Occurrence and variation of *Fusarium* free and masked mycotoxins in maize from agriculture regions of South Africa

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DECLARATION

I, the undersigned, declare that this PhD Thesis entitled "**Occurrence and variation of *Fusarium* free and masked mycotoxins in maize from agriculture regions of South Africa**", hereby submitted by me to the North-West University is my own work and has not previously been submitted by me to another university. All materials contained herein have duly been referenced.

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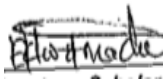
SIGNATURE**:** 

DATE: **20/11/2018**

DEDICATION

This research work is dedicated to almighty God, for being my God every step of the way.

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I wish to express my profound gratitude to the entire academic and support staff members of the Department of Biological Sciences and the Department of Animal Health, North-west University for their contributions in one way or the other towards the completion of this study. I also wish to thank the North West University, for their financial support towards this study.

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Dankie!

Thank you!

ABSTRACT

In the past years, it has become very clear that in mycotoxin-contaminated foodstuffs, many structurally related compounds generated from plant metabolism or during food processing can coexist with the native mycotoxins. The presence of mycotoxins in cereal grain is a very important food safety issue with the occurrence of masked mycotoxins extensively investigated in recent years. This study investigated the occurrence and variation of different *Fusarium* fungal species and their mycotoxins (free and masked) in maize grains from different maize producing regions of South Africa. The risk of exposure associated with consumption of *Fusarium* mycotoxin contaminated maize grains was also conducted and a relationship between the maize producing regions, the maize type and the occurrence of different *Fusarium* fungi as well as their mycotoxins was established.

A total of 123 maize samples harvested during the 2015/2016 season were obtained from randomly selected silo sites in the two (western and eastern) agriculture regions of South Africa. Fungal contamination of samples was investigated using conventional (macroscopic and microscopic) and molecular methods for species identification. Mycological analyses revealed that the maize samples were contaminated with different *Fusarium* species. Most of the samples were contaminated with at least one fungal species, while co-contamination with different *Fusarium* spp. occurred in a majority of the samples. Seven *Fusarium* species found to contaminate the maize in both the western and eastern regions were *Fusarium verticiloides, Fusarium oxysporum, Fusarium subglitans, Fusarium proliferatum, Fusarium napiforme, Fusarium fujikuroi* and *Fusarium graminearum* with total incidence rate of 96 %, 84 %, 66 %, 83 %, 25 %, 24 % and 34 %, respectively. *Fusarium verticiloides* was the predominant *Fusarium* species irrespective of the agricultural regions. Screening of the *Fusarium* isolates for the presence of *Fum13, Tri 6* and *Zea13* genes, which underlie *Fusarium* mycotoxins production showed that the isolates have the biosynthetic genes. The outcome of mycotoxin analysis showed that maize types were contaminated with a mixture of both free and masked mycotoxins across the maize producing regions of South Africa. Generally, all the maize samples analysed were contaminated with an average of 5 and up to 24 out of 42 mycotoxins, including 1 to 3 masked forms at the same time. Data obtained (Table 4.3) highlights the relevance of fumonisin B_1, B_2, B_3, B_4 and A_1 vorstufe in

the samples with 98 %, 91 %, 80 %, 82 % and 54 % of 123 samples contaminated with maximum contamination levels 8907.7, 3383.3, 990.4, 1014.4 and 51.5 µg/kg, respectively. Deoxynivalenol occurred in 50 % of the samples with mean concentration of 152 µg/kg (max 1380 µg/kg). Thirty-three percent of the samples were contaminated with zearalenone at a mean concentration of 13.6 µg/kg (max 145.6 µg/kg). Occurrences of HT-2 and T-2 in the samples were at very low levels at 0.8 % each and at maximum concentration of 40.2 µg/kg and 148.0 µg/kg, respectively, while nivalenol occurred in 11 % of samples at mean concentration of 14.2 µg/kg. Of the masked mycotoxins, DON-3-glucoside occurred at a high incidence rate of 53 % than the others. Among emerging toxins, moniliformin, fusarinolic acid and beauvericin showed high occurrences at 98 %, 98 % and 83 %, respectively. High incidences of these toxins in maize, which serves as a staple food in South Africa is an important cause for concern as not much has been done about the occurrence of these mycotoxins in food in South Africa and neglecting them increases the risk of exposure to humans and animals.

Also, all the 42 *Fusarium* toxins and metabolites investigated in the maize samples across the agricultural regions (AR) were detected and quantified except for the emerging toxin, enniantin B_2 which was only detected in 2 % of the samples from the western region. Of the major mycotoxins, HT-2 was not detected at all in the eastern region but was quantified only in 2 % of the maize from the western region. Of the fumonisin Bs, fumonisin B_1 (FB_1) occurred at more frequently than FB_2, FB_3 and FB_4. Fumonisin B_1 was the main contaminating mycotoxin, occurring at mean concentration of 752.46±1469 µg/kg from the warm western region and of 439.88±514 µg/kg in the cold eastern region with only 3 % (2 samples) not contaminated. Fumonisin B_2 was the second most occurring contaminant at mean concentration levels of 290.08±188 and 151.16±513 µg/kg from the western and eastern regions, respectively. Of the masked mycotoxins detected in the samples included DON-3-glucoside, occurred at a high incidence rate of 53% than the others. Although there was no significant difference in their distribution across the agriculture regions, there seems to be no data on the occurrence of some of these masked mycotoxins in South Africa. Hence, this is the first report on zearalenone-sulphate and HT-2-glucoside on South African maize.

Exposure assessment for adults calculated through maize intake for deoxynivalenol (DON), fumonisin B_1 and B_2 across the AR showed that probable daily intake (PDI) for DON was within the maximum limit of 2000 µg/kg across the ARs. The PDI for the sum of fumonisin B_1 and fumonisin B_2 in the WR was above the maximum limit of 4000 µg/kg as stated in South African

regulation while that in the ER is within the maximum limit. This suggests high exposure of the population to these mycotoxins especially in the WR and which calls for public health concern. In addition, the higher incidence of the fumonisins and most of the *Fusarium* free and masked mycotoxins in the WR may be explained by the higher susceptibility of the maize samples to mycotoxin producing *Fusarium* species. However, significant differences in contamination pattern were observed between the agricultural regions. Therefore, this study has shown that there is higher risk of *Fusarium* mycotoxin exposure, especially fumonisin Bs with consumption of maize grown in the western than with the eastern agriculture regions of South Africa. White maize samples from the western region (WR) had significantly higher mean levels of fumonisins. It also showed that there is no significant difference in the occurrence of the masked toxins across the agriculture regions. Although toxicological data are still limited, the occurrence or presence of masked mycotoxins will add substantially to the overall mycotoxin load and toxicity. This invariably will increase the toxic health effects by these masked mycotoxins, which may be either direct or indirect through hydrolysis, or released from the matrix during digestion into the free mycotoxins (De Boevre *et al*., 2015).

Generally, the high prevalence and at high levels (for some) of these *Fusarium* mycotoxins on maize may have serious health implications on the consumers since maize constitute a major dietary staple in South Africa. There is therefore, a need to carry out periodic surveys and awareness campaigns in the higher-risk regions (WR) to educate farmers as well as other agricultural stakeholders on the benefits of good agricultural practices (GAP) in relation to reducing mycotoxin exposure.

However, most of the *Fusarium* mycotoxin research in South Africa has mainly focused on the free mycotoxins, but the novelty of this study is that very limited data are available so far, on the impact of climatic differences on these fungi and their mycotoxins (free and masked), in the different agriculture regions of South Africa. Futhermore, literature is also minimal of information on the risk assessment of maize consumers in South Africa to contaminated maize grains.

Keywords: Fungi, *Fusarium*, maize, masked mycotoxins, LC-MS/MS, molecular methods, chromatography, agricultural regions, South Africa.

