within the *Fg*SC the type B trichothecenes which are produced differ amongst the lineages. DON is the most widely produced *Fg*SC mycotoxin, but is largely associated with *F. boothii* (lineage 3) on maize. Furthermore *F. boothii* is known to produce 15-A-DON and *F. graminearum sensu strictu* is known to produce 3-A-DON. NIV and ZEA are mycotoxins produced by *F. acaciae-mearnsii* (lineage 5) and *F. meridionale* (lineage 2) on sorghum (Table 1) (Miller, 1995; Bandyopadhyay *et al*., 2000; Mavhunga, 2013).

The accumulative effect of mycotoxins cannot be confirmed but is suspected to include various effects, depending on the mycotoxin involved. Mycotoxicosis is the general term which is used for disease caused by mycotoxins (Bhat *et al.,* 2010). General effects include cancers, neurological disorders, reproductive complications, immune suppression and extensive organ damage (Bandyopadhyay *et al.*, 2000). Severity of mycotoxicosis varies depending on the mycotoxin present, quantity and extent of exposure, age and immunity of the individual affected. Developing countries will have higher exposure in quantity and extent, to mycotoxins, due to traditional methods of producing and consuming grain and leguminous staples as these are not regulated as strictly as mycotoxins in developed countries (WHO/FAO, 2012).

Trichothecenes

Over 200 toxins form part of the trichothecene family (McCormick *et al.,* 2011). Trichothecenes can be differentiated by the pattern of their 12,13-epoxytrichothec-9-ece (EPT) core structure and the substitution of the C-8 positions. Based on the above chemical structure they can be classified into four distinct groups, Types A, B, C and D. DON and NIV are Type B trichothecenes as they have a jet (carbonyl) function at the C-8 position (Figure 6). Uniquely *Fusarium* species specific Type B trichothecenes also have a hydroyxl group at C-7. It must be noted that Type A trichothecenes are of greater concern, as listed in Figure 6, due to the higher level of toxicity than Type B trichothecenes (Köppen *et al.,* 2010). Trichothecenes can be further classified by their structural arrangements and reactivity of the functional groups. DON and NIV are simple trichothecenes belonging to Group III as they contain a keto at C-8. An alternate method of classifying trichothecenes is genetically based on the addition of oxygens, *Fusarium* species specifically are derived from isotrichotriol (t-type) as there are four oxygens added, while trichothecenes from other fungal genera are derived from isotrichodiol (d-type) as there are three additional oxygens (McCormick *et al.,* 2011).

Fusarium graminearum only has four chromosomes and 15 genes which are associated with trichothecene biosynthesis, are distributed among these chromosomes (Woloshuk & Shim, 2012). Trichothecenes functioning as phytotoxins result in chlorosis, dwarfism and root elongation inhibition. Trichothecenes also contribute to the virulence factor in wheat head scab. In humans, trichothecenes from contaminated grains move passively through membranes and are easily absorbed and thus rapidly affect integumentary and gastrointestinal systems (McCormick *et al.,* 2011). Furthermore, when trichothecene contaminated grain is consumed, neurotoxic, immunosuppressive and nephrotoxic effects are observed (Woloshuk & Shim, 2012).



	Trichothecene	MW	R_1	R_2	R_3	R_4	R_5
Type A	T-2 toxin	466.5	OH	OAc	OAc	H	OCOi-Bu
	HT-2 toxin	424.5	OH	OH	OAc	H	OCOi-Bu
	15-Monoacetoxyscirpenol	324.4	OH	OH	OAc	H	H
	Diacetoxyscirpenol	366.4	OH	OAc	OAc	H	H
	Neosolaniol	382.4	OH	OAc	OAc	H	OH
Type B	Deoxynivalenol	296.3	OH	H	OH	OH	=O
	Nivalenol	312.1	OH	OH	OH	OH	=O
	3-Acetyldeoxynivalenol	338.4	OAc	H	OH	OH	=O
	15-Acetyldeoxynivalenol	338.4	OH	H	OAc	OH	=O
	Fusarenon-X	354.4	OH	OAc	OH	OH	=O

Figure 6. Structure of type A and B trichothecene mycotoxins and derivatives (Köppen *et al.*, 2010)

Bhat *et al.* (2010) stated that within hours of consuming contaminated trichothecene feed or food, symptoms of mycotoxicosis can be observed. DON is more commonly known as vomitoxin due to the common symptoms associated with DON poisoning. Consumption of contaminated feed has resulted in reduced dairy production in cattle and feed refusal and vomiting in pigs. Immunosuppressive and reproductive inhibition has been observed in multiple species, including humans (Pestka, 2007; Bhat *et al.,* 2010).

DON is most commonly found in contaminated wheat and maize (Audenaert *et al.,* 2013). Low amounts have been recorded in eggs indicating that DON can be transferred embryonically if lay hens consume contaminated feed (Bhat *et al.,* 2010). DON is most frequently distributed in wheat bran fractions, and can be double or more than that found in flour. Since DON is commonly found in hyphae of fungal colonised grains, concentrations of DON have been highly correlated with ergosterol concentrations (Lancova *et al.,* 2008). The effects of DON are not completely understood and more detailed research on the effects on humans is vital for legislation (Bhat *et al.,* 2010).

The Babylonians and the Hittites (~1500 BC) were amongst the first known civilizations to set legislations to monitor and regulate food safety and fraud (Köppen *et al.*, 2010). Over 70 countries have legislative measures with strict levels on the minimum allowance of mycotoxins present in grain which is intended for human and livestock consumption (Kumar *et al.,* 2008; Bhat *et al.,* 2010). Currently South African legislation regulates two major mycotoxins, aflatoxin and patulin. Aflatoxin is regulated in all food products, but special attention is paid to milk and groundnut products. Patulin in apple juice based products is also regulated (Rheeder *et al.*, 2009). However, the USA's Food and Drug Administration (FDA) has set maximum limits for DON on wheat contaminated bran, flour and germ products for human consumption at 1000 $\mu g.kg^{-1}$. The European Union however have set maximum limits for DON contaminated raw cereals at 1250 $\mu g.kg^{-1}$, flours at 750 $\mu g.kg^{-1}$ and bread at 500 $\mu g.kg^{-1}$ (Lancova *et al.,* 2008; Bhat *et al.,* 2010).

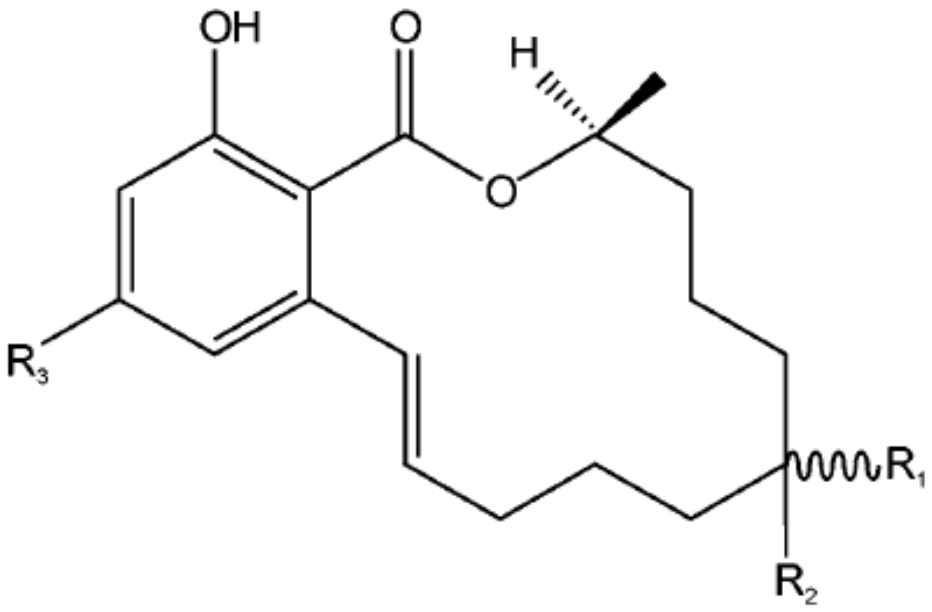
NIV is more commonly found at lower concentrations than that of DON although it is also produced by *Fg*SC (Pestka, 2007). NIV has commonly been found in grains such as barley, wheat, oats, rye and sorghum. NIV is stable during storage and processing and is commonly found in products made from the above grains. Inhibition of protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis in humans and livestock causes cell necrosis and toxicosis to lymphoid and intestinal organs. Acute symptoms of NIV toxicity range from potential DNA damage, genotoxicity to leucopenia, the reduction of white blood cells and sensitivity of the immune system. Currently there are no regulations for NIV in contaminated grain or products (De Lucca, 2007).

Zearalenone

Zearalenone and its derivatives (Figure 7) can be classified as a phytoestrogen and forms part of a class of naturally occurring dietary estrogens, known as resorcylic acid lactone, with antagonistic reproductive consequences (Turcotte *et al.,* 2005; Bhat *et al.,* 2010). *Fusarium graminearum* and *F. culmorum* are primary producers of ZEA which is known to co-exist with DON (Richard *et al.,* 2007).

The most frequently encountered contaminated cereals include barley, maize, oats, sorghum and ZEA has been detected in bread which was produced from

contaminated wheat. Pigs are known to be particularly sensitive to ZEA contaminated foods. Hyperestrogenic effects in female pigs are due to the excess of estrogen, leading to genital or urinary problems which include hyperemia and edematous swelling of the vulva. This is caused by ZEA increasing blood circulation and excess fluids being trapped by bodily tissues or organs. In male pigs symptoms such as decreased libido and loss of testicular functions has been observed. Poultry are the least affected by ZEA and there are no known health hazards associated with dairy cattle produce (Bhat *et al.,* 2010). Children are the most sensitive to ZEA where premature pubertal changes have been observed (Bhat *et al.,* 2010). CAST (2003) have set lowest maximum allowable limits of ZEA to 100 $\mu g.kg^{-1}$. The European food safety authority has set the tolerable daily intake limit for ZEA to 0.25 $\mu g.kg^{-1}$ body weight (Marroquin-Cardona *et al.,* 2014).



Zearalenone and derivatives	MW	R_1	R_2	R_3
Zearalenone	318.4	-	=O	OH
α-Zearalenol	320.4	OH (R)	H	OH
β-Zearalenol	320.4	OH (S)	H	OH
Zearalenone-4-sulfate	398.4	H	H	OSO_3H

Figure 7. Structure of zearalenone and derivatives (Köppen *et al*., 2010)

4. Epidemiology of *Fusarium graminearum* Species Complex

4.1 Saprophytic Fitness

Saprophytic fitness is correlated with pathogen vigour. Aggressiveness of the isolate during the pathogenic phase affects DON production during the saprophytic phase. The reproductive phase and DON production are known to have overlapping cellular factors. A gene encoding protein, FgStuA, has been known to influence spore development and DON biosynthesis (Audenaert *et al.,* 2013).

The protein biosynthesis-inhibition ability of DON acts as an antimicrobial agent that can reduce other eukaryotic soil organisms. Therefore the DON-producing members of *Fg*SC are given the advantage to proliferate in the soil (Audenaert *et al.,* 2013). Other *Fusarium* spp. that are present in contaminated grain influence the content of DON. The highest DON concentrations are recorded when *Fg*SC is found unaccompanied by other *Fusarium* spp., especially other non-DON producing *Fusarium* spp. (Landschoot *et al.,* 2012).

4.2 Weather Variables

Survival by FgSC

The development of disease is dependent on the overwintering capabilities of primary inoculum in the soil and on harvested residues (Aldred & Magan, 2004). Survival of the pathogen and decomposition of previous crop residues are affected by soil moisture and soil temperature. Dispersal of spores is favoured by strong winds, warm air and rainfall. Temperature at the beginning of the growing season has a significant influence on disease incidence due to the pathogen favouring warm, dry weather for inoculum build up and mycelium growth (Landschoot *et al.,* 2012).

Colonisation by FgSC

High rainfall during grain development promotes SGM colonisation (Reddy *et al.,* 2010). *Fg*SC is favoured by temperatures ranging between 26 and 28°C, high precipitation and high relative humidity (RH) and water activity (a_w) > 0.88 (Trigo-Stockli *et al.,* 1996; Marroquín-Cardona *et al.,* 2014). High seed moisture ranging

between 20 and 25% promotes the growth of *Fg*SC and other sorghum seedling colonising fungi (WHO/FAO, 2012).

Two rainfall events which are critical to allow spore germination and initiation of FHB infection on wheat are associated with two days prior to anthesis and three days within post anthesis (Hooker *et al.,* 2002). The optimum temperature for the infection and colonisation of *Fg*SC on wheat is 25°C coinciding with leaf wetness. Leaf wetness can be a result of rain which on the first day is ≥ 0.2 mm and provides RH over 81% and on the second day provides a RH ≥ 78% (Wegulo, 2012; Brustolin *et al.,* 2013).

Mycotoxin Production

Seasonal variation had a more significant effect on DON produced than that of geographical variation in a region (Landschoot *et al.,* 2012). The meteorological variables which favour the development of mycotoxins pre- or post-harvest include temperature, moisture, a_w and RH. The minimum a_w requirements for *Fusarium* spp. range from 0.85 to 0.87 a_w (Bhat *et al.,* 2010). Weather conditions during flowering, the use and timing of fungicides and moisture content at harvest are critical aspects for the accumulation of DON (Aldred & Magan, 2004). In wheat, high humidity, ≥90%, and rainfall around heading greatly influences DON concentrations (Landshcoot *et al.,* 2012; Wegulo, 2012; Audenaert *et al.,* 2013).

4.3 Agronomic Practices

Survival of FgSC

Survival of inoculum is influenced by crop rotation, weed management, nitrogen fertilization schemes and soil structure and biota. Ploughing, inverting the upper 10-20 cm of soil, has been known to remove, reduce or bury *Fusarium* inoculum, reducing the incidence of disease. While minimum tillage and no-till, planting directly into residue of previously harvested host crops are known to increase *Fusarium* inoculum and disease development (Edwards, 2004; Audenaert *et al.,* 2013).

Colonisation by FgSC

Increasing applications of nitrogen promote the colonisation and incidence of FHB. The form of nitrogen applied also influences the incidence and symptoms shown. Urea applications reduced symptoms in wheat infected with FHB while no reductions where seen with ammonium nitrate applications. However the form of nitrogen applied did not affect the accumulation of DON (Edwards, 2004).

The role of nitrogen in host plant defense varies over three distinct pathways. The first is the use of nitrogen as an energy source, second is their involvement in the induction of the hypersensitive response and lastly in the hosts evasion or endurance mechanisms. Evasion mechanisms are responsible for transporting nitrogen away from the infected area this being most effective against biotrophic fungi. Endurance mechanisms move nitrogen from non-infected tissues to infected tissues to ensure tissue survival. *Fusarium* spp. that produce DON have the ability to take over and use the above defense mechanisms to their own advantage (Audenaert *et al.,* 2013).

Mycotoxin Production

Prevention of fungal infection and concomitant mycotoxin contamination can be initiated at field level through crop rotation and removal of crop debris from fields after harvest (Bhat *et al.,* 2010).

Various responses of DON contamination have been observed in interactions within tillage and crop rotation systems. Crop rotations that include hosts of *Fusarium* spp. showed an increased level of DON accumulation (Landschoot *et al.,* 2012). Wheat fields which were rotated with maize or followed on wheat the next season and also had no till had significant DON increases. Minimum till systems in wheat fields previously planted to maize resulted in DON contamination increasing ten-fold. However in wheat fields which had previously been planted to soya beans and no-till had been applied, no effect on the levels of DON were recorded (Edwards, 2004).

5. Control

Factors which reduce or restrict *Fg*SC on host crops include the use of resistant cultivars, chemical control, biological control, agronomic practices and post-harvest practices. However the complexity of SGM and the *Fg*SC requires an integrated pest management program (Hall *et al.,* 2000).

5.1 Resistance

Selecting resistant cultivars is a management practice of great value to the reduction of multiple diseases globally and not only the prevention of SGM. However, conventional breeding has not resulted in SGM resistant cultivars. Tolerant genotypes have been identified but these have inadequate control of infection. The degree of expression of cultivar resistance is limited by the disease pressure present in field. Resistance breeding is generally based on three primary mechanisms in sorghum; phenolic compounds, grain hardness and panicle and flower structure (Hall *et al.,* 2000).

Important phenolic compounds (found mainly in pericarp, glumes, and leaf sheaths) which contribute to grain mold resistance include phenolic acids, flavonoids, and tannins. A coloured pericarp in grain is associated with flavan-4-ols while condensed tannins and proanthocyanidins are found in grains with a pigmented testa (Waniska, 2000). Not only do tannins reduce fungal colonisation but also bird and insect predation due to the bitter taste (Taylor, 2003). White grain cultivars are not associated with flavan-4-ols or condensed tannins and thus lack this barrier against infection (Hall *et al.,* 2000). Chandrashekar & Satyanarayana (2006) reported lower mycotoxin and ergosterol concentrations associated with red genotypes and higher amounts of both occur in white genotypes. Therefore, the assumption is that a red pericarp is associated with resistance to *Fg*SC. Resistance genes specifically associated with SGM can be identified, but *Fg*SC is a fungal complex, therefore gene pyramiding or stacking would be required for adequate resistance. This could be complex and very time consuming, therefore phenolic compounds are used as a selection criterion in breeding programs more readily (Waniska, 2000).

Anti-fungal proteins (AFPs) which are more commonly situated in the endosperm are known to inhibit fungal growth. However grain mold resistance by AFPs is only

initiated during stress periods when higher AFPs levels are synthesised (Waniska, 2000).

Harder grains are more resistant to grain mold pathogens, these are associated with poor grain characteristics for food quality. Therefore, when developing resistant cultivars nutritional and processing properties need to be considered (Hall *et al.,* 2000; Waniska, 2000).

An inconsistent relationship between resistance and panicle and glume morphology as well as flower structure has been reported (Hall *et al.,* 2000). Studies have indicated that glume colour in white grain cultivars have the ability to reduce SGM infections (Hall *et al.,* 2000). The development of modern breeding techniques such as marker assisted selection (MAS) could assist in facilitating combinations of the above mechanisms. Currently there are no transgenic sorghum varieties which could be a route to finding new sources of genetic resistance against SGM (Hall *et al.,* 2000).

5.2 Chemical Control

According to Marley & Ajayi (1999), reduction of SGM infections could be possible if fungicides are sprayed at the milk stage and 10 days thereafter (Figure 4). However the use of fungicides may be uneconomical or even impractical in certain regions of the world (Hall *et al.*, 2000). Furthermore fungicide applications effective in the control of fungal contamination at field level can induce stress that initiates or stimulates the production of mycotoxins by colonising fungi (Bhat *et al.,* 2010).

Effects of fungicides on the biosynthesis of DON vary greatly with the fungicide applied, the dosage and the associated weather variables. Variable results of weather increasing or decreasing levels of DON have been observed for the strobilurin fungicide, azoxystrobin, and carbendazim and thiram. Azole fungicides also have varied results with respect to reducing DON, but are the most important fungicides used to date to control Fusarium head blight (FHB). Field trials completed in Belgium indicated that application of azole fungicides were not able to reduce DON by more than 75% of the control fields. This result suggested that the application of fungicides in DON infested fields which are above legislative limits will not save the harvest (Audenaert *et al.,* 2014).

Mycotoxin chemotype responses vary in their sensitivity towards fungicides. This was evident with the application of carbendazim fungicides. Where these fungicides were applied at rates lower than the registered rate, the trichothecene gene expression (*Tri*4*, Tri*5 *and Tri*11*)* of *Fg*SC isolates was higher than that of untreated controls. This would suggest that stress on the pathogen without completely removing the pathogen, allows for higher levels of trichothecene production (Audenaert *et al.,* 2013). Fungicide applications in fields where lodging occurred were not effective against reducing DON in FHB infected wheat. Wheat plants which had undergone drought stress are more susceptible to infection by FHB and production of DON. However, sorghum is known to be a drought tolerant crop and therefore may not have the same responses to stress (Aldred & Magan, 2004).

5.3 Biological Control

Selecting biological control agents (BCA) requires attention to potential interactions between BCA and the pathogen, environment, host and agronomic practices (Palazzini *et al.,* 2007). The use of *Trichoderma viridae* and *T. harzianum* as a treatment for increased germination of sorghum seed infected with SGM was greater than that of chemical seed treatments (Thakur *et al.,* 2006). Application of BCAs to wheat heads during anthesis may reduce FHB and concomitant DON accumulation (Gilbert & Fernando, 2004). Argentinian researchers have selected native bacterial strains of *Streptomyces* spp. and *Brevibacillus* spp., BRC263, as potential BCAs against FHB on wheat and DON production (Palazzini *et al.,* 2007). *In vitro* studies with *T. harizianum* application on post-harvest plant residue reduced perithecial formation on stubble (Gilbert & Fernando, 2004). Waniska (2000) however stated that, although there are a number of potential BCAs, these are not feasible and reliable methods of epidemic control in sorghum.

5.4 Agronomic Practices

In 1993 a FHB epidemic in Argentina caused 50% crop loss and DON contamination in areas where no-till was implemented and maize was planted previously. Subsequently, although the use of crop rotation and tillage practises has been shown to reduce epidemics, these are not able to reduce the total impact of epidemics and concomitant mycotoxin contamination (Palazzini *et al.,* 2007).

Removal of alternate hosts, maize, soybean, wheat and wild oats, as rotational crops or crop debris can reduce sources of *Fg*SC inoculum. However, *Fg*SC inoculum has been found in great concentrations annually in the South Brazilian atmosphere suggesting long distance dispersal and thus crop rotation must be combined with other FHB control practices (Lori *et al.,* 2009). Epidemics were aggravated by agronomic practices including increased areas of maize being planted and soil conservation techniques. The above practices directly contributed to an elevated inoculum risk factor (De Wolf *et al.,* 2003).

Nitrogen deficiencies in soil place host plants at high risk to *Fg*SC infection due to plant weakness and nitrogen abundancies resulted in increased susceptibility. Therefore balanced nitrogen and nitrogen forms in the soil are key to cultural control of FHB infections (Lori *et al.,* 2009).

5.5 Post-Harvest Practices and Processing

Post-harvest techniques which are known to reduce *Fg*SC inoculum and prevent mycotoxin contamination include proper drying, storage and transporting procedures (Bhat *et al.,* 2010). The only method to control mycotoxin contamination is to prevent colonisation by the fungi responsible for their production. Due to the diversity and magnitude of mycotoxin producing fungi and their extent in the food chain this can be seen as a major challenge (Köppen *et al.*, 2010).

Post-harvest inactivation of mycotoxins is being consistently researched due to the increased awareness of mycotoxin health threats posed by contamination of consumed produce (Bhat *et al.,* 2010). Levels of processing on traditional, household or industrial scales will variably influence the reduction or accumulation of toxins occurring in products (Lancova *et al.,* 2008).

Mycotoxins are all very stable compounds and are not readily removed by heating or other processes used in industry (Miller, 1995; Sweeney & Dobson, 1998). DON, NIV and ZEA are robust and relatively stable to physical cleaning, heat, moisture and aqueous solutions at variable pH levels (Lauren & Smith, 2001). Physical cleaning practices, such as sorting, trimming, milling and decortication, reduce or redistribute mycotoxins during processing of bulk grain for food production (Lauren & Smith, 2001). The highest concentrations of DON are found in the pericarp, thus mycotoxins

are most commonly concentrated in the germ and the bran fractions of grain (Bullerman & Bianchini, 2007; Woloshuk & Shim, 2012). Relatively high fractions of trichothecenes are present in the bran and are reflected as high concentrations in waste product of the milling process. Presence and prevalence of *Fg*SC in aleurone and pericarp tissues can be attributed to this observation. However, approximately 40% of original mycotoxins remained in the flour. The above observation could be due to *Fg*SC hyphae present in the central endosperm of colonised grains (Trigo-Stockli *et al.,* 1996; Lancova *et al*., 2008).

Changes in DON concentrations have been observed during the baking process of bread, however the reduction or accumulation of the content was variable, and this could be due to masked forms being revealed. Dough quality and stability are negatively influenced by the presence of DON (Lancova *et al.,* 2008).

DON and NIV in contaminated maize are relatively stable in heat and aqueous buffer solutions over the pH range of 1–10. Observations of reduced DON have been recorded with sodium bisulphate treatments, however masked DON is revealed in alkaline treatments. Additions of heat treatments, 80 and 110°C, to bicarbonate treatments were significantly effective at reducing mycotoxins. Exposure to the above treatment for prolonged periods of 12 days reduced DON and NIV by over 75%. The addition of moisture did not significantly reduce DON or NIV levels. However with reductions observed in DON and NIV, no significant reductions were seen in ZEA in any of the pH, heat or moisture treatments. Thus, it can be suggested that ZEA is more robust and stable than its trichothecene counterparts, DON and NIV (Lauren & Smith, 2001).

Chemical treatments, abiotic or biotic, for the removal or detoxification of mycotoxins post-harvest have been researched but results are highly variable. Methods for the removal of DON and ZEA include phosphate ethanol extractions and the use of fermentative bacteria. Autoclaving an 8.33% aqueous sodium bisulphate solution at 121°C for an hour has been shown to reduce DON in maize by 95%, although this method does unmask less toxic DON derivatives. Lactic and propionic acid bacteria have the capability to remove up to 55% DON and 88% ZEA, the propionic acid bacteria were determined to be the most successful. Further studies have clarified

that fermentative bacteria have the ability to detoxify mycotoxins associated with *Fg*SC (Bhat *et al.,* 2010).

6. Quantitative Analysis of Colonisation and Mycotoxins

A sampling plan for research based analysis is critical for an accurate analysis of fungal colonisation and mycotoxin content in feed and food. Biological repetitions of an original bulk sample should be made up of randomized sampling of the consignment to ensure that the heterogeneous nature of fungal and mycotoxin contamination is taken into account. The bulk sample should be homogenised and divided to a laboratory scale, and sub samples should be used as technical repetitions to ensure reduced variation. It must be noted that sampling for the commercial control of contamination, fungal or mycotoxins, in food and feed commodities may vary from that used for research based studies (Köppen *et al.*, 2010).

Errors in mycotoxin analysis have socio-economic impacts that affect producers and consumers of food commodities. Economical risk can be observed when false-positive results occur, with the implication that consignments below the regulatory levels will be rejected. False-negative results impact the buyers of food commodities, as the consignments are over the legislative limits but are accepted into the market (Rahmani *et al.*, 2009).

6.1 Colonisation

Quantitative analysis of fungal colonisation of SGM and *Fg*SC can be done through traditional techniques or modern molecular techniques. Traditional techniques which estimate the degree of colonisation involve visual ratings in the field or post-harvest, and the use of ergosterol or chitin estimation (Seitz *et al.,* 1979; Rao *et al.,* 2012).

Field and Post-Harvest Ratings

In-field or post-harvest disease assessment of plant disease intensity, defined as disease incidence or severity, was traditionally done visually. The general principle of manual assessments is described when an individual person, known as the rater, observes a specimen, plant material, and estimates the diseased area.

Considerations affecting the assessments are the frequency, timing and sampling size (Madden *et al.,* 2007). When considering the timing of assessments the growth stage of the host and the pathogens "window of opportunity" should be taken into account. The frequency of the sampling is dependent of the type of data interpretation which must be made. The more detailed the data required for statistical use and model development the higher the specific frequency requirement will be (Campbell & Madden, 1990). The most common visual grain rating is that of threshed grain disease ratings, allowing for descriptive and a visual estimate to be made regarding the level of infection (Bandyopadhyay & Mughogho, 1988). Sorghum grain mold screening has been widely applied using visual estimations, represented in Table 2.

Table 2. Visual grain mold severity ratings (Rao *et al.,* 2012).

Rating	Visual grain mold severity (%)
1	0
2	1-5
3	6-10
4	11-20
5	21-30
6	31-40
7	41-50
8	51-75
9	>75

A disadvantage of visual estimation, whether it is with standard area diagrams or image pixel analysis, is that it is reliant on a rater. Each rater has different capabilities and levels of plant pathological understanding. Therefore accuracy and precision will vary between different raters (Madden *et al.,* 2007). Accuracy is defined as the closeness to the actual diseases severity that a rater can estimate to and precision of the rater is defined as the degree of reliability a rater has during estimation (Raikes & Burpee, 1998). Visual assessment only offers limited quantitative data and further quantification of disease is required in advanced plant pathology and this component of phytopathometry requires the use of molecular and chemical techniques to assess and quantify disease.

Chromatographic Methods

Quantifying disease can be by means of the correlation between fungal biomass and chitin or ergosterol present in host tissues (Doohan *et al.,* 1999). Chitin analysis requires that chitin can be hydrolysed to glucosamine, followed by deamination to an aldehyde and quantification by colorimetric analysis, gas chromatography (Seitz *et al*., 1977). This is possible because chitin is an important component of fungal cell walls. A disadvantage of chitin assay is that chitin is also a component of insect exoskeleton and bacteria, interfering in the measurement of fungal biomass.

Ergosterol is a sterol found in fungal pathogens in the Ascomycetous and Deuteromycetes classes. The use of ergosterol to quantify fungal biomass was first described by Seitz *et al*. (1977). The use of a high performance liquid chromatography (HPLC) has improved the accuracy of ergosterol quantification. The procedure of ergosterol assay requires the extraction of the ergosterol using an alcoholic base followed by separation using a non-polar solvent such as hexane. Evaporation follows extraction with the precipitate being added to a standardised solvent. The final separation of the ergosterol is done through HPLC (Jambunathan *et al.,* 1991; Gessner & Schmitt, 1996). Ergosterol detection and quantification has been used to indicate sorghum grain mold resistance in various coloured grains hybrids (Rao *et al.,* 2012). Ergosterol analysis has been done on a variety of plant pathogens including *Fusarium* spp.*, A. alternata, Rhizopus stolonifer, A. flavus, A. candidus* and ecto-mycorrhizae (Seitz *et al*., 1979; Montgomery *et al.,* 2000).

Respective advantages and disadvantages of chitin and ergosterol assays are that where the chitin assay can detect metabolically active or inactive fungi while ergosterol assay can only detect the metabolically active fungal biomass. Therefore these describe the fungal biomass which is present in total and the fungal biomass when the crop was harvested respectively. Ergosterol is more sensitive, specific and rapid than that of chitin assay and therefore is more widely used. The initial costs of these techniques are high but are cost effective for the accurate detection and quantification of pathogens. Experience is required to run these assays but there are multiple procedures available for the various crops and pathogens (Gessner & Schmitt, 1996).

Molecular Analysis

Molecular techniques for disease assessment have the advantage of being qualitative and quantitative. The techniques include polymerase chain reaction (PRC) and real-time quantitative polymerase chain reaction (qPCR). The advantage of these techniques is that they are rapid, sensitive and can be used to detect and quantify many pathogens (Henson & French, 1993).

The PCR technique allows for the amplification of minute amounts of DNA at a rapid speed and qualitative identification of pathogens. There are three basic steps involved in the PCR technique; separating the two DNA strands at 95°C (melting), primers bind at specific regions of the target DNA that require amplification between 40-65°C (annealing) and lastly the primer extension to provide a second strand of DNA to be synthesized. The last stage requires the use of a thermostable DNA polymerase and deoxyribonucleoside triphospahtes (dNTPs). These three steps are done repeatedly for approximately 50 cycles; this allows the multiplication of the specific DNA to an amount which allows for the DNA to be detected and quantified (Henson & French, 1993; Ward *et al.,* 2004). Detection of DNA is achieved through the process of Southern Blot, agarose gel electrophoresis, colorimetric and fluorometric assays or DNA Detection Test Strips^{TM} (Ward *et al.,* 2004).

Real-time qPCR is the modified PCR process where target DNA is measured throughout the cycles, allowing for qualitative and quantitative analysis. This technique is rapid, sensitive and specific. The specificity is however determined by the primer sequence or assay which is developed (Schaad & Frederick, 2002). The measurement of the DNA throughout the cycles is achieved through the use of fluorescent dyes or probes which bind to the target DNA as it is amplified (Henson & French, 1993). There are three types of dyes and probes which are used; the first being Taqman^{R} probes, fluorescent resonance energy transfer (FRET) probes and molecular beacons (Schaad & Frederick, 2002). At each level where the target DNA binds to the dye or probe during amplification a “cycle threshold” (Ct) value is calculated. The amount of target DNA decreases with an increase in Ct, a calibration curve is created and the initial amount of unknown target DNA can be quantified (Ward *et al.,* 2004). Currently there are three known techniques specifically for qPCR analysis of *Fg*SC, Waalwijk *et al.* (2004), Nicolaisen *et al.* (2009) and Boutigny *et al.*

(2012). The first protocol makes use of the TaqMan technique and the latter two protocols make use of elongation factor primer. However it must be noted that the Boutigny *et al.* (2012) protocol is primarily based on the previously described method. A limitation associated with qPCR is the availability of probes, although PCR techniques are able to be adapted for qPCR use if the correct probes and assays are available (Schaad & Frederick, 2002).

The above molecular techniques are a great advantage to plant pathology, other plant sciences and agricultural disciplines. The advance of technology allows these molecular techniques to be rapid and more widely used allowing plant pathologists to make more accurate decisions surrounding breeding, control methods and development of pathosytem models.

6.2 Mycotoxins

Sampling and Sample Preparation

Prior to the detection and quantification of mycotoxins accurate sampling and sample preparation is required. Due to the heterogeneous nature of mycotoxins in a consignment, care should be taken to ensure the correct representation of the mycotoxins present in the consignment is addressed. The error in original sampling technique can contribute up to 90% of the mycotoxin analysis error. The mycotoxin which is being detected and the matrix will determine the protocol of the analytical technique and the mode of detection (Turner *et al.*, 2009).

Sample preparation is dependent on the sample matrices and the mycotoxins which are to be extracted. Liquid extractions (LE) and solid phase extractions (SPE) are the most widely used techniques. Polar solvents are used to dissolve and extract mycotoxins in LE. The use of non-polar solvents is frequently used for the removal of inhibitory agents, for example the use of hexane for the removal of lipids. To remove the effect that matrices might have on extraction, SPE can be used as a purification procedure. However SPE is also applied to extraction techniques to increase the concentration of mycotoxin (Rahmani *et al.,* 2009). Mycotoxin analysis should be done in replications of three to five to conform to actual concentrations present (Bhat *et al.,* 2010).

To determine the presence of mycotoxins in produce, qualitative and quantitative assessments can be done. Qualitative assessments determine whether or not mycotoxins are present in the commodity, whereas quantitative methods determine the extent of the commodities contamination. The most valuable assessments are those which are quantitative, even more so if they are rapid methods. Rapid methods are techniques which can be done in the field, relatively fast and are easy to use (Zheng *et al.,* 2006).

Enzyme-Linked Immunosorbent Assay (ELISA)

Qualitative and quantitative ELISA allows for low cost easy application and detection of mycotoxins. ELISA detection is an immunochemical technique based on a competitive assay format, the use of specific antibodies and antigens or an enzyme that requires a target (Köppen *et al.,* 2010). Antibodies developed for mycotoxin detection require carrier molecules, usually proteins, to be incorporated in order to invoke a detection response (Turner *et al.,* 2009).

The most common mycotoxins from multiple matrices can be detected using ELISA, however multiple mycotoxins cannot be run simultaneously. Mycotoxins that have validated protocols include aflatoxins, DON and derivatives, fumonisins, orchratoxin, T-2 toxin and ZEA. Detection kits are available in multiple formats such as microlitre plates and bead based assays or membranes. Advantages of ELISA are rapid detection, multiple matrices and their ability to be portable and used outside of the laboratory. This technique is one of the most widely used detection methods for mycotoxins. Limitations associated with ELISA include limited detection due to the sensitivity of the antibodies and the cost of bulk screening as ELISA kits may only be used once (Turner *et al.,* 2009).

Chromatographic Methods

Analytical techniques which are considered classical chromatographic methods include Thin Layer Chromatography (TLC), Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC). Recently coupling these techniques with more sophisticated detectors has greatly enhanced these methods. The advancement of the traditional techniques include GC - mass spectrometry (GC-MS)

and liquid chromatography tandem mass spectrometry (LC-MS-MS) (Bhat *et al.,* 2010).

In the past TLC was the most frequently used technique to detect and quantify mycotoxins. Although TLC is not as advanced as other techniques, it is still a popular method. This technique is based on the ability of compounds to separate in the presence of specific solvents and the distance travelled on the TLC matrix plate is used to determine the mycotoxins present (Bhat *et al.,* 2010). Mycotoxins which can be detected and quantified with HPLC include aflatoxin, fumonisins, ochratoxin, patulin, trichothecenes and ZEA. However the lack of chromophores in some mycotoxins may serve as a limitation due to the dependence of flourescense detection of HPLC (Köppen *et al.,* 2010). The innovative addition of mass spectrometric (HPLC-MS) or liquid chromatography tandem mass spectrometry (LC-MS-MS) detection has changed mycotoxin research. The use of mass spectrometry is described by electro-ionisation or atmospheric pressure chemical ionisation allowing for increased sensitivity and efficiency. Both HPLC-MS and LC-MS-MS allow for multiple mycotoxins from various matrices to be detected simultaneously following one extraction procedure. Protocols which have been validated for LC-MS-MS detection include those described by De Kok *et al.* (2007), Spanjer *et al.* (2008), Santini *et al.* (2008), Vendl *et al.* (2009), Kokkonen & Jestoi (2009) and Ediage *et al.* (2011). Techniques which are employed for commercial sample analysis will vary to those used for research purposes due to high-throughput and automation, but vary primarily due to cost and competitiveness (Turner *et al.*, 2009).

Matrix effects are defined as the interference of the sample origin in the detection of the mycotoxins. Minimizing matrix effects is essential to the use of chromatographic techniques to reduce interference during separation. Matrix matched standards and materials are required where possible to allow for matrix effects to be excluded from variation in mycotoxin quantification. A limitation to chromatographic methods is a lack of matrix matched materials that are completely free of mycotoxins (Köppen *et al.,* 2010).

7. Plant Disease Models

7.1 Principals

Epidemiology as defined by Kranz (1974) is “the study of dynamic interactions between pathogen and host, and the disease that results, as influenced by man and environment.” The interaction between pathogen, host and the environment is explained in the disease triangle. To many the disease triangle is no longer feasible when working with epidemiological models. A modified disease triangle is required to include the influence humans have on plant disease epidemics (Teng & Bowen, 1985).

Teng & Bowen (1985) describe the systems approach as “holistic”. Epidemiological systems are defined as the outcome of biological systems and their sub-systems. Sub-systems are affected by the environment either to favour the development or suppression of disease. Epidemiology is a discipline which not only deals with biological systems but mathematical and statistical systems. This deems epidemiology a qualitative and quantitative practice as relationships and their respective absolute values can be determined (Kranz & Hau, 1980).

The three steps of creating a pathosystem model include model development, model analysis and hypothesis testing. Model development requires data collection and determining relationships which can be quantified. Model analysis is the use of mathematical and statistical algorithms to generate a model which can predict pathogen and disease development. The validation of models is the final step which is required prior to application of the model on a larger scale (Prandini *et al.,* 2009). Schaafsma & Hooker (2007) stated that predictive models are only beneficial if they meet the need of the end-user.

The development and application of a model is dependent on the purpose of its use and the amount or detail required for the specific outcome (Campbell & Madden, 1990). All the biological components in epidemiology have spatial and temporal properties and limits which will be included in a model (Teng & Bowen, 1985). Model development varies according to model type. A mechanistic model starts with a hypothesis or a problem which must be solved. An empirical model is developed with data and the relationships which can be identified in the data. The model is then

developed in terms of the hypothesis and then experiments are done to observe the level of accuracy. A model with good fit, measured by R^2>0.75, can be used; however a model with a poor fit must be re-evaluated and the hypothesis revised. Once a good fit is established in a model, prior to the model being used it must be validated (Campbell & Madden, 1990; Van Maanen & Xu, 2003; Magarey & Sutton, 2007). Model validation is done either commercially or biologically before it can be used in practise. Commercial validation will ensure that the outcome of the forecast will allow correct management options and economic benefit for all end users. Biological validation ensures that the model developed, forecasts the correct disease incidence and severity (Prandini *et al.,* 2009).

Van Maanen & Xu (2003) describe the ideal model as "sufficiently complex but no more necessary to answer the posed question." In general for a model to be successful the following factors should be considered, reliable and simple, relevant to pathogen-host-environment dynamic, available to public and relatively easy to apply and use, have multiple purposes and be cost effective (Campbell & Madden, 1990).

Factors promoting *Fg*SC survival, colonisation and mycotoxin production differ due to the different mechanisms involved in each stage of the life cycle. The complexity of environments involved in promoting *Fg*SC is extensive and ranges from micro- to macro- environments (Woloshuk & Shim, 2012). The point where the host plant is most susceptible to the pathogen during critical weather periods or growth phases is described as the window of opportunity. Therefore, understanding stimuli associated with host plant phylogeny, agronomic practises which influence host and pathogen and meteorological variables which can affect both host and pathogen responses and are vital to understanding the *Fg*SC epidemiology and creating successful models (Bandyopadhyay *et al.,* 2000; Woloshuk & Shim, 2012).

7.2 *Purpose of Modelling*

Modelling is the tool which simulates epidemiological systems through the collection of disease-related data, and mathematically and statistically applying the data collected, biotic or abiotic, to understand the processes and interactions that drive the system. Plant pathogens are driven by environmental factors, climatic or

agronomic, to cause diseases and therefore modelling for disease colonisation and mycotoxin risk and accumulation is ideal (Magarey & Sutton, 2007). Models can be used as tools for understanding a system and provide a method for decision making to manage risks associated with epidemics (Teng & Bowen, 1985).

Globally numerous models have been developed for the prediction of *Fusarium graminearum* on wheat, the cause of FHB which indicates the scale and the importance of FHB. These range from dynamic or descriptive models that indicate the relationships between stimuli and response to comprehensive or explanatory models that not only indicate the relationships but explain the system between the stimuli and responses (Table 3; Prandini *et al.,* 2009).

Table 3. Summary of parameters used in forecasting models for incidence and severity of FHB of wheat and concomitant mycotoxin production of deoxynivalenol (DON).

Model type	Country	Weather parameters	Agronomic parameters	Description
Colonisation Models	Argentina	temperature, relative humidity, rainfall	growth stage	Moschini & Fortugno, 1996
	Argentina	temperature, rainfall, HWD	*	Brustolin *et al.,* 2013
	USA	temperature, relative humidity, rainfall	growth stage	De Wolf *et al.,* 2003 Shah *et al.,* 2013
	Brazil	temperature, relative humidity, rainfall, head wetness	inoculum present on host crop, dispersal rate, infection rate and frequency	Del Ponte *et al.,* 2005
Colonisation and Mycotoxin Model	Netherlands	temperature, relative humidity, rainfall	resistance level (rating 0 – 10), flowering date, harvest date and use of fungicides	Van der Fels-Klerx *et al.,* 2010 Van der Fels-Klerx *et al.,* 2014
Mycotoxin Models	Switzerland	temperature, relative humidity, rainfall	resistance level, previous crop, soil and straw management, growth stage, seedbed tillage	Musa *et al., 2007*
	Canada	temperature, rainfall	*	Hooker *et al.,* 2002

HWD head wetness duration

* no agronomic factors were taken into account

7.3 Colonisation Models

A model for FHB epidemic potentials in Argentina was developed by Moschini & Fortugno (1996) to assist wheat producers with selecting methods of control. This model is reliant on critical growth stages of the wheat plant, temperature, RH and precipitation. The plant growth interval that had the strongest correlation between disease incidence and weather started eight days prior to heading and ended 530 degree days (DD; DD= average temperature – base temperature; base temperature at 0^oC) later when the daily average temperature had been accumulated. The predicted *Fusarium* incidence (PFI) was greater than 45% if more than four consecutive, two day periods of precipitation > 0.2 mm and RH > 81% on the first day and RH > 78% was observed on the second day (NPPRH). Two models were selected from a range of similar models based on the best fits using R^2 and root mean square error (RMSE) i.e.

$$\text{PFI\%} = 20.37 + 8.63\ \text{NPPRH} - 0.49\ \text{DDXNT}, \text{ where } R^2 = 0.8604, \text{RMSE} = 8.89$$

$$\text{PFI\%} = 16.39 + 5.43\ \text{NPPRH} - 0.45\ \text{DDXNT} + 2.95\ \text{DPRH}, \text{ where } R^2 = 0.8863, \text{RMSE} = 8.35$$

Where NPPRH is precipitation > 0.2 mm and RH $> 81\%$ on the first day and the second day's RH $> 78\%$, DDXNT = DDMAXT + DDMINT, where DDMAXT is Σ^d(MaxT- 26°C) and DDMINT is Σ^d(9°C- MinT), where d is days of the period, DPRH is the co-occurrence of > 0.2 mm precipitation and RH $> 83\%$.

Relative actual disease incidences, from data collected from 1991-1993, and predicted values from the above models were plotted against one another to determine the accuracy of both models. The models both performed with a $R^2=1$ indicating that the relationship between the actual and predicted data is a perfect fit.

Combination of two existing models developed by Moschini & Fortugno (1996) and Zoldan (2008) resulted in Brustolin *et al.* (2013) determining the minimum head wetness duration (HWD) and temperature for FHB development and the potential of using rainfall forecasts as a predictive tool for the need for fungicide application on wheat produced in Brazil. Meteorological data collected from the start of anthesis to grain dough stage included temperature and HWD. Actual disease severity data, head incidence (HI) and spikelet incidence (SI), was collected during the 2005 and 2006 growing period and compared to daily values of infection favourability (DVIF, Table 4).

The trigger for FHB infection was calculated with respective values of HWD and temperature as 64.1 hours and 16.9°C. In combination with research completed by Moschini & Fortugno (1996) using the critical period of NPPRH and 48-72 hour rainfall forecasts, the FHB trigger of HWD would be met and FHB onset can be expected if the mean air temperature is at 16.9°C during this period. This provides a recommendation of applying chemical control if rainfall had been forecasted 48 - 72 hour period while a no rainfall forecast means no infection can be expected and thus no fungicide application is required.

Table 4. Interactions between air temperature (at 3°C intervals) and head wetness duration (HWD) which give different daily values of infection favourability (DVIF) for *Gibberella zeae* in wheat spikelets (Zoldan, 2008).

DVIF	[z]0	1	2	3	4
	T (^{0}C) Spikelet incidence (%)				
	0	1-15	16-40	41-60	>60
	HWD (h)				
10-12	< 12	> 13	-	-	-
13-15	< 8	9-28	> 29	-	-
16-18	< 6	7-23	24-42	> 43	-
19-21	< 6	7-21	22-36	> 37	-
22-24	< 5	6-20	21-34	35-44	> 46
25-27	< 5	6-20	21-33	34-44	> 45
28-30	< 5	6-20	21-33	34-45	> 46
31-33	< 6	7-22	23-37	> 38	-
34-35	< 8	9-29	> 30	-	-

[z]0 =no infection; 1 =1-15%; 2 =16-40%; 3 =41-60%; 4 >60%;
- incidence not apparent

De Wolf *et al.* (2003) developed logistic regression models to predict FHB epidemics in the USA. Four models (A-D) were selected due to their prediction accuracy of 84%. A fifth model (I) was selected as it solely contained pre-anthesis variables and had a prediction accuracy of 70%. Model A takes into consideration the duration of temperature between 15 and 30°C and RH ≥90%, 10 days following the start of anthesis (TRH9010).

$$FHB = -3.3756 + 6.8128TRH9010 \qquad A$$

Model B uses the interaction between the duration of temperature between 15 and 30°C, 7 days prior to anthesis and the duration of temperature between 15 and 30°C and RH ≥ 90%, 10 days following the start of anthesis (INT3).

$$FHB = -3.7251 + 10.509INT3 \qquad B$$

The interaction between the duration of temperature between 15 and 30°C and duration of precipitation 7 days prior to anthesis (INT1), the interaction between the duration of temperature between 15 and 30°C and duration of precipitation 7 days prior to anthesis and duration of temperature between 15 and 30°C and RH ≥ 90%, 10 days following on the initiation of anthesis (INT4), makes up Model C.

$$FHB = -1.0585 + -14.2400INT1 + 39.4590INT4 \qquad C$$

Model D uses the predictor variables of the interaction between the duration of temperature between 15 and 30°C and duration of precipitation 7 days prior to anthesis and duration of temperature between 15 and 30°C and RH ≥ 90%, 10 days following the start of anthesis (INT4) and duration of precipitation (DPPT7).

$$FHB = -1.5424 + 31.7868INT4 + -5.8085DPPT7 \qquad D$$

The lower prediction accuracy (70%) model which was included because it only consisted of pre-anthesis variables was referred to as Model I. The predictor variable is the duration of temperature between 15 and 30°C (T15307) and the duration of precipitation 7 days prior to anthesis (DPPT7). Model I, therefore allows for management decisions to be made early i.e. before the epidemic becomes severe.

$$FHB = -8.2175 + 8.4358T15307 + 4.7319DPPT7 \qquad I$$

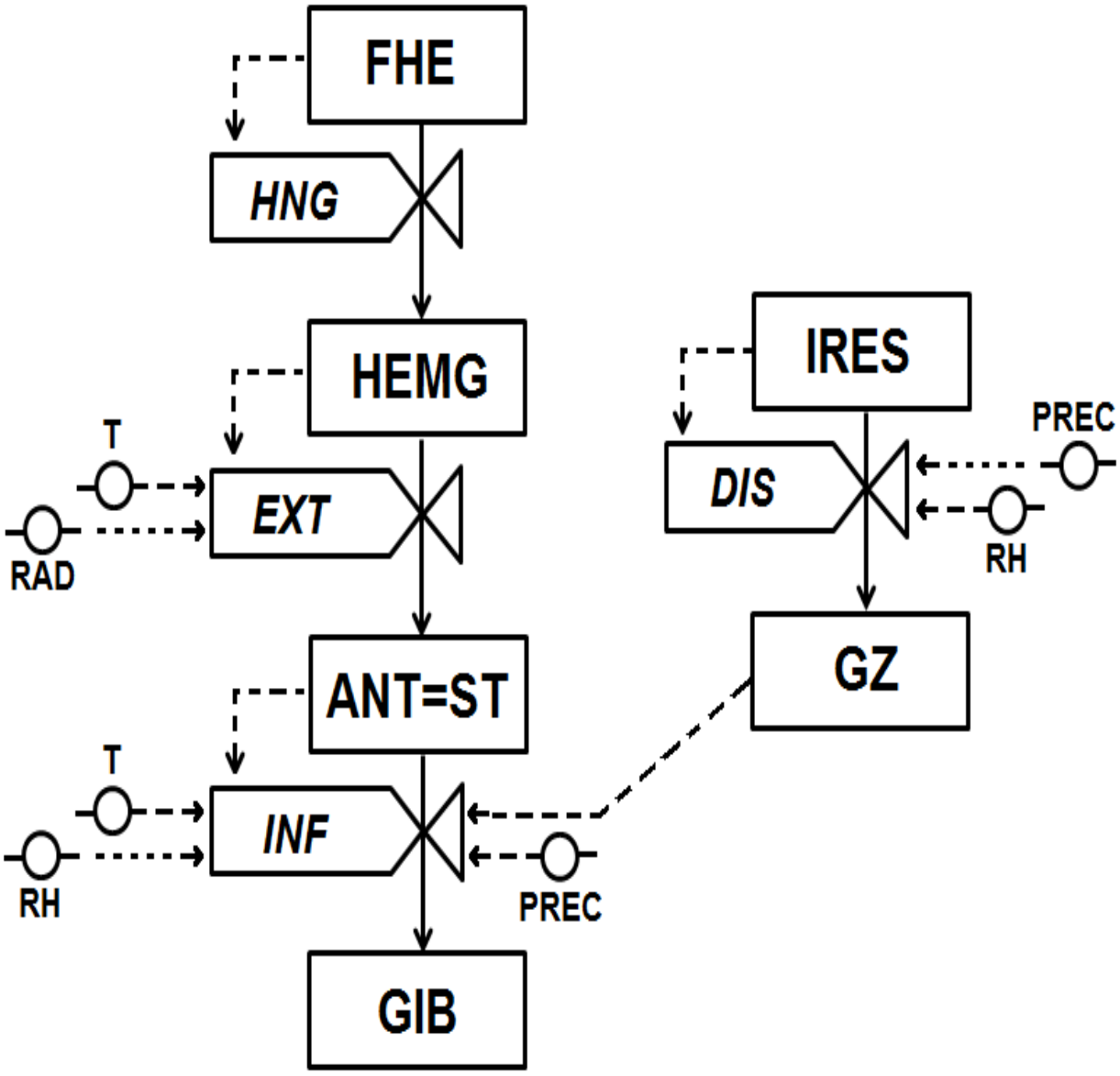
After external validation of the above models, Models A and B correctly classified seven of the nine risk areas and Model I correctly classified six of the nine risk areas. However, the latter model has a higher tendency to predict non-epidemic years and thus the assumption can be made that multiple false negative and false positive errors can occur. Model A and B are highly dependent on accurate and reliable weather data during anthesis and could account for errors associated with model A and B. However model I is not limited by weather data during anthesis but prior to

anthesis and thus model I's errors did not correspond with A and B. De Wolf *et al.* (2003) subjugated that future research combining models A, B and I could result in fewer errors. Accuracy of the model could also be increased if data of crop residues and local inoculum is taken into account.

Shah *et al.* (2013) used larger data sets on the above models to expand potential variables associated with the window of opportunity for FHB development. Subsequently, the combination of models developed by Shah *et al.* (2013) and De Wolf *et al.* (2003) produced an online warning system which allows for FHB risk to be predicted and the timely application of fungicides can be suggested to producers using the FHB Risk Assessment Tool (http://www.wheatscab.psu.edu). The purpose of this prediction goes beyond monitoring the risk of an epidemic but also to the extent of mycotoxin risk area identification and economic benefits.

Del Ponte *et al.* (2005) created a simulation model, GIBSIM, for calculating the proportion of wheat tissue infected with *Fg*SC or the daily infection index of FHB infected wheat, grown in Brazil. The dynamics of host-inoculum-environment were evaluated and applied into a system analysis that was driven by a process based model (Figure 8) and not an empirical model. Host factor variables ranged from first head emergence to anthers exposed, inoculum variables included inoculum present on host crop, dispersal rate, infection rate and frequency. Environmental data, also known as driving variables, collected included rainfall, relative humidity, head wetness and temperature between 10 and 30°C.

Figure 8. Relational diagram of GIBSIM a mechanistic model estimating risk infection index of FHB of wheat (Del Ponte *et al.*, 2005).



Where state variables are *FHE* = First heads emerged; *HEMG* = proportion of heads emerged; *ANT* = daily proportion of anthers present; *ST* = proportion of susceptible tissue based on *ANT* and coefficients for susceptibility after peak flowering up to 14 days after flowering ends; *IRES* = inoculum present on crop residues; *GZ*= relative density of a spore cloud; *GIB* = daily risk infection index. Rate variables: *HNG* = daily heading rate; EXT = daily anther's extrusion rate; *INF* = daily infection frequency; *DIS* = daily inoculum dispersal rate; Driving variables: *T* =daily mean temperature (°C); *RAD* = daily solar radiation (MJ/m2/dia); *RH* = daily mean relative humidity (%) and *PREC* = daily precipitation (mm).

Sub-routines were applied as follows;

$$HNG=1\text{-}\exp(\text{-}0.027.t^{2.4352}),$$

where t=1 day

$$ANText=1\text{-}\exp^{(a.tb)},$$

where t=1 day, $a=0.255\text{-}0.029T+0.0009T^2$, $b=\text{-}5.773+0.966T\text{-}0.0278T^2$

There is a clear indication that inoculum and environment dynamics interacted, hence meteorological factors such as RH and days with more than 3 mm rain were taken into account for predicting the density of a spore cloud (CDR).

$$GZ=(\text{-}0.6306+0.0152RH+0.1076CRD)^2$$

$$INF=0.001029\exp^{(0.1957T)}$$

Daily infection index (GIB) and accumulated infection index (GIB%) was modelled by various interactions of variables; anthers present daily, infection frequency, spore cloud density, and susceptible tissue available.

$$GIB1=ANT*INF$$

$$GIB2=ANT*INF*GZ$$

$$GIB3=ST*INF$$

$$GIB4=ST*INF*GZ$$

$GIB\%=\Sigma(GIB*100)$, daily infection index for each of the four models

By including spore cloud density, the predictive value of the model was improved. The use of a correction factor to extend the hosts susceptibility period and the daily spore cloud density enabled the model to account for 93% of the variation in disease severity and explain 69% of the variation in disease incidence, using regression analysis. GIBSIM allows for the regression to be a successful way to validate a process based model, to estimate severity ranges and alert producers of potential FHB infections in Brazil. If it is to be applied in other geographic or agronomic situations research and recalibration should be specific for those sites. Not only is

this model crucial to the prediction of infection indexes, but future research and applications of this model could provide a fungicide application prediction tool, mycotoxin risk forecaster or predictor of the potential impact of climate change on FHB in wheat producing regions of South America (Del Ponte *et al.,* 2005).

7.4 Mycotoxin Models

Van der Fels-Klerx *et al.* (2010) and Van der Fels-Klerx (2014) developed a descriptive model in the Netherlands using historical weather variables, agronomic factors and DON concentrations at harvest to predict FHB associated DON in winter wheat.

Meteorological variables collected closest to the field, from 2001 to 2008, includes data from the sum of hourly rainfall, number of hours where temperature ≥25°C, average hourly temperature and the sum of hours that RH ≥80% (RHh80), sum of hourly temperatures where RH ≥85% for at least two consecutive hours (ThRH85_2) and the sum of hours that RH ≥85% for at least four consecutive hours (ThRH85_4). However correlations between RHh80, ThRH85_2 and ThRH85_4 were high and thus RHh80 was selected as the variable to proceed with in the model development, as it is the simplest to measure. Wheat variety and resistance level (rating 0 – 10), flowering date, harvest date and the use of fungicides were agronomic data collected. The above data parameters were analysed using multiple regression to calculate the best set of variables that explains variation in DON concentrations. Model A (Table 5) was developed for use by wheat producers and can be applied up to 10 days prior to wheat flowering to assist producers whether or not to apply fungicides. Model B (Table 6) considers the entire cultivation period and is used by post-harvest parties, millers and food safety authorities.

Table 5. Model A parameters for the prediction of DON[1] contamination in mature winter wheat in the Netherlands (Van der Fels-Klerx, 2014).

Model variable	Parameter estimate	Standard error	Parameter significance
Constant	-16.040	7.620	0.036
REG N	-0.337	0.142	0.018
REG W	-1.654	0.167	<0.001
REG S	-0.812	0.312	<0.010
SPRAY 1	0.502	0.188	0.008
SPRAY 0	0.720	0.175	<0.001
FD	-0.123	0.009	<0.001
Resistance level	-0.403	0.048	<0.001
Rain A1	-0.136	0.031	<0.001
Tavg A3	-0.041	0.042	0.329
Interactive effect rain A1 * Tavg A3	0.009	0.002	<0.001
Rain linear A4	-0.098	0.012	<0.001
Rain quadratic A4	0.002	0.000	<0.001
Tavg linear A0	1.165	0.384	0.003
Tavg quadratic A0	-0.032	0.013	0.013
Tavg linear A1	0.779	0.364	0.033
Tavg quadratic A1	-0.030	0.011	0.005
Tavg linear A4	3.494	0.673	<0.001
Tavg quadratic A4	-0.112	0.020	<0.001
RHh80 A0	0.022	0.003	<0.001
RHh80 A3	0.015	0.005	0.002

[1]The model predicts ln(DON) concentrations (in μg kg^{-1}). DON values (in μg kg^{-1}) can be obtained by back transformation: exp(model A).

The model's parameters include; Northern region (REG N), Western region (REG W) and Southern region (REG S), SPRAY 1 and SPRAY 0 are number of fungicide sprayings at/after Zadoks GS 59 (0, 1, or 2 times), flowering date (FD),harvesting date (HD), length between FD and HD (LengthFH), wheat resistance level: resistance of the variety against *Fusarium* spp. infection (from 0 to 10); total rainfall (RAIN; in mm) and average temperature (Tavg; in ◦C). Time blocks are represented relative to FD (day 0) were; −17 to −10 days FD (A0), −10 to −3 days FD (A1), −3 to FD (A2), FD to +3 days FD (A3), and +3 to +10 days FD (A4) .

Table 6. Model B parameters for the prediction of DON[1] contamination in mature winter wheat in the Netherlands (Van der Fels-Klerx, 2014).

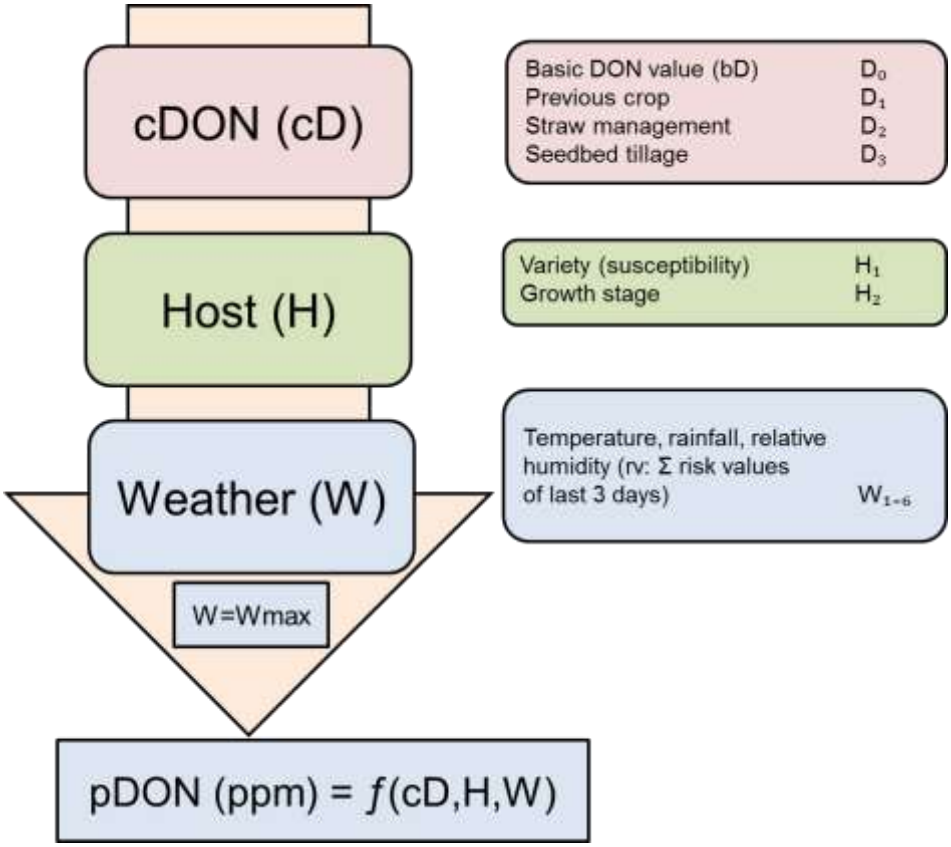
Model variable	Parameter estimate	Standard error	Parameter significance
Constant	-31.390	9.030	<0.001
REG N	0.246	0.194	0.206
REG W	-0.729	0.159	<0.001
REG S	0.305	0.260	0.241
SPRAY 1	0.688	0.165	<0.001
SPRAY 0	0.842	0.155	<0.001
FD	-0.065	0.010	<0.001
Resistance level	-0.428	0.046	<0.001
Length FH	0.089	0.017	<0.001
Rain B0	0.039	0.006	<0.001
RHh80 B0	0.006	0.003	0.012
Tavg B0	0.108	0.048	0.025
Tavg B1	-0.066	0.040	0.104
Tavg linear B3	2.614	0.965	0.007
Tavg quadratic B3	-0.078	0.027	0.004
Tavg linear B4	2.180	1.020	0.033
Tavg quadratic B4	-0.057	0.028	0.040

[1]The model predicts ln(DON) concentrations (in μg kg^{-1}). DON values (in μg kg^{-1}) can be obtained by back transformation using exp(model B).

The model's parameters include; Northern region (REG N), Western region (REG W) and Southern region (REG S), SPRAY 1 and SPRAY 0 are number of fungicide sprayings at/after Zadoks GS 59 (0, 1, or 2 times), flowering date (FD),harvesting date (HD), length between FD and HD (LengthFH), wheat resistance level: resistance of the variety against *Fusarium* spp. infection (from 0 to 10); total rainfall (RAIN; in mm) and average temperature (Tavg; in ◦C). Time blocks are represented relative to FD (day 0) were; −17 to −10 days FD (A0), −10 to −3 days FD (A1), −3 to FD (A2), FD to +3 days FD (A3), and +3 to +10 days FD (A4).

Actual DON concentrations were collected after harvesting grain and LC-MS-MS quantification of DON concentrations. DON concentration thresholds for the models were set at 1250 µg.kg^{-1} which is the regulation limit. Reference situations were calculated from forecasted meteorological data to represent actual field situations. The reference situation was plotted against the predicted DON scenario using the two models for external validation. Model A accurately predicted 95% of the situations with an R^2=64.4% and Model B accurately predicted 94% of the situations with an R^2=65.6%. Both the goodness of fit and the prediction accuracy are comparable to other models which exist. The reference scenario which best predicts DON for model A was the reference scenario which took seven days of forecasts from three to 10 days post-anthesis into consideration. Furthermore, of the 86 samples validated by Model A, one was a false positive and three were false negatives and of the total 83 samples run by Model B, two were false positives and three false negatives were observed. False positives would result in the application of fungicides and testing of mycotoxins unnecessarily. False negatives would result in fields which are above regulatory limits not sprayed with fungicides and contaminated harvested wheat not tested for contamination. This model has been validated for use of predicting DON concentrations in winter wheat produced in the Netherlands. Future research could include the response that these models would have if DON was above the regulated thresholds.

Musa *et al.* (2007) developed an internet based decision support system, *FusaProg,* for winter wheat producers who are challenged with severe infections of *F. graminearum* and concomitant DON grain contamination in Switzerland. The driving variables of this model include cropping factors, previous crop data, soil and straw management and the susceptibility of the variety planted to *Fg*SC infections. Furthermore the fundamental weather conditions at growth stages are combined with the above data to predict DON accumulation prior to harvest. A flowchart of key factors and parameters which are considered for the program are listed below in Figure 9.



Where dD = basic DON value, cD= cropping system related DON value and pDON=predicted DON value and rv= risk value.

Figure 9. Flow chart of the key factors and parameters which are considered in the Internet-based FusaProg system (Musa *et al.,* 2007).

The model is run on a threshold based method. The outputs for this model allow for plot specific, local and regional *Fg*SC infection risks, the correct fungicide timing to be predicted to reduced *Fg*SC infections and forecasts for DON during the flowering period. *FusaProg* was validated with data collected over 2004 and 2005 winter wheat season, where the critical DON threshold was below or above 0.5 µg.g^{-1} 78% of the cases predicted the DON concentrations correctly. The model was evaluated in 2006 and became publically available in 2007 online at www.fusaprog.ch.

Data accumulated from 750 farm fields in Ontario, Canada, between 1996 and 2000 were used for the development of a DON prediction model in mature wheat kernels (Hooker *et al.,* 2002). The use of meteorological data, rainfall and temperature, was collected based on the correlation with wheat heading timing, disease parameters and quantified mycotoxins. Critical periods of weather correlating with growth periods

relative to heading dates using multiple regression analysis were completed to reveal three predictive equations.

The first equation predicts DON from weather data collected four to seven days prior to heading,

$$DON1 = \exp[-0.3 + 1.84RAINA - 0.43(RAINA)^2 - 0.56TMIN] - 0.1$$

Where DON is the predicted concentration of DON ($\mu g.g^{-1}$), RAINA is the number rainy days where > 5 mm per day in the 4 to 7 days before heading period and TMIN is the number of days where temperature < 10°C between the 4 to 7 days before heading period.

The two equations below predict DON from weather data collected from seven days prior to heading to 10 days after heading,

$$when\ RAINB > 0$$

$$DON2 = \exp[-2.5 + 2.21RAINA - 0.61(RAINA)^2 + 0.85RAINB + 0.52RAINC - 0.30TMIN - 1.1TMAX] - 0.1$$

$$When\ RAINB = 0$$

$$DON3 = \exp(-0.84 + 0.78RAINA + 0.4RAINC - 0.42TMIN) - 0.1$$

Where DON is the predicted concentration of DON ($\mu g.g^{-1}$), RAINA is the number rainy days where > 5 mm per day in the 4 to 7 days before heading period, RAINB is the number of days of rain > 3 mm per day in the period 3 to 6 days after heading, RAINC is the number of rain >3 mm per day in the period 7 to 10 days after heading and TMAX is the number of days with temperature >32°C between 4 and 7 days before heading.

The first equation explains 55% of the DON variation and the second and third equations explain, 79% and 56% respectively. The above equations are accompanied by a model flowchart which starts with wheat stage observations (Figure 10).

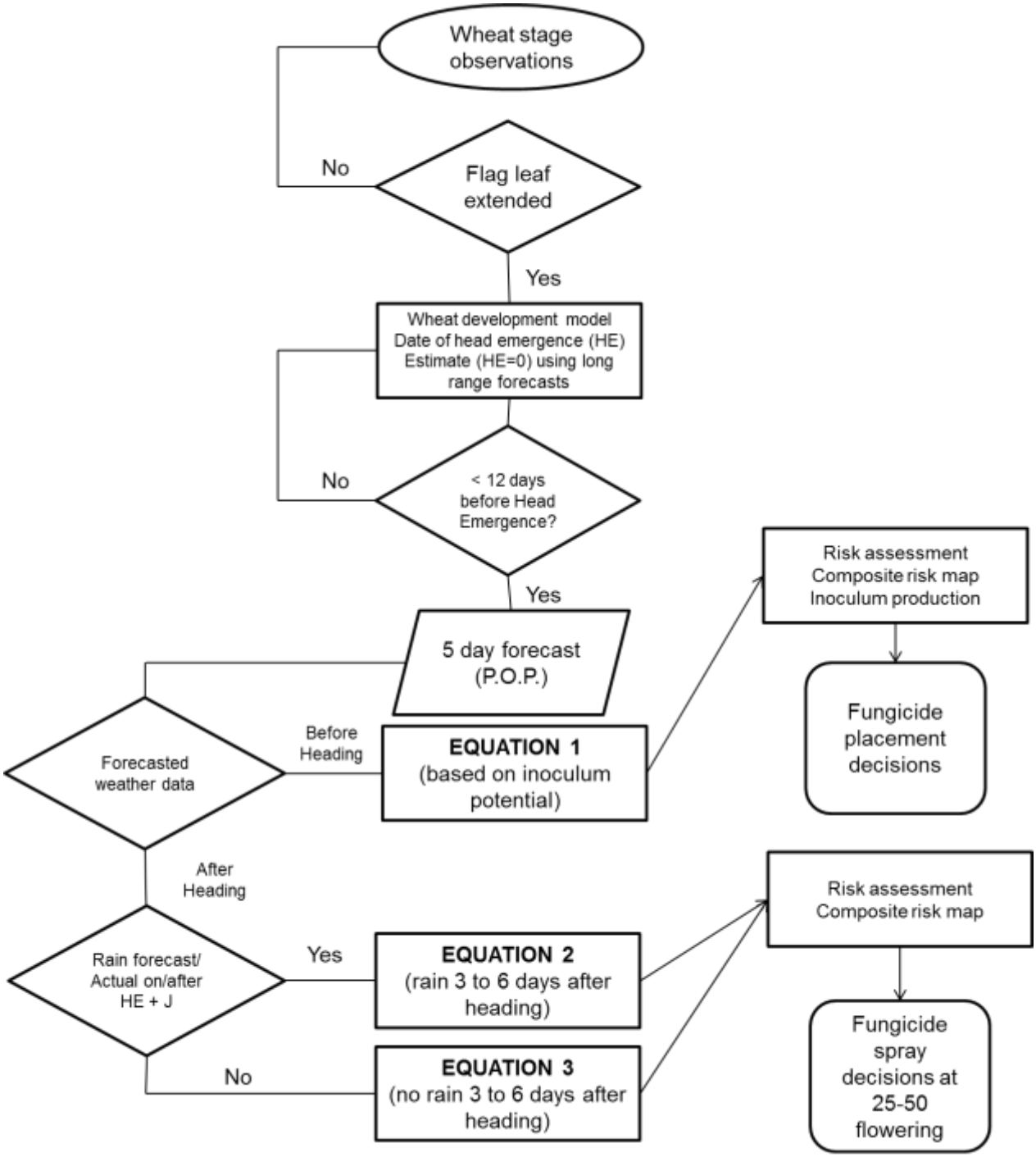


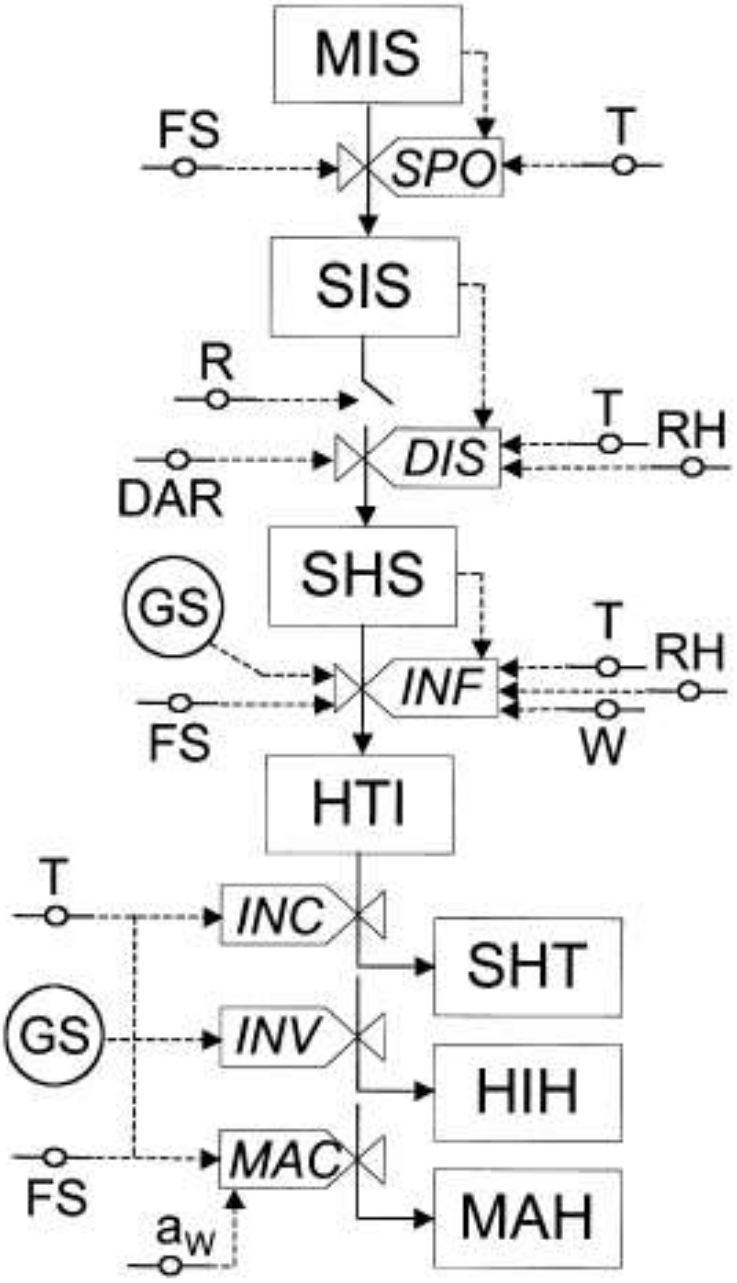
Figure 10. Flow chart for implementing the weather-based model at heading to predict the concentration of deoxynivalenol (DON) at grain harvest (Hooker *et al.,* 2002).

The model had an overall ability to predict 73% of the variation in DON across the five years of data collected. The model was validated in 2000, the model best predicted DON in situations where actual DON concentrations were < 100 µg.kg^{-1}. However, DON was accurately predicted in 13 of the 17 fields where DON < 100 µg.kg^{-1} and 15 of the 17 fields where DON was < 200 µg.kg^{-1}.

This model has been commercially available for five years and is easily accessed online. It is more commonly known as DONcast (http://www.doncast.eu/). The launch of the DONcast in 2000 made regional maps available online. Regional maps are sufficient for trends and expectations of DON accumulation in areas but are not field or site-specific. In 2005 DONcast was released online for field or site-specific predictions. These predictions utilise local and field or site specific inputs such as crop history, local meteorological data, crop rotation and wheat variety data. Not only is DONcast being applied in Canada but also in Uruguay, South America and the European DONcast® webpage was updated in 2014 and can be applied in France. The 2005 version of DONcast allows for producers to successfully predict their local DON contamination of wheat grain. It must be noted that this model is qualitative, however global validation of this model deems the model robust for DON accumulation at a threshold of 500 µg.kg^{-1} (Hooker *et al.,* 2002; Schaafsma & Hooker, 2007).

An explanatory model, based on systems analysis of a relational diagram (Figure 11) originally developed by Leffelaar & Ferrari (1989) was improved and validated by Rossi *et al.* (2003) as a preliminary model to predict FHB and mycotoxin accumulation. The original model assumes that inoculum at locations is equal in doses, and uses hourly meteorological data and host disease assessment as inputs. Weather data which was collected included temperature, RH, rainfall and leaf wetness. Disease incidence and severity was assessed at milk stage on a 300 head sample and the actual mycotoxin content was determined at harvest.

Figure 11. Relational diagram of the model for Fusarium head blight infection and mycotoxin contamination of wheat (Rossi *et al.*, 2003).



Where MIS = mycelium infecting the basal wheat organs or cereal straw, SIS = spores produced on inoculum sources, SPO = sporulation rate, SHS = spores landed on head surface, DIS = dispersal rate, INF = infection rate, HTI = head infected tissue, INC = incubation, SHT = scab on head tissue, HIH = hyphae invading heading tissue, INV = invasion rate, MAH = mycotoxin accumulation on heads, T = temperature, RH = relative humidity, R = rainfall, DAR = sequences of daily rainfall, W = wetness duration, a_w = free water in host tissue, FS = fungal species and GS = growth stage.

Subroutines for the model are given as follows;

$$SPO = [25.98 \times Teq^{8.59} \times (1 - Teq)^{0.24}]/[1 + \exp(5.52 - 0.51 \times t)]$$

Where Teq = (T-Tmin)/(Tmax-Tmin), T is temperature, Tmin = 5°C, Tmax = 35°C and t is incubation time, which is calculated on basis of a contour plot (Figure 12)

$$INF = -0.099 - 0.363 \times t + 0.07808 \times T \times t - 0.00591 \times T^2 \times t + 0.000199 \times T^3 \times t - 0.0000024 \times T^4 \times t$$

Where Teq = (T-Tmin)/(Tmax-Tmin), T is temperature, Tmin = 0°C, Tmax = 38°C and where Figure 13 is used in combination with T is temperature (°C) and t is incubation time (hours)

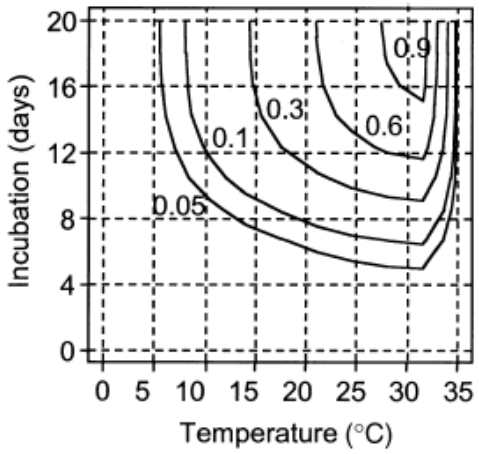


Figure 12. Contour plot of the equation quantifying the relationship between temperature, days of incubation and sporulation rate of *Gibberella zeae* (Rossi *et al.,* 2003). .

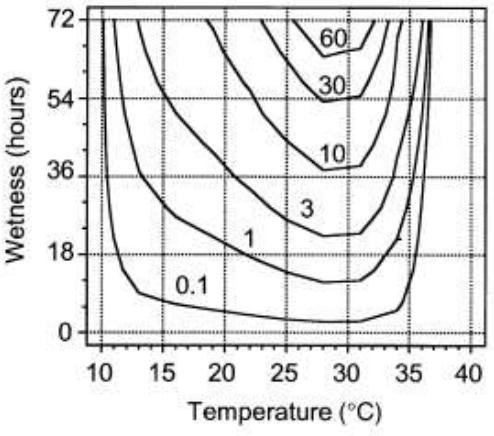


Figure 13. Contour plot of the equation quantifying the relationship between temperature, incubation time and frequency of infection by *Gibberella zeae* (Rossi *et al.,* 2003).

$$INV = [5.53 \times (Teq)^{1.55} \times (1 - Teq)]^{1.35}$$

There are two outputs to this model; the first is for FHB risk

$$FHB_{risk} = SPO \times DIS \times INF \times GS$$

and the second is toxin accumulation risk

$$TOX_{risk} = INF \times GS \times INV$$

Both the above equations are calculated daily and accumulated until harvest.

Regression analysis was completed to validate the model with data collected from northern Italy in 2002. Actual disease severity was compared with FHB risk and an exponential regression accounted for 83% of the variation. The exponential regression of the relationship between actual total mycotoxins and predicted toxin risk was R^2=0.90. The above relationships validate the original model developed to predict FHB and mycotoxin risk but future validations are required under a wider range of conditions to quantify environment-host-pathogen dynamics to ensure multiple locations in Italy can be accounted for by this model (Rossi *et al.,* 2003).

7.5. Limitations and Advantages

A limitation which is associated with plant disease models in general is that there is a lack of standardisation. Therefore, models cannot be adjusted for pathogens or to other locations easily. The initial costs to develop a disease model may also limit the production of these forecasting tools. An additional cost which must be considered is also the cost required to modify a model for a specific region (Magarey & Sutton, 2007). Limitations for descriptive and explanatory models differ as do their purposes. With few inputs descriptive models are easy to understand, use and apply, but these models require calibration for new sites. Explanatory models are most commonly used for genotype x environment interactions; with more inputs they are more complex and can be more unstable due to high input volumes (Prandini *et al.,* 2009).