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LIST OF ABREVIATIONS AND ACRONYMS

AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AOCS	American Oil Chemists' Society
APCI	Atmospheric Pressure Chemical Ionization
AR	Apparent Recovery
ARC-GCI	Agricultural Research Council-Grain Crops Institute
ARC-ISCW	Agricultural Research Council-Institute for Soil, Climate and Water
ATA	Alimentary Toxic Aleukia
BLAST	Basic Local Alignment Search Tool
B*w*	Body weight
CAC	Codex Alimentary Commission
CAPD	Continuous Ambulatory Peritoneal Dialyses
CAST	Council for Agricultural Science and Technology
CCFAC	Codex Committee on Food Additives and Contaminants
CRD	Completely Randomized Design
DAS	Diacetoxyscirpenol
DM	Dry matter
DNA	Deoxyribonucleic Acid
DOA	Department of Agriculture
DON	Deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
ELEM	Equine leucoencephalomalacia
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray Ionization
EU	European Union
FAO	Food and Agricultural Organization
FBs	Two or more of Fumonisin B_1, B_2, B_3, B_4

FB_1, FB_2, FB_3, FB_4	Fumonisin B_1, B_2, B_3, B_4 respectively
GAP	Good Agricultural Practices
GEMS	Global Environment Monitoring System
GIT	Gastro-intestinal Tract
GSA	Grain South Africa
HFB_1	Hydrolyzed Fumonisin B_1
HPLC	High Performance Liquid Chromatography
HT-2	HT-2 Toxin
IAC	Immuno affinity column
IARC	International Agency for Research on Cancer
IGS	Inter-genic Spacer
ITS	Internal Transcribed Spacer
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
LSD	Least Significant Difference
MAS	Monoacetoxyscirpenol
ME	Metabolisable Energy
MGA	Malachite Green Agar
NCBI	National Centre for Biotechnology Information
NDA	National Department of Agriculture
NTD	Neural Tube Defects
OPA	O-Phthaldialdehyde
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDI	Probable Daily Intake
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
SAGIS	South African Grain Laboratory
SD	Standard Deviation

TEF	Translocation Elongation Factors
T-2	T-2 Toxin
USDA	United States Department of Agriculture
WHO	World Health Organisation
ZON	Zearalenone

LIST OF UNITS

%	Per cent
°C	Degree Celsius
<	Less than
>	Greater than
≥	Greater or equal to
µg/kg	Microgram per kilogram
µg/ml	Microgram per millilitre
µg/g	Microgram per gram
Mcal/Kg	Megacalorie per kilogram
µl	Microlitre
µm	Micrometer
Bp	Base pair
G	Gram
Hrs	Hours
Kg	Kilogram
kPa	Kilopascal
L	Litre
Mins	Minutes
m/z	Mass per charge ratio
Ng/g	Nanogram per gram
Nm	Nano metre
Ng/ml	Nanogram per mililitre
Ppm	Parts per million
Ppb	Parts per billion
Psi	Pound-force per square inch
S	Seconds
s/n	signal to noise
V	Volume

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CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Mycotoxin-producing fungi are very common worldwide, occurring in varying amounts on agricultural commodities. These filamentous fungi often grow on edible plants, thus contaminating food and feed with mycotoxins in toxicologically relevant concentrations (Bennet and Klich, 2003). Mycotoxins contamination caused by fungal development usually results in a highly concentrated, localized and inhomogeneous distribution that can spoil an entire batch (Rivas Casado *et al.*, 2009). Fungi of agro-economic relevance are phytopathogenic fungi that infect plants while growing in the field or while in greenhouse and saprophytic fungi that colonize plant produces after harvest (Bhat *et al.*, 2010). *Fusarium* species are fungi of great importance owing to their ability to induce numerous devastating plant diseases, and cause economic losses and trade barriers, whereas potentially being able to produce a range of mycotoxins.

Mycotoxins can adversely affect human and animal health condition, productivity, economics and trade (Smith *et al*., 1995; Wild and Gong, 2010). The United Nations' Food and Agricultural Organization (FAO), made an estimate that there was significant contamination of about twenty-five percent of the world's food crops with mycotoxins leading to annual loss in the range of one million tons (Smith *et al*., 1994). Recently, studies suggest that the percentage of contaminated cereals is much higher at 72% (Streit *et al*., 2013). The difference may be due in part, to what levels are considered as contamination, in addition to advances in detection and monitoring (Berthiller *et al*., 2015).

It has come to be clearer that in mycotoxin contaminated produce, various structurally related compounds produced during plant metabolism or during after food processing can co-occur with the parent toxins (Galaverna *et al.*, 2009). These mycotoxin derivatives may have a very different chemical behaviour including polarity and solubility, compared to the precursor and thus, can easily escape routine analyses (Berthiller *et al*., 2013). Since they are undetectable by conventional analytical techniques because of their altered structures, there is thus generally an underestimation of the mycotoxin load. Also, despite their chemical alteration, coupled with the fact that they are generally not regulated by legislation, they may be considered as being masked (Berthiller *et al*., 2015). These conjugated or masked mycotoxins first came to the

attention of public health officials, when animals fed with apparently low mycotoxin contaminated feed, showed high severity of mycotoxicosis. The unanticipated high toxicity was ascribed to the presence of undetected, conjugated forms of mycotoxins (Berthiller *et al*., 2013).

Historically, Gareis *et al*., (1990) for the first time, used the term, 'masked mycotoxins' and it refers to the products that are formed when plants metabolise mycotoxins, as part of their natural defence system. These secondary metabolites are not detectable by conventional techniques because their structure has been altered in the plant, nor are they regulated. The metabolites are so-called masked as they become toxic again as soon as they cleave off their sugar molecule in the intestine of the humans and animals. The term conventional applies to the analytical detection methods that have previously or initially been developed for specific mycotoxins only. Then in 2013, researchers revisited the masked mycotoxin topic again and Berthiller *et al.,* (2013), made a clear definition of what masked mycotoxins are. The term "masked mycotoxins" is now widely accepted. However, in 2014, Michael Rychlik and his, research group came up with a comprehensive definition to include all modified forms of mycotoxins as well as masked mycotoxins as "modified mycotoxins" (Rychlik *et al.,* 2014). Actually, the latter is the umbrella term of all mycotoxins that are modified by some sort of process (for example food processing). Masked mycotoxins are sort of, part of this definition, but only entail the molecules that are formed by the plants.

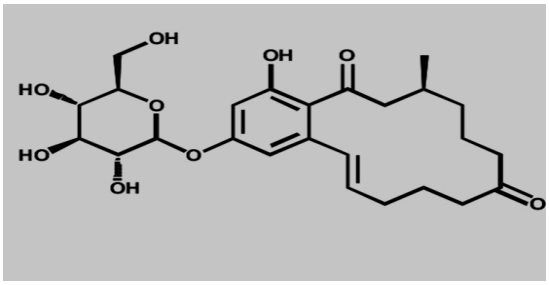
The likelihood of mobilisation of mycotoxins that interact with metabolically active plants in the field is the issue. As *Fusarium* infection usually occurs in the field (in contrast to *Aspergillus* and *Penicillium* infections), the *Fusarium* mycotoxins (deoxynivalenol, zearalenone, fumonisins, nivalenol, fusarenon- X, T-2 toxin, HT-2 toxin, fusaric acid) are the most prominent target for conjugation (Berthiller *et al*., 2013). Although, transformation of other mycotoxins e.g., ochratoxin A, patulin and destruxins, by plants has also been described. Specifically, deoxynivalenol-3-glucoside (D3G), zearalenone-14-glucoside (Z14G) and zearalenone-14-sulphate (Z14S) are the most commonly found masked mycotoxins occurring in food commodities (Berthiller *et al*., 2013). Currently, only glucoside and sulphate conjugates of DON, ZON, T-2 and HT-2 have been proven to occur in naturally infected cereals such as maize, wheat and barley (Figure 1.1). Though toxicological data are scarce since masked mycotoxins represent an emerging condition, but studies highlight the potential threat to consumer safety from these substances. However, even as it seems that derivatives can be less toxic than the precursor toxins (Poppenberger *et al*., 2003; Wu *et al*., 2007), the potential hydrolysis of masked mycotoxins back to their parent toxin in the digestive tract of mammals

raises concerns (Gareis *et al*., 1990). In particular, enzymatic cleaving of the attached functional groups like glycosylic or sulphate residues during digestion (Gareis *et al*. 1990; Plasencia and Mirocha, 1991) releases the unconjugated, free mycotoxins. Moreover, this could further add to the total mycotoxins content of the respective food or feed (Streit *et al*., 2013) with subsequent effects, increasing health risks to farm animals and humans.

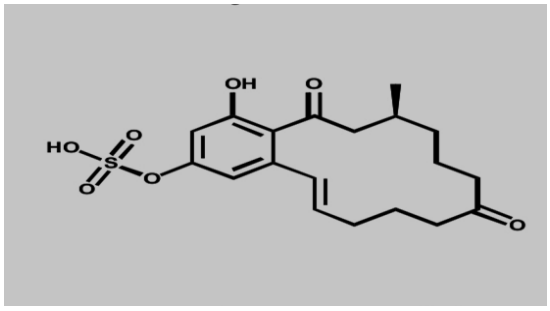
Masked mycotoxins might on the other hand, be more toxic than their parent compounds, e.g., when they are more bioavailable (Berthiller *et al*., 2013). It should be highlighted that currently there are no adequate toxicity investigations available to evaluate the likely hazard or risk assessment of the masked toxins as compared to their parent forms. Thus, accurate risk assessment of masked mycotoxins in foodstuff is difficult, owing to absence of contamination data as well as toxicological properties. The recognition of the toxicological relevance of masked mycotoxins, as well as the evaluation of the hazard of co-occurrence of target mycotoxins contaminating food products creats new big problem. This should be addressed by the food producers, food risk assessment bodies and food regulatory and monitoring bodies so as to guard consumers health and to evaluate human health hazard (Stoev and Denev, 2013; Stoev, 2015).

Very limited data are available on the occurrence and toxicity of most *Fusarium* mycotoxins and their masked forms in food and feed. For example, a study conducted on South African maize processing chain by Erasmus *et al*., (2012) on occurrence of *Fusarium* mycotoxins, dwelt only on fumonisins and their masked forms. Hence, this study investigated the occurrence and variation of different *Fusarium* fungi spp and their mycotoxins (free and masked forms) in maize grains from different maize producing regions of South Africa. To conduct a risk assessment of exposure, which is the magnitude and probability of harmful effect of consumption of *Fusarium* free and masked mycotoxins contaminated grains. And to establish the interaction between the maize producing regions, maize type and occurrence of different *Fusarium* fungi species and their mycotoxins.

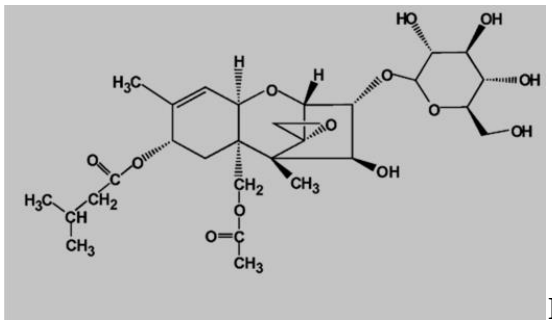
Yet, only glucoside and sulphate conjugates of ZON, DON, T-2 and HT-2 have been proven to occur in naturally infected cereals such as maize, wheat and barley (Figure 1.1).



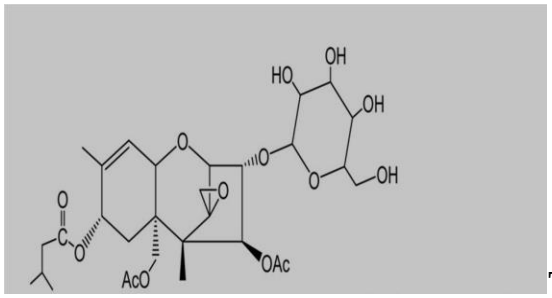
Zearalenone-14-glucoside



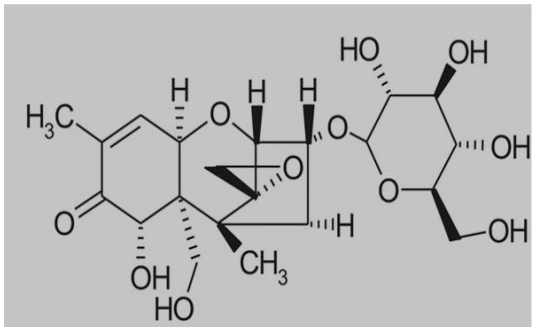
Zearalenone-14-sulphate



HT-2 glucoside



T-2 glucoside



DON-3-Glucoside

Figure 1.1: Structurally elucidated masked *Fusarium* mycotoxins.

1.2 Problem Statement

The likelihood of mobilisation of mycotoxins that interact with metabolically active plants in the field is the issue. As *Fusarium* infection usually occurs in the field (in contrast to *Aspergillus* and *Penicillium* infections), the *Fusarium* mycotoxins (deoxynivalenol,

zearalenone, fumonisins, nivalenol, fusarenon- X, T-2 toxin, HT-2 toxin, fusaric acid) are the most prominent target for conjugation (Berthiller *et al*., 2013). Though toxicological data are scarce since masked mycotoxins represent an emerging condition, but studies highlight the potential threat to consumer safety from these substances. However, the possible hydrolysis of masked mycotoxins back to their parent toxin during food/feed processing or during mammalian digestion raises concerns. To ensure the safety of agricultural products (food safety), there is a need for the identification and determination of mycotoxins and their masked forms in order to assess possible effects on consumers. Furthermore, since there are very few investigations on the impact of climatic differences on mycotoxins variation in the different agriculture regions of South Africa, a comprehensive study was imperative.

1.3 Aim of the Study

The aim of the study was to investigate the occurrence and variation of *Fusarium* fungi and their mycotoxins (free and masked forms) in maize from different maize producing regions of South Africa. To conduct a risk assessment of exposure associated with consumption of *Fusarium* mycotoxins contaminated maize grains. Furthermore, to establish some relationships between the maize producing regions, maize type and occurrence of different *Fusarium* fungi and their mycotoxins.

1.4 Objectives of the Study

The following specific objectives were addressed in order to achieve the main aim of the study:
a) To determine *Fusarium* contaminations of the samples using polyphasic approach including conventional (morphological) methods and to further validates the identification using PCR-based molecular methods
b) To detect the toxigenic isolates using species-specific PCR markers for the key mycotoxins biosynthetic gene cluster (fumonisin- fum*13*; trichothecenes - tri*6*; zearalenone, zea*13*) in *Fusarium* species by PCR technique.

c) To analyse for free and masked *Fusarium* mycotoxins, (FUM, ZON, DON, NIV, DON-3G and others on LC-MS/MS and other methods.

d) To conduct a risk assessment of exposure, this is the magnitude and probability of harmful effect of consumption of *Fusarium* free and masked mycotoxins contaminated grains.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Fusarium*: Overview and Taxonomy

The taxonomy of *Fusarium* started in 1809 when the genus *Fusarium* was first described by Link (Nelson, *et al*., 1981). It comprises of naturally ubiquitous species (Nelson *et al*., 1983; Logrieco *et al.*, 2003). *Fusarium* is a large group of filamentous fungi that occur predominantly in the air and soil which usually associates with plants and occasionally with humans. Some of the most important plant pathogenic fungal species known today are members of this genus. Worldwide, it is a concern that a large number of economically important plant species are susceptible to at least one or more *Fusarium* spp. (Leslie & Summerell, 2006). Fungi now included in the genus *Fusarium* were originally described and defined as *Fusisporium* based on the type *Fusisporium roseum* described by Link in 1809 (Summerell *et al*., 2010). Wollenweber & Reinking (1935) reclassified the two *F. roseum* type specimens as *F. sambucinum* and *F. graminearum* and currently accepting *F. sambucinum* as the type species for the genus. Although the taxonomy of *Fusarium* continues to undergo major changes, mainly on the basis of molecular classifications, the Wollenweber and Reinking classification system continues to form the foundation on which species are described (Leslie & Summerell, 2006).

Members of the genus *Fusarium* are characterised by the having septate, hyaline, delicately curved, elongate macroconidia (Moss & Thrane, 2004; Leslie & Summerell, 2006). Mycelia and spore masses are generally brightly coloured (Booth, 1971). In some species, smaller, 0 to 1 septate microconidia and chlamydospores are common, while some authors recognize a third conidial type known as mesoconidium. The *Fusarium* genus comprises around 70 recognized species, identified by means of a polyphasic approach, and about 300 putative species. Following phylogenetic species concepts, many putative species do not yet have formal names (Munkvold, 2017).