If a model is successfully developed and takes heed of the limitations many advantages of modelling for plant disease can be reaped. These include plant disease management decision making tools, reduced pesticide usage, integration of multiple disciplines and reduced plant disease management costs. The advantages may also be indirect which include the accuracy of the timing of fungicide applications and therefore, the fungicide being more effective. With fungicides or

other chemicals controls being more effective there are reduced costs, reduced negative impacts on the environment and reduced chemical control resistance build up amongst the plant disease causing agents. Natural enemies of the pathogens are also conserved if chemical control is reduced (Fry, 1977; Prandini *et al.,* 2007).

8. Conclusion

Current research into biofortification, increasing iron and zinc in grain, sorghum has the potential to reduce malnutrition in Sub-Saharan Africa and Asia (Reddy *et al.,* 2010). The current increase in *Fg*SC epidemics globally, and particularly in sorghum, means that the need to control not only the colonisation of the pathogen but also concomitant mycotoxin contamination is pivotal. The genus *Fusarium* has been widely researched and will continue to be studied as it is a complex genus and is still the focus of one of the broadest based research communities in the world (Summerell & Leslie, 2011). Approximately only 4.5% of fungal species have been reported and described, the remainder are yet to be discovered. This raises concern regarding the current status of known and unknown mycotoxins associated with fungal species which are yet to be identified, putting pressure on mycotoxin research and legislation (Bhat *et al.*, 2010).

Forecasting models are the future for controlling and managing *Fg*SC in an integrated disease control approach, as they form the basis for pre-emptive control. Currently in South Africa the most effective control for *Fg*SC is the application of fungicides. The high cost of these fungicides often raises a fear of unnecessary fungicide application with the result that fungicides are often applied when unnecessary or alternatively applied when the risk of disease is low. Models will therefore, assist in the application of timely chemical control and as a result in more effective control of the disease system and associated risks (Shaafsma & Hooker, 2007).

The majority of predictive models which have been developed use environmental factors which affect both the host crop and the pathogen at critical growth periods. The environmental factors are either weather parameters or agricultural practices which alter the environment of the pathogen. The integration of these techniques into a model for predicting *Fg*SC with data collected for South African situation of SGM would be of a great advantage to our sorghum producers and end users.

A balanced and integrated approach to control disease, using agronomic measures, breeding tools, chemical control, biological control, forecasts and other mechanisms of control is always empirical in a food production system. Although there is still much work to be done in plant disease modelling, the use of statistical and mathematical modelling is rapidly expanding in the plant pathological discipline. Therefore, more farmers should be informed of the advantages which the use of models can provide for them and sustainable agriculture, of not only the farm at hand but of the industry as a whole.

9. References

Aldred, D. & Magan, N. 2004. Prevention strategies for trichothecenes. *Toxicology Letters* **153**: 165-171.

Audenaert, K., Vanheule, A., Höfte, M. & Haesaert, G. 2013. Deoxynivalenol: a major player in the multifaceted response of *Fusarium* to its environment. *Toxins* **6**: 1-19.

Balota, M. 2012. Sorghum diseases, head mold. Virginia Polytechnic Institute and State University, Extension Program, Virginia State, Petersburg Accessed on 25.10.2013 from http://pubs.ext.vt.edu/AREC/AREC-20/AREC-20_pdf.

Bandyopadhyay, R. & Mughogo, L. K. 1988. Evaluation of field screening techniques for resistance to sorghum grain molds. *Plant Disease* **72**: 500-503.

Bandyopadhyay, R., Butler, D. R., Chandrashekar, A. Reddy, R.K., & Navi, S.S. 2000. Biology, epidemiology, and management of sorghum grain mold. Pages 34-71 in Technical and institutional options for sorghum grain mold management: proceedings of an international consultation, 18-19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Belton, P. S. & Taylor, J. R. N. 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science & Technology* **15**: 94-98.

Bhat, R., Rai, R. V. & Karim, A. A. 2010. Mycotoxins in food and feed: Present status and future concerns. *Comprehensive Reviews in Food Science & Food Safety* **9**: 57–81.

Boutigny, A-L., Ward, T. J., Van Coller, G. J., Flett, B., Lamprecht, S. C., O'Donnell, K. & Viljoen, A. 2012. Analysis of the *Fusarium graminearum* species

complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. *Fungal Genetics & Biology* **48**: 914-920.

Brustolin, R., Zoldan, S. M., Reis, E. M., Zanatta, T. & Carmona, M. 2013. Weather requirements and rain forecast to time fungicide application for Fusarium head blight control in wheat. *Summa Phytopathologica* **39**: 248-251.

Bullerman, L. B. & Bianchini, A. 2007. Stability of mycotoxins during food processing. *International Journal of Food Microbiology* **119**: 140-146.

Campbell, C. L. & Madden, L. V. 1990. Introduction to plant disease epidemiology. John Wiley & Sons, Canada.

Chandrashekar, A. & Satyanarayana, K. V. 2006. Disease and pest resistance in grains of sorghum and millets. *Journal of Cereal Science* **44**: 287-304.

Chukwukam O. K., Okoli, I. C., Opara, M. N., Omede, A. A., Ogbuewu, I. P. & Iheshiulor, O. O. M. 2010. The growing problems of mycotoxins in animal feed industry in West Africa: a review. *Asian Journal of Poultry Science* **4**: 122-134.

Cole, R. J. 1986. Etiology of Turkey "X" disease in retrospect: A case for the involvement of cyclopiazonic acid. *Mycotoxin Research* **2**: 3-7.

Council for Agricultural Science and Technology (CAST). 2003. Mycotoxins: Risks in plant, animal, and human systems. Accessed on 25.10.2013 from http://www.trilogylab.com/uploads/Mycotoxin_CAST_Report.pdf

De Kok, A., Spanjer, M., Scholten, J., Rensen, P. & Kearney, G. 2007. Rapid multi-mycotoxin analysis using ACQUITY UPLC and Quattro Premier XE. Waters Application Note, (720001996EN).

De Lucca, A. J. 2007. Harmful fungi in both agriculture and medicine. *Revista Iberoamericana de Micología* **24**: 3-13.

De Wolf, E. D., Madden, L. V & Lipps, P.E. 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. *Phytopathology* **93**: 428–35.

Del Ponte, E. M., Fernandes, J. M. C. & Pavan, W. 2005. A risk infection simulation model for Fusarium head blight of wheat. *Fitopatologia Brasileira* **30**: 634-642.

Department of Agriculture, Forestry and Fishery (DAFF). 2010. Sorghum: Production guideline. Accessed on 10/16/2012 from http://www.nda.agric.za/docs/Brochures/prodGuideSorghum.pdf

Doohan, F.W., Parry, D. W. & Nicholson, P. 1999. Fusarium ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control. *Plant Pathology* **48**: 209–217.

Du Plessis, J. 2008. Sorghum production. Department of Agriculture, ARC-Grain Crops Institute, South Africa.

Ediage, N. E., Mavungu, D. D. J., Monbaliu, S., Van Peteghem, C. & De Saeger, S. 2011. A validated multi-analyte LC–MS-MS method for quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples. *Journal of Agricultural & Food Chemistry* **59**: 5173-5180.

Edwards, S. G. 2004. Influence of agricultural practices on Fusarium infection of cereals and subsequent contamination of grain by trichothecene mycotoxins *Toxicology Letters* **153**: 29-35.

Fry, W. E. 1977. Integrated control of potato late blight-effects of polygenic resistance and techniques of timing fungicide applications. *Phytopathology* **67**: 415 – 420.

Gessner, M. O. & Schmitt, A. L. 1996. Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonised by fungi. *Applied Environmental Microbiology* **62**: 415–419.

Gilbert, J. & Fernando, W. G. D. 2004. Epidemiology and biological control of *Gibberella zeae/Fusarium graminearum*. *Canadian Journal of Plant Pathology* **26**: 464-472.

Hall, A.. J., Bandyopadhyay, R., Chandrashekar, A., & Shewry, P. R. 2000. Technical and institutional options for sorghum grain mold management and the potential for impact on the poor: overview and recommendations. Pages 7-33 in Technical and institutional options for sorghum grain mold management: proceedings of an international consultation, 18-19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Henson, J.M. & French, R. 1993. The polymerase chain reaction and plant disease diagnosis. *Annual Reviews of Phytopathology* **31**: 81–109.

Hooker, D. C., Schaafsma, A. W., & Tamburic-Ilincic, L. 2002. Using weather variables pre-and post-heading to predict deoxynivalenol content in winter wheat. *Plant Disease* **86**: 611-619.

Jambunathan, R., Kherdekar, M. S. & Vaidyas, P. 1991. Ergosterol concentration in mold-susceptible and mold-resistant sorghum at different stages of grain development and its relationship to flavan-4-olst. *Journal of Agriculture & Food Chemistry* **39**: 1866-1870.

Kokkonen, M. K. & Jestoi, M. N. 2009. A multi-compound LC-MS-MS method for the screening of mycotoxins in grains. *Food Analytical Methods **2***:128-140.

Köppen, R., Koch, M., Siegel, D., Merkel, S., Maul., R. & Nehls, I. 2010. Determination of mycotoxins in foods: current state of analytical methods and limitations. *Applied Microbiology & Biotechnology* **86**: 1595–612.

Kranz, J. 1974. Epidemics of plant diseases: mathematical analysis and modeling (Vol. 13). Springer Science & Business Media, Berlin.

Kranz, J. & Hau. B. 1980. Systems analysis in epidemiology. *Annual Review of Phytopathology* **18**: 67-83.

Kumar, V., Basu, M. S. & Rajendran, T. P. 2008. Mycotoxin research and mycoflora in some commercially important agricultural commodities. *Crop Protection* **27**: 891-905.

Lancova, K., Hajslova, J., Kostelanska, M., Kohoutkova, J., Nedelnik, J., Moravcova, H. & Vanova, M. 2008. Fate of trichothecene mycotoxins during the processing: milling and baking. *Food Additives & Contaminants* **25**: 650-659.

Landschoot, S., Waegeman, W., Audenaert, K., Vandepitte, J., Baetens, J. M., De Baets, B., & Haesaert, G. 2012. An empirical analysis of explanatory variables affecting Fusarium head blight infection and deoxynivalenol content in wheat. *Journal of Plant Pathology* **94**: 135-147.

Lauren, D. R. & Smith, W. A. 2001. Stability of the *Fusarium* mycotoxins nivalenol, deoxynivalenol and zearalenone in ground maize under typical cooking environments. *Food Additives & Contaminants* **18**: 1011-1016.

Leffelaar, P. A. & Ferrari, T. J. 1989. Some elements of dynamic simulation. Pages 19–45 in Simulation and systems management in crop protection (Ed. Rabbinge, R., Ward, S.A. & van Laar, H. H.), Wageningen Publishers, Netherlands.

Leslie, J. F., & Summerell, B. A. (eds). 2006. The Fusarium laboratory manual (Vol. 2, No. 10). Blackwell Publishing, Ames, Iowa, USA.

Little, C. R. 2000. Plant responses to early infection events in sorghum grain mold interactions. Pages 169-182 in Technical and institutional options for sorghum grain mold management: proceeding of an international consultation, 18-19 May 2000, ICRISAT, Patancheru, India. (Chandrashekar, A., Bandyopadhyay., R, and Hall, A. J., eds.) Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Lori, G. A., Sisterna, M. N., Sarandon, S. J., Rizzo, I., & Chidichimo, H. 2009. Fusarium head blight in wheat: impact of tillage and other agronomic practices under natural infection. *Crop protection* **28**: 495-502.

Madden, L. V., Hughes, G., & van den Bosch, F. 2007. The study of plant disease epidemics. APS Press, St. Paul, MN.

Magarey, R. D. & Sutton, T. B. 2007. How to create and deploy infection models for plant pathogens. Pages 3-25 in General Concepts in Integrated Pest and Disease Management (Eds. Ciancio, A. & Mukerji, K.G.), Springer, Netherlands.

Marley, P. S. & Ajayi, O. 1999. Sorghum grain mold and the influence of head bug, *Eurystylus oldi*, in West and Central Africa. *Journal of Sustainable Agriculture* **13**: 35-44.

Marroquín-Cardona, A. G., Johnson, N. M., Phillips, T. D. & Hayes, A. W. 2014. Mycotoxins in a changing global environment - a review. *Food & Chemical Toxicology* **69**: 220-230.

Mavhunga, M. 2013. *Fusarium graminearum* mycotoxins associated with grain mold of maize and sorghum in South Africa (Masters dissertation, University of the Free State Bloemfontein, South Africa).

McCormick, S. P., Stanley, A. M., Stover, N. A. & Alexander, N. J. 2011. Trichothecenes: from simple to complex mycotoxins. *Toxins* **3**: 802–814.

Menkir, A., Ejeta, G., Butler, L. G., Melake-Berhan, A. & Warren, H. L. 1996. Fungal invasion of kernels and grain mold damage assessment in diverse sorghum germ plasm. *Plant Disease* **80**: 1399-1402.

Miller, J. D. 1995. Fungi and mycotoxins in grain: Implications for stored product research. *Journal of Stored Production Research* **31**: 1-16.

Montgomery, H. J., Monreal, C. M., Young, J. C., Seifert, K. A., 2000. Determination of soil fungal biomass from soil ergosterol analyses. *Soil Biology & Biochemistry* **32**: 1207–1217.

Moschini, R. C. & Fortugno, C. 1996. Predicting wheat head blight incidence using models based on meteorological factors in Pergamino, Argentina. *European Journal of Plant Pathology* **102**: 211–218.

Mtisi, E. & McLaren, N. W. 2002. Diseases of sorghum and pearl millet in some southern African countries. Pages 427-430 in Sorghum and millet diseases (Ed. Leslie, J. F.), Iowa State Press, Ames.

Musa, T., Hecker, A., Vogelgsang, S. & Forrer, H. R. 2007. Forecasting of Fusarium head blight and deoxynivalenol content in winter wheat with FusaProg. *EPPO bulletin **37***: 283-289.

Navi, S. S., Bandyopadhyay, R., Reddy, R. K., Thakur, R. P., and Yang, X. B. 2005. Effects of wetness duration and grain development stages on sorghum grain mold infection. *Plant Disease* **89**: 872-878.

Nicolaisen, M., Supronienė, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H. & Justesen, A. F. 2009. Real-time PCR for quantification of eleven individual Fusarium species in cereals. *Journal of Microbiological Methods* **76**: 234-240.

O'Donnell, K., Ward, T. J., Geiser, D. M., Kistler, H. C., & Aoki, T. 2004. Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade*. Fungal Genetics and Biology* **41**: 600-623.

Obanor, F. & Chakraborty, S. 2014. Aetiology and toxigenicity of *Fusarium graminearum* and *F. pseudograminearum* causing crown rot and head blight in Australia under natural and artificial infection. *Plant Pathology* **63**: 1218-1229.

Palazzini, J. M., Ramirez, M. L., Torres, A. M. & Chulze, S. N. 2007. Potential biocontrol agents for Fusarium head blight and deoxynivalenol production in wheat. *Crop Protection* **26**: 1702-1710.

Pan African Agribusiness and Agro Industry Consortium (PANAAC). 2012. Sorghum growing, a solution for food security and poverty. Accessed on 26/11/2012 from www.hortfreshjournal.com/archive/sorghum_growing.pdf.

Pestka, J. J. 2007. Deoxynivalenol: toxicity, mechanisms and animal health risks. *Animal Feed Science & Technology* **137**: 283-298.

Pioneer. 2014. Grain Sorghum Management. Accessed on 03/09/2015 from https://www.pioneer.com/CMRoot/Pioneer/US/Non_Searchable/agronomy/cropfocus_pdf/grain_sorghum_mgmt.pdf.

Prandini, A., Sigolo, S., Filippi, L., Battilani, P. & Piva, G. F. 2009. Review of predictive models for Fusarium head blight and related mycotoxin contamination in wheat. *Food and Chemical Toxicology* **47**: 927-931.

Prom, L. K., Waniska, R. D., Kollo, A. I. & Rooney, W. L. 2003. Response of eight sorghum cultivars inoculated with *Fusarium thapsinum, Curvularia lunata* and a mixture of the two fungi. *Crop Protection* **22**: 623-628.

Rahmani, A., Jinap, S. & Soleimany, F. 2009. Qualitative and quantitative analysis of mycotoxins. *Comprehensive Reviews in Food Science & Food Safety* **8**: 202-251.

Raikes, C. & Burpee, L. L. 1998. Use of multispectral radiometry for assessment of Rhizoctonia blight in creeping bentgrass. *Phytopathology* **88**: 446-449.

Rampho, E. T. 2005. Sorghum bicolor (L.) Moench. Accessed on 16/10/2012 from http://www.plantzafrica.com/plantqrs/sorghum.htm.

Rao, V. T., Reddy, P. S., Reddy, B. V. S. & Kumar, A. A. 2012. Ergosterol as an indicator of mold resistance in white and colored grain sorghum hybrids. *Crop Protection* **37**: 7-12.

Reddy, B. V. S., Kumar, A. A. & Reddy, P. S. 2010. Recent advances in sorghum improvement research at ICRI- SAT, Kasetsart. *Natural Science* **44**: 499-506.

Rheeder, J. P., Shephard, G. S., Vismer, H. F., & Gelderblom, W. C. A. 2009. Guidelines on mycotoxin control in South African foodstuffs: from the application of the Hazard Analysis and Critical Control Point (HACCP) system to new national mycotoxin regulations. *Medical Research Council Policy Brief.* Accessed on 26/11/2012 from http://www.mrc.ac.za/policybriefs/Mycotoxinguidelines.pdf

Richard, J. L. 2007. Some major mycotoxins and their mycotoxicoses-an overview. *International Journal of Food Microbiology* **119**: 3-10.

Rossi, V., Giosuè, S., Pattori, E., Spanna, F., & Del Vecchio, A. 2003. A model estimating the risk of Fusarium head blight on wheat. *EPPO Bulletin* **33**: 421-425.

South African Grain Information Service (SAGIS). 2014. Crop estimates committee: The final production estimate for summer crops for 2014. Accessed on 29.06.2015 from www.sagis.org.za/CEC

Santini, A., Ferracane, R., Somma, M. C., Aragon, A & Ritienia, A. 2009. Multitoxin extraction and detection of trichothecenes in cereals: an improved LC-MS-MS approach. *Journal of the Science of Food & Agriculture* **89**: 1145–1153.

Sarver, B. A., Ward, T. J., Gale, L. R., Broz, K., Kistler, H. C., Aoki, T., Nicholson, P., Carter, J. & O'Donnell, K. 2011. Novel Fusarium head blight pathogens from Nepal and Louisiana revealed by multilocus genealogical concordance. *Fungal Genetic Biology* **12**: 1096-107.

Schaad, N. W. & Frederick, R. D. 2002. Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology* **24**: 250-258.

Schaafsma, A. W. & Hooker, D.C. 2007. Climatic models to predict occurrence of Fusarium toxins in wheat and maize. *International Journal of Food Microbiology* **119**: 116–25.

Seitz, L. M., Mohr, H. E., Burroughs, R. & Sauer, D. B. 1977. Ergosterol production as an indicator of fungal invasion in grains. *Cereal Chemistry* **54**: 1207-1217.

Seitz, L. M., Sauer, D. B., Burroughs, R., Mohr, H. E. & Hubbard, J. D. 1979. Ergosterol as a measure of fungal growth. *Phytopathology* **69:** 1202-1203.

Shah, D. A., Molineros, J. E., Paul, P. A., Willyerd, K. T., Madden, L. V. & De Wolf, E. D. 2013. Predicting Fusarium head blight epidemics with weather-driven pre-and post-anthesis logistic regression models. *Phytopathology **103***: 906-919.

Sharma, R., Thakur, R. P., Senthilvel, S., Nayak, S., Reddy, S. V., Rao, V. P. & Varshney, R. K. 2011. Identification and characterization of toxigenic *Fusaria* associated with sorghum grain mold complex in India. *Mycopathologia* **171**: 223–230.

Sheorain, V., Banka, R. & Chavan, M. 2000. Ethanol production from sorghum. Pages 228– 239 in Technical and institutional options for sorghum grain mold management: proceedings of an international consultation, 18–19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Spanjer, M. C., Rensen, P. M. & Scholten, J. M. 2008. LC–MS-MS multi-method for mycotoxins after single extraction, with validation data for peanut, pistachio, wheat, maize, cornflakes, raisins and figs. *Food Additives & Contaminants* **25**: 472-489.

Summerell, B. A. & Leslie, J. F. 2011. Fifty years of *Fusarium*: how could nine species have ever been enough? *Fungal Diversity* **50**: 135-144.

Sweeney, M. J. & Dobson, A. D. W. 1998. Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. *International Journal of Food Microbiology* **43**: 141-158.

Taylor, J. R. N. 2003. Overview: Importance of sorghum in Africa. Accessed on 26/11/2012 from: http://www.afripro.org.uk/papers/Paper01Taylor.pdf.

Taylor, J. R. N., Tilman, J. S. & Bean, S. R. 2006. Novel food and non-food uses for sorghum and millets. *Journal of Cereal Sciences* **44**: 252-271.

Teng, P. S. & Bowen, K. L. 1985. Disease modelling and simulation. Pages 435-466 in The Cereal Rusts. Volume II. (Eds. Roelfs, A. P. & Bushnell, W. R.) Academic Press, New York

Thakur, R. P., Reddy, B. V. S., Indira, S., Rao, V. P., Navi, S. S., Yang, X. B. & Ramesh, S. 2006. Sorghum grain mold: Information Bulletin No. 72. International Crops Research Institute for the Semi-Arid Tropics. Patancheru 502324, Andhra Pradesh, India: 32 pp. ISBN 92-9066-488-6.

Trigo-Stockli, D. M., Deyoe, C. W., Satumbaga, R. F. & Pedersen, J. R. 1996. Distribution of deoxynivalenol and zearalenone in milled fractions of wheat. *Cereal Chemistry* **73**: 389-391.

Turcotte, J. C., Hunt, P. J. & Blaustein, J. D. 2005. Estrogenic effects of zearalenone on the expression of progestin receptors and sexual behavior in female rats. *Hormones & Behaviour **47***: 178-184.

Turner, N. W., Subrahmanyam, S. & Piletsky, S. A. 2009. Analytical methods for determination of mycotoxins: a review. *Analytica Chimica Acta* **632**: 168-180.

Van der Fels-Klerx, H. J. 2014. Evaluation of performance of predictive models for DON in wheat. *Risk Analysis* **34**: 380–90.

Van der Fels-Klerx, H. J. Burgers, S. L. G. E. & Booij, C. J. H. 2010. Descriptive modeling to predict deoxynivalenol in winter wheat in The Netherlands. *Food Additives & Contaminants* **27**: 636-643.

Van der Lee, T., Zhang, H., van Diepeningen, A., & Waalwijk, C. 2015. Biogeography of *Fusarium graminearum* species complex and chemotypes: A review. *Food Additives & Contaminants*: **32**: 453-460.

Van Maanen, A. & Xu, X. M. 2003. Modelling plant disease epidemics. *European Journal of Plant Pathology* **109**: 669–82.

Vendl, O., Berthiller, F., Crews, C. & Krska, R. 2009. Simultaneous determination of deoxynivalenol, zearalenone, and their major masked metabolites in cereal-based food by LC–MS–MS. *Analytical & Bioanalytical Chemistry* **395**: 1347-1354.

Waalwijk, C., Van der Heide, R., De Vries, P. M., Van der Lee, T. A. J., Schoen, C. D., Costrel-de Corainville, G., Häuser-Hahn, I., Kastelein, P., Köhl, J., Lonnet, P., Demarquet, T. & Kema, G. H. J. 2004. Quantitative detection of *Fusarium* species in wheat using TaqMan. *European Journal of Plant Pathology* **110**: 481-494.

Wang, J.-H., Ndoye, M., Zhang, J.-B., Li, H.-P. & Liao, Y.-C. 2011. Population structure and genetic diversity of the *Fusarium graminearum* species complex. *Toxins* **3**: 1020 –1037.

Waniska, R. D. 2000. Structure, phenolic compounds, and antifungal proteins of sorghum caryopses. Pages 18-19 in *Technical and institutional options for sorghum grain mold management: proceedings of an international consultation*, 18–19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Ward, E., Foster, S. J., Fraaije, B. A. & McCartney, H. A. 2004. Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *Annals of Applied Biology* **145**: 1-16.

Warrick B. E. 2000. How a Sorghum Plant Develops, Texas AgriLife Research & Extension Center. Accessed on 03/09/2015 from http://sanangelo.tamu.edu/extension/agronomy/agronomy-publications/how-a-sorghum-plant-develops/

Wegulo, S. N. 2012. Factors influencing deoxynivalenol accumulation in small grain cereals. *Toxins* **4**: 1157-1180.

WHO/FAO. 2012. Codex alimetarius commission: Discussion paper on fungi and mycotoxins in sorghum. Accessed on 27/11/2012 from www.codexalimentarius.net.

Woloshuk, C. P. & Shim, W. B. 2013. Aflatoxins, fumonisins, and trichothecenes: a convergence of knowledge. *FEMS Microbiology Reviews* **37**: 94-109.

Woolf, A. 2000. Witchcraft or mycotoxin? The Salem witch trials. *Journal of Toxicology: Clinical Toxicology* **38**: 457-460.

Zheng, M. Z., Richard, J. L. & Binder, J. 2006. A review of rapid methods for the analysis of mycotoxins. *Mycopathologia* **161**: 261-273.

Zoldan, S. M. 2008 (abs). Risk areas, anthesis characterization in winter cereals and warning system to head blight in wheat. Doutorado em fitopato- logia. Universidade de Passo Fundo. Passo Fundo.

Chapter 2

Stability of sorghum cultivar responses to the *Fusarium graminearum* species complex, grain mold severity and mycotoxin accumulation

Abstract

Grain mold pathogens of sorghum cause reductions in grain size, kernel mass, nutritional quality and complete destruction of the entire grain, resulting in lower yield, milling quality of sorghum grains and poor germination. Starch granules, soluble carbohydrates and proteins are degraded which reduces fermentation quality. In addition, certain grain mold fungi are capable of producing mycotoxins which are potentially harmful to humans and livestock. Grain mold of sorghum is attributed to a complex of fungi, including *Alternaria* spp., *Curvularia* spp., *Phoma* spp., and *Fusarium* spp. The latter genus includes the *Fusarium graminearum* species complex (*Fg*SC) which is associated specifically with economically important sorghum grain mycotoxins, deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) and this was therefore, the focus of this study. Grain from nine sorghum cultivars, harvested from Cedara (2010/11, 11/12 and 12/13) and Alma (2012/13 and 13/14) were evaluated for grain mold severity and the stability of the grain mold response over changing environmental conditions was quantified. Evaluation criteria included threshed grain disease ratings (tgdr), ergosterol analysis, *Fg*SC DNA concentration and the mycotoxins DON, NIV and ZEA. Cultivar tgdr ranged from 1.58 in NS5511 to 2.66 in PAN8553W on a 0-5 rating scale. Ergosterol content in grain ranged from 30.90 $\mu g.g^{-1}$ in NS5511 to 86.43 $\mu g.g^{-1}$ in PAN8609. There was no correlation between tgdr and ergosterol content (R^2=0.46, df=7). Ergosterol is regarded as the more reliable criterion since it measures total fungal biomass in grains while tgdr can be confounded by superficial colonisation. Real time PCR was used to determine the specific quantities of *Fg*SC present in the grains**.** *Fg*SC DNA content in grain ranged from 5.52 $ng.\mu l^{-1}$ in PAN8625 to 55.43 $ng.\mu l^{-1}$ in PAN8806. Only three of the 162 grain samples had DON concentrations exceeding 10 $\mu g.kg^{-1}$, therefore DON was excluded from further analysis. However, NIV and ZEA were present in all but four and two samples, respectively. AMMI analysis of *Fg*SC DNA concentrations, NIV and ZEA indicated a relatively stable response in cultivars to

changing environments with most cultivars yielding an IPCA1 score <1. PAN8706W, PAN8553W and PAN8806 clustered together and indicated a higher potential to accumulate NIV at Cedara. In contrast, PAN8625, NS5655, NS5511 and PAN8816 had a stable response to NIV accumulation. Furthermore NS5655 showed the most stable response to ZEA production. Robust regression was applied to quantify the rate of NIV increase relative to the *FgSC* DNA concentration within grains. Cultivars differed in the relationship between the accumulation of mycotoxins and quantity of *Fg*SC DNA, demonstrating that host genotype influences mycotoxin production despite similar colonisation levels. Results indicate the need for the inclusion of environmental variation in the screening and selection for resistance to sorghum grain molds in sorghum genotypes, to ensure quality grain and human and animal health.

1. Introduction

Sorghum is one of the most widely cultivated grains in Africa, providing a staple diet for many communities in semi-arid and sub-tropical countries (Belton & Taylor, 2004). Sorghum grain mold (SGM) proliferates in these regions, particularly where sorghum matures during wet periods (Bandyopadhyay *et al.,* 2000). The *Fusarium graminearum* species complex (*FgSC*) is one of the most important SGM causal agents. The species complex is globally distributed and has the ability to produce economically important mycotoxins (Tesfaendrias *et al.,* 2011).

Symptoms associated with *FgSC* on sorghum include white to pink discolorations and physical deterioration of grains (Chandrashekar & Satyanarayana, 2006). However, *FgSC* is mostly regarded as important for the mycotoxins which are produced, the most important being deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA) (Thakur *et al.,* 2006; Köppen *et al*., 2010). Deoxynivalenol and NIV are associated with reproductive anomalies, immune response inhibition and intestinal haemorrhaging. Zearalenone is an estrogenic toxin which influences the fertility and reproductive ability of livestock. European Union legal maximum limit associated with DON is 500 $\mu g.kg^{-1}$ for processed cereals. The lowest maximum limit for ZEA is 100 $\mu g.kg^{-1}$ in cereal based products for human consumption. Currently no worldwide legislation has been set for NIV, however Japan has set a total daily allowance for NIV of 0.4 $\mu g.kg^{-1}$ of body weight per day (CAST, 2003; Köppen *et al*., 2010; EFSA, 2013).

Agronomic factors that influence inoculum survival include crop rotation, weed management, nitrogen fertilization, soil structure and biota. The onset of an epidemic depends on the capability of the primary inoculum to overwinter in soil and crop residues (Aldred & Magan, 2004; Edwards, 2004; Audenaert *et al*., 2013). High rainfall and relative humidity (75-100%) and moderate temperatures (21-27°C) during grain development promote colonisation of grain by *FgSC* (Melake-Berhan *et al.,* 1996; Trigo-Stockli *et al*., 1996; Reddy *et al*., 2010). Seasonal variation, and thus short term fluctuations in these variables, has been observed to be more predominant in promoting variation in mycotoxin levels than geographical variation in a region (Landschoot *et al*., 2012).

The epidemiological application of the *FgSC* lifecycle, the host plant and interactions with factors that promote inoculum survival, fungal colonisation, development and concomitant mycotoxin production and accumulation can be applied into the mathematical and statistical models that can assist in disease risk management (Campbell & Madden, 1990; Prandini *et al.,* 2009). The purpose of this study was to evaluate grain mold severity in the most widely planted commercial sorghum cultivars grown locally under diverse environmental conditions, with particular reference to *FgSC*, and the mycotoxins DON, NIV and ZEA using (i) threshed grain disease ratings to measure disease severity visually, (ii) ergosterol quantification as a measure of general grain colonisation by grain mold pathogens and (iii) *FgSC* DNA quantification to determine pathogen-specific internal fungal biomass. A further goal was to quantify the stability of genotype x environment (GxE) interactions using AMMI analysis and the above techniques as disease and mycotoxin determinates.

2. Methods and Materials

Field Samples

Sorghum grain mold evaluation blocks, consisting of nine cultivars, were planted at Cedara (KwaZulu Natal) over three seasons (2009/2010, 2010/2011 and 2011/2012) and at Alma (Limpopo) over two seasons (2012/13 and 2013/14). All plantings at Cedara were done in mid-November. Alma 2012/13 consisted of two plantings i.e. mid-November and mid-December while Alma 2013/14 had a single planting in mid-November. Sorghum cultivars at both localities included four red cultivars (PAN8806, PAN8609, PAN8816, PAN8420), three brown cultivars (NS5511, NS5655, PAN8625) and two white cultivars (PAN8533W and PAN8706W) to include a range of plant and grain characteristics. The nine cultivars were planted in a randomized block design with three replicates at both Cedara and Alma, over all the respective plantings.

Plots were fertilized with 600 $kg.ha^{-1}$ 2:3:2 (28) prior to planting. Herbicides included pre-emergence DUAL GOLD (Metalachlor) applied at 500 $ml.ha^{-1}$ and post-emergence applications of BASAGRAN (480 $g.l^{-1}$ Bentazone) at 1.5 $l.ha^{-1}$ as required. Stalk-borers and aphids were controlled using 250 ml DECIS per hectare (deltamethrin, 25 $g.l^{-1}$ a.i.) at the observed onset of infestations.

A total of 162 sorghum grain samples from Cedara and Alma were collected at maturity (12% water) for this study.

Threshed Grain Disease Rating

At maturity (approximately 12% grain moisture content) panicles were hand harvested and threshed. A threshed grain disease rating (tgdr) was conducted on each of the sorghum grain samples using descriptive as well as qualitative estimates of visual disease according to Frederiksen *et al*. (1991), where 0 = none (0%), 2 = scant (1-10%), 3 = moderate (11-25%), 4 = considerable (26-50%) and 5 = significant (>50%).

Quantification of Total Fungal Biomass

The modified method of Jambunathan *et al.* (1991) was used to quantify total fungal biomass in grain using ergosterol concentration. Ergosterol was extracted from the grains of all nine cultivars. Five grams of grain from each cultivar-replicate were ground into a fine powder using a Mellerware® Coffee Bean Grinder. The powder was added to 25 ml of extraction methanol (ROMIL-SpS™) in a 50 ml test tube with a screw cap and mixed at 1900 cycles.min^{-1} with a Heidolph MultiReax Shaker (Labotec) for 30 min. The mixture was allowed to settle and 12.5 ml of the clear supernatant was removed using a pipette and added to a 50 ml test tube with a screw cap, containing 1.5 g of potassium hydroxide pellets (KOH, Merck Pty Ltd). The mixture was stirred in a vortex mixer (Vortex Genie 2, Scientific Industries) to dissolve the KOH. Five ml of n-hexane (Merck Pty Ltd) was then added and the mixture was incubated for 30 min in a water bath at 75°C and subsequently, allowed to cool to room temperature. During cooling, test tubes were covered with foil to prevent ergosterol degradation as ergosterol is light-sensitive and degrades when exposed to UV light (Robine *et al*., 2005). Distilled water (2.5 ml) was added, the mixture was shaken with a vortex mixer and the hyper-thermal reaction was cooled to room temperature. The upper hexane layer was transferred to a screw cap test tube. Five ml n-hexane was added to the remaining aliquot in the screw-cap test tube and mixed well. The hexane layer was again removed and added to the earlier aliquot. This step was repeated. The hexane extract was evaporated in the glass test tube until dry in a water bath at 75°C. The residue was re-suspended in 2.5 ml

HPLC-grade methanol (Sigma-Aldrich) and filtered through 0.45 µl syringe filter. The filtrate was analysed using high performance liquid chromatograph (HPLC) with a SIL-20A auto sampler (Perkin Elmer). The extract was loaded onto a reverse phase column (C18 125 A 10 µm particle size, 150 x 4.6 mm) at 50°C. The mobile phase was methanol:water (96:4) at a flow rate of 1.2 $ml.min^{-1}$. Standard ergosterol (>75% assay; Sigma-Aldrich) was used to calibrate the equipment. Ergosterol was determined from the peak area determined at 282 nm using a Perkin Elmer PDA UHPLC detector at a retention time of approximately 7 minutes.

Quantification of Fusarium graminearum species complex

DNA extraction

Reference strains were purchased from the Plant Protection Research Institute of the Agricultural Research Council (ARC-PPRI) as well as the PROMEC Unit of the Medical Research Council (MRC) culture collections, South Africa.

Total DNA was extracted from 400 mg milled grain samples and 100 mg fungal mycelia of the reference cultures. Genomic DNA from milled grain samples as well as fungal mycelia was extracted using a Wizard ® Genomic DNA Purification Kit (Promega Corporation, USA) according to the manufacturer's instructions. The DNA concentrations and purity were determined using a NanoDrop® spectrophotometer (ND1000) (Thermo, Waltham, MA, USA) by absorbance at 260 nm (OD260). All the DNA samples were diluted to 10 $ng.µl^{-1}$ and aliquots of 100 µl sub-samples were stored at -20°C.

Quantitative Real Time PCR

Quantitative detection of *F. graminearum* in the sorghum grains was determined by real time quantitative PCR (qPCR) as described by Nicolaisen *et al.* (2009). Primers used for the detection of *F. graminearum* were FgramB379 fwd (CCATTCCCTGGGCGCT) and complimentary, FgramB411 rev (CCTATTGACAGGTGGTTAGTGACTGG) (Inqaba Biotechnical Industries (PTY) Ltd) as developed by Nicolaisen *et al.* (2009). Real-time PCR reactions were carried out in a total volume of 10 µl consisting of 5 µl iTaq™ Universal SYBR® Green Mix (BIO-RAD), 0.5 µl FgramB379 fwd (2 µM) and 0.5 µl FgramB411 rev (2 µM), 3.2 µl

Ultrapure DNASE and RNASE Free Water (18.2 M, Bioline) and 0.8 µl template DNA (10 ng.µl^{-1}). Each qPCR plate contained a triplicate of each biological template DNA, no template control, positive control and a standard curve of matrix DNA. The standard curve matrix was made up of *F. graminearum* DNA diluted four-, 16-, 64-, 256-fold in sorghum free of *F. graminearum* DNA (10 ng.µl^{-1}).

Quantitative PCR was performed on a Rotor-gene TM 6000 (Corbett Life Science) using the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s, and a melting curve analysis from 72°C to 95°C, increasing by 1°C each step.

Mycotoxin Detection and Quantification

The modified protocol of Small *et al.* (2012) was used to extract DON, NIV and ZEA. Five gram sub-samples of grain were milled and placed into 50 ml Falcon tubes and 20 ml of methanol/water (70:30 v/v) was added. The flasks were shaken at 200 rpm on a Heidolph MultiReax Shaker (Labotec, South Africa) for 30 min at room temperature. The samples were centrifuged for 10 min at 4000 rpm. A syringe was used to draw up 2 ml of the supernatant which was filtered with a 0.45 µm RC syringe filter (Acrodisc ® PALL PSF Syringe Filter, Premium Glass Fiber Prefilter). The filtered supernatant was placed into a 2 ml Eppendorf and left overnight at 4°C. The samples were centrifuged for 10 min at maximum acceleration (*g*). Nivalenol and ZEA samples were diluted ten-fold by placing 100 µl supernatant to 900 µl distilled water and vortexed prior to analysis. Deoxynivalenol samples remained undiluted and were placed in 1.8 µl vials.

Standards of DON, NIV and ZEA were obtained from Sigma-Aldrich. A known mycotoxin free sorghum sample was extracted using the above mentioned protocol and 1 ml undiluted supernatant was added to the 1.8 ml LC-MS-MS vial. This was done to take into consideration the matrix effect associated with mycotoxin extractions. The 1 ml mycotoxin free matrix was evaporated under a gentle air flow. The evaporated samples were reconstituted with a calibration standard solution ranging from 1600 µg.kg^{-1} to 50 µg.kg^{-1} for DON and NIV and 3200 µg.kg^{-1} and 50 µg.kg^{-1} for ZEA. The samples were vortexed prior to analysis. The samples were extracted and reconstituted in triplicate to ensure repeatability and accuracy. The

concentrations of the DON, NIV and ZEA standards were determined at the University of the Free State, Department of Microbial, Biochemical and Food Biotechnology. The lowest level of quantification for DON, NIV and ZEA was 25 $\mu g.kg^{-1}$.

Samples were analysed using a 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer (AB Sciex) and Shimadzu UFLC stack with LC-20AB binary pump and SIL-20A HT autosampler as front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software.

Twenty microliters of each sample was separated on a C18 (150 x 2.1 mm x 5 µ, Discovery C18, Supelco) column at a flow rate of 300 $\mu l.min^{-1}$ using a stepwise gradient between 10mM ammonium acetate (mobile phase A) and methanol 10 mM ammonium acetate (mobile phase B). The column was equilibrated and loaded at 2%B, rapidly increased to 75%B and maintained for 4 min, followed by re-equilibration at 2% for a total runtime of 9 min. Eluting analytes were ionised in negative electrospray mode with a 4500 V ion spray voltage and 500°C heater temperature to evaporate excess solvent, 60 psi nebuliser gas, 60 psi heater gas and 25 psi curtain gas.

A targeted Multiple Reaction Monitoring (MRM) workflow was followed on the instrument to analyse the sample. During an MRM scan type the instrument is used in triple quadrupole mode where every ionised analyte (the precursor) eluting off the column is fragmented in the collision cell to produce fragment masses. A set of masses, the precursor mass and one fragment mass constitutes a transition. The instrument jumps between different transitions in an MRM transition list during an analysis cycle, each cycle typically lasting less than a second. If a transition is detected the instrument's response is registered and this ion intensity value is plotted as a chromatogram. Additional mass spectrometer settings are given in Table 1.

All compound and source dependant parameters were optimised using compound optimization in Analyst 1.5.2. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the other transitions are used as qualifiers. The qualifier serves as an additional level of

confirmation for the presence of the analyte and the retention time for these three transitions needs to be the same.

Meteorological Data

Total radiation (MJ/m2), average maximum and minimum temperature (°C), average relative humidity (%) and total rainfall (mm) for the 2009/2010, 2010/2011, 2011/2012, 2012/13 and 2013/14 seasons for the respective localities were monitored in this study. The data were supplied by the South African Weather Services (SAWS) and by the ARC-Institute for Soil Water and Climate's meteorology office.

Data Analysis

Genstat 15th Edition (2012) was used to conduct analysis of variance (ANOVA) on all variables measured as well as Additive Main Effect and Multiplicative Interaction analysis (AMMI). Fishers unprotected test was used to determine the least significant difference's (LSD's) at the 5% significance level to compare means. Correlations between weather data and disease parameters were conducted using Pearson's Correlation (Hintze, 2007). Ordinary least square regression analysis (SAS 9.4, 2013) was used to determine the relationship between variables. Where data were contaminated by outliers and influential observations, robust regression was applied (Hintze, 2007; SAS 9.4, 2013).

3. Results

Threshed grain disease rating

Analysis of variance (ANOVA) of the threshed grain disease ratings (tgdr; Table 2A) indicated significant differences due to cultivar and environment but no significant cultivar x environment interaction effect was recorded. Threshed grain disease ratings were lowest in PAN8706W (mean tgdr=1.3) and NS5511 (mean tgdr=1.5) compared with the highest ratings in PAN8625 (mean tgdr =2.7) and PAN8553W (mean tgdr=2.6) (Table 3). Fischer's LSD ($P<0.05$) applied to cultivar effects indicated that both extreme groups were significantly different from the remaining five cultivars which clustered together in a single grouping (Table 3). The highest threshed grain disease ratings (mean tgdr=2.3) was recorded during the 2011/2012

season at Cedara while the lowest threshed grain disease rating was recorded in 2012/13 at Alma during the first planting (mean tgdr=1.4) (Table 3). With the exception of these two extremes, all threshed grain disease ratings in the remaining seven plantings did not differ significantly from one another. Based on partitioning of total sum of squares genotypes accounted for 34% of observed variation in threshed grain disease rating and environment for 14%.

Quantification of Total Fungal Biomass

Highly significant (R^2=0.98) linear relationships were recorded between detected peak areas and the actual concentrations of the standards to provide a reliable standard curve. Analysis of variance (Table 2C) indicated significant differences in the ergosterol content of grains from the different cultivars. NS5511 had the lowest mean ergosterol concentration (mean ergosterol concentration=30.9 $\mu g.g^{-1}$) although this did not differ from PAN8625, PAN8816, PAN8553W and PAN8420 according to Fischer's LSD (P<0.05) (Table 3). The highest fungal biomass was associated with PAN8609 (mean ergosterol concentration=86.43 $\mu g.g^{-1}$) although this cultivar clustered with PAN8420, NS5655, PAN8706W and PAN8806 (P<0.05) (Table 3). No significant differences in ergosterol concentrations across localities were recorded (Table 2C). Analysis of variance for ergosterol concentration indicated no significant GxE interaction ($P=0.73^{ns}$).

Quantification of Fusarium graminearum species complex

Regression analysis yielded a highly significant relationship (R^2=0.99) between the matrix-matched standards for *Fg*SC DNA and actual standard concentrations, thus providing a reliable standard curve against which to quantify DNA extracted from grain from the nine test cultivars. No *Fg*SC DNA was detected in the "no-template control" and *Fg*SC DNA was detected in the positive control.

Analysis of variance (Table 2A) indicated significant differences in the *FgSC* DNA content of grains of different cultivars (P<0.001). The cultivars which had the lowest *FgSC* DNA quantities were PAN8625 (mean *FgSC* DNA concentration=3.8 $pg.mg^{-1}$) and NS5511 (mean *FgSC* DNA concentration=10.0 $pg.mg^{-1}$; Table 4). *FgSC* DNA concentrations in PAN8625 and NS5511 did not differ significantly (P<0.05) from one another but were significantly different from all the remaining cultivars. The *Fg*SC

DNA content in the remaining cultivars were not significantly different from one another as indicated by Fischer's LSD (Table 4).

There were significant differences in *FgSC* DNA concentration across environments (P<0.001). Cedara 2012/13 was significantly more conducive to colonisation of grain by *Fg*SC (mean *FgSC* DNA concentration=145.3 pg.mg^{-1}) while Cedara 2009/10 was least favourable for grain colonisation by the *Fg*SC (mean *FgSC* DNA concentration=3.0 pg.mg^{-1}; Table 4). A significant GxE interaction (P<0.001) was observed with the AMMI analysis of variance (Table 2A) and IPCA1 scores in most cultivars exceeding 1 (Figure 1). NS5511 and PAN8625 responded to the lower potential environments which enhanced the resistance displayed by these cultivars (Figure 1). The response in PAN8806 was higher at the Cedara 2012/13 environment which enhanced the susceptibility to *Fg*SC. This tendency was also evident in PAN8816, PAN8553W and NS5655 (Figure 1). PAN8609, PAN8420 and PAN8706W displayed stable responses to the *Fg*SC across environments as indicated by low IPCA1 scores.

Mycotoxin Detection and Quantification

Regression analyses provided highly significant relationships between the matrix matched standards for DON, NIV and ZEA (R^2=0.98, R^2=99 and R^2=0.84, respectively) and hence reliable standard curves for the quantification of these mycotoxins in sorghum grain. DON was detected in 23 of the 54 interactions although only three yielded concentrations exceeding 10 µg.kg^{-1}, DON was therefore excluded from further analysis due to an absence of variance.

NIV was absent in four of the 54 GxE interactions while two were ZEA free. Analysis of variance (Table 2B) indicated significant differences (P<0.001) in NIV accumulation in grains from the different cultivars. According to Fischer's LSD analysis NIV concentrations in PAN8806, PAN8706W and PAN8533W did not differ significantly from one another but were significantly higher than the remaining cultivars, indicating two distinct response groups (Table 5). The highest NIV accumulation was observed was PAN8806 (mean NIV concentration=11.9 µg.kg^{-1}) while the lowest NIV accumulation (mean NIV concentration=4.0 µg.kg^{-1}) was recorded in PAN8625. There were significant differences (P<0.001) in NIV

concentrations across seasons (Table 2B). NIV accumulation at the Alma environments did not differ significantly from one another but were significantly different from the Cedara environments. Cedara 2009/10 and 2011/12 were not significantly different from one another but were significantly different from Cedara 2010/11 (Table 5). The environment which had the lowest NIV quantity in the grains (mean NIV concentration=2.9 $\mu g.kg^{-1}$) was the Alma 2012/13 season first planting while the highest NIV accumulation was recorded at Cedara 2011/12 (mean NIV concentration=13.9 $\mu g.kg^{-1}$) (Table 5).

As with *Fg*SC levels, AMMI analysis of variance indicated significant GxE interactions for NIV level in grain ($P<0.001$). Only IPCA1 scores were significant and biplots (Figure 2) indicated that PAN8625, NS5655, NS5511 and PAN8816 clustered together and responded to low disease potential environments by producing significantly lower levels of NIV were produced whereas PAN8706W, PAN8553W and PAN8806 cluster together and responded to the higher disease potential, Cedara environments, resulting in higher NIV accumulation. Based on the ratios of the sum of squares, 29.5% of the variation in NIV concentration can be attributed to environmental variation and 10.0% due to cultivar (Table 2B).

Analysis of variance (Table 2B) indicated significant differences ($P<0.001$) in ZEA accumulation in grains from different cultivars. Based on Fischer's LSD, cultivars grouped into two distinct groups with PAN 8652, NS5655, PAN8816 and NS5511 displaying the lowest ZEA concentrations within the range of 2.3-6.6 $\mu g.kg^{-1}$ and PAN8420, PAN8553W and PAN8706W with higher concentrations within the range of 10.31-11.82 $\mu g.kg^{-1}$ (Table 6).

Significant differences ($P<0.001$) in ZEA concentrations across seasons (Table 2B) were recorded. ZEA accumulation at Cedara 2009/10 and Cedara 2010/11 did not differ significantly and were the most conducive environments for ZEA production (mean zearalenone concentration=13.0 and 13.8 $\mu g.kg^{-1}$, respectively) (Table 6). The drier, warmer Alma environments clustered together and yielded the lowest levels of ZEA (range=2.3-3.8 $\mu g.kg^{-1}$) (Table 6). AMMI analysis of variance indicated significant ($P<0.001$) GxE interaction effects on ZEA concentration. Based on AMMI model biplots (Figure 3) most cultivars reacted to the lower potential environments with reduced levels of ZEA while PAN8816 responded strongly to the high potential

conditions at Cedara 2010/11. AMMI ANOVA indicated significant responses to a second principle component with most genotypes yielding a strong response with IPCA score ≥ 2 (Figure 4). The weakest IPCA scores were recorded in PAN8816 and PAN8553W.

NS5655 showed the most stable response to ZEA production (Figure 3). Based on the ratios of the sum of squares, 13.5% of the variation in ZEA concentration can be attributed to environmental variation and 6.7% due to cultivar (Table 2B).

Ordinary linear regression analysis conducted on pooled data from all cultivars and environments gave no significant relationships between the colonisation of grain by *Fg*SC as indicated by qPCR and NIV and ZEA levels in grain. Improved results were obtained with robust regression using the Tukey-Biweight model (Hintze, 2007), where a significant relationship was obtained between *Fg*SC-target DNA concentration and NIV (R^2=0.78, 52 df). However, no relationship was recorded with ZEA (R^2=0.04, 52 df). Further improvement was obtained by splitting data according to cultivar where, despite reduced df, robust regression yielded highly significant relationships between *Fg*SC target DNA concentration and NIV (the exception being PAN8625; Figure 5) and ZEA (the exception being PAN8816; Figure 6).

Three distinct response groups in NIV production were observed within cultivars. The F-test used to compare regression models from different data sets where,

$$F = [\{(SScombined - (SSreg1 + SSreg2))/(DFcombined - (DFreg1 + DFreg2))\}/\{(SSreg1 + SSreg2)/(DFreg1 + DFreg2)\}]).$$

The rate of NIV increase relative to the *FgSC* DNA concentration in PAN8806 was significantly higher (P<0.05) than PAN8553W, PAN8706W, PAN8609 and PAN8420 that grouped together. A significantly reduced rate of NIV production in response to colonisation by *FgSC* was recorded in NS5511, PAN8816, PAN8625 and NS5655. (Figure 5). Although less distinct, a similar response was recorded with ZEA production where the response rate to colonisation in NS5511 and NS5655 was significantly greater than the remaining cultivars (Figure 6). These results indicated that mycotoxin production is not only a result of fungal colonisation but also significantly influenced by host genotype, despite similar colonisation levels.

Correlation analysis was conducted to determine relationships between disease/mycotoxin parameters and monthly mean meteorological data for the months January to March for the respective November plantings and February to April for the Alma 2012/13 second planting. These periods correspond with flowering and grain fill. No significant correlations were recorded between threshed grain rating and meteorological factors (Table 7). Significant positive correlations were recorded between ergosterol and *Fg*SC content of grain and mean maximum and minimum temperatures (MaxT; MinT) and mean total radiation (RAD), while a negative relationship was recorded with mean relative humidity (avgRH) during January of the respective seasons. In contrast, significant negative correlations were recorded between NIV and ZEA content of grain and mean MaxT and MinT and mean RAD, while a positive relationship was recorded with mean avgRH during January of the respective seasons. These disease and mycotoxin parameters were similarly correlated with mean avgRH during February of the respective seasons which could suggest that avgRH could be a major variable in the colonisation and mycotoxin production during advanced grain development stages. No relationship was recorded with total rainfall (RF) with the exception of ergosterol during March of the respective seasons which may be associated with the superficial colonisation of grain during the maturation phase.

4. Discussion

The qualitative measurement of grain mold severity using threshed grain disease ratings allows for a descriptive and visual estimate to be made of the level of infection of sorghum grain by grain mold fungi and has been in general use by researchers and sorghum breeders (Bandyopadhyay & Mughogho, 1988). Jambunathan *et al.* (1991) reported a significant relationship between visual ratings and ergosterol which measures total internal fungal biomass and assumed that cultivars which have less severe external symptoms, have a lower internal grain mold fungal biomass. In contrast Audilakshmi *et al.* (1999) found that bias arises due to superficial external symptoms being more readily visible on white grained cultivars and as a result these could be rated more disease susceptible than red and brown grain cultivars. In the current study the rank correlation between cultivar threshed grain rating and ergosterol concentration was $0.017^{(ns)}$ indicating that these criteria are not related in the cultivars used in this study. This notion is further supported by

the expectation that the white grained PAN8706W and PAN8553W, would have high threshed grain disease ratings due to the increased visibility of symptoms and low tannin content (and thus greater susceptibility to colonisation by fungi; Jambunathan *et al.,* 1991) relative to the brown, high tannin cultivar NS5511. However, PAN8706W had one of the lower threshed grain disease ratings with an intermediate ergosterol content equivalent to NS5655, while PAN8625 had one of the highest threshed grain disease ratings but significantly lower ergosterol content than most cultivars used in this study. Therefore, the antithesis of the expectations suggested by Jambunathan *et al.* (1991) was observed, indicating that threshed grain ratings are not reliable measures of sorghum grain mold and that superficial external symptoms are not indicators of internal pathogen colonisation. Most resistance breeding programmes make use of field ratings as indicators of resistance and the results of this study highlight inherent limitations of this selection criterion. Ergosterol quantification should be considered the more powerful tool to indicate general resistance to both internal and external (superficial) grain mold pathogens.

Mpofu & McLaren (2014) reported that *Phoma sorghina, Fusarium thapsinum* and *Curvularia lunata* are more likely to be responsible for higher levels of ergosterol content in grains than *Fg*SC and advocated the use of ergosterol for general grain mold assessment. Furthermore field ratings may vary between assessors due to bias (Campbell & Madden, 1990), whereas ergosterol as a biochemical assay is quantitative and thus reduces bias. Ergosterol is a sterol unique to metabolically active fungi. The quantification of ergosterol is a sensitive, rapid detection technique. In the current study PAN8609 was the cultivar with the highest ergosterol content, and grouped with PAN8806 and PAN8706W. Thus these cultivars can be assumed to be more susceptible to grain mold pathogens. Using this argument, therefore NS5511, PAN8625 and PAN8816 may be regarded as cultivars with most resistance to colonisation by grain mold fungi.

Specific fungal biomass detection and quantification of the *Fg*SC is possible through sensitive and specific quantitative real time PCR (qPCR). This study used the protocol as described by Nicolaisen *et al.* (2009) to detect and quantify the *Fg*SC DNA concentrations present in sorghum grain from nine cultivars from multiple plantings at two localities in South Africa. Since 86.5% of the sorghum grain samples

contained *Fg*SC DNA, it can be assumed that sorghum is generally susceptible to colonisation by the *Fg*SC. Differences in colonisation were, however, recorded and NS5511 and PAN8625, cultivars with condensed tannins and a red pericarp, had the lowest concentrations of *FgSC* DNA. Bueso *et al*. (2000) found that a pigmented testa and presence of tannins were associated with less grain deterioration than those that lacked a pigmented testa.

AMMI analysis indicated that the colonisation of grain by the *FgSC* occurs differentially to environmental conditions. The importance of measuring stability is reflected by NS5511 and PAN8625 that had the lowest *Fg*SC DNA concentrations but also displayed unstable responses to environment with high IPCA1 scores. Thus, by implication, the resistance response could break down under specific conditions as for example those experienced in Alma 2013/14, although further studies are required to verify this. The low IPCA scores of PAN8609, PAN8420 and PAN8706W despite having high mean *Fg*SC DNA levels are expected to give consistent susceptible responses over a wide range of environmental conditions.

The *Fg*SC is the group of pathogens which are associated specifically with economically important sorghum grain mycotoxins such as DON, NIV and ZEA (Köppen *et al.,* 2010). LC-MS-MS detection and quantification of mycotoxins allows for multiple mycotoxins to be detected and quantified simultaneously through a single extraction protocol, making the modified Small *et al.* (2012) method a high-throughput procedure. Measurement of the mycotoxins in grains is essential to understanding the risk of mycotoxins in food and feed, to potentially take action to reduce the effects on human and livestock health (CAST, 2003). Mycotoxin-producing *FgSC*, fungi which are of particular importance to sorghum, include *F. acaciae-mearnsii* and *F. meridionale*. These two pathogens are known to produce ZEA and NIV and are specifically associated with sorghum grain (Mavhunga, 2013). The exceptionally low levels of DON detected in the current study and NIV and ZEA being detected in over 60 and 80% of the sorghum grain samples, respectively, supports the findings of the NIV-producer being the predominant member of the *Fg*SC in local sorghum grain.

AMMI analysis indicated the NIV response varied with host and environment interaction. PAN8553W and PAN8816 had susceptible reactions to NIV and IPCA>1

suggests cultivars are highly reactive to environments. In contrast PAN8706W, although susceptible to the accumulation of NIV, had an IPCA<1 indicating that the cultivar is less reactive to environments. The above susceptible reactions were observed in cultivars which did not contain tannins, supporting Bueso *et al*. (2000).

Weather affected mycotoxin concentrations, the season which had the highest levels of NIV was Cedara 2011/12 where the rainfall was lowest during flowering and grain fill. However mean average relative humidity was highest of all the planting dates. This could possibly suggest that NIV accumulation is related to wetness duration and not directly to precipitation. Schaafsma & Hooker (2007) stated that daily average temperature, relative humidity and total daily rainfall during and after anthesis are positively correlated with creating moisture periods for Fusarium head blight infection and have the ability to trigger the concomitant accumulation of DON in wheat. However Brustolin *et al.* (2013) suggest that heavy rainfall does not relate to duration of wetness. The above confirms the absence of correlations between mycotoxin levels and total rainfall in the present study and positive correlation with average relative humidity (Table 2B). Alma's first planting in 2012/13 had the lowest levels of observed NIV and also the hottest and driest weather, further supporting the assumption that wetness duration is a driving variable for NIV accumulation.

The role of host-genotype in the suppression or stimulation of NIV and ZEA assuming equal colonisation of grains by the *Fg*SC is a new finding and could explain the absence of a relationship between *Fg*SC DNA levels and mycotoxin concentrations in pooled genotype data. Genotype resistance to mycotoxin production is thus a selection criterion which could reduce mycotoxin accumulation and complement resistance to colonisation of grain by *Fg*SC. Genotype effect on NIV and ZEA where fungal biomass was similar has not previously been reported on sorghum. However, Janse van Rensburg *et al.* (2015) reported that cultivar differences have been observed in maize with respect to fumonisin accumulation. This could be of practical and economical value by shifting the focus of breeding programs to lower toxins as opposed to resistance to colonisation as has been applied in maize resistance breeding programs to *Aspergillus flavus* infection or aflatoxin production (Munkvold, 2003).

This study has highlighted the importance of multiple and specific disease evaluation criteria by illustrating the absence of correlation between TGDR, ergosterol and *Fg*SC DNA concentration. It also showed that understanding genotype reactions to environments could assist in selecting cultivars which are better suited for specific planting localities and stable resistance to grain molds and mycotoxin contamination. This study also indicated the importance of including the genotype as a driving variable that affects the accumulation of mycotoxins, specifically NIV and ZEA, into a resistance breeding program.

5. References

Aldred, D. & Magan, N., 2004. Prevention strategies for trichothecenes. *Toxicology Letters*. **153**: 165–71.

Audenaert, K., Vanheule, A., Höfte, M & Haesaert, G. 2013. Deoxynivalenol: a major player in the multifaceted response of *Fusarium* to its environment. *Toxins*. **6**: 1–19.

Audilakshmi, S., Stenhouse, J. W., Reddy, T. P. & Prasad, M. V. R. 1999. Grain mold resistance and associated characters of sorghum genotype. *Euphytica* **107**: 91-103.

Bandyopadhyay, R. & Mughogo, L. K. 1988. Evaluation of field screening techniques for resistance to sorghum grain molds. *Plant Disease* **72**: 500-503.

Bandyopadhyay, R., Butler, D. R., Chandrashekar, A. Reddy, R.K., and Navi, S.S. 2000. Biology, epidemiology, and management of sorghum grain mold. Pages 34-71 in Technical and institutional options for sorghum grain mold management: proceedings of an international consultation, 18-19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Belton, P. S. & Taylor, J. R. N., 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science & Technology* **15**: 94–98.

Brustolin, R., Zoldan, S. M., Reis, E. M., Zanatta, T. & Carmona, M. 2013. Weather requirements and rain forecast to time fungicide application for Fusarium head blight control in wheat. *Summa Phytopathologica* **39**: 248-251.

Bueso, F. J., Waniska, R. D., Rooney, W. L. & Bejosano, F. P. 2000. Activity of antifungal proteins against mold in sorghum caryopses in the field. *Journal of Agricultural and Food Chemistry* **48**: 810-816.

Campbell, C. L. & Madden, L. V. 1990. Introduction to plant disease epidemiology. John Wiley & Sons, Canada.

Chandrashekar, A. & Satyanarayana, K. V. 2006. Disease and pest resistance in grains of sorghum and millets. *Journal of Cereal Science* **44**: 287-304

Council for Agricultural Science and Technology (CAST). 2003. Mycotoxins : Risks in Plant , Animal , and Human Systems. Accessed on 25.10.2013 from http://www.trilogylab.com/uploads/Mycotoxin_CAST_Report.pdf

Edwards, S. G., 2004. Influence of agricultural practices on *Fusarium* infection of cereals and subsequent contamination of grain by trichothecene mycotoxins. *Toxicology Letters* **153**: 29–35.

European Food Safety Authority (EFSA; 2013). Scientific opinion on risks for animal and public health related to the presence of nivalenol in food and feed. *EFSA Journal* **11**: 3262.

Frederiksen, R. A., Rosenow, D. T., Teetes, G. L., Peterson, G. C., Odvody, G. N., Miller, F. R., & Collins, S. D. 1991. Disease and insect rating schemes for sorghum. *Sorghum Newsletter* ***32:*** 30-36.

Hintze, 2007. NCSS 2007. NSCC, LLC. Kaysville, Utah, USA. www.ncss.com.

Jambunathan, R., Kherdekar, M. S. & Stenhouse, J.W., 1991. Sorghum grain hardness and its relationship to mold susceptibility and mold resistance. *Journal of Agriculture and Food Chemistry* **40**: 1403–1408.

Janse Van Rensburg, B., McLaren, N. W., Flett, B. C., & Schoeman, A. 2015. Fumonisin producing *Fusarium* spp. and fumonisin contamination in commercial South African maize. *European Journal of Plant Pathology* **141**: 491-504.

Köppen, R, Kock, M., Siegel, D., Merkel, S., Maul., R. & Nehls, I. 2010. Determination of mycotoxins in foods: current state of analytical methods and limitations. *Applied Microbiology & Biotechnology* **86**: 1595–612.

Landschoot, S., Waegeman, W., Audenaert, K., Vandepitte, J., Baetens, J.M., De Baets, B., Haesaert, G. 2012. An empirical analysis of explanatory variables affecting Fusarium head blight infection and deoxynivalenol content in wheat. *Journal of Plant Pathology* **94**: 135–147.

Mavhunga, M. 2013. *Fusarium graminearum* mycotoxins associated with grain mold of maize and sorghum in South Africa (Masters dissertation, University of the Free State Bloemfontein, South Africa).

Melake-Berhan, A., Butler, L. G., Ejeta, G. & Menkir, A. 1996. Grain mold resistance and polyphenol accumulation in sorghum. *Journal of Agricultural & Food Chemistry* **44**: 2428-2434.

Mpofu, L. T. & McLaren, N. W. 2014. Ergosterol concentration and variability in genotype-by-pathogen interaction for grain mold resistance in sorghum. *Planta* **240**: 239-250.

Munkvold, G. P. 2003. Cultural and genetic approaches to managing mycotoxins in maize. *Annual Review of Phytopathology* **41**: 99-116.

Nicolaisen, M., Suproniene, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H., Justesen, A. F. 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *Journal of Microbiological Methods* **76**: 234–40.

Prandini, A., Sigolo, S., Filippi, L., Battilani, P. & Piva, G. 2009. Review of predictive models for Fusarium head blight and related mycotoxin contamination in wheat. *Food & Chemical Toxicology* **47**: 927–31.

Reddy, B. V. S., Kumar, A. A. & Reddy, P. S. 2010. Recent Advances in Sorghum Improvement Research at ICRI- SAT, Kasetsart. *Natural Science* **44**: 499-506.

Robine, E., Lacaze, I., Moularat, S., Ritoux, S. & Boissier, M. 2005. Characterization of exposure to airborne fungi: Measurement of ergosterol. *Journal of Microbiological Methods* **63**: 185-192.

SAS Institute, Inc. 2013. SAS/STAT User's Guide, Version 9, 1st printing, Volume 2. SAS Institute Inc, SAS Campus Drive, Cary, North Carolina, USA.

Schaafsma, A. W. & Hooker, D.C. 2007. Climatic models to predict occurrence of Fusarium toxins in wheat and maize. *International Journal of Food Microbiology* **119**: 116–25.

Small, I. M., Flett, B. C., Marasas, W. F. O. McLeod, A., Stander, M. A. and Viljoen, A. 2012. Resistance in maize inbred lines to *Fusarium verticilliodes* and fumonisin accumulation in South Africa. *Plant Disease* **96**: 881-888.

Tesfaendrias, M. T., McLaren, N. W. & Swart, W. J. 2011. Grain mold fungi and their effect on sorghum grain quality. *South African Journal of Plant & Soil* **28**: 172–180.

Thakur, R. P., Reddy, B. V. S., Indira, S., Rao, V. P., Navi, S. S., Yang, X. B. & Ramesh, S. 2006. Sorghum Grain Mold. Information Bulletin No. 72. International Crops Research Institute for the Semi-Arid Tropics. Patancheru 502324, Andhra Pradesh, India: 32 pp. ISBN 92-9066-488-6.

Trigo-Stockli D. M., Deyoe, C. W., Satumbaga, R. F., Pedersen, J. R. 1996. Distribution of deoxynivalenol and zearalenone in milled fractions of wheat. *American Association of Cereal Chemists* **73**: 388–391.

Table 1. Mass spectrometer settings for the detection of nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEA) in sorghum grain using LC-MS-MS.

Mass spectrometer settings	**NIV**	**DON**	**ZEA**
Precusor ion [M+H]+(m/z)	371	355	317
Product ion (m/z)	281	265	175
Declustering potential (V)	-1	-1	-16
Collusion 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 2. AMMI analysis of variance (ANOVA) for threshed grain disease ratings (tgdr, 0-5 rating) and FgSC DNA fungal biomass (ng.ul^{-1}) (A), accumulation of nivalenol and zearalenone (B) and ergosterol (C) in the nine cultivars tested in six environments in South Africa between 2009/10 and 2013/14.

A		tgdr[1]				Biomass of *Fg*SC[2]			
Source	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr**	**s.s.**	**m.s.**	**v.r.**	**F pr**
Total	**161**	80.58	0.50			755649	4693		
Treatments	**53**	51.08	0.96	3.66	<0.001	584605	11030	7.03	<0.001
Genotypes	**8**	27.07	3.38	12.85	<0.001	45873	5734	3.65	<0.001
Environments	**5**	11.33	2.27	4.69	<0.001	392009	78402	40.10	<0.001
Block	**12**	5.80	0.48	1.84	0.05	23464	1955	1.25	0.26
Interactions	**40**	12.68	0.32	1.20	0.23	146723	3668	2.34	<0.001
IPCA 1	**12**	6.75	0.56	2.13	0.02	131274	10939	6.97	<0.001
IPCA 2	**10**	2.42	0.24	0.92	0.52	8264	826	0.53	0.87
Residuals	**18**	3.51	0.20	0.74	0.76	7186	399	0.25	1.00
Error	**90**	23.70	0.26			147580	1570		

B		NIV[3]				ZEA[4]			
Source	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr**	**s.s.**	**m.s.**	**v.r.**	**F pr**
Total	161	12028	74.7			99198	616		
Treatments	53	8473	159.9	5.1	<0.001	64882	1224	3.93	<0.001
Genotypes	8	1202	150.3	4.79	<0.001	6635	829	2.67	0.01
Environments	5	3548	709.6	12.71	<0.001	13460	2692	5.67	<0.001
Block	12	670	55.8	1.78	0.063	5694	474	1.53	0.13
Interactions	**40**	**3723**	**93.1**	**2.97**	**<0.001**	44787	1120	3.60	<0.001
IPCA 1	12	2772	231	7.37	<0.001	31263	2605	8.37	<0.001
IPCA 2	10	536	53.6	1.71	0.0904	8228	823	2.64	0.01
Residuals	18	415	23.1	0.74	0.7666	5296	294	0.95	0.53
Error	92	2885	31.4			28622	311		

C		Ergosterol[5]			
Source	**d.f.**	**s.s.**	**m.s.**	**v.r.**	**F pr**
Total	**134**	555657	4147		
Treatments	**44**	172214	3914	2.31	<0.001
Genotypes	**8**	44186	5523	3.25	0.00
Environments	**4**	83452	20863	0.81	0.52
Block	**10**	257818	25782	15.19	<0.001
Interactions	**32**	44576	1393	0.82	0.73
IPCA 1	**11**	22919	2084	1.23	0.28
IPCA 2	**9**	18499	2055	1.21	0.30
Residuals	**12**	3158	263	0.16	1.00
Error	**74**	125625	1698		

[1] Threshed grain disease ratings of sorghum grain mold caused by FgSC complex on a scale from 0-5
[2] Absolute concentrations of FgSC DNA (ng.ul^{-1})
[3] Concentration of nivalenol (µg.kg^{-1})
[4] Concentration of zearalenone (µg.kg^{-1})
[5] Measurement of total fungal biomass by quantification of ergosterol content (µg.g^{-1})

Table 3. Means of threshed grain disease ratings (tgdr, rating 0-5) and ergosterol content (µg.g^{-1}) in nine sorghum commercial cultivars evaluated in six environments.

Cultivar	tgdr[1]	Ergosterol[2]		Environment	tgdr[1]	Ergosterol[2]
NS5511	1.58^{ab}	30.90^{a}		Cedara 2009/10	1.89^{b}	39.63^{a}
NS5655	1.93^{c}	72.39^{bcd}		Cedara 2010/11	2.00^{b}	60.01^{a}
PAN8420	1.87^{bc}	60.11^{abcd}		Cedara 2011/12	2.34^{c}	27.53^{a}
PAN8553W	2.66^{d}	55.98^{ab}		Alma 2012/13 (1)	1.46^{a}	85.23^{a}
PAN8609	1.97^{c}	86.43^{d}		Alma 2012/13 (2)	1.97^{b}	91.44^{a}
PAN8625	2.62^{d}	40.53^{a}		Alma 2013/14	2.12^{bc}	*
PAN8706W	1.33^{a}	77.53^{cd}				
PAN8806	1.87^{bc}	78.08^{cd}				

[1] Threshed grain disease ratings (tgdr) of sorghum grain mold on a 0-5 rating scale
[2] Measurement of total fungal biomass by quantification of ergosterol content (µg.g^{-1})
* No data collected in this season

Table 4. Two-way interactions between genotypes and environments in sorghum grain samples infected with *Fg*SC (ng.µl^{-1}) representing nine cultivars collected between 2009/10 and 2013/14 seasons in two South African localities.

	Environment						
Genotype	Cedara 2009/10	Cedara 2010/11	Cedara 2011/12	Alma 2012/13 (1)	Alma 2012/13 (2)	Alma 2013/14	**MEAN**
NS5511	0.3	0.4	5.0	1.2	12.1	41.0	**10.00^{a}**
NS5655	1.0	28.3	199.9	3.9	5.8	12.0	**41.82^{b}**
PAN8420	9.1	8.1	151.4	31.9	29.8	16.7	**41.17^{b}**
PAN8553W	1.8	23.9	190.3	3.1	39.9	20.6	**46.60^{b}**
PAN8609	1.8	0.8	144.1	13.2	23.6	29.6	**35.52^{b}**
PAN8625	0.2	0.0	12.8	0.0	1.8	18.3	**5.52^{a}**
PAN8706W	9.9	0.0	161.0	60.6	20.6	23.7	**45.97^{b}**
PAN8806	1.9	0.4	256.3	4.5	33.9	35.6	**55.43^{b}**
PAN8816	1.2	22.1	187.2	24.3	30.7	36.6	**50.35^{b}**
MEAN	**3.02^{a}**	**9.33^{ab}**	**145.33^{c}**	**15.86^{ab}**	**22.02^{b}**	**26.01^{b}**	**36.93**

***** Absolute concentrations of *Fusarium graminearum* species complex DNA (ng.ul^{-1})

LSD GxE = 64.06

Table 5. Two-way interactions between genotypes and environments in sorghum grain samples contaminated with nivalenol* from nine sorghum cultivars at six environments between 2009/10 and 2013/14 seasons at two South African localities.

	Environment						
Genotype	Cedara 2009/10	Cedara 2010/11	Cedara 2011/12	Alma 2012/13 (1)	Alma 2012/13 (2)	Alma 2013/14	**MEAN**
NS5511	11.7	4.8	3.1	4.8	5.2	3.6	**5.52^{a}**
NS5655	15.5	1.4	4.1	1.7	2.5	3.6	**4.77^{a}**
PAN8420	14.3	4.5	13.8	1.7	2.3	3.2	**6.63^{a}**
PAN8553W	11.5	21.6	22.0	0.0	2.0	4.8	**10.31^{b}**
PAN8609	14.9	4.0	13.8	2.4	0.0	1.7	**6.12^{a}**
PAN8625	7.2	0.1	4.5	5.1	5.7	1.5	**4.02^{a}**
PAN8706W	21.2	13.7	18.3	0.0	2.9	6.6	**10.44^{b}**
PAN8806	10.3	12.5	36.9	3.1	0.0	8.1	**11.82^{b}**
PAN8816	10.8	1.3	8.6	2.3	5.7	1.8	**5.07^{a}**
MEAN	**13.03^{c}**	**7.08^{b}**	**13.89^{c}**	**2.33^{a}**	**2.92^{a}**	**3.88^{a}**	**7.19**

* Concentration of nivalenol (µg.kg^{-1})

LSD GxE = 9.06

Table 6. Two-way interactions between genotypes and environments in sorghum grain samples contaminated with zearalenone* from nine sorghum cultivars at six environments between 2009/10 and 2013/14 seasons at two South African localities.

	Environment						
Genotype	Cedara 2009/10	Cedara 2010/11	Cedara 2011/12	Alma 2012/13 (1)	Alma 2012/13 (2)	Alma 2013/14	**MEAN**
NS5511	6.7	0.8	0.1	0.6	1.1	5.6	**2.48^{a}**
NS5655	37.5	26.8	30.2	1.0	0.7	4.0	**16.70^{bc}**
PAN8420	44.1	9.2	3.8	0.8	0.7	1.1	**9.95^{ab}**
PAN8553W	34.4	13.5	6.1	0.5	0.6	3.7	**9.80^{ab}**
PAN8609	19.7	1.6	0.0	39.0	0.7	1.6	**10.43^{ab}**
PAN8625	3.2	7.2	0.0	1.2	0.5	1.7	**2.30^{a}**
PAN8706W	50.3	3.0	1.2	0.9	0.5	2.6	**9.75^{ab}**
PAN8806	1.8	21.7	3.4	0.5	0.6	26.1	**9.02^{ab}**
PAN8816	14.1	119.5	1.1	0.4	1.0	2.3	**23.07^{c}**
MEAN	**23.53^{b}**	**22.59^{b}**	**5.10^{a}**	**4.99^{a}**	**0.71^{a}**	**5.41^{a}**	**10.39**

* Concentration of zearalenone (µg.kg^{-1})

LSD GxE = 28.51

Table 7. Correlations between disease parameters and weather factors in nine sorghum cultivars at six environments between 2009/10 and 2013/14 seasons at two South African localities.

Disease Parameter	MxT[1]			MnT[2]			RainT[3]			RadN[4]			Rhavg[5]		
	J	F	M	J	F	M	J	F	M	J	F	M	J	F	M
Rating[6]	-0.48	-0.79	-0.63	-0.67	-0.97	-0.37	-0.33	-0.68	0.59	-0.45	-0.68	-0.63	0.51	0.48	0.49
Erg[7]	0.91	0.71	0.41	0.90	0.15	-0.60	-0.51	-0.42	-0.94	0.96	0.89	0.90	-0.96	-0.93	-0.99
FgSC[8]	0.90	0.66	0.33	0.86	0.05	-0.68	-0.54	-0.51	-0.9	0.95	0.85	0.87	-0.94	-0.92	-0.96
NIV[9]	-0.83	-0.67	-0.59	-0.86	-0.25	0.44	0.59	0.29	0.98	-0.9	-0.86	-0.85	0.92	0.86	0.98
ZEA[10]	-0.99	-0.84	-0.19	-0.95	-0.18	0.68	0.23	0.41	0.84	-1.00	-0.94	-0.96	0.98	1.00	0.93

Where J = January, F = February and M = March

1 Average Maximum Temperature (°C)

2 Average Minimum Temperature (°C)

3 Total Rainfall (mm)

4 Total Radiation (MJ/m2)

5 Average Relative Humidity (%)

6 Threshed grain disease ratings (0-5)

7 Ergosterol ($\mu g.g^{-1}$)

8 FgSC fungal biomass ($ng.\mu l^{-1}$)

9 Nivalenol ($\mu g.kg^{-1}$)

10 Zearalenone ($\mu g.kg^{-1}$)

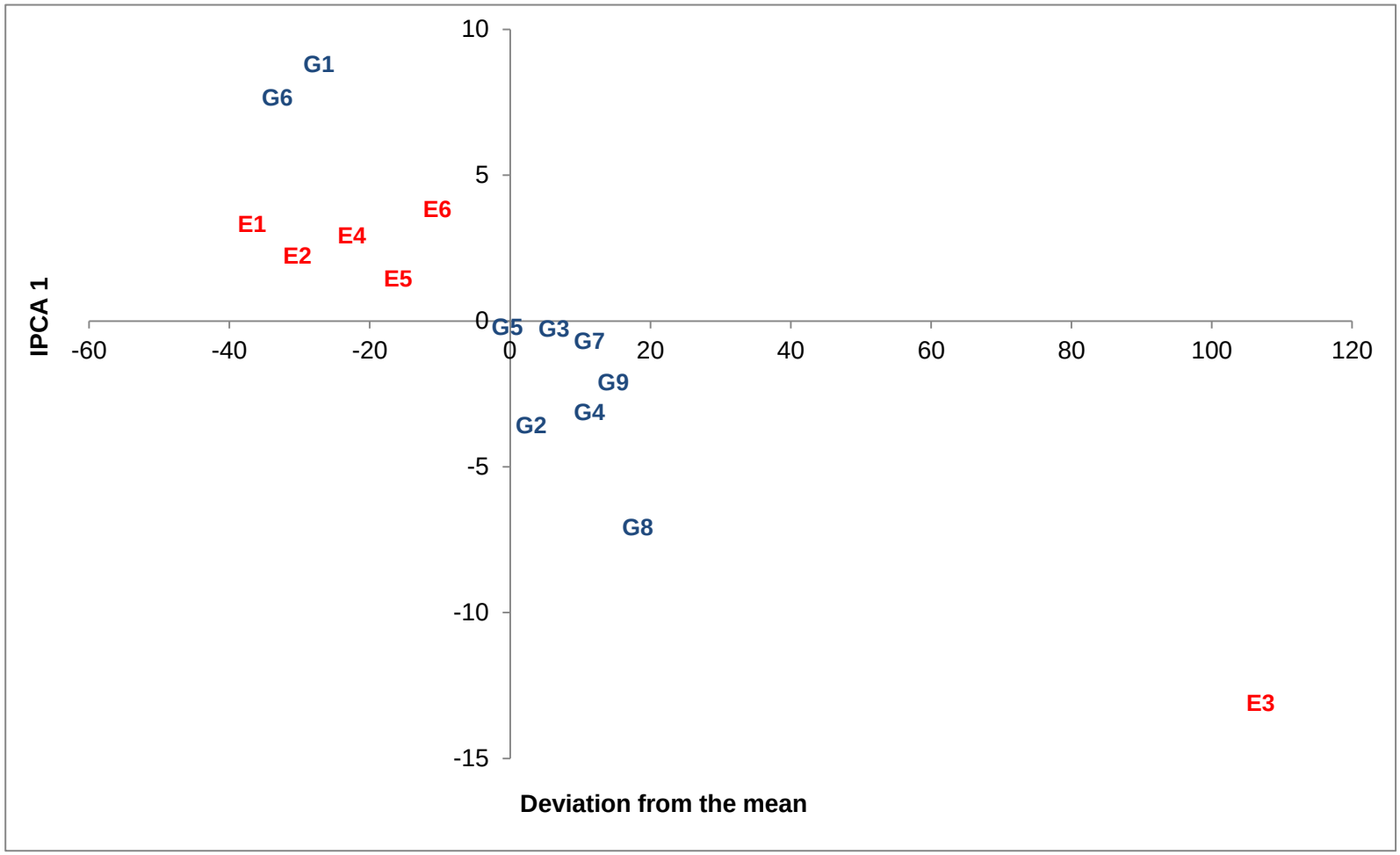
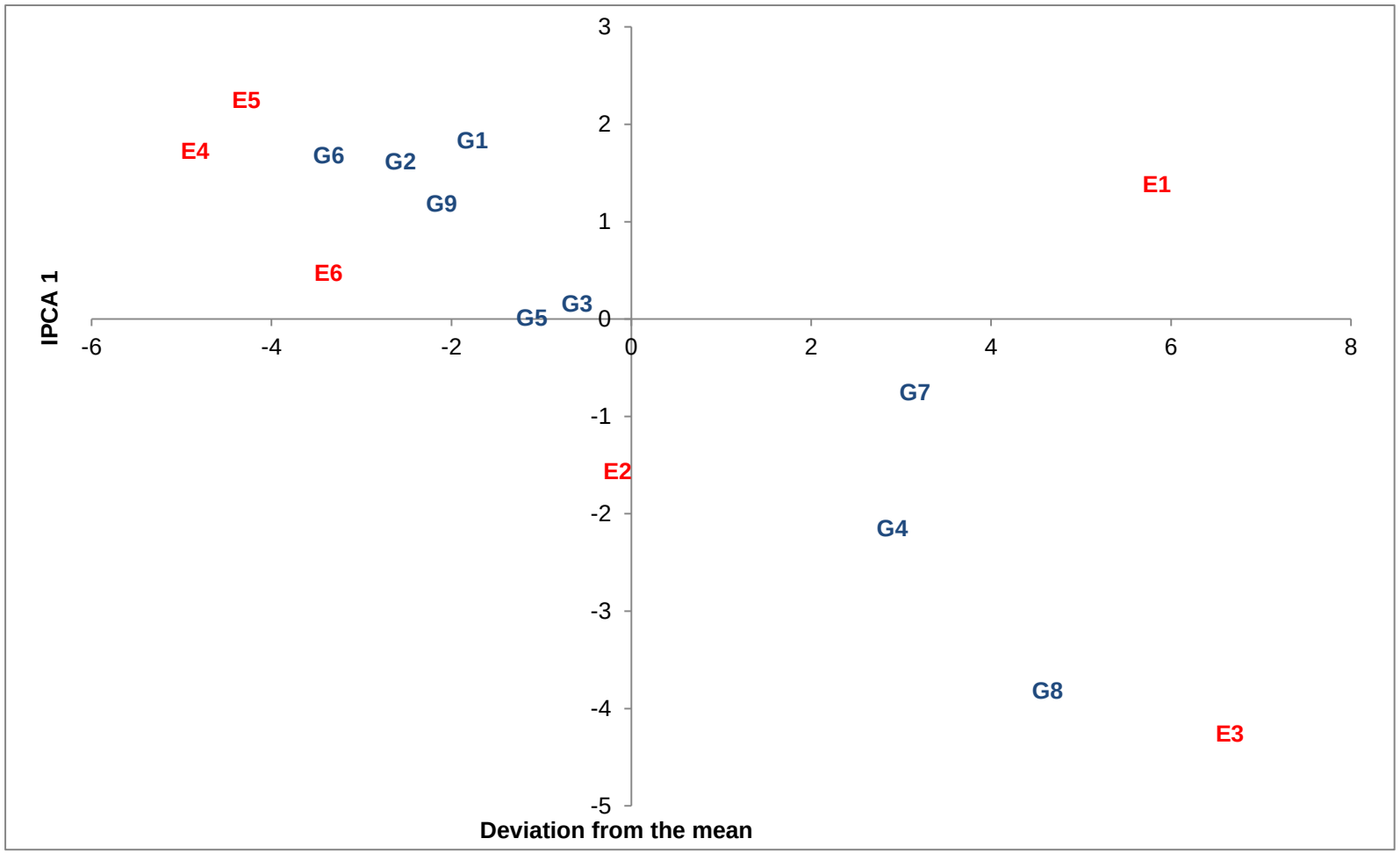


Figure 1. AMMI bi-plot of the colonisation of grain by the *Fusarium graminearum* species complex, determined by using *Fg*SC DNA concentrations (ng.ul^{-1}) in nine sorghum cultivars evaluated over six environments at two localities in South African sorghum grain producing areas.