2.1.1 Morphological characters for identifying *Fusarium* fungal species.

Morphological characters are considerably the most traditional criteria used to identify any fungal species. *Fusarium* produces a range of mycelia that are cottony in nature with shades of pink, yellow and purple. Some species produce either macroconidia or microconidia as asexual reproductive structures, while in some other species both are present (Jay, 1987). The

morphology of microscopic characteristics, generally the shape and dimensions of the macroconidia, the generating of microconidia, chlamydospores, sclerotia, sexual stages and pigmentation, are the primary means used for the identification of *Fusarium* species. Members of the genus are variable in cultural characteristics because changes in the environment wherein they grow can result in morphological changes both in culture and in conidia. However, the description of these characters is of necessity under very specific, environmental conditions because of distinctive cultural variability of morphological traits.

i) The genus, *Fusarium* is categorised by the production of septate, hyaline, delicately curved, elongate macroconidia, distinct macroconidia and chlamydospores along with other secondary characters like mycelial growth and pigmentation (Moss and Thrane, 2004).

ii) The macroconidium is the single most important cultural character for the identification of a culture of *Fusarium* species. The most distinctive character of macroconidia is the shape, followed by the size and number of septa and finally, the nature of apical, basal or foot cell (Leslie and Summerell, 2006). With reference to shape, most of the *Fusarium* produce sickle shaped macroconidia that can be characterised into three types (1) Straight macroconidia, which can appear virtually needle-like if they are thin eg *F. avenacum*. (2) Microconidial having dorsivental curvature along all or portion of the spore (these pores are almost of the same width along their entire length: eg *F. equiseti and* (3) Microconidia in which the dorsal side is more curved than the ventral side eg *F. crookwellence* (Figure 2.1). Macroconidia can be long (*F. armeniacum*) or short (*F. culmorum*), but usually, spore size is a relatively constant character and major variations indicate improper culture conditions. Typically, *Fusarium* macroconidia are 3-5 septate. The number of septa ought to be determined depending on the range and the average number of septa per spore (Leslie and Summerell, 2006).

Another important macroconidial character is the apical and basal cell forms. There are four common forms of apical cells: (1) Blunt e.g. *F. culmorum,* (2) Papillate eg *F. sambucinum*, (3) Hooked e.g. *F. lateritium* and (4) Tapering e.g. *F. equiseti* (Figure 2.1)

The apical cell length also can vary amongst species, but it is usually constant within a specie. The main diagnostic features of apical cells are the degree of curvature, relative length and general form. Microconidia are not produced by all *Fusarium* species; therefore, their presence is a potential diagnostic character in *Fusarium* identification. The major characters regarding microconidia are the microconidia, the coniogenous cells where they are born and their arrangement on or around the conidiogenous cell (Leslie and Summerell, 2006).

iii) Chlamydospores**:** Are verrucose (rough) or smooth-walled structures produced in single, e.g. *F. solani,* double or paired e.g. *F. compactum*, clumps, e.g. *F. scirpi* or chains e.g. *F. compactum*. Chlamydospores are produced rarely and take longer time (more than 6 weeks)

when compared to macro or microconidia. The presence of chlamydospores in the aerial mycelia or embedment on the agar surface is another important criterion used in the identification of *Fusarium* species.

2.1.1.1 Other important characters

The characters discussed above are universally found in almost all *Fusarium* species. There are some other characters which are restricted to only few species of *Fusarium* and which serve as important delimiting factor in their identification. Mesoconidia telemorphs and other characters like circulated (coiled) hyphae in the case of *F. circinatum*. In addition, formation of scelerotia-like structures (compact masses of hardened mycelium with stored reserve food material) etc. are some other relevant characters which help in the primary identification process.

Secondary Characters

The most important and diagnostically potential secondary character is pigmentation (Leslie and Summerel., 2006). The different *Fusarium* species produce colours ranging from yellow to orange to carmine red. (Joffe, 1974). The pattern of pigmentation is detectable on PDA and a 12:12 h light: dark cycle is usually preferred. Pigments produced by these fungi may be sensitive to light or pH., maybe diffusible or non-diffusible into the growth media and most of the evaluations are carried out a week after incubation. Another important character is the growth rate of the species, usually measured as colony diameter from PDA plates incubated with single spore culture and incubated at 25 or 30 for 3 days. There are slow growing species like *F. lateritium, F. merismoides* and fast growing species like *F. culmorum, F. graminearum etc.* (Leslie and Summerell, 2006). These characters if not properly analysed also may not be clear and are not usually preferred for identification of species. Secondary metabolites and mycotoxins are also characteristic features, which may influence a particular odour to the culture and serve as specific secondary characters. The chemical background of the metabolites or mycotoxins can be used to primarily group the fungi, which can further be analysed to finally assign the fungi to particular species. *Fusarium* is known to produce many toxins, which can be effectively used for their specific identification

Spore morphology

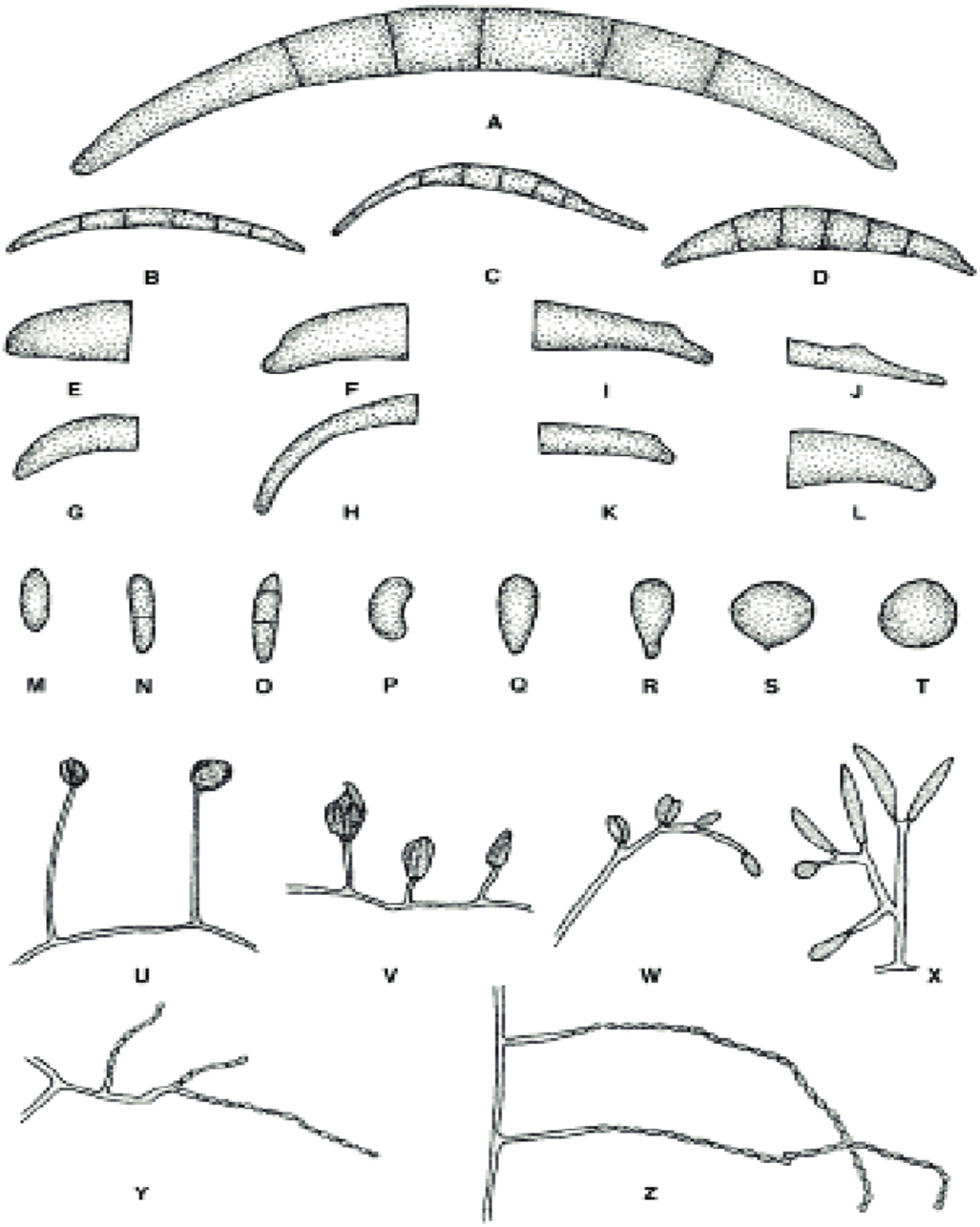


Figure 2.1: Spores morphological characters of *Fusarium spp*., **A-D**: Macroconidial shapes, **E-H**: Macroconidial apical cell shapes, **I-L**: Macroconidial basal cell shapes, **M-T**: Microconidial shapes, **U-X**: Phialide morphology, **Y-Z**: Microconidial chains
Adapted from (Leslie and Summerell, 2006).

2.1.2 Molecular tools for identifying *Fusarium* fungal species based on genetic diversity

Different molecular tools for example, PCR-based techniques aid to describe differences amongst species according to genetic diversity. Some examples such as ; random amplified polymorphic DNA (RAPD) (Arici and Koc., 2010), restriction fragment length polymorphism (RFLP) (Martínez-García *et al*., 2011), amplified fragment length polymorphism (AFLP) (Leissner, *et al*., 1997;Schmidt, *et al*., 2004), DNA sequences of inter-genic spacers (IGS) (Konstantinova and Yli-Mattila *et al*., 2004), *β*-tubulin (*tub2*) (Yli-Mattila *et al*., 2004), translation elongation factor-1 alpha (TEF-1α) (Knutsen *et al.* 2004) and internal transcribed spacers (ITS) (White *et al*., 1990), have been used to differentiate and diagnose fungal strains. Analyses of other genes such as calmodulin, topoisomerase II and cell biohydrolase-C have also been used for the identification of *Fusarium*. (Hatsch *et al*., 2004; Mule *et al*., 2004).

i) Random Amplified Polymorphic DNA (RAPD)

This is a PCR technique where primers (usually10-20 base pair (bp) in length randomly bind to complementary sequences of the genomic DNA of a given organism and leads to the generation of consensus sequence patterns which serve as fingerprints for the organism (Dassanayake and Samaranayake, 2003). This technique works in such a way that nucleotide sequence variation due to insertions, additions or base substitutions, inversion of priming site, conformational changes in the template DNA etc. in the PCR priming regions, especially at the 3' ends prevent primer annealing. This results in different sized PCR fragments that are highly specific for a particular specie (Kumar and Gurusubramanian, 2011). The RAPD assays have been effectively used for genome analyses of different bacteria and fungi (Nicholson *et al*., 1998 and Fungaro *et al*., 2004). In addition, RAPD has been used for the identification of other *Fusarium* species such as *F. oxysporum, F. avenaceum, F. poae, F. solani* and *F. moniliforme* (Yli-Mattila *et al*., 1996; Kernnyi *et al*., 1997; Hue *et al*., 1999; Paavanen *et al*., 1999). Despite the advantages of RAPD, it has been criticized due to poor reproducibility of results that affects its use in fungal taxonomy and there is need for fastidious PCR conditions. (Ellsworth, *et al*., 1993; Khanda, *et al*., 1997).

ii) Restriction fragment length polymorphism (RFLP)

This technique is based on restriction enzyme digestion of the pathogen DNA, and afterwards separation of the fragments by electrophoresis in agarose or polyacrilamide gels to identify differences in the sizes of DNA fragments (Capote *et al*., 2012). Polymorphisms within the restriction enzyme cleavage sites are meant to differentiate fungal species. Although DNA

restriction profile can directly be noticed by staining the gels, Southern blot analysis is usually needed. The DNA is transferred to appropriate membranes and hybridised with an appropriate probe (Capote *et al*., 2012). The RFLPs have been largely used for the study of the diversity of micorrhizal and soil fungal population/community (Thies, 2007; Kim, *et al*., 2010; Martínez-García *et al*., 2011). Although meant for differentiation of pathogenic fungi (Hyakumachi *et al.,* 2005), this early technique has been increasingly supplanted by other fingerprint techniques based on PCR. RFLP combines the amplification of a target region with the further digestion of the PCR products obtained.

iii) Amplified fragment length polymorphism (AFLP)

The AFLP technology (Vos *et al.,* 1995) is a modified version of RAPD, which is based on the use of restriction enzymes to digest total genomic DNA followed by ligation of restriction half-site specific adaptors to all restriction fragments. Then, a selective amplification of these restriction fragments is achieved with PCR primers that have in their 3' end the corresponding adaptor sequence and selective bases. The bands of the amplified fragments are visualized on denaturing polyacrylamide gels. The AFLP technology has the capability to amplify between 50 and 100 fragments at one time and to detect various polymorphisms in different genomic regions simultaneously. It is also very sensitive and reproducible. The disadvantages of AFLPs are that they need high molecular weight DNA, more technical expertise than RAPDs (ligations, restriction enzyme digestions, and polyacrylamide gels), and that AFLP analyses suffer the same analytical limitations of RAPDs (McDonald *et al*., 1997). AFLP has been used to differentiate fungal isolates at several taxonomic levels e.g. to differentiate *Monilinia laxa* that infect apple trees from isolates infecting other host plants (Gril *et al*., 2008) and to separate non-pathogenic strains of *Fusarium oxysporum* from those of *F. commune* (Stewart *et al*., 2006). AFLP profiles have also been widely used for the phylogenetic analysis of *Fusarium oxysporum* complex (Baayen *et al*., 2000; Groenewald *et al*., 2006; Fourie *et al*., 2011). Leissner *et al.,* (1997) also used AFLP to differentiate between isolates of *F. graminearum*.

iv) Inter-genic Spacers (IGS)

These are regions that separates nuclear ribosomal DNA repeat units, which consist of highly conserved genes and more variable spacer regions (Taylor *et al.,* 2000). The number of ribosomal DNA repeat units varies among different species, and this results in variations in the length and restriction sites of IGS (Hills and Dixon, 1991). IGS-RFLP has been used for the analysis of genetic variation within and between closely related species or communities (Mishra *et al*., 2006; Mbofung *et al*., 2007). Analysis of the IGS region with the RFLP

technique have been effectively used for phylogenetic analysis of closely related species of *Fusarium* such as *F. langsethiae* vs *F. sporotrichioides* and *F. poae* vs *F. kyushuense*. The result showed clear differentiation between the two species (Konstantinova and Yli-Mattila *et al*., 2004).

v) *β*-tubulin

The *β*-tubulin gene sequences have been widely used for phylogenetic investigations in various fungi (Samson *et al.,* 2004; Amrani and Corio-Costet, 2006). It has also been used in the phylogenetic investigations of *F. xylarioides* (Geiser *et al*., 2005). Schmidt *et al*., (2004) also used DNA sequences of *β*-tubulin along with other marker genes for the taxonomic study of *F. langsethiae, F. poae* and *F. sporotrichioides.*

vi) Translation elongation factors

TEF-1a has been widely used for *Fusarium* classification, because it is highly informative at the species level in *Fusarium* (Geiser *et al*., 2004). Also, universal primers have been designed that work across the identification of the genus (Geiser *et al*., 2004), to amplify a ~700 bp region of TEF, including three introns. These introns cover over half of the amplicon's length, in all known *Fusarium* species (O'Donnell *et al*., 1998). The TEF gene is a single copy in *Fusarium* and its sequence shows high variability among closely related species.

vii) Internal Transcribed Spacers (ITS)

Internal Transcribed Spacer (ITS) regions are useful tools for the identification of different fungal species (Bruns and Shefferson, 2004). Internal Transcribed Spacers, ITS1 and ITS2 spacers undergo more variations even within closely related species and hence are widely used for identification processes and for studying evolutionary events. The highly conserved priming site of the ITS region makes it easy to be amplified from practically all fungal species. The stretches of DNA between 18S, 5.8S and 28S rRNA regions make up the ITS regions (White *et al.,* 1990). The growing ITS sequence data is also an added advantage that helps in the identification of various fungi. This information can also be used for the development of species-specific primers for the detection of some fungi in a much reduced time instead of via the morphology method (Mule *et al*., 2004). For the identification of *Fusarium* species, ITS-RFLPs have been extensively used (Young-Mi *et al*., 2000). Variations occurring in ITS1 and ITS2 sequences have been used to study the genetic relationship between different *Fusarium* species (Young-Mi *et al*., 2000).