E1 – Cedara 2009/10
E2 – Cedara 2010/11
E3 – Cedara 2011/12
E4 – Alma 2012/13 (1)
E5 – Alma 2012/13 (2)
E6 – Alma 2013/14
G1 – NS5511
G2 – NS5655
G3 – PAN8420
G4 – PAN8553W
G5 – PAN8609
G6 – PAN8625
G7 – PAN8706W
G8 – PAN8806
G9 – PAN8816

Figure 2. AMMI bi-plot of nivalenol accumulation (µg.kg^{-1}) in nine sorghum cultivars evaluated over six environments at two localities in South African sorghum grain producing areas.

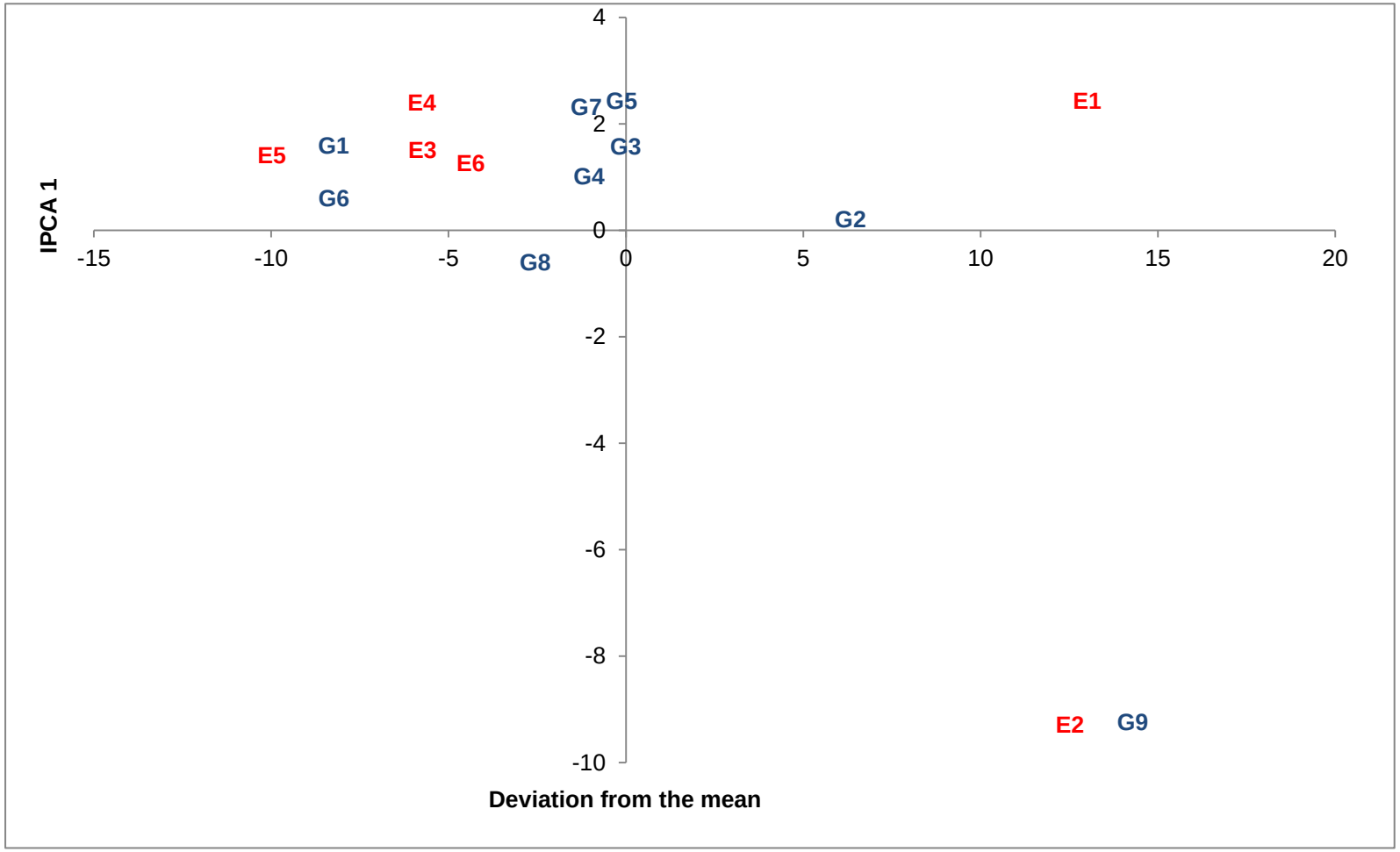


Figure 3. AMMI bi-plot of zearalenone accumulation (µg.kg^{-1}) in nine sorghum cultivars evaluated over six environments at two localities in South African sorghum grain producing areas, IPCA 1 versus deviation from the mean.

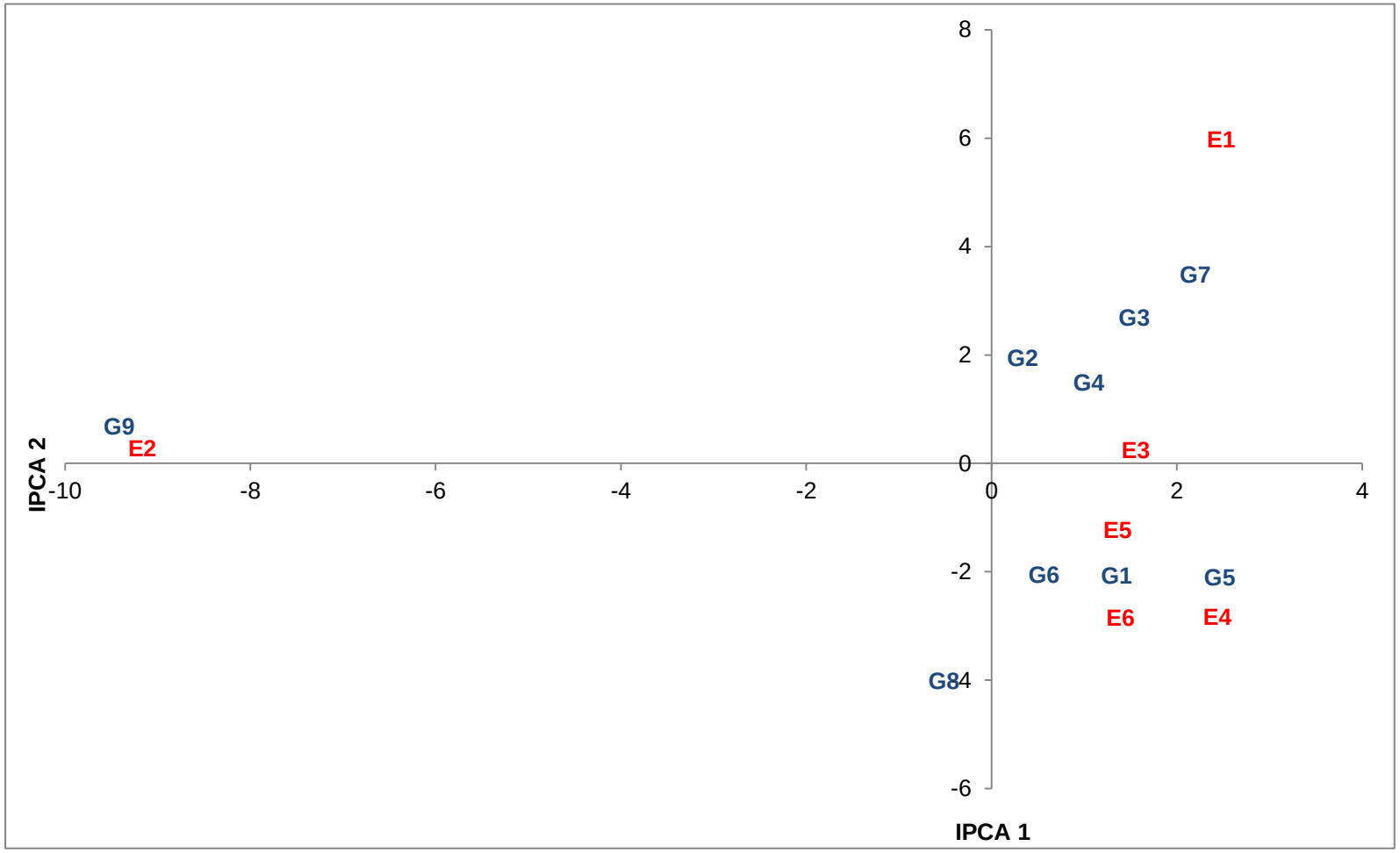


Figure 4. AMMI bi-plot of zearalenone accumulation (µg.kg^{-1}) in nine sorghum cultivars evaluated over six environments at two localities in South African sorghum grain producing areas, IPCA 1 versus IPCA 2.

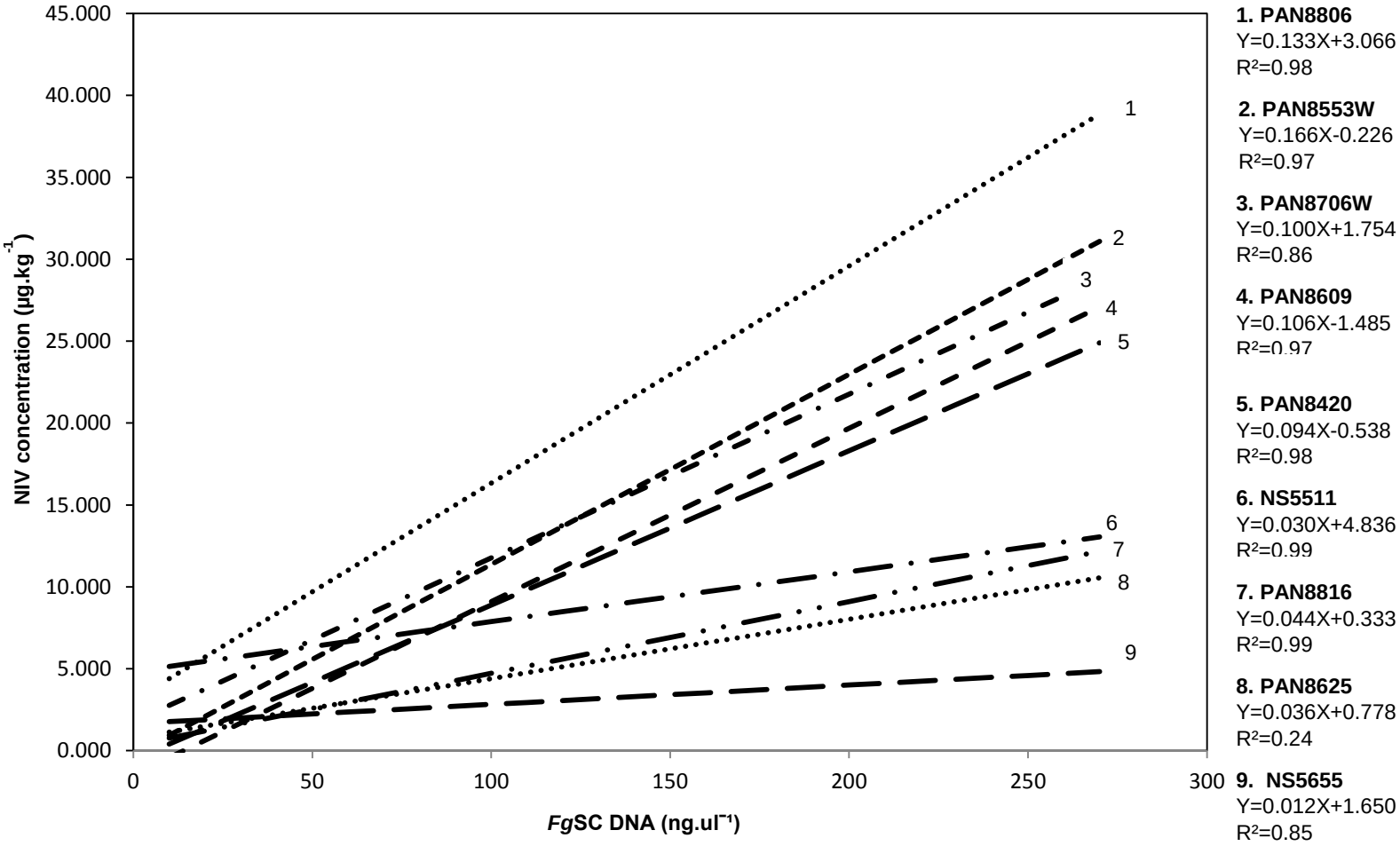


Figure 5. Relationship between *Fusarium graminearum* species complex DNA (*Fg*SC DNA; ng.µl^{-1}) concentration in sorghum grain and NIV production (NIV; µg.kg^{-1}) in nine sorghum cultivars grown at six environments.

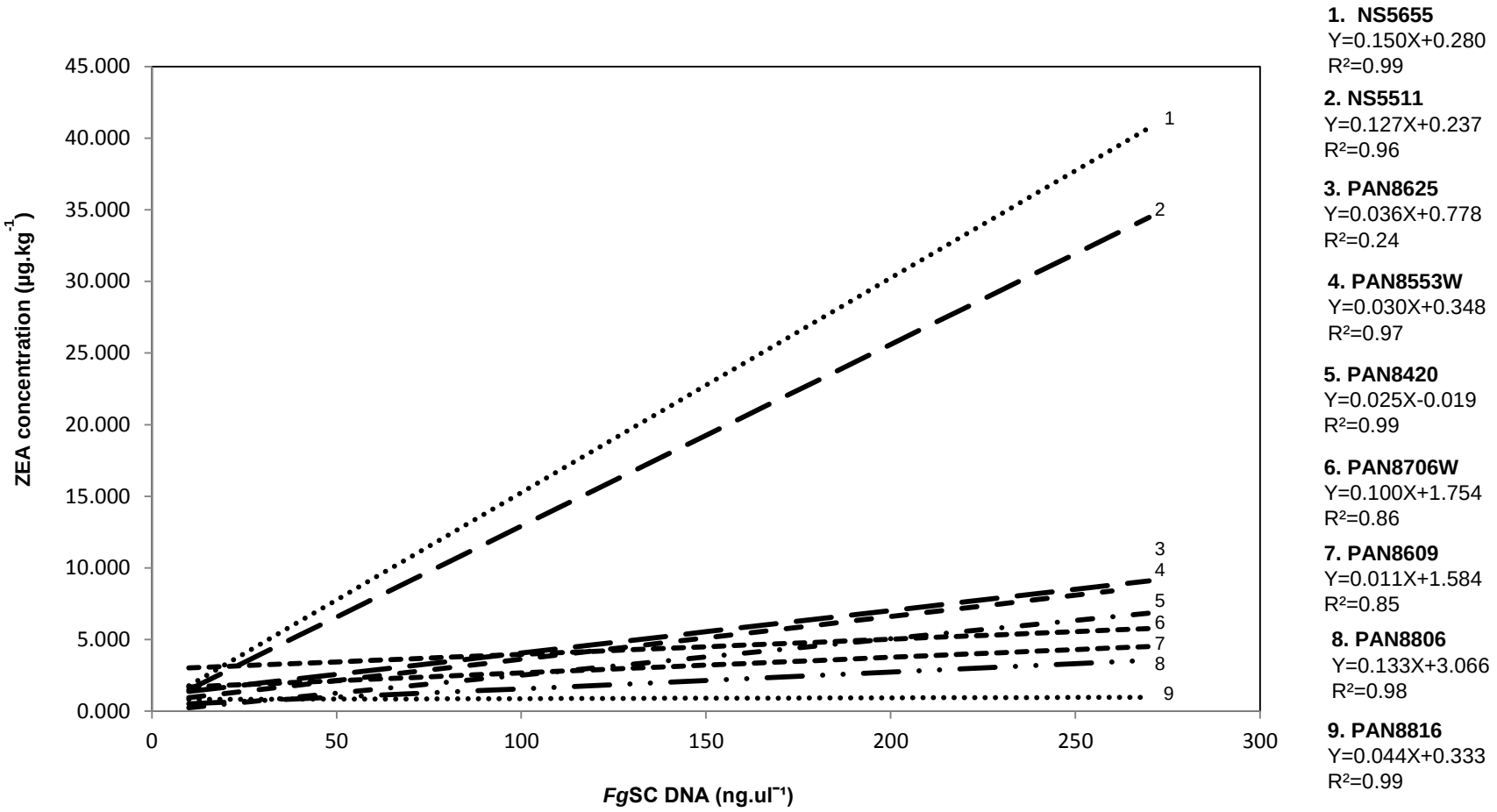


Figure 6. Relationship between *Fusarium graminearum* species complex DNA (*Fg*SC DNA; ng.µl^{-1}) concentration in sorghum grain and ZEA production (ZEA; µg.kg^{-1}) in nine sorghum cultivars grown at six environments

Chapter 3

Relationship between weather and the associated mycotoxins produced by *Fusarium graminearum* species complex on sorghum grain

Abstract

Sorghum grain mold (SGM) is the one of the most important biotic constraints in sorghum production, causing reductions in grain size, kernel mass, nutritional quality and complete destruction of the entire grain, resulting in lower yield, milling quality and poor germination. The *Fusarium graminearum* species complex (*Fg*SC) is responsible for the majority of important mycotoxins associated with SGM, including deoxynivalenol (DON), nivalenol (NIV) and zearalenone (ZEA). Control methods for *Fg*SC and concomitant mycotoxin accumulation are limited to tolerant cultivars and agronomic management practices. Development of an epidemiological model which identifies specific risk areas in sorghum production regions would enable producers to ensure that timely management decisions are made to reduce FgSC infection and mycotoxin contamination. The aim of this study is to identify climatic factors which promote disease development and increase severity of SGM, with specific reference to *Fg*SC colonisation and concomitant mycotoxin production of DON, NIV and ZEA for the future development of a risk forecasting model. Sorghum grain was collected from the National Cultivar Evaluation Trials over two seasons, 2010/2011 and 2011/2012, from seven and 15 South African sorghum production areas, respectively. Site specific weather data, maximum and minimum temperature (MaxT; MinT), maximum and minimum relative humidity (MaxRH; MinRH), evapotranspiration (Eto), radiation (RAD), rainfall (RF) were provided by the ARC-Institute for Soil Water and Climate's meteorology office. Grain samples were analysed for *Fg*SC colonisation using quantitative real-time PCR and mycotoxin contamination, specifically for DON, NIV and ZEA, with the L-MS-MS. *Fg*SC DNA concentrations ranged from 0-3790 $pg.mg^{-1}$ over the two seasons. DON, NIV and ZEA ranged from 0-263.42 ppm, 0-0.18 ppm and 0.28-18.82 ppm over the two seasons, respectively. *Fg*SC colonisation and concomitant mycotoxin accumulation coincided with weather conditions during early-post-flowering, and soft-dough stage of grain development, which are the critical periods for disease development. *Fg*SC

development and colonisation was significantly, positively correlated with MaxRH, 82-95 days after planting (d.a.p; R^2=0.59), and significantly, inversely correlated with MaxT and Eto, 82-95 d.a.p. (R^2=0.71 and R^2=0.69, respectively). DON, NIV and ZEA accumulation were significantly, positively related with *Fg*SC DNA concentration (R^2=0.66, R^2=0.63 and R^2=0.66, respectively). DON had borderline significant, positive relationship with MaxT, 101-115 d.a.p. (R^2=0.45). However, NIV and ZEA had significant inverse relationships with MinT 91-104 d.a.p and 100-113 d.a.p, respectively (R^2=0.72 and R^2=0.68, respectively). Preliminary models based on stepdown multiple regression analysis were developed. Future studies could include localities with more available and accurate weather data and the validation of the models developed.

1. Introduction

Sorghum production in South Africa is comparable to that of Australia with average yields of 2 t.ha^{-1} (DAFF, 2010). Sorghum is the fifth most important grain worldwide and is a staple food for over half a billion people in more than 30 countries (Reddy *et al.,* 2010). Beyond a staple food, sorghum is also used for livestock feed and fodder as well as for industrial purposes such as fuel, oils and building materials (Rampho, 2005). Sorghum production occurs mostly in the semi-arid and topical to sub-tropical regions of the world. It is under these climatic conditions that sorghum grain mold (SGM) proliferates (Bandyopadhyay *et al.,* 2000; Reddy *et al.,* 2010).

Sorghum grain mold is one of the most important pre-harvest biotic constraints associated with sorghum production. This disease is an amalgamation of pathogen species including members of the genera *Alternaria, Curvularia, Fusarium* and *Phoma* (Bandyopadhyay *et al.,* 1991; Tesfaendrias *et al.,* 2011). This pathogen complex has the ability to cause severe yield losses as a result of loss of seed mass, density and quality. In addition to economic implications, SGM is also associated with the risk of mycotoxin accumulation which is detrimental to human and livestock health (Thakur *et al.,* 2006). The *Fusarium graminearum* species complex (*Fg*SC*)* is the most important with regards to the latter due to their ability to produce some of the most harmful mycotoxins (Audenaert *et al.,* 2013).

Trichothecenes, deoxynivalenol (DON) and nivalenol (NIV), as well as zearalenone (ZEA) are the most frequent mycotoxins associated with the *Fg*SC. However, host specificity has been noted where *F. boothii* produces DON and is associated primarily with maize, while NIV and ZEA production is associated with *F. acaciae-mearnsii* and *F. meridionale* and are predominant on sorghum (Miller, 1995; Boutigny *et al.*, 2012; Mavhunga, 2013). Deoxynivalenol and NIV are highly correlated with cardiovascular and gastro-intestinal system defects and are known immune inhibitors. Zearalenone is suspected to be a carcinogen in humans, and is known to have hyperestrogenic effects and cause deformations of the reproductive systems in a variety of livestock (Magan *et al.,* 2011). The USA has legal limits for DON which may be no higher than 1000 µg.kg^{-1} in maize and wheat food crops (CAST, 2003). Legislation in the European Union states that the maximum level of DON in cereal products ranges from 500 to 1250 µg.kg^{-1}, depending on use (Bhat *et*

al., 2010). A maximum tolerated level of ZEA in grain products is 100 µg.kg^{-1} (FAO *et al.*, 2012). There is no legislation in South Africa which regulates the above mentioned mycotoxins. Only aflatoxins and patulin are regulated in South Africa, but recommendations for the addition for DON, ochratoxin A and fumonisins have been made (Rheeder *et al.*, 2009).

Grain mold incidence, severity and mycotoxin accumulation is dependent on seasonal, geographic and agronomic variations (Landschoot *et al.,* 2012). Meteorological variables, responsible for seasonal variation, which encourage the development of *Fg*SC include temperature, moisture and relative humidity (Wegulo, 2012). The greater the ability for *Fg*SC to overwinter and primary inoculum to persist will intensify the incidence and severity of the disease (Audenaert *et al.,* 2013). Timing of favourable meteorological variables with host crop growth stages is critical in the development of epidemics. Sorghum is the most sensitive to grain mold development from anthesis, during grain development until harvest (Menkir *et al.,* 1996).

Wegulo (2012) states that in wheat *Fg*SC biomass is significantly correlated with the amount of DON produced. This could indicate that conditions that favour higher fungal colonisation in grains should also favour higher accumulation of mycotoxins. The interaction of free water (a_w) and temperature contributes significantly to the production and accumulation of trichothecenes in grains (Ramirez *et al*., 2006). Furthermore, in wheat there has been evidence that light regulation, thus the number of sunshine days, can influence the regulation of the trichothecene regulator genes *Tri4, Tri5, Tri6* and *Tri10* (Audenaert *et al.,* 2013). Weak correlations between ZEA accumulation and temperature in maize have been observed, however rainfall during ear development and maturation are positively correlated with the production of ZEA (Sutton *et al.,* 1980).

The aim of this study is to identify climatic factors which promote disease development and increase severity of SGM, with specific reference to *Fg*SC colonisation and concomitant mycotoxin production, DON, NIV and ZEA.

2. Methods and Materials

Field Samples

Sorghum grains were harvested from selected cultivars in the National Cultivar Evaluation Trials at a range of localities during the 2010-2011 and 2011-2012 sorghum production season (Table 1). Cultivar trials were conducted using randomised complete block design with three replicates under dryland conditions. Trials were maintained according to best practice approaches appropriate to the respective production areas.

Quantification of Fusarium graminearum species complex

DNA extraction

Reference strains were purchased from the Plant Protection Research Institute of the Agricultural Research Council (ARC-PPRI) as well as the PROMEC Unit of the Medical Research Council (MRC) culture collections, South Africa.

Total DNA was extracted from 400 mg milled grain samples and 100 mg fungal mycelia of reference culture. Genomic DNA from milled grain samples as well as fungal mycelia was extracted using Wizard ® Genomic DNA Purification Kit (Promega Corporation, USA) according to the manufacturer's instructions. The DNA concentrations and purity were determined using a NanoDrop® spectrophotometer (ND1000) (Thermo, Waltham, MA, USA) by absorbance at 260 nm (OD260). All the DNA samples were diluted to 10 ng.μl^{-1}, aliquots of 100 µl sub-samples were and at -20 ºC.

Quantitative Real Time PCR

Quantitative detection of *F. graminearum* in the sorghum grains was determined by real time quantitative PCR (qPCR) as described by Nicolaisen *et al.* (2009). Primers used for the detection of *F. graminearum* were FgramB379 fwd (CCATTCCCTGGGCGCT) and complimentary, FgramB411 rev (CCTATTGACAGGTGGTTAGTGACTGG) (Inqaba Biotechnical Industries (PTY) Ltd) as developed by Nicolaisen *et al.* (2009). Real-time PCR reactions were carried out in a total volume of 10 µl consisting of 5 µl iTaq™ Universal SYBR® Green Mix

(BIO-RAD), 0.5 µl FgramB379 fwd (2 µM) and 0.5 µl FgramB411 rev (2 µM), 3.2 µl Ultrapure DNASE and RNASE Free Water (18.2 M, Bioline) and 0.8 µl template DNA (10 ng.µl^{-1}). Each qPCR plate contained a triplicate of each biological template DNA, no template control, positive control and a standard curve of matrix DNA. The standard curve matrix was made up of *F. graminearum* DNA diluted 4-, 16-, 64-, 256-fold in sorghum free of *F. graminearum* DNA (10 ng.µl^{-1}).

Quantitative PCR was performed on a Rotor-gene TM 6000 (Corbett Life Science) using the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s, and a melting curve analysis from 72°C to 95°C, increasing by 1°C each step.

Mycotoxin Detection and Quantification

The modified protocol of Small *et al. (*2012) was used to extract DON, NIV and ZEA. Five gram sub-samples of grain were milled and placed into 50 ml Falcon tubes and 20 ml of methanol/water (70:30 v/v) was added. The flasks were shaken at 200 rpm on a Heidolph MultiReax Shaker (Labotec, South Africa) for 30 min at room temperature. The samples were centrifuged for 10 minutes at 4000 rpm. A syringe was used to draw up 2 ml of the supernatant which was filtered with a 0.45 µm RC syringe filter (Acrodisc ® PALL PSF Syringe Filter, Premium Glass Fiber Prefilter). The filtered supernatant was placed into a 2 ml Eppendorf and left overnight at 4°C. The samples were centrifuged for 10 minutes at maximum acceleration (*g*). Nivalenol and ZEA samples were diluted ten-fold by placing 100 µl supernatant to 900 µl distilled water and vortexed prior to analysis. Deoxynivalenol samples remained undiluted and were placed in 1.8 µl vials.

Standards of DON, NIV and ZEA were obtained from Sigma-Aldrich. A known mycotoxin free sorghum sample was extracted using the above mentioned protocol and 1 ml undiluted supernatant was added to the 1.8 ml LC-MS-MS vial. This was done to take into consideration the matrix effect associated with mycotoxin extractions. The 1 ml mycotoxin free matrix was evaporated under a gentle air flow. The evaporated samples were reconstituted with a calibration standard solution ranging from 1600 µg.kg^{-1} to 50 µg.kg^{-1} for DON and NIV and 3200 µg.kg^{-1} and 50 µg.kg^{-1} for ZEA. The samples were vortexed prior to analysis. The samples were extracted and reconstituted in triplicate to ensure repeatability and accuracy. The

concentrations of the DON, NIV and ZEA standards were determined at the University of the Free State, Department of Microbial, Biochemical and Food Biotechnology. The lowest level of quantification for DON, NIV and ZEA was 25 $\mu g.kg^{-1}$.

Samples were analysed using a 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer (AB Sciex) and Shimadzu UFLC stack with LC-20AB binary pump and SIL-20A HT autosampler as front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software.

Twenty microliters of each sample was separated on a C18 (150 x 2.1 mm x5 µ, Discovery C18, Supelco) column at a flow rate of 300 $\mu l.min^{-1}$ using a stepwise gradient between 10mM ammonium acetate (mobile phase A) and methanol 10mM ammonium acetate (mobile phase B). The column was equilibrated and loaded at 2%B, rapidly increased to 75%B and maintained for 4 minutes, followed by re-equilibration at 2% for a total runtime of 9 minutes. Eluting analytes were ionised in negative electrospray mode with a 4500 V ion spray voltage and 500°C heater temperature to evaporate excess solvent, 60 psi nebuliser gas, 60 psi heater gas and 25 psi curtain gas.

A targeted Multiple Reaction Monitoring (MRM) workflow was followed on the instrument to analyse the sample. During an MRM scan type the instrument is used in triple quadrupole mode where every ionised analyte (the precursor) eluting off the column is fragmented in the collision cell to produce fragment masses. A set of masses, the precursor mass and one fragment mass constitutes a transition. The instrument jumps between different transitions in an MRM transition list during an analysis cycle, each cycle typically lasting less than a second. If a transition is detected the instrument's response is registered and this ion intensity value is plotted as a chromatogram. Additional mass spectrometer settings are given in Table 1.

All compound and source dependant parameters were optimised using compound optimization in Analyst 1.5.2. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the other transitions are used as qualifiers. The qualifier serves as an additional level of confirmation for the presence of the analyte and the retention time for these three transitions needs to be the same.

Meteorological Data

Total radiation (RAD; MJ/m2), evapotranspiration (Eto; mm), mean maximum and minimum temperature (MaxT; MinT;°C), mean maximum and minimum relative humidity (MaxRH; MinRH; %) and total rainfall (RF; mm) for the 2010/2011 and 2011/2012 seasons for the respective localities were monitored in this study during flowering and grain development, i.e. 70 days after planting (d.a.p.) to 120 d.a.p. The data were supplied by the ARC-Institute for Soil Water and Climate's meteorology office.

Data Analysis

Weather x colonisation/mycotoxin (DON, NIV and ZEA) production analyses were conducted on data collected during individual seasons as well as pooled data from the respective seasons. Weather data subsequent to flowering in each season were bulked into periodic running means (0, 5, 10 and 14 days) and correlation matrix analysis was used to determine clusters of significant relationships between bulked weather variables and *Fg*SC DNA concentrations and DON, NIV and ZEA production in the post-flowering period. Correlations between weather data and disease parameters were conducted using Pearson's Correlation. Non-linear regression was used to determine the relationship between variables. Stepwise multiple regression analysis (MRA) was also conducted for variable selection (Hintze, 2007).

3. Results

Fusarium graminearum species complex DNA concentrations in harvested sorghum grains ranged from 0-2700 $pg.mg^{-1}$ in 2008 and 0-3790 $pg.mg^{-1}$ in 2009.

Deoxynivalenol concentrations ranged from 0-258.75 ppm in 2008 and 0-263.42 ppm in 2009; NIV concentrations ranged from 0-0.18 ppm in 2008 and 0-0.14 ppm in 2009 and ZEA concentrations ranged from 0.35-18.82 ppm in 2008 and 0.28-15.75 ppm in 2009 in harvest sorghum grains.

Weather variable values for the period subsequent to flowering i.e. 70 d.a.p., were pooled into five-, 10- and 14- day running means. Inclusion of the day running means in correlation analysis indicated 14-day running means to be best correlated with

*Fg*SC DNA concentrations and subsequent analyses were standardised using these as respective weather variables.

*Fg*SC data from all localities were bulked into DNA concentration frequency classes with a class value of 50 pg.mg^{-1} and mean values of weather variables associated with each class were calculated. Scatter diagrams between *Fg*SC DNA concentrations and weather variables indicated non-linear relationships and non-linear regression was, therefore, used to determine those variables significantly related to grain colonisation by the species complex.

The Michaelis-Menten model was used to determine the relationship between *Fg*SC DNA and the 14 day means of maximum temperature (MaxT) and evapotranspiration (Eto), where,

$$Y = \frac{Ax}{B + x}$$

Where Y is *Fg*SC DNA concentrations; x is either MaxT or Eto.

while the exponential model below was applied to mean maximum relative humidity (MaxRH);

$$\mathrm{Y} = \mathrm{A}\mathrm{e}^{\mathrm{Bx}}$$

Where Y is *Fg*SC DNA concentrations; x is MaxRH.

A histogram of regression R^2-values for the period 75-85 d.a.p. is presented in Figure 1. R^2-values indicated improved relationships between *Fg*SC DNA and the weather variables, mean MaxT, MaxRH, Eto and rainfall (RF) as the 14 day mean for days 82-95 post planting was approached after which the relationship declined rapidly. No relationships were recorded with mean minimum temperature (minT), mean minimum relative humidity (minRH) and RF, using an array of non-linear models 82-95 d.a.p. *Fg*SC DNA concentrations had a significant relationship with mean MaxT, MaxRH and Eto, R^2=0.71; 0.59; 0.69, respectively. These relationships are presented in Figure 2.

Inclusion of these parameters in a stepdown MRA indicated temperature x moisture interactions as follows;

$$FgSC = 216.855MaxRH_{82-95} - 746.745MaxT_{82-95} \qquad R^2 = 0.79$$

Where *Fg*SC is *Fusarium graminearum* species complex DNA concentration, $MaxRH_{82-95}$ is average maximum relative humidity 82-95 days after planting (d.a.p.), $MaxT_{82-95}$ is average maximum temperature 82-95 d.a.p.

Variables MaxT and MaxRH were log transformed to linearize the relationship with *Fg*SC for inclusion in a stepdown multiple regression analysis. The relationship was not improved and thus the untransformed data was used to develop the above preferred model for the relationship between *Fg*SC DNA concentrations and MaxRH and MaxT. However, the relationship of *Fg*SC DNA concentrations with MaxT and Eto was improved on linearization thus the below model was preferred;

$$FgSC = 15171.779LnMaxT_{82-95} - 35746.558LnEto_{82-95} \qquad R^2 = 0.73$$

Where *Fg*SC is the *Fusarium graminearum* species complex DNA concentration, $MaxT_{82-95}$ is the average maximum temperature 82-95 days after planting (d.a.p.), Eto_{82-95} is the average evapotranspiration 82-95 d.a.p.

The above data bulking and regression approach was applied to DON, NIV and ZEA. Significant relationships were recorded between the *Fg*SC DNA concentrations and levels of mycotoxin contamination, represented in Figure 3A, 4A and 5A with R^2=0.66%, 0.63% and 0.66%, respectively. In addition, regression indicated best fits were obtained for NIV, DON, ZEA, and with $MinT_{91-104,}$ $MaxT_{101-115}$ and $MinT_{100-113}$, respectively and is represented in Figure 3B, 4B and 5B where R^2=0.45%, 0.72% and 0.68%, respectively. Multiple regression analysis yielded the following significant models, where log-linearization did not improve the model and thus normal data was applied;

$$\text{DON}=0.0626FgSC\text{-}0.0352\text{MaxT}_{101\text{-}115} \qquad R^2=0.84$$

$$\text{NIV}=3.684\text{E-}05FgSC+2.992\text{E-}03\text{MinT}_{91\text{-}104} \qquad R^2=0.83$$

$$\text{ZEA}=7.120\text{E-}03FgSC+0.2606\text{MinT}_{100\text{-}113} \qquad R^2=0.92$$

Where DON is deoxynivalenol, NIV is nivalenol, ZEA is zearalenone, *Fg*SC is the *Fusarium graminearum* species complex DNA concentration, $MaxT_{101-115}$ is the average maximum temperature 101-115 days after planting (d.a.p.), $MinT_{91-104}$ is the average minimum temperature 91-104 d.a.p. and $MinT_{100-113}$ is the average minimum temperature 100-113 d.a.p.

4. Discussion

According to the 2010/2011 National Sorghum Cultivar Trials, 50% flowering of local sorghum cultivars occurs between 76 and 82 d.a.p., therefore soft dough would occur approximately 100-110 d.a.p., hard dough would follow at 120-130 d.a.p. and physiological grain maturity at 160 d.a.p. The FHB window of infection of grain coincides with critical weather periods just prior to early- and post-anthesis (Schaafsma & Hooker, 2007). The optimum temperature for FHB infection and most damage is 25°C accompanied by RH≥90% over this growth period in wheat (Hooker *et al.,* 2002; De Wolf *et al.,* 2003; Wegulo, 2012).

Wegulo (2012) reported that the accumulation of mycotoxins in grain is attributed to fungal biomass in the grains. The significant relationship between *Fg*SC DNA concentration and DON, NIV and ZEA concentrations reported in this study, partly supports the findings of Wegulo (2012) as *Fg*SC was positively related to the accumulation of DON, NIV and ZEA, as seen in Figure 3A, 4A and 5A respectively. This study also indicates the role of temperature subsequent to colonisation on mycotoxin accumulation.