The ITS region is the perfect region for species identification, since even closely related species have sequence variations (White *et al*., 1990). In addition, these variable regions are flanked by conserved ribosomal RNAs, which give the option to use primers for PCR amplification of the variable regions that are recognized by the majority of fungi and fungal-like organism. For fungal identification most people use ITS1/ITS4 primer pair (White *et al.,* 1990) for amplification of the ITS region, which leads to another fact in favour of ITS as a barcode marker there are huge databases consisting of ITS sequences from the majority of known fungi. Though perfect for species identification, the ITS region is too variable to determine the phylogeny of higher ranks.

This region contains highly conserved areas adequate for genera- o species-consensus primer designing (RNA ribosomal genes), alternate with highly variable areas that allow discrimination over a wide range of taxonomic levels (ITS region) (White *et al*., 1990). The ITS region is ubiquitous in nature and found in all eukaryotes. In addition, the high copy numbers of rRNA genes in the fungal genome enable a highly sensitive PCR amplification. Furthermore, a large numbers of ribosomal sequences are publicly available in databases, facilitating the validation and the reliability of the detection assays. Traditionally, molecular identification of plant pathogenic fungi is accomplished by PCR amplification of ITS region followed by either restriction analysis (Durán *et al*., 2010) or direct sequencing and BLAST searching against GenBank or other databases (White *et al*., 1990).

2.2. The pathogen – *Fusarium*

The genus *Fusarium* comprises numerous toxigenic species that are pathogenic to plants and/or humans. They are capable of colonizing various environments on earth (Munkvold, 2017). *Fusarium* species as versatile fungi are found everywhere such as in air, water, soil, on plants and organic substrates. *Fusarium*'s widespread distribution is attributable to its ability to withstand a wide range of conditions, to grow on a broad range of substrates and their efficient mechanisms for dispersal (Tupaki-Sreepurna and Kindo, 2018). Often regarded as soil‑borne fungi, since they are abundant in soil and frequently associated with plant roots, they are also present in water as parts of water biofilms (Elvers *et.al*., 1998). *Fusarium* specie have been isolated from public swimming pools, shower drainage pipes and hospital water systems (Williams *et al*., 2013)

These field fungi require high moisture levels in order to colonise and contaminate grain (Placinta *et al.,* 1999; Gale *et al*., 2002). Aside from their ability to act as plant pathogens, *Fusarium* species have been linked to a wide range of diseases and infections, directly or indirectly in humans and animals (Nucci and Anaissie, 2007b). *Fusarium* is one of the most economically important

fungal genera because of yield loss due to plant pathogenic activity. Mycotoxins contamination of food and feed products, which often render them unacceptable for marketing, also add to the huge economic losses to the agricultural industry and posing a substagreat threat to human and animal health due to consumption of mycotoxins. (Nelson *et.al*., 1994; Nucci and Anaissie, 2007b; Guarro, 2013).

2.2.1 *Fusarium* as plant pathogen

Fusarium is known to contain a range of plant-pathogenic fungal species, with a record of devastating infections and has been in existence for the past two centuries (Leslie and Summerell, 2006). As common invaders of aerial plant parts, they can either be part of the normal mycoflora or act as plant pathogens on horticultural crops and cereal grains, such as maize, where they render them unfit for consumption. (LysØe *et al.*, 2006; Schollenberger *et al.*, 2008). *Fusarium* spp. can cause seedling, root and crown rot as well as stalk and ear rot at any stage through plant development (Marasas *et al.*, 1981; Rheeder *et al.*, 1992; Cotten and Munkvold, 1998).

Successful infection of plants by pathogenic *Fusarium* spp involves many different and highly regulated processes from initial infection to production of symptom of disease development in the host (Lucas 1998). An example is shown in figure 2.2

i) Adhesion:

Fungal infection commences after the recognition of roots through unknown host signals, and then infection hyphae adhere to the host root surface (Bishop and Cooper 1983a). This adhesion of fungi to the host surface is not a specific process, as they can adhere to the surface of both host and non-hosts (Vidhyasekaran, 1997). Site-specific binding may be crucial in anchoring of the propagules at the root surface, after which further processes required for colonization can proceed (Recorbet and Alabouvette 1997).

ii) Entry

During pathogenesis, the fungus penetrates the complex physical defense barriers of the host plant cell walls (Mengden *et al*. 1996 and Koretsky, 2001). Gaining entrance to plant cells requires hydrolytic degradation of physical host barriers such as the cell walls endodermis, whereby fungi secrete a mixture of hydrolytic enzymes including cutinases, cellulases,

pectinases and proteases (Knogge, 1996). This is in order for it to reach the vascular tissues where it lodges.

After penetration and once in the vascular tissues, it has to adapt to the hostile plant environment and tolerate plant antifungal compounds. The fungus tries suppressing and inactivating host defense responses, usually by secreting toxins or plant hormone-like compounds that manipulate the plants physiology to the benefit of the pathogen (Knogge 1996). This quite often is achieved through the production of phytotoxins with varying degrees of specificity toward different plants (Walton 1994).

iii) Colonization, Adaptation and Propagation

In the course of colonization, the mycelium spreads intracellularly through the root cortex until it reaches the xylem vessels and enters them through the pits. The fungus then remains solely within the xylem vessels, using them to colonize the host (Bishop & Cooper 1983b). Fungal colonization of the host's vascular system is often fast and frequently facilitated by the formation of microconidia inside the xylem vessel elements (Beckman *et al*. 1961) that are detached and taken upward in the sap stream (Bishop & Cooper 1983b). As soon as the perforation plates stop the spores, they ultimately germinate and germ tubes pierce the perforation plates. Subsequently hyphae, conidiophores and conidia are formed (Beckman *et al.* 1961; Beckman *et al*. 1962).

iv) Disease development

Wilting is almost certainly triggered by a combination of pathogen activities. These include buildup of fungal mycelium in the xylem tissue and/or production of toxin, host defense responses, comprising production of gels, gums, tyloses, and vessel crushing by multiplication of adjacent parenchyma cells (Beckman, 1987). The wilting symptoms seem to be a result of serious water stress, mainly due to vessel occlusion. Symptoms are somewhat variable, but involve combinations of vein clearing, leaf epinasty, wilting, chlorosis, necrosis, and abscission. Harshly infected plants may wilt, die, while plants affected to a lesser extent may become stunted, and unproductive (MacHardy & Beckman 1981).

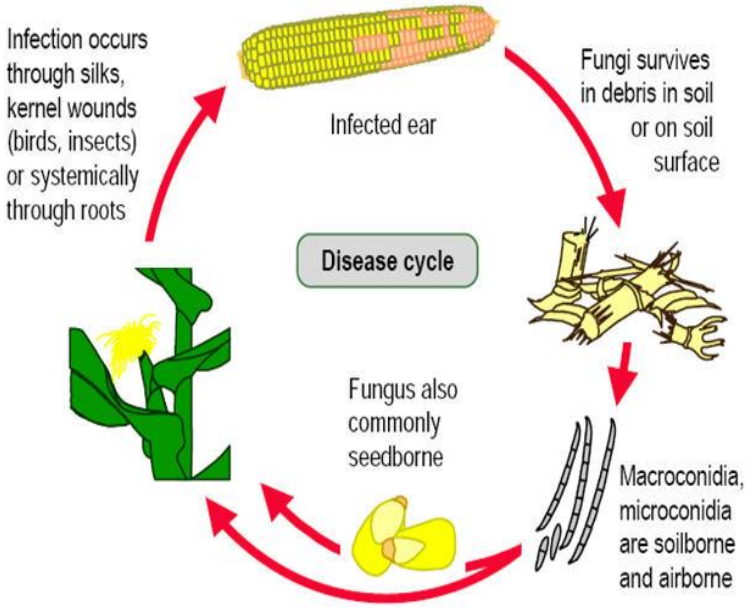


Figure 2.2: The disease cycle of (FER) *Fusarium* ear rot. (Adapted from Pioneer Agronomy Sciences, 2010) (https://www.pioneer.com)