The sequential critical weather periods associated with *Fg*SC and NIV, i.e. 82-95 d.a.p and 91-104 d.a.p. respectively, could be explained by the predominance of NIV chemotypes on sorghum. *Fg*SC lineages of *F. acaciae-mearnsii* and *F. meridionale* were the only lineages isolated from sorghum grain in South Africa (Mavhunga, 2013). It can therefore be assumed that NIV is the primary factor in the initiation of infection and colonisation of sorghum grain and hence, its earlier detection than DON and ZEA and its close association with the critical period for *Fg*SC infection.

Xu *et al.* (2007) stated that temperatures ranging from 20 to 25°C are sufficient to increase DON concentrations. In this study there was no significant relationship between weather variables and DON accumulation. However, a tendency for increased DON with mean MaxT at 101-115 d.a.p., as indicated in Figure 3B was recorded. Weather data was not the most reliable due to multiple missing data and may have confounded the relationship between MaxT and DON. Future research could include selecting localities with available and more reliable weather station data to improve quantification of the above relationship.

The critical point for NIV accumulation and mean MinT is at 91-104 d.a.p. This is approximately at the end of flowering and prior to soft dough. An increase of mean MinT resulted in a reduction of NIV concentrations, as represented in Figure 4B. According to Wagacha & Muthomi (2007), the optimum temperature for NIV is 20°C. Environmental stress, such as cold stress, can significantly influence the production and accumulation of mycotoxins, independent of *Fg*SC biomass produced (Sanchis & Magan, 2004). This may be an explanation for the increased production of NIV at lower temperatures reported by Wagacha & Muthomi (2007).

The optimum conditions for the production of ZEA by *Fg*SC in maize, rice and wheat are from 17-28°C, or a temperature series with two weeks at 25 - 28°C and between 12 and 15°C for three to four weeks combined with RH > 90% (Doohan *et al*., 2003). A significant positive relationship existed between *Fg*SC and ZEA, where ZEA accumulation would increase at higher *Fg*SC infections, shown in Figure 5A. Furthermore, where there is an increase of mean MinT there would be a subsequent reduction of ZEA concentrations, indicated in Figure 5B. The critical point for ZEA accumulation and mean MinT is from 100-113 d.a.p. The optimum temperature for ZEA accumulation, in this study, during this period was between 12 and 13°C, which corroborates with Doohan *et al.* (2003).

Current FHB and DON forecasting models include pathogen parameters and weather variables, temperature, RH, RF, head wetness duration (HWD) and interactions of the above variables, as predictive parameters. This study confirms the driving variables, of fungal biomass, temperature and RH, as well as interactions. Future data collection could include more accurate RF data as well as measurement of HWD to improve the accuracy of each model and potential predictive capability of the models.

5. References

Audenaert, K., Vanheule, A., Höfte, M & Haesaert, G. 2013. Deoxynivalenol: a major player in the multifaceted response of Fusarium to its environment. *Toxins*. **6**: 1–19.

Bandyopadhyay R., Mughogho L. K., Satyanarayana M. V. & Kalisz, M. E. 1991. Occurrence of air-borne spores of fungi causing grain mold over a sorghum crop. *Mycological Research* **95**: 1315–20.

Bandyopadhyay, R., Butler, D. R., Chandrashekar, A. Reddy, R. K., & Navi, S. S. 2000. Biology, epidemiology, and management of sorghum grain mold. Pages 34-71 in Technical and institutional options for sorghum grain mold management: proceedings of an international consultation, 18-19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Bhat, R., Rai, R. V & Karim, A. A., 2010. Mycotoxins in Food and Feed: Present Status and Future Concerns. *Comprehensive Reviews in Food Science & Food Safety* **9**: 57–81.

Boutigny, A-L., Ward, T. J., Van Coller, G. J., Flett, B., Lamprecht, S. C., O'Donnell, K. & Viljoen, A. 2012. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. *Fungal Genetics & Biology* **48**: 914-920.

Council for Agricultural Science and Technology (CAST). 2003. Mycotoxins : Risks in Plant, Anima, and Human Systems. Accessed from http://www.trilogylab.com/uploads/Mycotoxin_CAST_Report.pdf on 25.10.2013.

De Wolf, E. D., Madden, L. V & Lipps, P.E. 2003. Risk assessment models for wheat Fusarium head blight epidemics based on within-season weather data. *Phytopathology* **93**: 428–35.

Department of Agriculture, Forestry and Fishery (DAFF). 2010. Sorghum: Production guideline. Accessed from on 10/16/2012: http://www.nda.agric.za/docs/Brochures/prodGuideSorghum.pdf.

Doohan, F.M., Brennan, J., and Cooke, B.M. 2003. Influence of climatic factors on Fusarium species pathogenic to cereals. *European Journal of Plant Pathology* **109**: 755-768.

FAO, WFP and IFAD. 2012. The State of Food Insecurity in the World 2012. Economic growth is necessary but not sufficient to accelerate reduction of hunger and malnutrition. Rome, FAO.

Hintze, 2007. NCSS 2007. NSCC, LLC. Kaysville, Utah, USA. www.ncss.com.

Hooker, D. C., Schaafsma, A. W., & Tamburic-Ilincic, L. 2002. Using weather variables pre-and post-heading to predict deoxynivalenol content in winter wheat. *Plant Disease* **86**: 611-619.

Landschoot, S., Waegeman, W., Audenaert, K., Vandepitte, J., Baetens, J. M., De Baets, B., & Haesaert, G. 2012. An empirical analysis of explanatory variables affecting Fusarium head blight infection and deoxynivalenol content in wheat. *Journal of Plant Pathology* ***94***: 135-147.

Magan, N., Medina, a. & Aldred, D., 2011. Possible climate-change effects on mycotoxin contamination of food crops pre- and postharvest. *Plant Pathology* **60**: 150–163. Available at: http://doi.wiley.com/10.1111/j.1365-3059.2010.02412.x

Mavhunga, M. 2013. *Fusarium graminearum* mycotoxins associated with grain mold of maize and sorghum in South Africa (Masters dissertation, University of the Free State Bloemfontein, South Africa).

Menkir, A., Ejeta, G. & Butler, L. 1996. Fungal invasion of kernels and grain mold damage assessment in diverse sorghum germ plasm. *Plant Disease* **80**: 1399–1402.

Miller, J. D. 1995. Fungi and mycotoxins in grain: Implications for stored product research. *Journal of Stored Production Research* **31**: 1-16.

Nicolaisen, M, Suproniene, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H., Justesen, A. F. 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *Journal of Microbiological Methods* **76**: 234–40.

Ramirez, M. L., Chulze, S., & Magan, N. 2006. Temperature and water activity effects on growth and temporal deoxynivalenol production by two Argentinean strains of *Fusarium graminearum* on irradiated wheat grain. *International Journal of Food Microbiology* **106**: 291-296.

Rampho, E. T. 2005. Sorghum bicolor (L.) Moench. Accessed from http://www.plantzafrica.com/plantqrs/sorghum.htm on the 16/10/2012

Reddy, B. V. S., Kumar, A. A. and Reddy, P. S. 2010. Recent Advances in Sorghum Improvement Research at ICRI- SAT, Kasetsart. *Natural Scie*nce **44**: 499-506.

Rheeder, J. P., Shephard, G. S., Vismer, H. F., & Gelderblom, W. C. A. 2009. Guidelines on mycotoxin control in South African foodstuffs: from the application of the Hazard Analysis and Critical Control Point (HACCP) system to new national mycotoxin regulations. *Medical Research Council Policy Brief.* Accessed on 26/11/2012 from http://www.mrc.ac.za/policybriefs/Mycotoxinguidelines.pdf

Sanchis, V. & Magan, N. 2004. Environmental conditions affecting mycotoxins. Pages 174–189 in Mycotoxins in food: Detection and control, N. Magan & M. Olsen (Eds.), Boca Raton, CRC Press, USA.

Schaafsma, A. W. & Hooker, D.C. 2007. Climatic models to predict occurrence of *Fusarium* toxins in wheat and maize. *International Journal of Food Microbiology* **119**: 116–25.

Small, I. M., Flett, B. C., Marasas, W. F. O. McLeod, A., Stander, M. A. and Viljoen, A. 2012. Resistance in maize inbred lines to *Fusarium verticilliodes* and fumonisin accumulation in South Africa. *Plant Disease* **96**: 881-888.

Sutton, J. C., Baliko, W. & Funnell, H. S. 1980. Relation of weather variables to the incidence of zearalenone in corn in southern Ontario. *Canadian Journal of Plant Sciences* **60 :** 149 – 155.

Tesfaendrias, M. T., McLaren, N. W., & Swart, W. J. 2011. Grain mold fungi and their effect on sorghum grain quality. *South African Journal of Plant & Soil **28**:* 172-180.

Thakur, R. P., Reddy, B. V. S., Indira, S., Rao, V. P., Navi, S. S., Yang, X. B. & Ramesh, S. 2006. Sorghum grain mold: Information Bulletin No. 72. International Crops Research Institute for the Semi-Arid Tropics. Patancheru 502324, Andhra Pradesh, India: 32 pp. ISBN 92-9066-488-6.

Wagacha, J. M., & Muthomi, J. W. 2007. *Fusarium culmorum*: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. *Crop Protection* **26**: 877-885.

Wegulo, S. N. 2012. Factors influencing deoxynivalenol accumulation in small grain cereals. *Toxins* **4**: 1157-1180.

Xu, X. M., Monger, W., Ritieni, A. & Nicholson, P. 2007. Effect of temperature and duration of wetness during initial infection periods on disease development, fungal biomass and mycotoxin concentrations on wheat inoculated with single, or combinations of, *Fusarium* species. *Plant Pathology* ***56***: *943*-956.

Table 1. Localities and cultivars sampled over a two year period for the development of a model to predict colonisation of sorghum grain by *Fusarium graminearum* species complex and concomitant mycotoxin accumulation.

Season			
2007/08		**2008/09**	
Localities	**Cultivars**	**Localities**	**Cultivars**
Bethlehem	PAN8609	Amersfoort	PAN8609
Cedara	PAN8247	Cedara	PAN8247
Goedgedacht	PAN8625	Bethlehem	PAN8625
Holmdene	PAN8816	Dover	PAN8816
Klerksdorp	PAN8648	Goedgedacht	NS5511
Potchefstroom Irrigation	PAN8420	Klerksdorp	
Val	NS5511	Klipdrift	
		Leeuwkraal	
		Parys	
		Perdekop	
		Plantrand NorthEast	
		Potchefstroom 1	
		Potchefstroom 2	
		Potchefstroom 3	
		Potchefstroom Dryland	

Table 2. Mass spectrometer settings for the detection of nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEA) in sorghum grain using LC-MS-MS.

Mass spectrometer settings	**NIV**	**DON**	**ZEA**
Precusor ion [M+H]+(m/z)	371	355	317
Product ion (m/z)	281	265	175
Declustering potential (V)	-1	-1	-16
Collusion 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

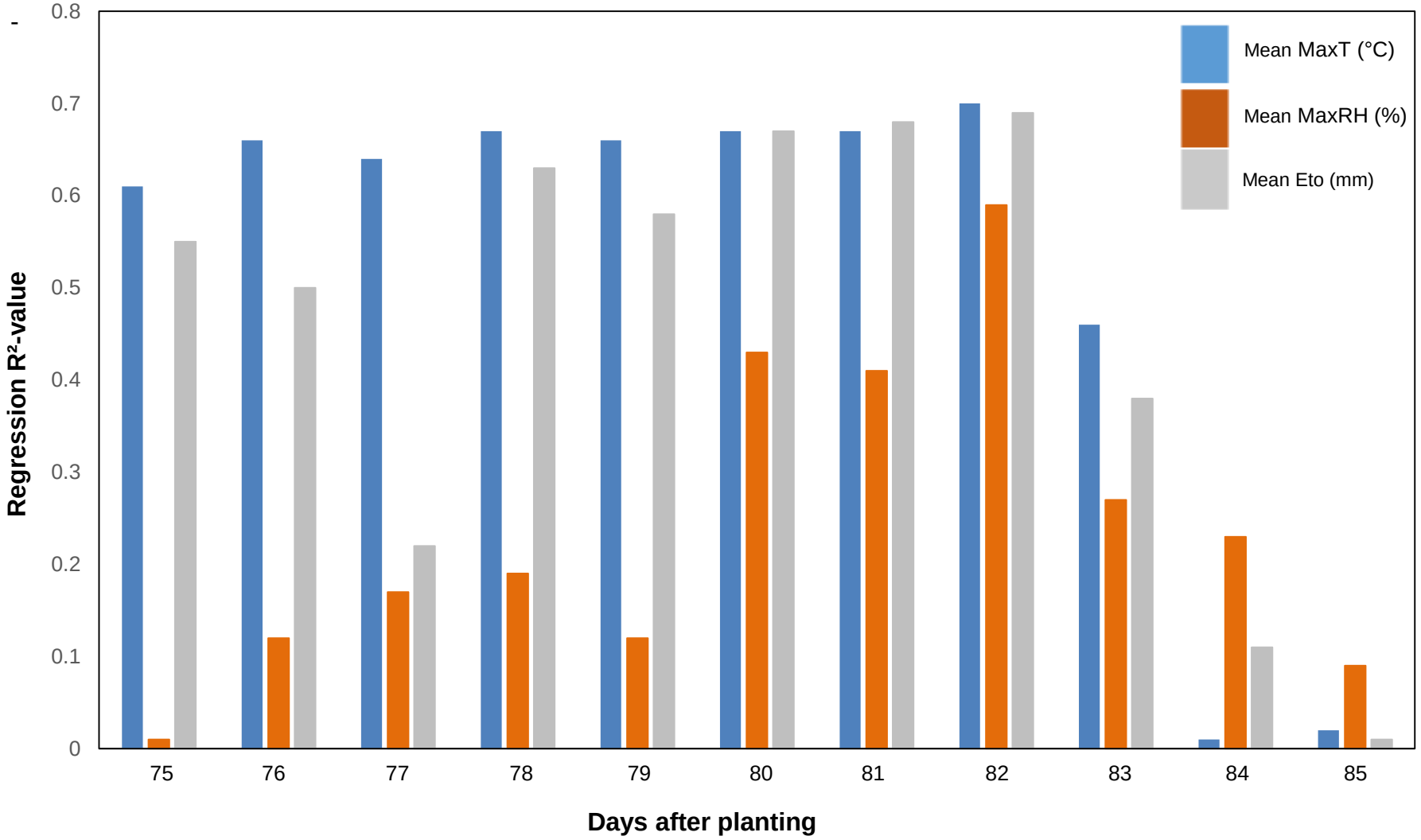


Figure 1. Regression R^2-values for mean maximum temperature (mean MaxT;°C); mean maximum relative humidity (mean MaxRH, %) and Mean evapotranspiration (mean Eto, mm) and critical periods from 75 to 85 days after planting.

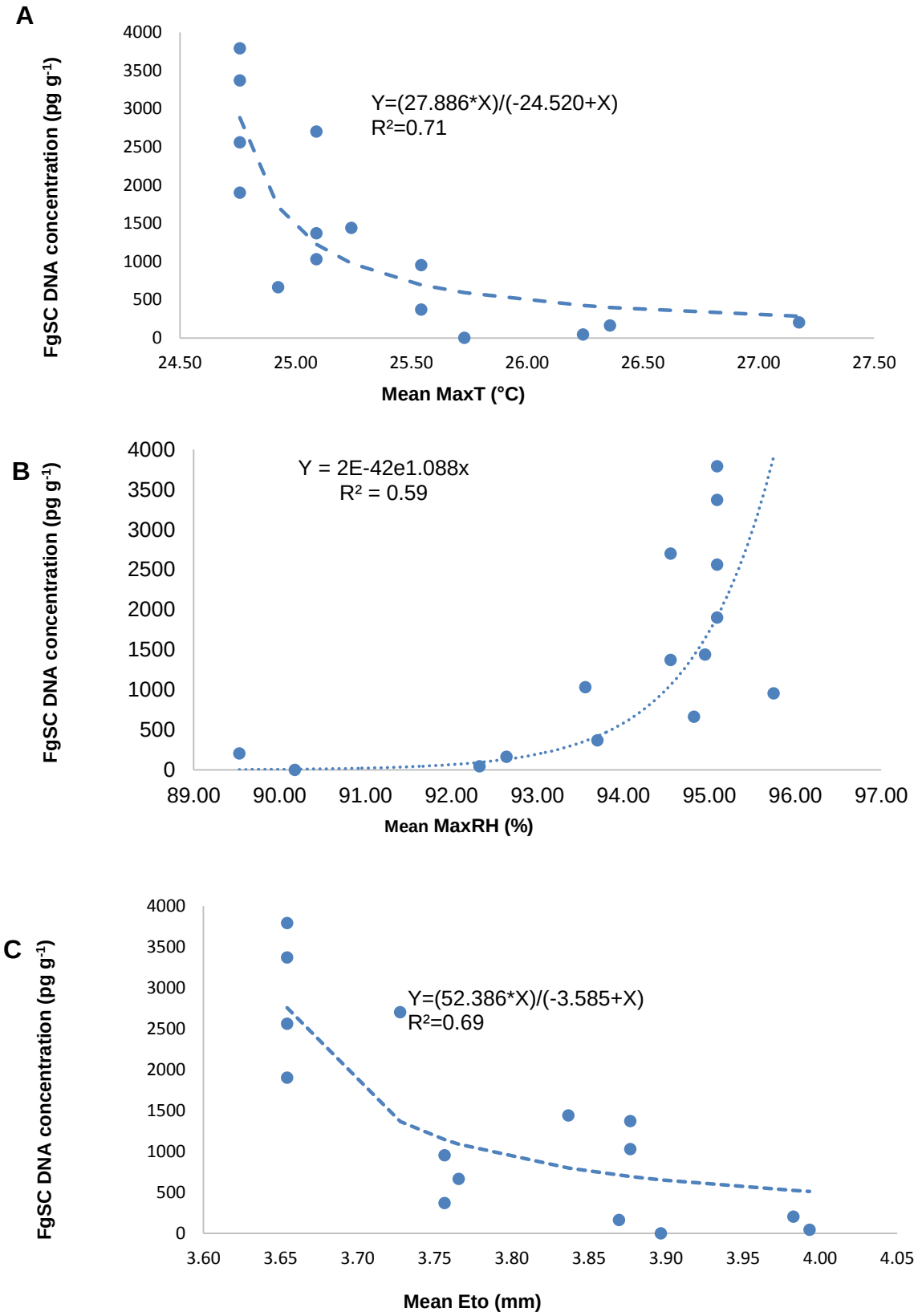


Figure 2. Regression analysis of *Fg*SC fungal biomass concentration (*Fg*SC DNA concentration; ng.ul^{-1}) quantified through quantitative PCR and mean maximum temperatures (°C)(A); mean maximum relative humidity (mean MaxRH; %)(B) and mean evapotranspiration (Eto, mm)(C) at 82-95 days after planting.

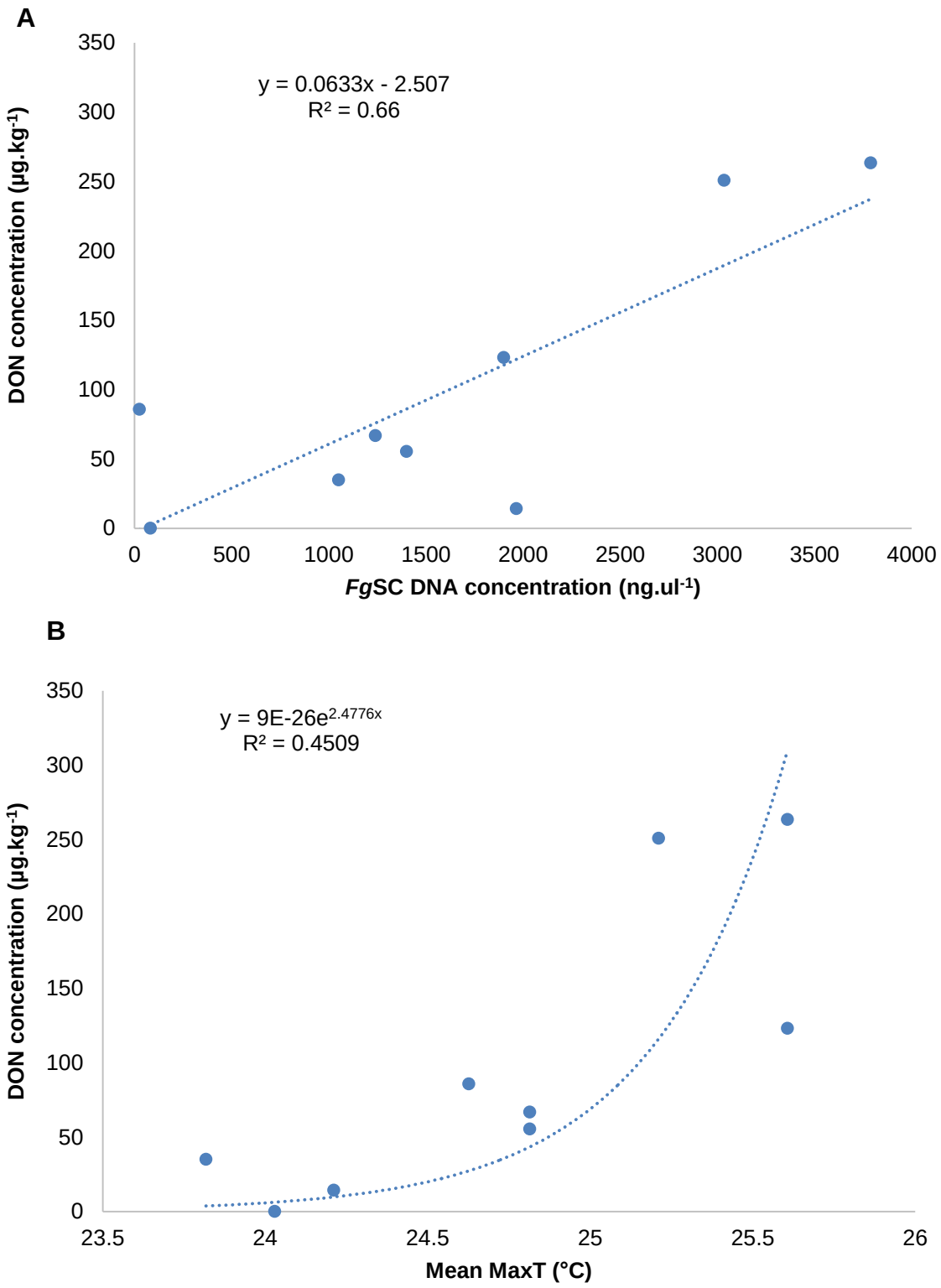


Figure 3. Regression analysis of deoxynivalenol (DON; µg.kg^{-1}) detected and quantified through LC-MS-MS and *Fg*SC fungal biomass concentration (*Fg*SC DNA concentration; ng.ul^{-1}) quantified through quantitative PCR (A) and mean maximum temperatures (°C) at 101-115 days after planting (B).

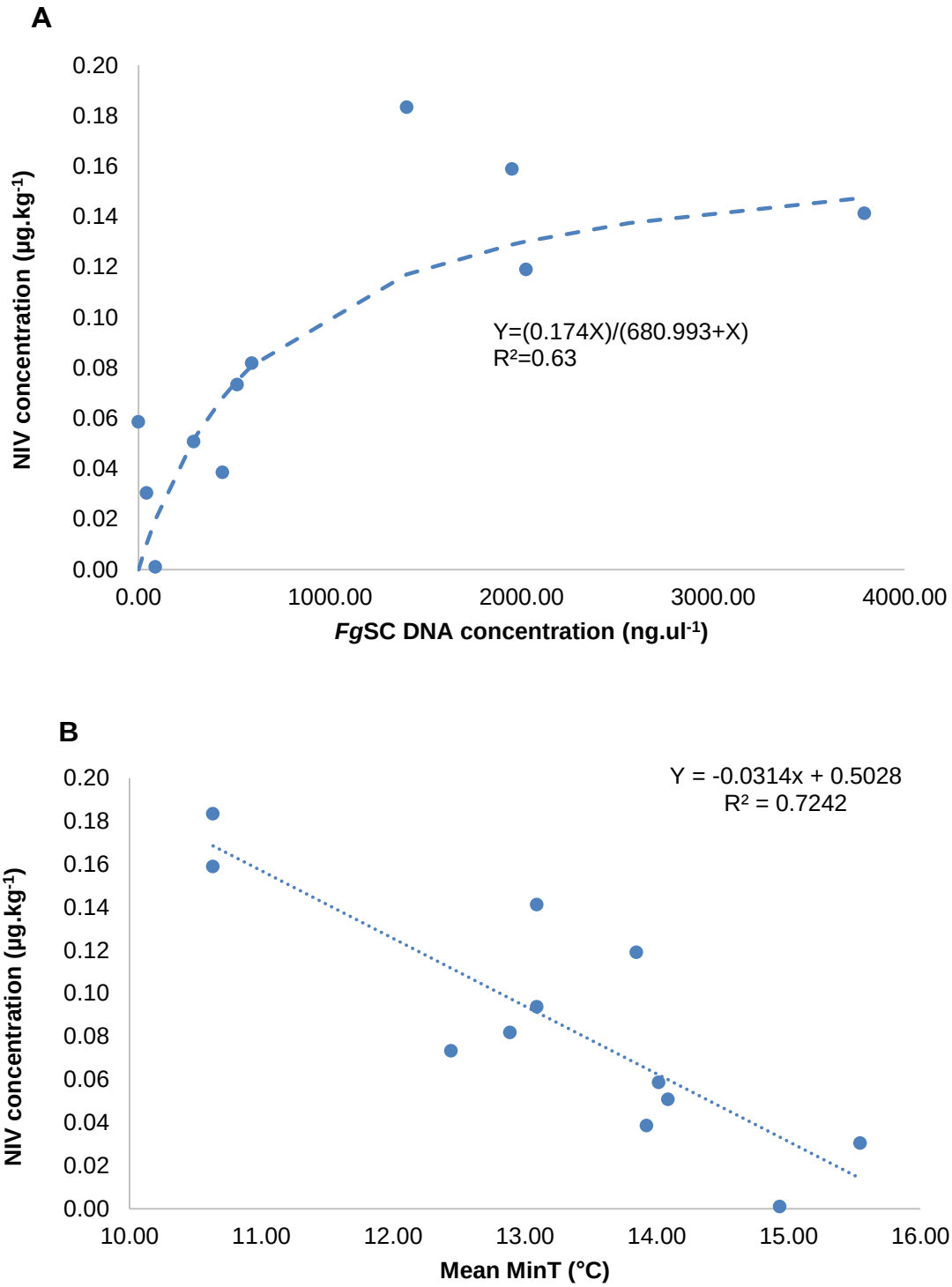


Figure 4. Regression analysis of nivalenol (NIV; µg.kg^{-1}) detected and quantified through LC-MS-MS and *Fg*SC fungal biomass concentration (*Fg*SC DNA concentration; ng.ul^{-1}) quantified through quantitative PCR (A) and mean minimum temperatures (°C) at 91-104 days after planting (B).

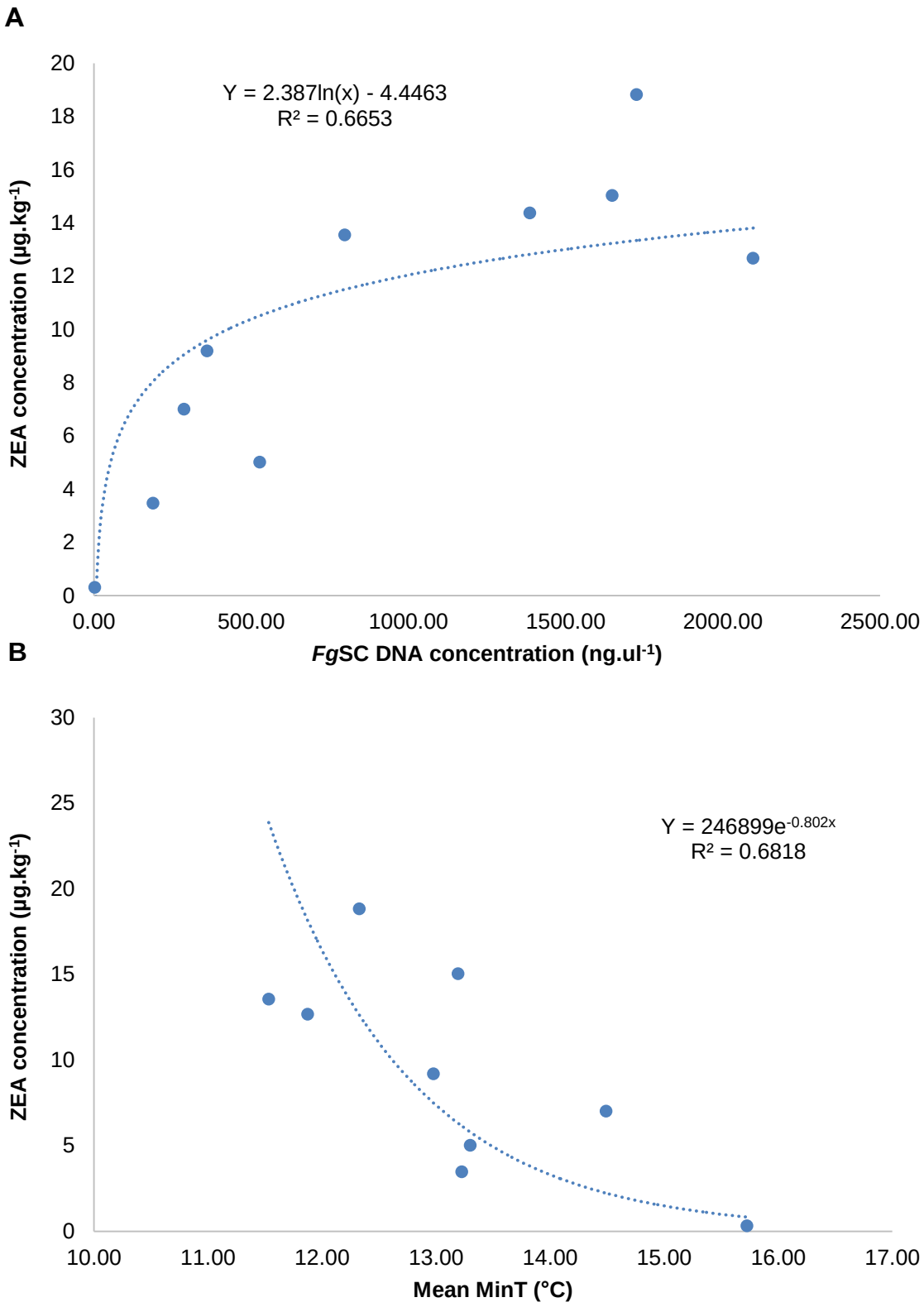


Figure 5. Regression analysis of zearalenone (ZEA; µg.kg^{-1}) detected and quantified through LC-MS-MS and *Fg*SC fungal biomass concentration (*Fg*SC DNA concentration; ng.ul^{-1}) quantified through quantitative PCR (A) and mean minimum temperatures (°C) at 100-113 days after planting (B).

Chapter 4

Effect of processing on mycotoxins associated with *Fusarium graminearum* species complex in sorghum grain products

Abstract

Sorghum grain mold (SGM) is the most important biotic constraint in sorghum grain production. Not only is it detrimental to the crop but also to human and livestock health, due to the production of mycotoxins. *Fusarium* spp. are responsible for the majority of mycotoxins including zearalenone (ZEA) and the trichothecenes nivalenol (NIV) and deoxynivalenol (DON), which are closely associated with SGM and the *Fusarium graminearum* species complex (*Fg*SC). The purpose of this study was to evaluate different phases of processing in the preparation of two final products from a commercial sorghum processor on FgSC, DON, NIV and ZEA contamination, and to assess removal of *Fg*SC colonisation and concomitant mycotoxins at various percentages of decortication using tangential abrasive dehulling device (TADD) in controlled conditions. Ergosterol concentrations were highest in sorghum bran (423.15 $\mu g.g^{-1}$) and lowest in 22% dehulled grain (322.09 $\mu g.g^{-1}$) indicating that a high proportion of grain fungal contamination lies in the outer grain layers. In contrast, *Fg*SC DNA concentrations were detected at lowest levels in sorghum bran (0.00 $pg.mg^{-1}$) and at highest levels in 22% dehulled grain (18.52 $pg.mg^{-1}$). This observation was supported by controlled decortication by TADD which rendered the highest levels of *Fg*SC DNA concentrations (214.5 $pg.mg^{-1}$) at two minutes decortication which equates to 23% decortication and lowest at four minutes decortication i.e. 35% decortication. The assumption can, therefore, be made that *Fg*SC infections were deep-seated within the grain endosperm. This assumption is further supported by NIV concentrations in commercial samples which ranged from 8.58 $\mu g.kg^{-1}$ and 4.95 $\mu g.kg^{-1}$ in the sorghum bran and 4% dehulled grain, respectively, to 0.00 $\mu g.kg^{-1}$ in the 22% dehulled grain. Similarly, NIV concentrations from TADD analysis ranged from 10.83 $\mu g.kg^{-1}$ at one minute decortication to 5.9 $\mu g.kg^{-1}$ at six minutes decortication which equate to 10 and 49% decortication, respectively. In contrast, ZEA concentrations from TADD analysis ranged from

59.78 µg.kg^{-1} at nil minutes decortication to a low of 8.13 µg.kg^{-1} at six minutes decortication. Similarly, DON, which was only detected in one commercial sample compared with 60% of the field samples used for controlled TADD analysis, was also removed from grain by short periods of decortication. The assumption can be made that ZEA and DON are associated with superficial *Fg*SC infections and accumulate in the outer layers of the grain while NIV is associated with pathogenesis in the deeper endosperm layers. Cultivars differed in hardness with the lowest mean decortication percentage over all times in grain from NS5511 at 19.3% and the highest in grain from PAN8609 at 26.6% decortication. Variation in hardness of grains was associated with prevailing weather conditions at each locality. Lower colonisation and mycotoxin levels were recorded in the harder grain. The highest *Fg*SC DNA concentrations, DON, NIV and ZEA accumulation was recorded in grain from Greytown where weather conditions during the critical grain development stages promoted the development of infection and the contamination of grains by mycotoxins. Understanding the effects of decortication on *Fg*SC DNA concentrations and the accumulation of DON, NIV and ZEA could assist commercial processors to make the best management decisions for the removal of these harmful mycotoxins.

1. Introduction

Annually, 20 million tons of sorghum is produced in Africa. Thus, Africa is accountable for a third of the sorghum produced globally (Taylor, 2003). Sorghum is a drought tolerant crop which also has the advantage of being able to resist water-logging for extended periods of time (Reddy *et al*., 2012). However the importance of sorghum should not only be calculated by yield and production adaptability. Fifteen of the 16 most food insecure nations of the world are found in Africa, Haiti being the exception (FAO, WFP and IFAD, 2012). As the majority of African countries have semi-arid and subtropical climates sorghum is crucially important to food security (Taylor, 2003). Sorghum products include staple foods such as injera (gluten-free pancake-like product) in Ethiopia, mabele (instant sorghum porridge) in South Africa, and togwa (porridge for toddlers under age of five). Furthermore sorghum can be used for non-alcoholic beverages, beer and malt (Belton & Taylor, 2003).

Sorghum grain mold (SGM) is one of the most important biotic constraints on pre-harvest sorghum production (Bandyopadhyay *et al.,* 2000). One of the most pathogenic and widely distributed fungi responsible for SGM is the *Fusarium graminearum* species complex (*Fg*SC). Symptoms associated with *Fg*SC grain mold include white or pink grains, reduced grain filling, size and density. Furthermore there is a health risk associated with accumulation of *Fg*SC specific mycotoxins which include the trichothecenes deoxynivalenol (DON) and nivalenol (NIV) as well as zearalenone (ZEA). These mycotoxins are regarded as economically important (Thakur *et al.,* 2006; Summerell & Leslie, 2011; FAO, 2012).

The European Union's legislative maximum limit is set at 500 $\mu g.kg^{-1}$ for DON contaminated grains for human consumption and derivative products. Currently there are no legislative limits associated with NIV. Zearalenone's lowest maximum level set by CAST is 100 $\mu g.kg^{-1}$ (CAST, 2003; FAO, 2012). Detrimental effects of DON and NIV include immune-suppressive disorders, neurological toxins and gastrointestinal haemorrhaging while zearalenone has the potential to cause estrogenic malformations (Köppen *et al.,* 2010).

Prior to the manufacturing of sorghum products, physical cleaning, sorting and trimming of bulk consignments of sorghum are required (Lauren & Smith, 2001;

Bullerman & Bianchini, 2007). Diseased and contaminated grains are generally lighter than healthy grains and separation of healthy and damaged grains can occur during combine harvesting and on grain elevators. Further removal of contamination can occur during dry milling (Wegulo, 2012). Dry milling can be divided into two steps, debranning and flour formation. Debranning is also known as dehulling or decortication. The removal of the testa and pericarp (seed coat) reduces polyphenolic compounds making the flour more palatable. The unique characteristic of sorghum grain is that the pericarp is highly friable, which is defined as crumbly by nature. As a result the bran could contaminate the flour during dry milling of sorghum. Traditional and cultural dry milling is completed through the use of a pestle and mortar. However with industrial requirements for high throughput milling a tangential abrasive dehulling device (TADD) has become available (Taylor, 2003; Hazel & Patel, 2004).

The purpose of this study was to evaluate different phases of processing in the preparation of two final products from a commercial sorghum processor on *Fg*SC, DON, NIV and ZEA contamination, and to assess removal of *Fg*SC colonisation and concomitant mycotoxins at various percentages of decortication using TADD.

2. Methods and Materials

2.1 Commercial Processing Unit

Commercial Samples

Raw sorghum, puffed sorghum (heat extrusion), 4 and 22% de-hulled grain and sorghum bran were collected from the same commercial platform at a sorghum processing company. Two finished products associated with this processing sequence were also taken from storage. The first product was a fine meal sorghum porridge and the second was a course sorghum porridge.

Quantification of Total Fungal Biomass

The modified method of Jambunathan *et al.* (1991) was used to quantify total fungal biomass in grain and meal using ergosterol concentration as the evaluation criterion. Ergosterol was extracted from the grains of all seven commercial samples. Grain and coarse samples were ground into a fine powder using a Mellerware® Coffee

Bean Grinder. Five g of the respective powders were added to 25 ml of extraction methanol (ROMIL-SpS™) in a 50 ml test tube with a screw cap and mixed at 1900 cycles.min^{-1} with a Heidolph MultiReax Shaker (Labotec) for 30 minutes. The mixture was allowed to settle and 12.5 ml of the clear supernatant was removed using a pipette and added to a 50 ml test tube with a screw cap, containing 1.5 g of potassium hydroxide pellets (KOH, Merck Pty Ltd, South Africa). The mixture was stirred in a vortex mixer (Vortex Genie 2, Scientific Industries) to dissolve the KOH. Five ml of n-hexane (Merck Pty Ltd) was added and the mixture was incubated for 30 min in a water bath at 75°C and subsequently, allowed to cool to room temperature. During cooling, test tubes were covered with foil to prevent ergosterol degradation as ergosterol is light-sensitive and degrades when exposed to UV light (Robine *et al*., 2005). Distilled water (2.5 ml) was added, the mixture was shaken with a vortex mixer and the hyper-thermal reaction was cooled to room temperature. The upper hexane layer was transferred to a glass test tube. Five ml n-hexane was added to the remaining aliquot in the screw-cap test tube and mixed well. The hexane layer was again removed and added to the earlier aliquot. This step was repeated. The hexane extract was evaporated in the glass test tube until dry in a water bath at 75°C. The residue was re-suspended in 2.5 ml HPLC-grade methanol (Sigma-Aldrich) and filtered through 0.45 µl syringe filter. The filtrate was analysed on high performance liquid chromatograph (HPLC) with a SIL-20A auto sampler (Perkin Elmer). The extract was loaded onto a reverse phase column (C18 125 A 10 µm particle size, 150 x 4.6 mm) at 50°C. The mobile phase was methanol:water (96:4) at a flow rate of 1.2 ml.min^{-1}. Standard ergosterol (>75% assay; Sigma-Aldrich) was used to calibrate the equipment. Ergosterol was determined from the peak area determined at 282 nm using a Perkin Elmer PDA UHPLC detector at a retention time of approximately 7 minutes.

Quantification of Fusarium graminearum species complex

DNA extraction

Reference strains were purchased from the Plant Protection Research Institute of the Agricultural Research Council (ARC-PPRI) as well as the PROMEC Unit of the Medical Research Council (MRC) culture collections, South Africa.

Total DNA was extracted from 400 mg milled grain and meal samples and 100 mg fungal mycelia of reference culture. Genomic DNA from milled grain samples as well as fungal mycelia was extracted using Wizard ® Genomic DNA Purification Kit (Promega Corporation, USA) according to the manufacturer's instructions. The DNA concentrations and purity were determined using a NanoDrop® spectrophotometer (ND1000) (Thermo, Waltham, MA, USA) by absorbance at 260 nm (OD260). All the DNA samples were diluted to 10 ng.µl^{-1}, aliquots of 100 µl sub-samples were and at -20ºC.