Table 2.1: Phytopathogenic *Fusarium* fungal species

Pathogen	Host plant	Infection	Reference
F. avenaceum	Wheat	*Fusarium* head blight (FHB)	Moradi *et al*., 2010
F. oxysporium	Oriental lilium plant	Root and bulb disease	Prados-Ligero *et al*., 2008
F. graminearum	Wheat and Barley	*Fusarium* head blight (FHB)	Brandfass & Karlovsky,2006
F. proliferatum	Oriental lilium plant	Root and bulb disease	Prados-Ligero *et al*., 2008
F. proliferatum	Chillipepper (*Capsiumannuum* L.)	Fruit rot	Rampersad &Teelucksingh,2011
F. solani	Paprika	*Fusarium* rot	Jee *et al*., 2005
F. oxysporum	Potato	Stem-end rot	Aktaryzzaman *et al*., 2014
F.oxysporum	Banana	*Fusarium* wilt	Viljoen, 2002
F. verticilloides	Maize	*Fusarium* ear rot	Boutigny, *et al.*, 2011
F. commune	Chinese water (*Eleocharis* dulcis)	*Fusarium* wilt	Zhu *et al*., 2014

2.2.2 *Fusarium* as human and animal pathogen

Conventionally, *Fusarium* has been more of an agronomic threat than a medical one, but over the last thirty years, due to a variety of contributing factors, this scenario has undergone a radical change. This made *Fusarium* spp. emerging as major opportunistic human pathogens, causing an expansive range of infections with high morbidity and mortality (Fluckiger, 2006; Stempel *et al*., 2015; Tupaki-Sreepurna and Kindo, 2018). Infections caused by *Fusarium* species are generally referred to as fusariosis which is largely dependent on the immune status of the host and the route of entry of the infection (Nucci and Anaissie, 2002; Nucci and Anaissie, 2007b). Among immunocompetent hosts, the common *Fusarium* infections are keratitis and onychomycosis with other less common conditions such as sinusitis, pneumonia, thrombophlebitis and fungemia (Nucci and Anaissie, 2007b).

It is not all species of the genus that possess the ability to induce disease or infection with only a few causing infections on humans and animals. *Fusarium* human pathogens of growing importance include; *F. oxysporum, F. moniliforme* and *F. solani*, although infections by *F. proliferatum* and *F. napiforme* have also been reported recently (Pontón *et al*., 2000; Tupaki-Sreepurna and Kindo, 2018). According to Sullivan and Moran, (2014) and Shagi, (2016), the types of diseases found in animals especially, pets are often the same as those that are found in people. Although not much is known as regards fungal pathogenesis, it involves complex and interplay of many factors (Kobayashi, 1996).

The disease mechanisms of *Fusarium* human/animal pathogens include;

i) Adhesion

Fungal hyphae must adhere to the host surfaces both as a commensal to avoid being washed out of the various niches and during the onset of infections (van Burik and Magee, 2001). Fungal pathogens have the ability to adhere to host cells by way of specialised cell wall glycoproteins.

ii) Entry

A major factor in the pathogenesis of invasive fusariosis involves the disruption of the mucosa or cutaneous barrier of the host cell. Fungi rarely cause disease in healthy, immuno-competent hosts, even though we are constantly exposed to infectious propagules. It is only when fungi accidentally penetrate barriers such as intact skin and mucous membrane linings, or once immunologic deficiencies and other devastating conditions occur in the host, that fungi can

colonize and grow (Kobayashi, 1996). Hence, the ability of fungi to penetrate host cells is crucial for progression of infection in the setting of intact skin or gut barriers (van Burik and Magee, 2001). *Fusarium* from soil or water gains entry into the body making contact with minute breaks in the skin or mucous membranes, causing infections. These sites serve these organisms as cutaneous portals of systemic entry during periods of immunosuppression, allowing for dissemination of infection (Vennewald and Wollina., 2005). Infectious agents may also gain entry into the body because of extensive skin breakdown, such as in burns and wounds, wherein even air-borne conidia may be the source (Łukaszuk and Kułak, 2011) or due to presence of foreign bodies, such as keratitis in contact lens wearers or peritonitis in patients receiving continuous ambulatory peritoneal dialysis (CAPD).

iii) Colonisation, Adaptation and Propagation

In order to effectively colonise the host, these organisms must be able to survive at the elevated body temperature and either evades phagocytosis, counteract the hostility they come across, or adapt in a way that will make them to multiply. A number of factors contribute to infection and pathogenesis of these organisms. Ability to secrete enzymes e.g., keratinase, their ability to grow at 37 °C, dimorphism, and other as yet undefined factors contribute to fungal pathogenesis which involves a complex interplay of many fungal and host factors. Fungi often develop both virulence mechanisms that facilitate their multiplication within the host (Kobayashi, 1996).

iv) Disease development, Dissemination

Propagation of fungi in the body shows a breach or paucity of host defenses. Endocrinopathies or immune conditions may cause such a breach or it may be by iatrogenic induction (Kobayashi, 1996). Effective infection may result in disease, defined as an abnormality or interruption of the normal structure or function of body parts, organs, or systems (or combinations thereof) that is marked by a characteristic set of symptoms and signs and whose etiology, pathology, and prognosis are known or unknown (Kobayashi, 1996).

2.3 *Fusarium* species and associated mycotoxins

The genus *Fusarium* produce a number of mycotoxins of diverse chemical structures. Fusariotoxins are secondary metabolites produced by toxigenic fungi of the genus *Fusarium* (Čonková, *et al.*, 2003). The important and commonly encountered fusariotoxins are trichothecenes, fumonisins and zearalenone. A large and complex fungi family produces these

mycotoxins mainly in the field before harvest with species adapted to a wide range of habitats and, although having a special affinity for moderate climates, they contaminate crops all over the world. This fact makes fusariotoxins probably the most economically significant grain mycotoxins worldwide. *Fusarium* species might endanger human health through the action of their toxic metabolites such as mycotoxins (De Boevre *et al.*, 2013). Members of the genus produce mycotoxins, which have varying toxicities to humans and/ or animals after consumption of contaminated grain and can cause acute or chronic illness and in some cases death. For instance, a range of *Fusarium* mycotoxins can alter the different intestinal defence mechanisms such as the epithelial integrity, cell proliferation, mucus layer, immunoglobulins and cytokine production (Figure 2.8) (Biomin.net, 2015).

These *Fusarium* mycotoxins have attracted worldwide attention because of their impact on human and animal health, animal productivity and the associated economic losses (Gitu, 2006).

2.3.1 Trichothecenes

The trichothecenes are a very large family of structurally related fungal secondary metabolites produced mainly, but not exclusively by *Fusarium* species. (Zhou *et al*., 2008). It is a family of naturally occurring tetracyclic sesquiterpenoids. Also, a class of terpenes, which consists of three isoprene units. Trichothecenes share a common core structure consisting of an olfenic group, an epoxide group and varying numbers of hydroxyl and acetyl groups (Figure 2.3). Depending on their functional groups, they can further be classified into one of four groups (A to D), of which groups A and B are the most toxic (Wu *et al*., 2011b; Shank *et al*., 2011) and are the most important in the context of food (Desjardins, 2006). Type A trichothecenes, mainly include the highly toxic T-2 toxin (T-2), its deactylated form HT-2 toxin (HT-2), diacetoxyscirpenol (DAS) and neosolaniol (NEO) (Thrane *et al.*, 2004; Rocha *et al*., 2005). Type B trichothecenes, deoxynivalenol (DON), nivalenol (NIV), and their acetylated derivatives, 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), as well as fusarenon-X (FUS-X) are of great concern for cereal growing regions worldwide (Jurado *et al*., 2005). Trichothecenes generally, are a global concern as they are found in cereals such as maize, barley, oats and wheat usually consumed by livestock and humans (Erikson and Pettersson, 2004; Wu *et al*., 2011b).They are potent inhibitors of eukaryotic protein synthesis (CAST, 2003), interfering with initiation, elongation and termination stages (Bennett & Klich, 2003). Some of the diseases associated with these toxins in humans and animals, include feed refusal, nausea, vomiting, abortions, weight loss, inflammation of the skin, haemorrhaging of internal organs, blood disorders, immunosuppression and disturbance of the nervous system (Bennett and Klich, 2003; Logrieco *et al.,* 2003; Desjardins, 2006; Kumar *et al.*, 2008).