Quantitative Real Time PCR

Quantitative detection of *F. graminearum* in the sorghum samples was determined by real time quantitative PCR (qPCR) as described by Nicolaisen *et al*. (2009). Primers used for the detection of *F. graminearum* were FgramB379 fwd (CCATTCCCTGGGCGCT) and complimentary, FgramB411 rev (CCTATTGACAGGTGGTTAGTGACTGG) (Inqaba Biotechnical Industries (PTY) Ltd) as developed by Nicolaisen *et al*. (2009). Real-time PCR reactions were carried out in a total volume of 10 µl consisting of 5 µl iTaq™ Universal SYBR® Green Mix (BIO-RAD), 0.5 µl FgramB379 fwd (2 µM) and 0.5 µl FgramB411 rev (2 µM), 3.2 µl Ultrapure DNASE and RNASE Free Water (18.2 M, Bioline) and 0.8 µl template DNA (10 ng.µl^{-1}). Each qPCR plate contained a triplicate of each biological template DNA, no template control, positive control and a standard curve of matrix DNA. The standard curve matrix was made up of *F. graminearum* DNA diluted 4-, 16-, 64-, 256-fold in sorghum DNA free of *F. graminearum* DNA (10 ng.µl^{-1}).

Quantitative PCR was performed on a Rotor-gene TM 6000 (Corbett Life Science) using the following cycling conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 15 s, and a melting curve analysis from 72°C to 95°C, increasing by 1°C each step.

Mycotoxin Detection and Quantification

The modified protocol of Small *et al.* (2012) was used to extract DON, NIV and ZEA. Five gram sub-samples of commercial samples were milled and placed into 50 ml Falcon tubes and 20 ml of methanol/water (70:30 v/v) was added. The flasks were shaken at 200 rpm on a Heidolph MultiReax Shaker (Labotec, South Africa) for 30

min at room temperature. The samples were centrifuged for 10 minutes at 4000 rpm. A syringe was used to draw up 2 ml of the supernatant which was filtered with a 0.45 µm RC syringe filter (Acrodisc ® PALL PSF Syringe Filter, Premium Glass Fiber Prefilter). The filtered supernatant was placed into a 2 ml Eppendorf and left overnight at 4°C. The samples were centrifuged for 10 minutes at maximum acceleration (*g*). Nivalenol and zearalenone samples were diluted ten-fold by placing 100 µl supernatant to 900 µl distilled water and vortexed prior to analysis. Deoxynivalenol samples remained undiluted and were placed in 1.8 µl vials.

Standards of DON, NIV and ZEA were obtained from Sigma-Aldrich. A known mycotoxin free sorghum sample was extracted using the above mentioned protocol and 1 ml undiluted supernatant was added to the 1.8 ml LC-MS-MS vial. This was done to take into consideration the matrix effect associated with mycotoxin extractions. The 1 ml mycotoxin free matrix was evaporated under a gentle air flow. The evaporated samples were reconstituted with a calibration standard solution ranging from 1600 $\mu g.kg^{-1}$ to 50 $\mu g.kg^{-1}$ for DON and NIV and 3200 $\mu g.kg^{-1}$ and 50 $\mu g.kg^{-1}$ for ZEA. The samples were vortexed prior to analysis. The samples were extracted and reconstituted in triplicate to ensure repeatability and accuracy. The concentrations of the DON, NIV and ZEA standards were determined at the University of the Free State, Department of Microbial, Biochemical and Food Biotechnology. The lowest level of quantification for DON, NIV and ZEA was 25 $\mu g.kg^{-1}$.

Samples were analysed using a 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer (AB Sciex) and Shimadzu UFLC stack with LC-20AB binary pump and SIL-20A HT autosampler as front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software.

Twenty microliters of each sample was separated on a C18 (150 x 2.1 mm x 5 µ, Discovery C18, Supelco) column at a flow rate of 300 $\mu l.min^{-1}$ using a stepwise gradient between 10mM ammonium acetate (mobile phase A) and methanol 10 mM ammonium acetate (mobile phase B). The column was equilibrated and loaded at 2%B, rapidly increased to 75%B and maintained for 4 minutes, followed by re-equilibration at 2% for a total runtime of 9 minutes. Eluting analytes were ionised in negative electrospray mode with a 4500 V ion spray voltage and 500°C heater

temperature to evaporate excess solvent, 60 psi nebuliser gas, 60 psi heater gas and 25 psi curtain gas.

A targeted Multiple Reaction Monitoring (MRM) workflow was followed on the instrument to analyse the sample. During an MRM scan type the instrument is used in triple quadrupole mode where every ionised analyte (the precursor) eluting off the column is fragmented in the collision cell to produce fragment masses. A set of masses, the precursor mass and one fragment mass constitutes a transition. The instrument jumps between different transitions in an MRM transition list during an analysis cycle, each cycle typically lasting less than a second. If a transition is detected the instrument's response is registered and this ion intensity value is plotted as a chromatogram. Additional mass spectrometer settings are given in Table 1.

All compound and source dependant parameters were optimised using compound optimization in Analyst 1.5.2. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the other transitions are used as qualifiers. The qualifier serves as an additional level of confirmation for the presence of the analyte and the retention time for these three transitions needs to be the same.

2.2 Effect of decortication of grain on fungal biomass and mycotoxin contamination

Field Samples

Sorghum grain of four cultivars, were collected from evaluation blocks in Greytown (Kwa-Zulu Natal), Standerton (Mpumalanga) and Potchefstroom (North West) in 2013/14, to provide for a range of weather conditions during anthesis through to soft dough and harvest, as well as a genotype diversity. Sorghum cultivars included three red cultivars PAN8816, PAN8609 and PAN8911 and one brown cultivar, NS5511. The four cultivars were planted in a randomized block design (RBD) with three replicates and maintained according to best practice at each location until harvest. Mean weather variables for January and February at each locality were obtained from the South African Weather Services (Table 2).

Decortication by TADD

The tangential abrasive dehulling device (TADD) was used to decorticate grain from each of the sorghum cultivars and respective replicates. The device consists of eight sample cups set at 1 mm above the abrasive surface. The abrasive surface used was a horizontally rotating disc (1725 rpm) which had sand paper attached to the surface. Triplicate samples of 70 g from each cultivar-locality replicate were decorticated at 1, 2, 4 and 6 minutes and the sample was then bulked. The total weight after decortication was recorded and the percentage grain removed was calculated for each time interval. Each sorghum cultivar had a control which was not decorticated; this was recorded as nil minutes.

Fusarium graminearum species complex DNA and mycotoxin detection and quantification were determined in each sample using the methods described above.

Data Analysis

Genstat 15th Edition (2012) was used to conduct analysis of variance (ANOVA) on all variables measured and two- and three-way interaction tables were drawn up. Correlation analysis was used to determine a relationship between decortication and disease parameters using Pearson's Correlation Matrix (Hintze, 2007). Regression analysis was conducted to quantify the relationships. Regression parameters were compared using t-test where,

$$t = \frac{b_1 - b_2}{\sqrt{{S_{b1}}^2 + {S_{b2}}^2}} \qquad \sim T(n_1 + n_2 - 4)$$

Where b_1 and b_2 are the regression parameters and S_{b1}^2 and S_{b2}^2 are the error variances of the respective regression models.

3. Results

3.1 Commercial Processing Unit

Quantification of Total Fungal Biomass

Analysis of variance (ANOVA; Table 3A) indicated significant differences in the ergosterol content of grains from the different commercial samples (P<0.001). The lowest ergosterol content was found in 22% dehulled sorghum grain (mean ergosterol concentration=322.1 $\mu g.g^{-1}$, Table 4). The highest ergosterol content was found in the sorghum bran (mean ergosterol concentration=423.2 $\mu g.g^{-1}$, Table 4). The coarse finished product did not significantly differ from the sorghum bran according to Fischer's LSD. However, raw sorghum, 4% and 22% dehulled sorghum, puffed sorghum and fine finished product were grouped together according to Fischer's LSD and did not significantly differ from one another (Table 4). Based on the ratios of the sum of squares, 84% of the variation in total fungal biomass concentrations can be attributed to the form of sorghum processing (Table 3A)

FgSC Fungal Biomass Quantification

Analysis of variance (Table 3B) indicated significant differences in the *FgSC* DNA content of grains of commercial samples (P<0.001). Sorghum bran was associated with the lowest *Fg*SC fungal biomass concentrations (mean *Fg*SC DNA concentration=0.0 $pg.mg^{-1}$; Table 4). In contrast the highest *Fg*SC fungal biomass concentrations were found in 22% dehulled sorghum (mean *Fg*SC DNA concentrations=18.5 $pg.mg^{-1}$; Table 4). Bran, puffed sorghum, 4% dehulled sorghum and fine finished product were grouped together by Fischer's LSD (Table 4). Raw sorghum and the coarse finished product were grouped together by Fischer's LSD and were significantly different to the above and to 22% dehulled sorghum (Table 4). Based on the ratios of the sum of squares, 45% of the variation in *Fg*SC fungal biomass quantities was attributed to type of sorghum processing (Table 3B).

Mycotoxin Detection and Quantification

A total of 35 commercial samples were analysed for DON, NIV and ZEA detection and quantification. Only one sample had DON present (DON concentration=32.8

µg.kg^{-1}), but 25% and 45% of the samples contained NIV and ZEA, respectively. DON analysis was thus subsequently ignored.

NIV

Analysis of variance (Table 3C) indicated borderline significant differences in the quantity of NIV in commercial grain samples (P=0.05). The lowest NIV concentrations were associated with 22% dehulled sorghum (mean NIV concentrations=0.0 µg.kg^{-1}; Table 4). The highest NIV concentrations were found in sorghum bran (mean NIV concentration=8.5 µg.kg^{-1}; Table 4). Fischer's LSD grouped raw sorghum, 22% dehulled sorghum and the fine and coarse finished product together. Puffed sorghum and 4% dehulled sorghum were significantly different to one another and were uniquely grouped individually. Sorghum bran was the most significantly different from all samples according to LSD Fisher and was not grouped (Table 4). According to the ratios of the sum of squares, 53% of the variation in NIV concentrations can be attributed to type of sorghum processing (Table 3C).

ZEA

Analysis of variance (Table 3C) indicated significant differences in the *FgSC* DNA content of grains of commercial samples (P<0.001). The lowest mean ZEA concentration was found in the 22% dehulled sorghum sample (mean ZEA concentration=0.0 µg.kg^{-1}; Table 4). The highest mean ZEA concentration was found in the sorghum bran (mean ZEA concentration=1.5 µg.kg^{-1}; Table 4). Raw sorghum, 4% and 22% dehulled sorghum were grouped together by Fischer's LSD. In contrast, puffed sorghum and the fine and coarse sorghum samples were significantly different to the above and grouped together by Fischer's LSD. Sorghum bran was the most significantly different according to Fischer's LSD and was not placed into either group (Table 4). According to the ratios of the sum of squares, 74% of the variation in ZEA concentrations can be attributed to form of sorghum processing (Table 3C).

3.2 Effect of decortication of grain on fungal biomass and mycotoxin contamination

Decortication by TADD

Analysis of variance (Table 5A) indicated significant differences in the degree of decortication by TADD due to cultivar, location and time. Two-way interactions between cultivar x location, cultivar x time and location x time were recorded. No significant three-way interactions were recorded between variables.

PAN8609 and PAN8911 had the highest percentage grain decorticated when decortication times were pooled (mean decortication=26.6% and 25.8% respectively), in contrast to NS5511, which had the lowest mean decortication (mean decortication=19.3%; Table 6) indicating significant differences in hardness associated with cultivar genotype. The highest levels of decortication were recorded in grain from Potchefstroom and Greytown with mean decortications of 24.8% and 24.9%, respectively. Fischer's LSD indicated that grain from these locations reacted similarly to decortication. In contrast grain from Standerton had the lowest level of decortication (mean decortication=21.1%) and this was significantly different from the former two locations. Fischer's LSD indicated that the degree of decortication with each time, from nil to six minutes, differed significantly.

FgSC Fungal Biomass Quantification

A total of 180 decortication samples were analysed for *Fg*SC DNA contamination and 96% of the grain samples tested positive for the presence of the pathogen complex. Analysis of variance of *Fg*SC DNA concentration (Table 5A) indicated significant differences due to cultivars, location and the interaction between cultivar x location. Lowest mean *Fg*SC DNA concentration was associated with Standerton (mean *Fg*SC DNA biomass=48.8 $pg.mg^{-1}$) followed by grain from Potchefstroom (mean *Fg*SC DNA biomass=70.1 $pg.mg^{-1}$). Highest mean *Fg*SC DNA concentration was associated with grain from Greytown (mean *Fg*SC DNA biomass=419.6 $pg.mg^{-1}$). Fischer's LSD indicated that Standerton and Potchefstroom are not significantly different from one another but are significantly different from Greytown.

Regression analysis was used to calculate the rates of decortication in grain from cultivars from the different localities (Figure 1). General tendencies in Potchefstroom

and Greytown were similar. T-tests (Table 7) to identify differences in the rate of decortication indicated significant differences between NS5511 which had rates of 7.1 and 6.9% per minute at Greytown and Potchefstroom respectively and PAN8816 and PAN8906 that had rates >9.5% per minute at both localities. Grain from Standerton appeared harder than that at the other localities as indicated by the lower rates of decortication with the range of 6.5 and 8.7% per minute (Figure 1) with significant reactions, particularly relative to Potchefstroom in all the cultivars (Table 7).

Although ANOVA did not reveal overall effects of time on the degree of decortication, regression analysis using the logistic 4 model (Figure 2; Hintze, 2007) indicated a distinct decrease in *Fg*SC DNA levels in grain from Greytown which was heavily colonised by the pathogen. Figure 2 illustrates the relationship in pooled data from all cultivars. A distinct decrease in *Fg*SC DNA was observed at decortication percentages >25%. In contrast, no effect of decortication over time was evident in the grain from Potchefstroom and Standerton.

Mycotoxin Detection and Quantification

A total of 180 decortication samples were analysed for DON, NIV and ZEA detection and quantification. In the 60% of samples containing DON, only four samples had DON levels that exceeded the 750 µg.kg^{-1}, EU maximum legislative level. Nivalenol was present in 34% of the samples and over 78% of the samples contained ZEA. Ten samples (5.6% of the total samples analysed) contained ZEA concentrations that exceeded the 100 µg.kg^{-1} ZEA, EU maximum legislative limit. All the samples, except one from Standerton, that contained mycotoxins that exceeded the EU maximum legislative limits came from Greytown.

DON

Analysis of variance of DON (Table 5B) indicated significant differences due to cultivar, location, time and interactions between cultivar x location and location x time. However there was no significant interaction between all three variables. Lowest levels of DON were associated with Potchefstroom (mean DON concentration=1.8 µg.kg^{-1}) while the highest DON levels were found at Greytown (mean DON concentration=136.9 µg.kg^{-1}). Lowest levels of DON were detected at

four minutes decortication (mean DON concentration=20.66 µg.kg^{-1}) and the highest levels of DON were detected at one minute decortication (mean DON concentration=24.55 µg.kg^{-1}).

Since no response to decortication was recorded in grain from Potchefstroom regression analysis was only conducted on data from Greytown and Standerton. Exponential regression models fitted to these data indicated an immediate decline in DON concentrations with decortication, implying that this mycotoxin is produced on the outer layers of grain, as most DON was removed at < 10% decortication (Figure 3).

NIV

Analysis of variance of NIV (Table 5B) indicated significant differences due to cultivar, location, time and significant interactions between all three variables. Grain from Potchefstroom and Standerton had the lowest mean NIV concentrations of 0.3 µg.kg^{-1} and 0.6 µg.kg^{-1}, respectively, while Fischer's LSD indicated that these are significantly different to NIV concentrations in grain from Greytown, where the highest mean NIV concentration of 23.6 µg.kg^{-1} was observed.

Grain from PAN8816 and NS5511 react similarly to decortication and the removal of NIV according to Fischer's LSD. They also had the lowest mean NIV concentrations. The highest mean NIV concentration was found in PAN8609 grain (mean NIV concentration=15.5 µg.kg^{-1}) and was significantly different to the former two cultivars (Table 10).

Six minutes of decortication yielded the lowest NIV concentrations of 5.9 µg.kg^{-1}. The highest concentration of NIV was associated with one minute of decortication (mean NIV concentration=10.8 µg.kg^{-1}). The times differed significantly according to Fischer's LSD (Table 10).

Regression analysis (Figure 4) indicated that NIV responded similarly to decortication as did *Fg*SC DNA biomass, where a decline in NIV was observed at decortication percentages >10% when infection levels were high at Greytown (R^2=0.90). This could suggest that this mycotoxin is deep seated in the grain and directly related to the pathogen.

ZEA

Analysis of variance of ZEA (Table 5B) indicated significant differences due to cultivars, location, time, however there were no significant interactions. The lowest mean ZEA concentrations were found in grain from Potchefstroom and Standerton, mean ZEA concentrations of 0.8 µg.kg^{-1} and 4.5 µg.kg^{-1} respectively. The highest mean ZEA concentrations were found in grain from Greytown (mean ZEA concentration=82.3 µg.kg^{-1}). Fischer's LSD's indicated that ZEA concentrations in grain from Standerton and Potchefstroom were significantly different from ZEA concentrations in grain from Greytown.

Grain from NS5511 had the lowest concentrations of ZEA (mean ZEA concentrations of 2.3 µg.kg^{-1}). Fischer's LSD indicated that grain from PAN8906 was significantly different from cultivars NS5511, PAN 8816 and PAN8911, with the highest ZEA concentration of 70.2 µg.kg^{-1}.

The lowest concentration of ZEA was associated with six minutes decortication (mean ZEA concentration=8.1 µg.kg^{-1}). No decortication (nil minutes) had the highest concentration of ZEA (mean ZEA concentration=59.7 µg.kg^{-1}) and was significantly different from one, two, four and six minutes according to Fischer's LSD. Exponential regression analysis indicated that the low concentrations of ZEA in grain from Potchefstroom and Standerton did not respond to decortication (R^{ns}), in contrast to the response in grain from Greytown where high concentrations of ZEA occurred. There was a constant removal of ZEA over time in grain from Greytown (R^2=95), which could suggest that ZEA is distributed uniformly throughout the grain (Figure 5).

4. Discussion

Controlled decortication using the tangential abrasive dehulling device (TADD) is a common practice for plant breeders and pathologists to test grain hardness and milling quality (Reichert *et al.,*1986). However, commercially, decortication is associated with the removal of tannin-containing layers, testa and to some extent the pericarp, which adversely affects digestibility. Tannins and phenolic compounds in the grain pericarp act as a resistance mechanism to prevent grain molding. However tannin-containing grains are known to have lower nutritional value (Mwasaru et al., 1988). Grains used for commercial feed or food purposes are most commonly tannin

free (Waniska *et al.,* 2000). Various time intervals permit the device to remove certain percentages of the grain's pericarp, aleurone layer and, if long enough, the endosperm and germ (Mwasaru *et al.*, 1988). In this study linear regression analysis indicated that for every one minute of decortication approximately 10% of grain is removed. Analysis of variance and Fischer's LSD of decortication percentage and time decorticated supports the regression analysis.

Ergosterol is the indicator for metabolically active fungal biomass and is more accurate than that of chitin assays due to reduced risk of non-fungal entomological biomasses being detected (Jambunathan *et al.,* 1991). However, this study focuses more specifically on *Fg*SC contamination of grains. Thus, a more specific molecular technique, qPCR, was used to detect *Fg*SC DNA concentrations in grain (Nicolaisen *et al.,* 2009; Boutigny *et al.,* 2012). Spatial distribution of fungal biomasses and mycotoxins in grain research has been limited. However, Schnürer (1991) stated that fungal biomasses were mostly located in the outer layers of barley grain seed coat. Commercial samples in the current study had the lowest ergosterol levels at 22% decortication and the highest levels in the bran. The inverse was however recorded with the quantity of *Fg*SC in commercial samples. Superficial grain molding may occur on the outer layers of the grain and therefore be decorticated to the bran while the more deep seated infection of *Fg*SC required greater decortication. Controlled decortication for two minutes rendered the highest *Fg*SC concentration. This is equated to 23% decortication and thus decortication supports the commercial samples of 22% decorticated with highest *Fg*SC content. Grain from Greytown did not show reduced levels of *Fg*SC DNA until 23% of the grain had been decorticated. This supports the assumption that *Fg*SC infection was deep-seated. Thus the colonisation of sorghum by FgSC is the antithesis of the suggestion by Schnürer (1991). Based on the results of the current study, decortication of approximately 30% or three to four minutes is required to reach the primary site of infection by *Fg*SC and reduce this fungal biomass where infection levels are higher. This level of decortication is not economically viable.

The required levels of decortication to effectively reduce *Fg*SC differed according to cultivar and locality. Grain from PAN8609 and PAN8911 had the highest decortication over time, thus the assumption can be made that these cultivars had

softer grains, whereas grain from NS5511 was the hardest cultivar as indicated by the lowest mean decortication over time. Hardness is related to endosperm texture and grains with more corneous endosperm have been shown to have higher resistance to mold due to kafirin content (Waniska *et al.,* 2001). Grain from NS5511 had the lowest *Fg*SC content which is in agreement with Waniska *et al.* (2001). This observation is the antithesis of findings Mwasaru *et al.* (1988) who reported that high tannin containing grains were characterised by soft endosperms. Grain from PAN8609 is a tannin free cultivar with softer grains which are readily decorticated in contrast to grain from NS5511 which is a condensed tannin cultivar and was the hardest to decorticate.

Difference in hardness of grain associated with the different localities was probably due to the environmental conditions which prevailed at each locality (Figure 3). Weightman *et al.* (2007) stated that grain hardness increases with prolonged dry periods. Grain from Standerton was harder due to a cooler and drier environment. In contrast, grain from Greytown and Potchefstroom yielded softer grains as these environments were wetter and warmer.

Hooker *et al.* (2002) indicated that extended periods of moisture would result in higher levels of FHB in wheat. The cooler and drier environment in Standerton could explain the lower levels of *Fg*SC DNA in grain. The moderate temperatures and wetter environments found in Greytown and Potchefstroom explain the higher *Fg*SC fungal contamination in the grain.

Measuring the concentrations of mycotoxins present in grains prior to and after processing is essential for preventing intake by humans and livestock, thus prioritising food and feed quality (CAST, 2003). However, the fate of trichothecenes and ZEA during processing is dependent on two pathways; the redistribution during milling and/or chemical changes in the mycotoxin during processing (Scudamore & Patel, 2008).

Mycotoxin producing *Fg*SC fungi which are of particular importance to sorghum include *F. acaciae-mearnsii* and *F. meridionale*. These two pathogens are known to produce ZEA and NIV, and are specifically associated with sorghum grain (Mavhunga, 2013). This explains the low concentrations and lack of significant

differences in the quantity of DON in commercial grain samples. However DON was detected in the controlled decortication samples. Lamprecht *et al.* (2011) first reported *F. boothii* on maize crowns and roots in South Africa. Controlled decortication samples were taken from fields which had maize as the previous crop. Worldwide *F. boothii* is known for the production of DON and this could explain the contamination of the controlled decortication samples (Goswami & Kistler, 2005; Mavhunga, 2013).

Two processes can result in mycotoxins at higher concentrations in grain endosperm; *viz* proliferation of surface infection internally or infection of the endosperm (Pinson-Gadais *et al.,* 2007). The highest concentration of NIV, excluding bran, was removed at 4% decortication of commercial samples. Similarly in the controlled decortication study, highest NIV concentrations were associated with one minute decortication which is approximately 10% decortication. It is therefore possible to speculate that NIV is associated with endosperm infections and that the increase in NIV concentration with 4 to 10% decortication can be attributed to an increase in the NIV to grain ratio. In contrast, the highest ZEA concentrations were found in the bran of the commercial samples and in the samples which were not decorticated under controlled conditions. Further research is required to substantiate the above findings.

Highest levels of DON, NIV and ZEA contamination in grains were found at Greytown. The assumption can be made that the highest levels of *Fg*SC at grain from Greytown were responsible for the highest levels of mycotoxins present in the grains from this locality. Temperatures at this locality also favoured mycotoxin production. The minimum temperature for DON production is 11°C and the optimum occurs at maximum temperature of 28°C. The optimum temperature for NIV and ZEA development is 20°C (Wagacha & Muthomi, 2007). The temperatures at Greytown were closest to the optimums, i.e. minimum and maximum temperatures of 16.6°C and 29.6°C respectively for DON production and an average temperature of 20.6°C at Greytown promoting the production of NIV and ZEA. This locality also had higher rainfall and relative humidity, where total rainfall during February of 79.7 mm and average relative humidity of 64.6% was recorded, which would also contribute to increased levels of mycotoxins.

This study indicated decortication of grain can remove superficial colonisation of grain by mold fungi. However, *Fg*SC colonisation is deep-seated within the endosperm of sorghum grain, requiring higher levels of decortication, i.e. 23-30% decortication, which would be economically non-viable. NIV and ZEA accumulation occurred at different parts of the grain and were differentially affected by decortication. Understanding the effects of decortication on *Fg*SC DNA concentrations and the accumulation of DON, NIV and ZEA could assist commercial processors to make the best management decisions for the removal of these harmful mycotoxins.

5. References

Bandyopadhyay, R., Butler, D. R., Chandrashekar, A. Reddy, R. K., & Navi, S. S. 2000. Biology, epidemiology, and management of sorghum grain mold. Pages 34-71 in Technical and institutional options for sorghum grain mold management: proceedings of an international consultation, 18-19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Belton, P. S. & Taylor, J. R. N. 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science & Technology* **15**: 94-98.

Boutigny, A-L., Ward, T. J., Van Coller, G. J., Flett, B., Lamprecht, S. C., O'Donnell, K. & Viljoen, A. 2012. Analysis of the *Fusarium graminearum* species complex from wheat, barley and maize in South Africa provides evidence of species-specific differences in host preference. *Fungal Genetics & Biology* **48**: 914-920.

Bullerman, L. B. & Bianchini, A. 2007. Stability of mycotoxins during food processing. *International Journal of Food Microbiology* **119**: 140-146.

Council for Agricultural Science and Technology (CAST). 2003. Mycotoxins: Risks in Plant, Animal, and Human Systems. Accessed on 25.10.2013 from http://www.trilogylab.com/uploads/Mycotoxin_CAST_Report.pdf

FAO, 2012. Discussion paper on fungi and mycotoxins in sorghum. In joint FAO/WHO food standards programme codex committee on contaminants in

foods sixth session Maastricht, the Netherlands, 26–30 March, 2012 discussion paper on fungi and mycotoxins in sorghum.

FAO, WFP & IFAD. 2012. The State of Food Insecurity in the World 2012. Economic growth is necessary but not sufficient to accelerate reduction of hunger and malnutrition. Rome, FAO.

Goswami, R. S. & Kistler, H. C. 2005. Pathogenicity and in planta mycotoxin accumulation among members of the Fusarium graminearum species complex on wheat and rice. *Phytopathology* **95**: 1397-1404.

Hazel, C. M. & Patel, S. 2004. Influence of processing on trichothecene levels. *Toxicology Letters* **153**: 51–59.

Hintze, 2007. NCSS 2007. NSCC, LLC. Kaysville, Utah, USA. www.ncss.com.

Hooker, D. C., Schaafsma, A. W. & Tamburic-Ilincic, L. 2002. Using weather variables pre-and post-heading to predict deoxynivalenol content in winter wheat. *Plant Disease* **86**: 611-619.

Jambunathan, R., Kherdekar, M. S. & Vaidyas, P. 1991. Ergosterol concentration in mold-susceptible and mold-resistant sorghum at different stages of grain development and its relationship to flavan-4-olst. *Journal of Agriculture & Food Chemistry* **39**: 1866-1870.

Köppen, R., Kock, M., Siegel, D., Merkel, S., Maul., R. & Nehls, I. 2010. Determination of mycotoxins in foods: current state of analytical methods and limitations. *Applied Microbiology & Biotechnology* **86**: 1595–612.

Lamprecht, S. C., Tewoldemedhin, Y. T., Botha, W. J. & Calitz, F. J. 2011. *Fusarium graminearum* species complex associated with maize crowns and roots in the KwaZulu-Natal province of South Africa. *Plant Disease* **95**: 1153-1158.

Lauren, D. R. & Smith, W. A. 2001. Stability of the *Fusarium* mycotoxins nivalenol, deoxynivalenol and zearalenone in ground maize under typical cooking environments. *Food Additives & Contaminants* **18**: 1011-1016.

Mavhunga, M. 2013. *Fusarium graminearum* mycotoxins associated with grain mold of maize and sorghum in South Africa (Masters dissertation, University of the Free State Bloemfontein, South Africa).

Mwasaru, M. A., Reichert, R. D., & Mukuru, S. Z. 1988. Factors affecting the abrasive dehulling efficiency of high-tannin sorghum. *Cereal Chemistry* **65**: 171-174.

Nicolaisen, M., Suproniene, S., Nielsen, L. K., Lazzaro, I., Spliid, N. H. & Justesen, A. F. 2009. Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *Journal of Microbiological Methods* **76:** 234–40.

Pinson-Gadais, L., Barreau, C., Chaurand, M., Gregoire, S., Monmarson, M. & Richard-Forget, F. 2007. Distribution of toxigenic Fusarium spp. and mycotoxin production in milling fractions of durum wheat. *Food Additives & Contaminants* **24**: 53-62.

Reddy, B. V. S., Kumar, A. A., Sharma, H. C., Rao, P. S., Blümmel, M., Reddy, C. R., Sharma, R., Deshpande, S., Saikat, S. M. & Dinakaran, E. 2012. Sorghum improvement (1980–2010): Status and way forward. *Journal of SAT Agricultural Research* **10**: 1-14.

Reichert, R. D., Tyler, R. T., York, A. E., Schwab, D. J., Tatarynovichp, J. & Mwasaru, M. 1986. Description of a production model of the tangential abrasive dehulling device and its application to breeders' samples. *Cereal Chemistry* **63**: 201-207.

Schnürer, J. 1991. Distribution of fungal biomass among fine bran, coarse bran, and flour from wheat stored at four different moisture levels. *Cereal Chem*istry **68**: 434-437

Scudamore, K & Patel, S. 2009. *Fusarium* mycotoxins in milling streams from the commercial milling of maize imported to the UK, and relevance to current legislation. *Food Additives & Contaminants **26***: 744-753.

Summerell, B. A. & Leslie, J. F. 2011. Fifty years of *Fusarium*: how could nine species have ever been enough? *Fungal Diversity* **50**: 135-144.

Small, I. M., Flett, B. C., Marasas, W. F. O. McLeod, A., Stander, M. A. and Viljoen, A. 2012. Resistance in maize inbred lines to *Fusarium verticilliodes* and fumonisin accumulation in South Africa. *Plant Disease* **96**: 881-888.

Taylor, J. R. N. 2003. "Overview: Importance of sorghum in Africa." Afripro: Workshop on the Proteins of Sorghum and Millets: Enhancing Nutritional and Functional Properties for Africa, Pretoria. Vol. 2. No. 4.

Thakur, R. P., Reddy, B. V. S., Indira, S., Rao, V. P., Navi, S. S., Yang, X. B. & Ramesh, S. 2006. Sorghum Grain Mold. Information Bulletin No. 72. International Crops Research Institute for the Semi-Arid Tropics. Patancheru 502324, Andhra Pradesh, India: 32 pp. ISBN 92-9066-488-6.

Wagacha, J. M., & Muthomi, J. W. 2007. *Fusarium culmorum*: Infection process, mechanisms of mycotoxin production and their role in pathogenesis in wheat. *Crop Protection* **26**: 877-885.

Waniska, R. D. 2000. Structure, phenolic compounds, and antifungal proteins of sorghum caryopses. Pages 18-19 in *Technical and institutional options for sorghum grain mold management: proceedings of an international consultation*, 18–19 May 2000, ICRISAT, Patancheru, India (Chandrashekar, A., Bandyopadhyay, R., and Hall, A.J., eds.). Patancheru 502 324, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics.

Wegulo, S. N. 2012. Factors influencing deoxynivalenol accumulation in small grain cereals. *Toxins* **4**: 1157-1180.

Weightman, R. M., Millar, S., Alava, J., Foulkes, M. J., Fish, L. & Snape, J. W. 2008. Effects of drought and the presence of the 1BL/1RS translocation on grain vitreosity, hardness and protein content in winter wheat. *Journal of Cereal Science* **47**: 457-468.

Table 1. Mass spectrometer settings for the detection of nivalenol (NIV), deoxynivalenol (DON) and zearalenone (ZEA) in sorghum grain using LC-MS-MS.

Mass spectrometer settings	**NIV**	**DON**	**ZEA**
Precusor ion [M+H]+(m/z)	371	355	317
Product ion (m/z)	281	265	175
Declustering potential (V)	-1	-1	-16
Collusion 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 2. Mean meteorological data for months of anthesis (January & February) for the 2013/14 season in three sorghum producing areas in South Africa.

Weather Variable	Greytown			Potchefstroom			Standerton		
	Jan[1]	Feb[2]	**MEAN**	Jan	Feb	**MEAN**	Jan	Feb	**MEAN**
MaxT[3]	29.7	29.6	**29.65**	32.1	30.1	**31.10**	27.8	26.3	**27.06**
MinT[4]	16.8	16.6	**16.70**	16.7	16.5	**16.60**	15.0	14.5	**14.74**
AvgT[5]	20.9	20.6	**20.75**	23.3	21.8	**22.55**	20.8	19.8	**20.28**
RH[6]	76.1	79.7	**77.90**	57.5	68.3	**62.89**	67.8	72.0	**69.86**
RF[7]	53.6	64.6	**59.10**	61.2	85.0	**73.10**	73.6	45.5	**59.56**

Jan[1] January
Feb[2] February
MaxT[3] Maximum temperature (°C)
MinT[4] Minimum temperature (°C)
AvgT[5] Average temperature (°C)
RH[6] Average relative humidity (%)
RF[7] Total rainfall (mm)

Table 3. Analysis of variance (ANOVA) for commercial processing unit samples and ergosterol (µg.g^{-1}) (A), *Fg*SC DNA fungal biomass (ng.µl^{-1}) (B), accumulation of nivalenol (NIV; µg.kg^{-1})(C) and zearalenone (ZEA; µg.kg^{-1}) (C) from South African sorghum processor.

A	**Ergosterol**					**B**	***Fg*SC**				
Source		Sum of	Mean		Prob	**Source**		Sum of	Mean		Prob
Term	**DF**	Squares	Square	F-Ratio	Level	**Term**	**DF**	Squares	Square	F-Ratio	Level
Commercial Sample	**6**	15168.26	2528.04	5.36	0.03	**Commercial Sample**	**6**	477.22	79.54	3.52	0.03
Rep	**1**	24.24	24.24	0.05	0.83	**Rep**	**3**	288.85	96.28	4.26	0.03
Residual	**6**	2828.94	471.49			**Residual**	**12**	248.65	22.60		
Total (Adjusted)	**13**	18021.43				**Total (Adjusted)**	**20**	1066.56			
Total	**14**					**Total**	**21**				

C	**NIV**					**D**	**ZEA**				
Source		Sum of	Mean		Prob	**Source**		Sum of	Mean		Prob
Term	**DF**	Squares	Square	F-Ratio	Level	**Term**		Squares	Square	F-Ratio	Level
Commercial Sample	**6**	122.77	20.46	2.88	0.05	**Commercial Sample**	**6**	4.17	0.69	9.70	0.00
Rep	**3**	5.69	1.90	0.27	0.85	**Rep**	**3**	0.62	0.21	2.89	0.08
Residual	**12**	85.34	7.11			**Residual**	**12**	0.86	0.07		
Total (Adjusted)	**20**	233.63				**Total (Adjusted)**	**20**	5.64			
Total	**21**					**Total**	**21**				

Table 4. Mean values of samples from commercial processing unit representing; ergosterol (µg.g^{-1}), *Fg*SC DNA fungal biomass (ng.µl^{-1}) (A), accumulation of nivalenol (NIV; µg.kg^{-1}) and zearalenone (ZEA; µg.kg^{-1}) (B) from South African sorghum processor.

Product	Ergosterol (µg.g^{-1})	*Fg*SC (pg.mg^{-1})	NIV (µg.kg^{-1})	ZEA (µg.kg^{-1})
Raw Sorghum	342.23^{a}	6.44^{b}	1.04^{a}	0.13^{a}
De-hulled 4%	324.17^{a}	3.17^{a}	4.95^{c}	0.05^{a}
De-hulled 22%	322.09^{a}	18.52^{c}	0.00^{a}	0.00^{a}
Bran	423.15^{b}	0.00^{a}	8.58^{d}	1.48^{c}
Puffed Sorghum	325.41^{a}	0.44^{a}	3.29^{b}	0.75^{b}
Finished Product (Fine)	352.92^{a}	0.23^{a}	1.01^{a}	0.67^{b}
Finished Product (Coarse)	360.67^{b}	9.32^{b}	1.28^{a}	0.66^{b}

* Mean values followed by the same letter, in each column, do not differ significantly according to Fishers LSD (P>0.05)

Table 5. Analysis of variance (ANOVA) for Tangential Abrasive Dehulling Device (TADD; %), *Fg*SC DNA fungal biomass (ng.ul^{-1}) (A), accumulation of deoxynivalenol (DON; µg.kg^{-1}), nivalenol (NIV; µg.kg^{-1}) and zearalenone (ZEA; µg.kg^{-1}) (B) in four cultivars tested in three environments in South Africa.

A		**TADD**						***Fg*SC**	
Term	**DF**	Sum of Squares	Mean Square	F-Ratio	Prob Level	Sum of Squares	Mean Square	F-Ratio	Prob Level
Cultivar	**3**	1476	492	15.02	0.00*	629734.6	209911.5	3.9	0.01*
Location	**2**	596.39	298.2	9.1	0.00*	5202589	2601294	48.39	0.00*
Cultivar x Location	**6**	740.45	123.41	3.77	0.00*	1204087	200681.2	3.73	0.00*
Time	**4**	55799.83	13949.96	425.76	0.00*	223943.3	55985.84	1.04	0.39
Cultivar x Time	**12**	1440.6	120.05	3.66	0.00*	366041.1	30503.43	0.57	0.86
Location x Time	**8**	1074.11	134.26	4.1	0.00*	496964.3	62120.54	1.16	0.33
Cultivar x Location x Time	**24**	1040.84	43.37	1.32	0.16	737638.1	30734.92	0.57	0.94
Rep	**2**	533.94	266.97	8.15	0.00*	26444.5	13222.25	0.25	0.78
Error	**118**	3866.28	32.77			6343745	53760.55		
Total (Adjusted)	**179**	66568.44				15231190			
Total	**180**								

B				**DON**				**NIV**				**ZEA**	
Source		Sum of	Mean	F-Ratio	Prob	Sum of	Mean	F-Ratio	Prob	Sum of	Mean	F-Ratio	Prob
Term	**DF**	Squares	Square		Level	Squares	Square		Level	Squares	Square		Level
Cultivar	**3**	170140.5	56713.49	3.42	0.02*	4999.44	1666.48	9.79	0.00*	118924	39641.32	3.34	0.02*
Location	**2**	627250.2	313625.1	18.92	0.00*	21335.64	10667.82	62.68	0.00*	254630.1	127315	10.74	0.00*
Cultivar x Location	**6**	260635.5	43439.24	2.62	0.02*	8619.23	1436.54	8.44	0.00*	211104.8	35184.13	2.97	0.01*
Time	**4**	747765.6	186941.4	11.28	0.00*	501.24	125.31	0.74	0.57	61354.82	15338.71	1.29	0.28
Cultivar x Time	**12**	292980.5	24415.04	1.47	0.14	2911.36	242.61	1.43	0.16	55833.36	4652.78	0.39	0.96
Location x Time	**8**	922123.3	115265.4	6.95	0.000*	1440.91	180.11	1.06	0.4	135774.7	16971.84	1.43	0.19
Cultivar x Location x Time	**24**	309963.8	12915.16	0.78	0.76	6860.68	285.86	1.68	0.04*	126266.4	5261.1	0.44	0.99
Rep	**2**	106246.4	53123.18	3.21	0.04*	801.14	400.57	2.35	0.1	43139.62	21569.81	1.82	0.17
Error	**118**	1955761	16574.24			20083.13	170.2			1399297	11858.45		
Total (Adjusted)	**179**	5392867				67552.76				2406324			
Total	**180**												

Table 6. Percentage decortication (TADD, %); representing four sorghum cultivars collected in the 2013/14 season in three South African sorghum production localities.

Time	Locality														
	Greytown					Potchefstroom					Standerton				
	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean
0	0	0	0	0	**0**	0	0	0	0	**0**	0	0	0	0	**0**
1	14.98	12.86	7.53	5.82	**10.3**	8.44	11.02	11.65	7.22	**9.58**	9.54	11.97	7.81	13.63	**10.74**
2	25	24.08	24.18	19.87	**23.28**	32.51	26.03	25.47	13.58	**24.4**	18.81	24.71	22.4	20.73	**21.66**
4	37.95	52.17	45.82	30.47	**41.6**	34.95	40.7	45.03	24.89	**36.39**	25.51	33.35	26.59	26.94	**28.1**
6	47.75	60.73	51.11	39.56	**49.79**	48.2	61	63.71	43.31	**54.06**	37.6	40.94	56.41	44.66	**44.9**
Mean	**25.14**	**29.97**	**25.73**	**19.14**		**24.82**	**27.75**	**29.17**	**17.8**		**18.29**	**22.19**	**22.64**	**21.19**	

Main effects:					
Time	**TADD %**	**Cultivar**	**TADD %**	**Locality**	**TADD %**
0	0	PAN8816	22.75	Greytown	24.99
1	10.21	PAN8906	26.64	Potchefstroom	24.89
2	23.11	PAN8911	25.85	Standerton	21.08
4	35.36	NS5511	19.38		
6	49.58				

$LSD_{0.05}$	TADD
Cultivar	2.39
Locality	2.07
Time	2.67
Cultivar x Locality	4.14
Cultivar x Time	5.34
Locality x Time	4.63
Cultivar x Locality x Time	9.25

Table 7. Comparison of decortication rates using Fischers T-test determined from regressions of decortication in time.

		Greytown				Potchefstroom				Standerton		
		PAN8906	**PAN8911**	**NS5511**	**PAN8816**	**PAN8906**	**PAN8911**	**NS5511**	**PAN8816**	**PAN8906**	**PAN8911**	**NS5511**
Greytown	**PAN8816**	1.96	0.92	0.71	0.07	2.06	**2.77***	0.57	1.46	0.76	0.72	0.70
	PAN8906		0.83	**2.79***	1.37	0.46	0.04	2.97	**3.59***	**2.53***	1.06	**2.72***
	PAN8911			1.58	0.64	0.60	1.08	1.55	2.22	1.50	0.20	1.55
	NS5511				0.55	**3.35***	**4.36***	0.32	0.82	0.13	1.37	0.02
Potchefstroom	**PAN8816**					1.24	1.63	0.43	1.02	0.60	0.48	0.56
	PAN8906						0.87	**4.21***	**4.74***	**2.73***	0.87	**3.16***
	PAN8911							**6.10***	**6.13***	**3.40***	1.38	**4.07***
	NS5511								1.48	0.39	1.33	0.33
Standerton	**PAN8816**									0.51	2.02	0.75
	PAN8906										1.32	0.11
	PAN8911											1.35

Table 8. Two way interactions between location and cultivars representing four cultivars collected in the 2013/14 season in three South African localities of sorghum grain samples contaminated with *Fg*SC fungal DNA.

Time	Locality														
	Greytown					Potchefstroom					Standerton				
	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean
0	347.29	672.04	910.69	112.59	**510.65**	95.38	51.78	71.25	49.01	**66.85**	51.41	47.42	60.82	18.96	**44.65**
1	553.06	746.84	644.38	57.66	**500.49**	69.77	72.23	43.87	84.38	**67.56**	62.21	49.13	41.26	7.91	**40.13**
2	426.35	709.83	796.39	129.69	**515.57**	75.48	55.11	29.77	150.89	**77.81**	41.91	103.76	43.54	11.23	**50.11**
4	333.77	351.15	236.81	218.00	**284.93**	97.34	68.73	70.48	44.56	**70.27**	52.63	51.09	52.08	51.41	**51.80**
6	278.66	405.51	242.40	220.60	**286.79**	81.54	81.46	56.45	53.96	**68.35**	44.37	71.08	47.29	67.58	**57.58**
Mean	**387.83**	**577.07**	**566.13**	**147.71**		**83.90**	**65.86**	**54.36**	**76.56**		**50.51**	**64.5**	**49.00**	**31.42**	

Main effects:					
Time	***Fg*SC DNA pg.mg^{-1}**	**Cultivar**	***Fg*SC DNA pg.mg^{-1}**	**Locality**	***Fg*SC DNA pg.mg^{-1}**
0	207.39	PAN8816	174.08	Greytown	419.68
1	202.73	PAN8906	235.81	Potchefstroom	70.17
2	214.50	PAN8911	223.16	Standerton	48.85
4	135.67	NS5511	85.23		
6	137.57				

$LSD_{0.05}$	
Cultivar	68.78
Locality	59.57
Time	76.90
Cultivar x Locality	119.13
Cultivar x Time	153.80
Locality x Time	133.20
Cultivar x Locality x Time	266.39

Table 9. Two way interactions between location and cultivars representing four cultivars collected in the 2013/14 season in three South African localities of sorghum grain samples contaminated with DON.

Time	Locality														
	Greytown					Potchefstroom					Standerton				
	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean
0	615.062	540.367	657.983	43.327	**464.18**	0	0	0	0	**0**	73.2	0	270.92	0	**86.03**
1	146.785	56.833	44.142	3.695	**62.86**	0	0	4.637	20.006	**6.16**	8.23	2.79	7.52	0	**4.63**
2	167.94	46.833	48.175	1.757	**66.18**	0	0	3.692	1.853	**1.39**	2.01	2.67	1.1	0	**1.44**
4	142.102	19.417	35.953	1.009	**49.62**	0	0	3.394	1.186	**1.14**	0.64	42.27	1.89	0	**11.2**
6	116.292	16.98	34.613	0	**41.97**	0	0	1.64	1.383	**0.76**	1.15	78.01	2.24	0	**20.35**
Mean	**237.64**	**136.09**	**164.17**	**9.96**		**0**	**0**	**2.67**	**4.89**		**17.05**	**25.15**	**56.73**	**0**	

Main effects:					
Time	**DON µg.kg^{-1}**	**Cultivar**	**DON µg.kg^{-1}**	**Locality**	**DON µg.kg^{-1}**
0	183.4	PAN8816	84.89	Greytown	136.96
1	24.55	PAN8906	135.67	Potchefstroom	1.89
2	23.00	PAN8911	74.53	Standerton	24.73
4	20.66	NS5511	4.95		
6	21.03				

LSD$_{0.05}$	
Cultivar	38.19
Locality	33.07
Time	42.70
Cultivar x Locality	66.15
Cultivar x Time	85.40
Locality x Time	73.96
Cultivar x Locality x Time	147.91

Table 10. Two way interactions between location and cultivars representing four cultivars collected in the 2013/14 season in three South African localities of sorghum grain samples contaminated with NIV.

Time	Locality														
	Greytown					Potchefstroom					Standerton				
	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean
0	7.56	48.76	35.67	13.22	**26.3**	0	2.91	0	0	**0.73**	0	0	0	1.6	**0.4**
1	7.19	63.27	56.48	3.03	**32.49**	0	0	0	0	**0**	0	0	0	0	**0**
2	7.29	66.57	21.02	0.00	**23.72**	0	0	0	0	**0**	0	0	0	0	**0**
4	23.2	34.04	18.32	1.62	**19.29**	0	3	1.23	0	**1.06**	0	1.81	3.06	1.65	**1.63**
6	2.48	14.46	17.7	30.51	**16.29**	0	0	0	0	**0**	1.32	4.39	0	0	**1.43**
Mean	**9.54**	**45.42**	**29.84**	**9.67**		**0**	**1.18**	**0.25**	**0**		**0.26**	**1.24**	**0.61**	**0.65**	

Main effects:					
Time	**NIV µg.kg^{-1}**	**Cultivar**	**NIV µg.kg^{-1}**	**Locality**	**NIV µg.kg^{-1}**
0	9.14	PAN8816	3.27	Greytown	23.62
1	10.83	PAN8906	15.53	Potchefstroom	0.36
2	7.91	PAN8911	10.23	Standerton	0.69
4	7.33	NS5511	3.44		
6	5.90				

$LSD_{0.05}$	
Cultivar	3.87
Locality	3.35
Time	4.33
Cultivar x Locality	6.70
Cultivar x Time	8.65
Locality x Time	7.49
Cultivar x Locality x Time	14.99

Table 11. Two way interactions between location and cultivars representing four cultivars collected in the 2013/14 season in three South African localities of sorghum grain samples contaminated with ZEA.

Time	Locality														
	Greytown					Potchefstroom					Standerton				
	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean	PAN8816	PAN8906	PAN8911	NS5511	Mean
0	140.66	329.23	227.31	7.47	**176.17**	1.87	0.68	1.35	0.87	**1.19**	1.55	2.45	1.57	2.33	**1.97**
1	7.83	277.05	125.35	1.76	**103**	0.83	0.49	1.18	0.63	**0.78**	1.82	10.26	2.16	1.01	**3.81**
2	3.606	300.4	42.74	1.8	**87.14**	0.9	0.64	0.95	0.43	**0.73**	2	2.24	2.77	1.24	**2.06**
4	34.176	41.04	19.95	0.8	**23.99**	0.85	0	1.47	0.46	**0.69**	4.42	31.59	13.8	0.96	**12.69**
6	2.491	54.68	16.84	12.59	**21.65**	1.16	0.16	0.88	0.72	**0.73**	1.83	2.1	1.72	2.37	**2.01**
Mean	**37.75**	**200.48**	**86.44**	**4.89**		**1.12**	**0.39**	**1.17**	**0.62**		**2.32**	**9.73**	**4.4**	**1.58**	

Main effects:					
Time	**ZEA µg.kg^{-1}**	**Cultivar**	**ZEA µg.kg^{-1}**	**Locality**	**ZEA µg.kg^{-1}**
0	59.78	PAN8816	13.73	Greytown	82.39
1	35.86	PAN8906	70.2	Potchefstroom	0.83
2	29.98	PAN8911	30.67	Standerton	4.51
4	12.46	NS5511	2.36		
6	8.13				

LSD$_{0.05}$	
Cultivar	32.3
Locality	27.98
Time	36.12
Cultivar x Locality	55.95
Cultivar x Time	72.23
Locality x Time	62.56
Cultivar x Locality x Time	125.11

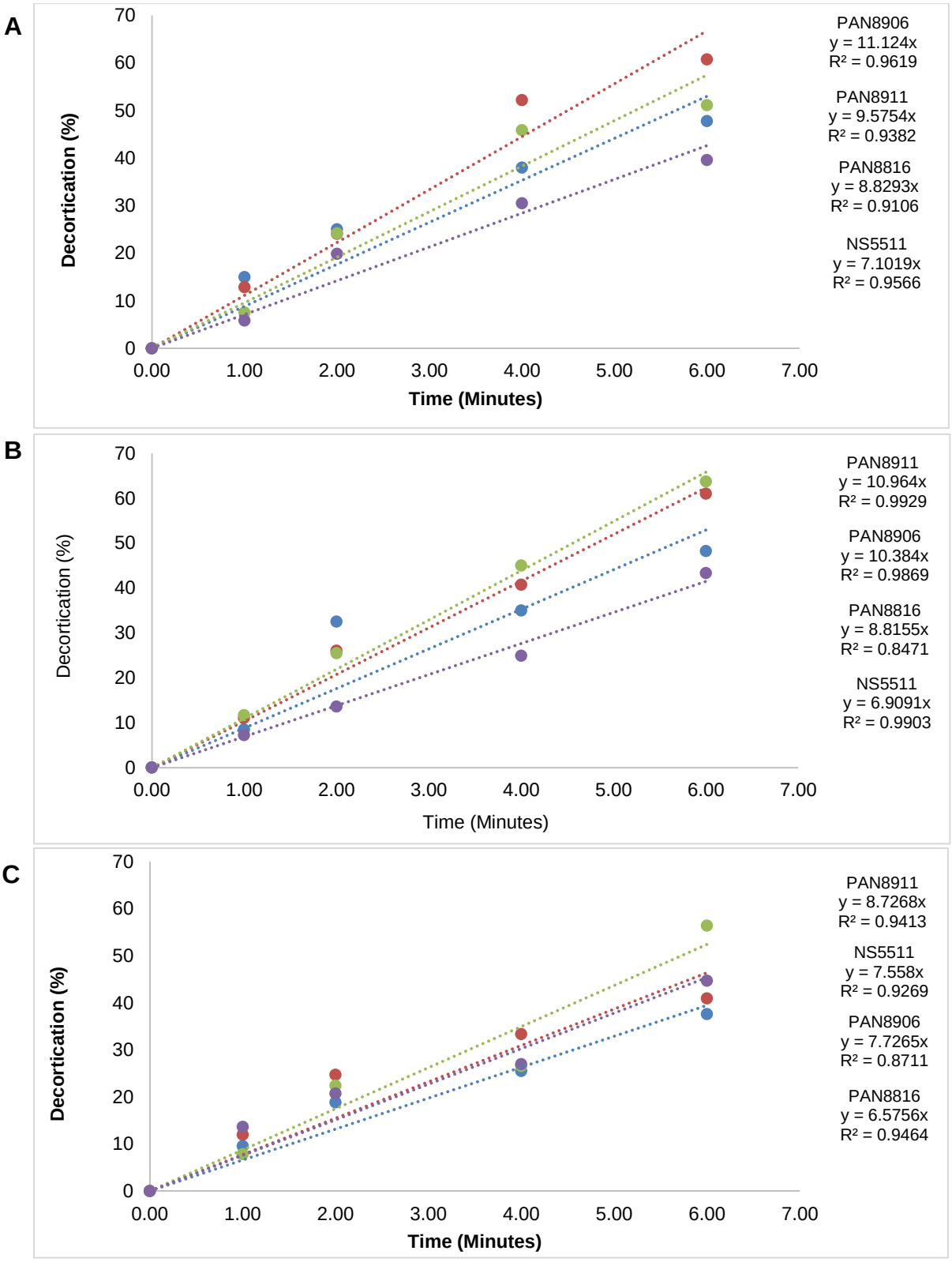


Figure 1. The relationship between percentage decortication by TADD and time decorticated in minutes for each cultivar, PAN8816, PAN8906, PAN8911 and NS5511 and three sorghum producing locations; Greytown(A), Potchefstroom (B) and Standerton (C).

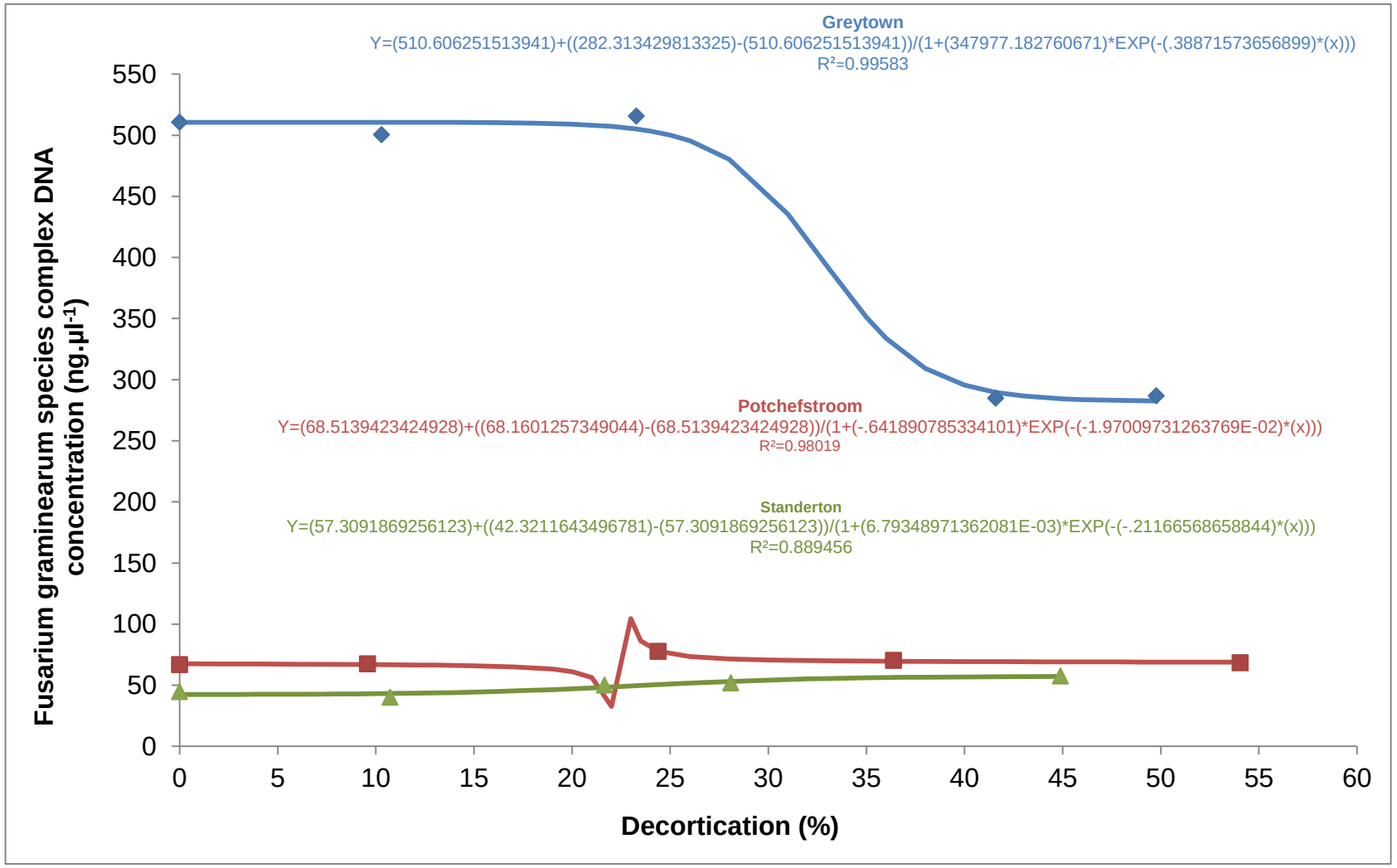


Figure 2. The relationship between *Fg*SC biomass, determined using qPCR, and percentage decortication by TADD.

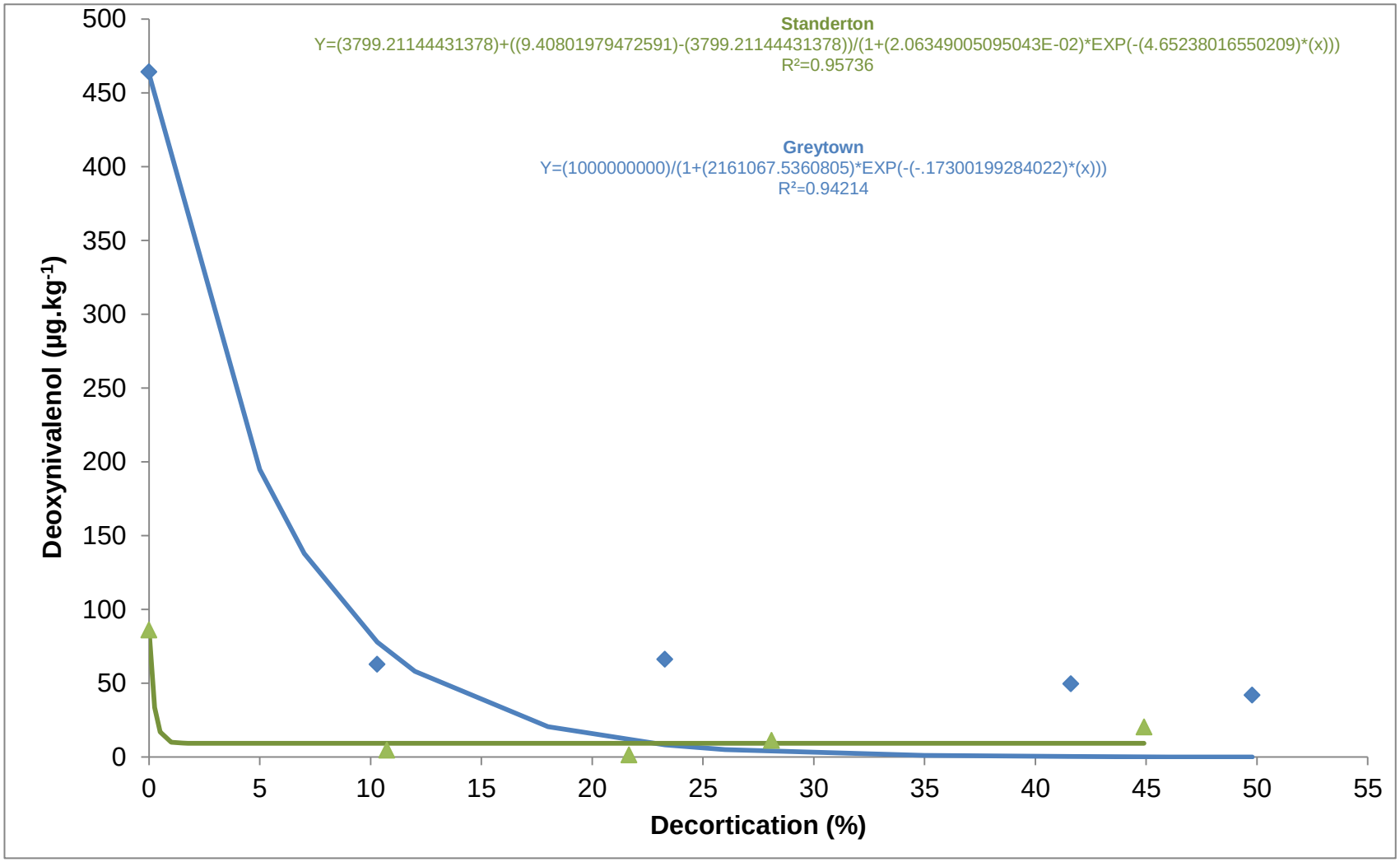


Figure 3. The relationship between percentage decortication by TADD and deoxynivalenol (DON; µg.kg^{-1}), determined by LC-MS-MS.

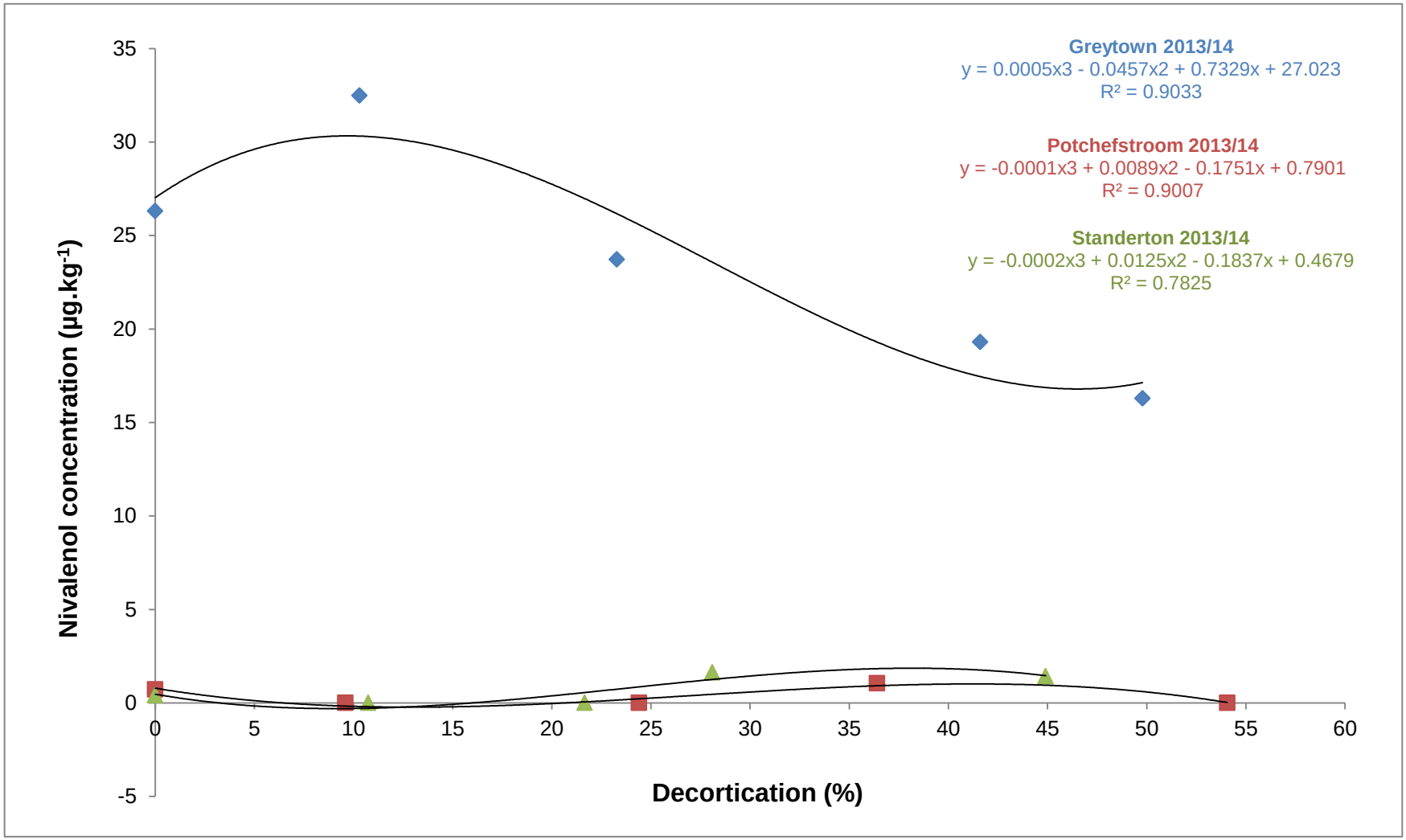


Figure 4. The relationship between percentage decortication by TADD and nivalenol (NIV; µg.kg^{-1}), determined by LC-MS-MS.

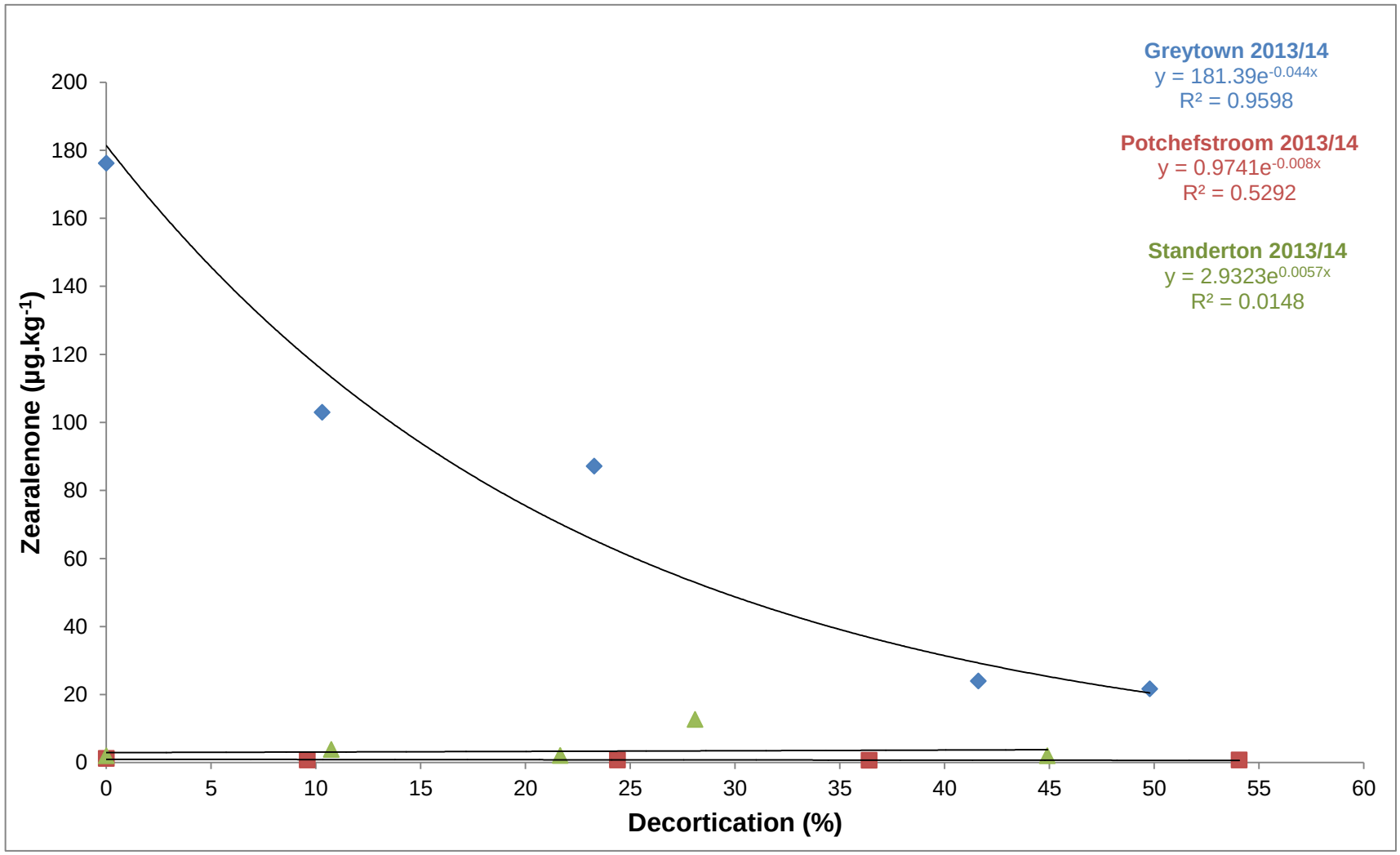


Figure 5. The relationship between percentage decortication by TADD and zearalenone (ZEA; µg.kg^{-1}), determined by LC-MS-MS.

Summary

Sorghum is the fifth most grown cereal worldwide, and is a staple food in 30 countries that sustains 500 million people in the semi-arid tropics. Sorghum grain mold (SGM) is the one of the most important pre-harvest biotic constraints in sorghum production. Over 40 genera of pathogenic fungi occur on sorghum grain and cause SGM. *Fusarium graminearum*, a causal agent of SGM, is responsible for the majority of economically and medically important mycotoxins associated with the disease. This, however, is not an individual pathogen, but a complex of species or a combination of related *Fusarium* species and is referred to as the *Fusarium graminearum* species complex (*Fg*SC). The *FgSC* formed the focus of this study.

Grain from nine sorghum cultivars, harvested over three seasons at Cedara and two seasons at Alma, was evaluated for grain mold severity, mycotoxin contamination and the stability of the grain mold response over changing environmental conditions. Lower visual threshed grain disease ratings and total fungal biomasses, determined using ergosterol analysis, were observed in grains with an elevated tannin content. However, no correlation between threshed grain disease ratings and ergosterol content was observed indicating that the former criterion is not a reliable measure of grain colonisation by grain mold fungi. Quantitative PCR analysis indicated a *Fg*SC DNA content in grain over a range of 5.52 $ng.\mu l^{-1}$ in PAN8625 to 55.43 $ng.\mu l^{-1}$ in PAN8806 with significant differences between cultivars. Only three of the 162 grain samples had deoxynivalenol (DON) concentrations that exceeded 10 $\mu g.kg^{-1}$ and DON was therefore excluded from further analysis. However, nivalenol (NIV) and zearalenone (ZEA) were present in all but four and two samples, respectively. Additive Main Effect and Multiplicative Interaction (AMMI) analysis of *Fg*SC DNA, NIV and ZEA concentrations indicated a relatively stable response in cultivars to changing environments with most cultivars yielding an IPCA1 score <1. Robust regression was applied to quantify the relationship between NIV and ZEA accumulation in grain relative to the *Fg*SC DNA concentration and indicated that host genotype influences mycotoxin production despite similar colonisation levels. Results indicate the need for the inclusion of environmental variation in the screening and

selection for resistance to SGM in sorghum genotypes, to ensure quality grain and human and animal health.

The development of an epidemiological model which quantifies the risk of grain molds and mycotoxins in sorghum production areas could enable producers to ensure that timely management decisions are made to reduce *Fg*SC infection and mycotoxin contamination. Sorghum grain collected over two seasons from 18 South African sorghum production areas were analysed for *Fg*SC colonisation and DON, NIV and ZEA contamination. *Fg*SC colonisation and concomitant mycotoxin accumulation coincided with weather conditions during early-post flowering, 82-95 days after planting (d.a.p.) and soft dough stage, 92-115 d.a.p., which are the critical periods for grain colonisation and mycotoxin accumulation. *Fg*SC development and colonisation were significantly, positively correlated with maximum relative humidity 82-95 d.a.p. and significantly inversely correlated with maximum temperature and evapotranspiration 82-95 d.a.p. DON, NIV and ZEA accumulation were significantly positively related to *Fg*SC DNA concentration. DON had borderline significant positive relationship with maximum temperature 101-115 d.a.p., however NIV and ZEA had significant inverse relationship with minimum temperature 91-104 and 100-113 d.a.p., respectively. Preliminary models based on stepdown multiple regression analysis were developed. Future studies could include localities with more available and accurate weather data to further calibrate and validate the models developed.

A range of commercial sorghum grain samples were collected from a sorghum processing company as well as two finished products were taken from storage. In addition a total of 180 sorghum grain samples consisting of four cultivars from three localities in South Africa (Greytown, Standerton and Potchefstroom) were collected during one production season and decorticated using a tangential abrasive dehulling device (TADD) for five time periods. Ergosterol concentrations were highest in sorghum bran and lowest in 22% dehulled grain, indicating that a high proportion of fungal contamination lies in the outer grain layers. In contrast, *Fg*SC DNA concentrations were detected at lowest levels in sorghum bran and at highest levels in 22% dehulled grain. The assumption was therefore, made that *Fg*SC infections were deep-seated within the grain endosperm. Furthermore, controlled decortication by TADD only resulted in a significant reduction in *Fg*SC DNA content after four

minutes i.e. 35% decortication. The assumption is further supported by NIV concentrations in both commercial and controlled samples which were relatively high in sorghum bran and 4% dehulled grain, but reduced to 0.00 µg.kg^{-1} in both the 22% dehulled grain and grain from the six minutes decortication. In contrast with NIV, ZEA and DON, were removed from grain by short periods of decortication. The assumption can be made that ZEA and DON are associated with superficial *Fg*SC infections and accumulate in the outer layers of the grain while NIV is associated with pathogenesis in the deeper endosperm layers. Cultivars differed in hardness and variation in hardness of grains was associated with prevailing weather conditions at each locality, as well as endosperm texture with lower colonisation and mycotoxin levels in the harder grain. The highest *Fg*SC DNA concentrations and DON, NIV and ZEA accumulation was recorded in grain from Greytown where weather conditions during the critical grain development stages promoted infection and the contamination of grains by mycotoxins. Understanding the effects of decortication on *Fg*SC DNA concentrations and the accumulation of DON, NIV and ZEA could assist commercial processors to make the best management decisions for the removal of these harmful mycotoxins.

KEY WORDS: *Fusarium graminearum* species complex, Sorghum, Mycotoxins, AMMI, Climatic Variables, Decortication

Opsomming

Sorghum is wêreldwyd die vyfde mees geplante graan en in 30 lande vir 500 miljoen mense in die semi-aride gebiede. Tydens produksie is sorghum graanskimmel (SGS) een van die belangrikste voor-oes biotiese stremmings faktore en meer as 40 genera patogeniese swamme word op graan aangelief. *Fusarium graminearum*, 'n veroorsakende organisme van SGS is verantwoordelik vir die belangrikste ekonomise en medise mikotoksiene wat met die siekte geassosieer word. Dit is nie 'n enkel patogeen nie, maar 'n kompleks van individuele spesies of 'n kombinasie van verwante *Fusarium* spesies en word na verwys as die *Fusarium graminearum* spesies kompleks (*Fg*SK). Die *Fg*SK was die basis van hierdie studie.

Graan van nege sorghum kultivars, wat oor drie seisoene op Cedara en twee seisoene op Alma geoes is, is geëvalueer vir graan skimmel strafheid, mikotoksien produksie en die stabiliteit van die graan skimmel reaksie oor veranderende omgewingstoestande. Minder visuele sikte waarnemings en totale fungus biomassa, soos bepaal met ergosterol analise, is in grane met 'n hoër tannien inhoud waargeneem. Geen korrelasie is tussen visuele siekte lesings en ergosterol inhoud waargeneem nie wat daarop dui dat eersgenoemde nie 'n betroubare maatstaf is vir die bepaling vir graan kolonisering nie. Kwantatiewe PCR analise het betekenisvolle verskille tussen kultivars aangedui, waar die *Fg*SK DNA inhoud van graan vanaf 5.52 $ng.\mu l^{-1}$ in PAN8625 tot 55.43 $ng.\mu l^{-1}$ in PAN8806 varieer het. Slegs drie van 162 graan monsters het meer as 10 $\mu g.kg^{-1}$, deoksinivalenol (DON) bevat dus is geen verdere DON analises gedoen nie. Nivalenol (NIV) en zearalenone (ZEA) was deurgaans teenwoordig met die uitsondering van vier by NIV en twee by ZEA. "Additive Main Effect and Multiplicative Interaction (AMMI)" ontledings van *Fg*SK DNA, NIV en ZEA konsentrasies het relatief stabiele waarneem op kultivars teen veranderende omgewingstoestande aangedui met "IPCA1" tellings <1 in meeste van die kultivars. Onsensitiewe regressie is toegepas om die verhouding tussen NIV en ZEA produksie in graan teenoor die *Fg*SK DNA konsentrasie te kwantifiseer wat 'n aanduiding is dat gasheer genotipe mikotoksien produksie beïnvloed ongeag eweredige kolonisasie vlakke. Resultate dui aan dat dit belangrik is om omgewingstoestande as 'n veranderlike in te sluit tydens weerstandseleksie van

SGS om sodoende kwaliteit graan te produsêer en menslike- en dieregesondheid te verseker.

Die ontwikkeling van ’n epidemiologiese model om die risiko van graan skimmel en mikotoksiene in die sorghum produksie areas te kwantifiseer, kan produsente in staat stel om bestuurspraktyke aan te pas om *Fg*SK infeksie en mikotoksien produksie te verminder. Sorghum graan is oor twee seisoene in 18 produksie gebiede versamel en vir analises *Fg*SK kolonisering, DON, NIV en ZEA produksie gedoen. Resulte van *Fg*SK kolonisering en gepaardgaande mikotoksien produksie ooreenstem met die twee kritiese tye (vroë blom, 82-95 dae na plant (d.n.p) en sagte deeg stadium, 92-115 d.n.p.) en mikotoksien akkumulasie. Weerstoestande is belangrik deurdat F*g*SK kolonisering van wat betekenisvol gekorreleer is met maksimum relatiewe humiditeit, 82-95 d.n.p. en betekenisvol negatief gekorreleerd is met maksimum temperatuur en verdamping, 82-95 d.n.p. Produksie van DON, NIV en ZEA is betekenisvol gekorreleer met *Fg*SK DNA konsentrasie bepalings. DON het ’n betekenisvolle positiewe verwantskap aangedui met maksimum temperatuur, 101-115 d.n.p., terwyl NIV en ZEA ‘n betekenisvolle negatiewe verwantskap met minimum temperatuur 91-104 en 100-113 d.n.p. Voorlopige modelle gebaseer op stapsgewyse regressie analise is onwikkel. Verdere studies, wat lokaliteite insluit waar betroubare weerdata oor tyd beskikbaar is word benodig om die modelle verder te verfyn en te bekragtig.

Verder is ’n aantal kommersieel goeste sorghum graan monsters in ’n sorghum verwerkings maatskappy versamel en twee stoor produkte is ingesluit vir ontledings. ’n Addisioneel is 180 sorghum graan monsters, wat vier kultivars vanaf drie lokaliteite (Greytown, Standerton en Potchefstroom) insluit versamel gedurende een produksieseisoen en met ’n “tangential abrasive dehulling device (TADD)" vir vyf tyd periodese ontdop. Sorghum semels het die hoogste ergosterol konsentrasies aangedui en 22% bewerkte graan die laagste, wat daarop dui dat die meeste fungus kontaminasie op die buitenste graanlaë van die saad voorkom. Die teenoorgestelde is aangedui deur *Fg*SK DNA konsentrasies waar die laagste vlakke in sorghum semels en die hoogste konsentrasies by 22% TADD bewerkte graan waargeneem is. Die aanname dat FgSK binne die endosperm voorkom, was gemaak. Beheerde bewerking van graan met TADD het eers na vier minute d.w.s. op 35% ’n

betekenisvolle afname in *Fg*SK voorkoms aangedui. Die aanname word verder ondersteun deur NIV produksie in albei kommersiële en beheerde monsters wat relatief hoë konsentrasies in sorghum semels en 4% TADD bewerkte graan en die afname na 0.00 µg.kg^{-1} in beide 22% en in die ses minute TADD bewerkte graan. In vergelyking met NIV, was DON en ZEA deur kort TADD bewerkings periodes vanaf die graan verwyder. Die aanname kan dus gemaak word dat ZEA en DON produksie met oppervlakkige infeksies van *Fg*SK gepaardgaan, terwyl NIV met patogenese in die dieper endosperm lae gepaardgaan. Kultivars verskil in hardheid en herdie variasie word geassosier met weerstoestande van die onderskeie lokaliteite sowel as endosperm tekstuur. Laer koloniserings en mikotoksien vlakke is bepaal in die harder graan, die hoogste *Fg*SK konsentrasies en DON, NIV en ZEA produksie is in graan vanaf Greytown waargeneem. Dit word toegeskyfaan gunstige weerstoestande tydens die kritiese graan ontwikkelings stadiums wat infeksie en ontwikkeling van die patogeen kompleks en dus kontaminasie deur mikotoksiene bevoordeel het. Kommersiële verwerkers kan die beste bestuurs besuite maak waneer hulle die invloed van TADD bewerkings effek op *Fg*SK DNA konsentrasies en die produksie van DON, NIV en ZEA verstaan om sodoende die skadelike miktoksiene vanaf graan te verwyder.