Emesis, reduced weight gain and other gastro-intestinal disorders are the most sensitive functional manifestations of type B trichothecenes, while immunotoxicity, cytotoxicity and neurotoxicity are caused by type A trichothecenes (van der Fels-Klerx and Stratakou, 2010). For emphasies, the intestinal epithelium is the first target tissue of food contaminants, consequently clinical gastro-intestinal outcomes are in most cases observed (Pinton *et al.*, 2012). Trichothecenes have been shown showed to be toxic to all species. However, sensitivity varied considerably between toxins and species with swine being the most sensitive farm animal. Some of the commonly occurring trichothecenes of *Fusarium* origin will follow.

2.3.1.1 Deoxynivalenol (DON)

Deoxynivalenol (DON), which is referred also as vomitoxin is produced mainly by Fusarium graminearum while in some geographic locations by Fusarium culmorum (Richard, 2000). Deoxynivalenol might co-occur with zearalenone, since both toxins can be produced by same Fusarium specie. Small grains are the main crops affected, for example oats, wheat and barley however DON can also occur in maize (CAST, 2003). Deoxynivalenol has been designated in Group III, that is to say they are not classifiable with regard to their carcinogenicity on humans (IARC, 1993). In dairy cattle, it was linked to reduced milk turn out. In addition, it caused vomiting in swine consuming contaminated feed or feed refusal because the feed was unpalatable. Moreover, reduction in feed intake may lead to serious weight loss which may impede reproductive performance as well as causing immune system disorder in various animal species (Agag, 2005; Pestka and Amuzie, 2008).

Humans are believed to display similar vomiting symptoms when they consume grains that are contaminated with DON (Richard, 2007). Furthermore, increased prevalence of infections of the upper respiratory tract has been described in children who ingested DON contaminated wheat bread for over a week. Then, the illness reduced when ingestion of the bread stopped (Hopton *et al.*, 2010). Deoxynivalenol's toxicity is assumed to occur through modulating the innate immune system (Pestka, 2010). Chronic exposure to low dosage of DON may result in anorexia, reduced weight gain, fluctuation in the production of growth hormone and IgA, while acute exposure to high dosage may induce gastroenteritis, emesis and a shock-like condition (Pestka, 2010). Deoxynivalenol is unlikely to appear as residues in the tissues or fluids of animals exposed to toxic levels, but baking and malting using DON-contaminated wheat and barley can have adverse effects (Richard, 2007).

2.3.1.2. Nivalenol (NIV)

Nivalenol are trichothescence mycotoxins produced by the *Fusarium* fungi, and they can cause problems to pig farmers. Nivalenol-contaminated feed causes feed refusal as the feed was not palatable and could result in vomiting (Agag, 2005). Maize rations for pigs should not contain in excess of 5 %-contaminated kernels. Cattle can resist some effects due to nivalenol contamination but that do not appear to affect chickens. There are no reports published so far on the effects of nivalenol on humans. Nivalenol was designated in Group III, that is to say they are not classifiable with regard to the carcinogenicity on humans (IARC, 1993).

2.3.1.3. T-2 toxins

These toxins are produced by Fusarium fungi on grains that are left unharvested in the farm. They can cause irritation. haemorrhage and also necrosis all the way through the gastrointestinal tract (GIT). T-2 toxins also delay cells regeneration of the bone marrow, spleen and weaken immune system functions as well as cause changes thru the reproductive organs. Infected animals demonstrate signs of losing weight, poor feed utilisation, loss of appetite, as well as vomiting, bloodstained diarrhoea, abortion and in severe cases, leads to death (Agag, 2005). T-2 toxin was the cause of Alimentary Toxic Aleukia (ATA) that killed thousands of people in Russia. Symptoms of Alimentary Toxic Aleukia include fever, nose bleeding and also bleeding from the throat, skin and gums. It also causes necrosis and other than the cytotoxic effects, suppresses the immune system. (Bennett and Klich, 2003).

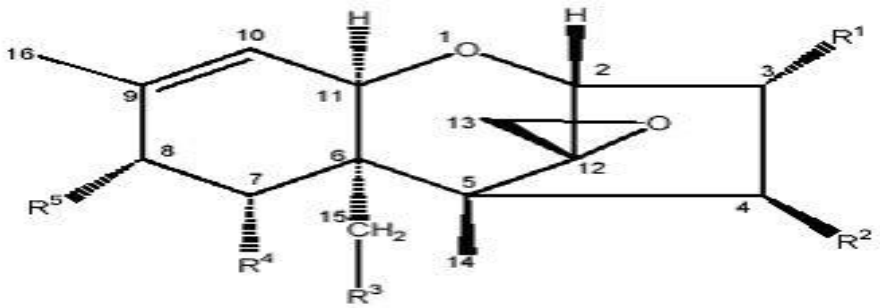


Figure 2.3: Basic structure of trichothecenes and their respective structures

Table 2.2: Trichothecenes and their structures

Trichothecene	R1	R2	R3	R4	R5
			Type A		
HT-2 toxin	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 toxin	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Diacetoxyscirpentriol	OH	OAc	OAc	H	H
			Type B		
Deoxynivalenol	OH	H	OH	OH	O
3-acetyl- deoxynivalenol	OAc	H	OH	OH	O
15-acetyl- deoxynivalenol	OH	H	OAc	OH	O
Nivalenol	OH	OH	OH	OH	O
Fusarenon X	OH	OAc	OH	OH	O

2.3.2 Fumonisins

Fumonisins were firstly discovered in 1988, and they are some of the cytotoxic and carcinogenic mycotoxins (Gelderblom *et al*., 1988). The fumonisin analogues are divided into four types, fumonisin A, B, C, P series. To date, 28 structurally related fumonisin analogues have been described, and fumonisin B (Figure 2.3) are most important and naturally widespread in the contaminated maize. Fumonisin B analogues comprises of toxicologically significant fumonisin B_1, B_2, as well as B_3. These fumonisin Bs occur naturally in abundance and fumonisn B_1 predominates at highest concentration (Marasas, 1996). They are produced by *Fusarium verticillioides* which was formerly known as *Fusarium moniliforme*, a common fungal pathogen of maize. *Aspergillus niger* in the other hand, has now been found to produce fumonisin B_2 (FB_2) (Frisvad *et al*., 2007). FumonisinB_1 is implicated in human oesophageal cancer in the Transkei region of South Africa. This was associated with the ingestion of fumonisin-contaminated maize. Similar observation was also made in China and in North East Italy (Peraica *et al*., 1999; Bennett and Klich, 2003). As stated by Marasas *et al.*, (2004), since FB_1 is capable of reducing folate absorption in different cell lines, it is likely to be connected with neural tube defects in infants. FumonisinB_1 has also been associated with high incidence of neural tube defects in babies of mothers consuming fumonisin-contaminated maize near Texas-Mexico boundary (Missmer *et al.*, 2006). Chronic intake of fumonisin mycotoxins has also been associated with impaired growth in children (Kimanya *et al*., 2010). The IARC designated FB_1 in Group II B as 'possibly carcinogenic to humans' (IARC, 2002). Fumonisin B_1 structurally resembles the sphingoid bases which can explain why the biosynthesis of sphingolipid complexes is inhibited and leads to cell destruction, ensuing cell death (Richard, 2007; Rodrigues *et al*., 2011). Fumonisins are somewhat heat stable, however in heat-processed foods, FB_1 exists in covalently bound forms (Richard, 2007). Nevertheless, not like very well-

known mycotoxins, that can dissolve in organic solvents, fumonisins are water soluble and this makes them a challenge to be studied (Bennett and Klich, 2003).

Then again, although fumonisin has a somewhat simple chemical structure, its blockage of sphingolipid metabolism can have diverse and complex effects on various animal systems (Desjardins, 2006). Fumonisin damages functions of the immune system, liver and kidney, causes weight reductions and increases mortality rates. The most sensitive animals to fumonisin toxicity are horses and levels at the range of 0.2-126 ppm FB_1 were found in feed samples associated with outbreaks of leukoencephalomalacia (LEM) (hole in the head syndrome), which is a necrosis of the brain in North America, South America and South Africa (Wilson *et al.*, 1985; Jackson, 2004). Porcine pulmonary oedema is associated with the consumption of fumonisin-contaminated feed (Harrison *et al.*, 1990; Marasas, 2001). It has also been shown that fumonisin B_1 is hepato carcinogenic to rats. Susceptibility to fumonisins may be higher in dairy cattle than in beef cattle due to major production stress (Sultana and Hanif, 2009).

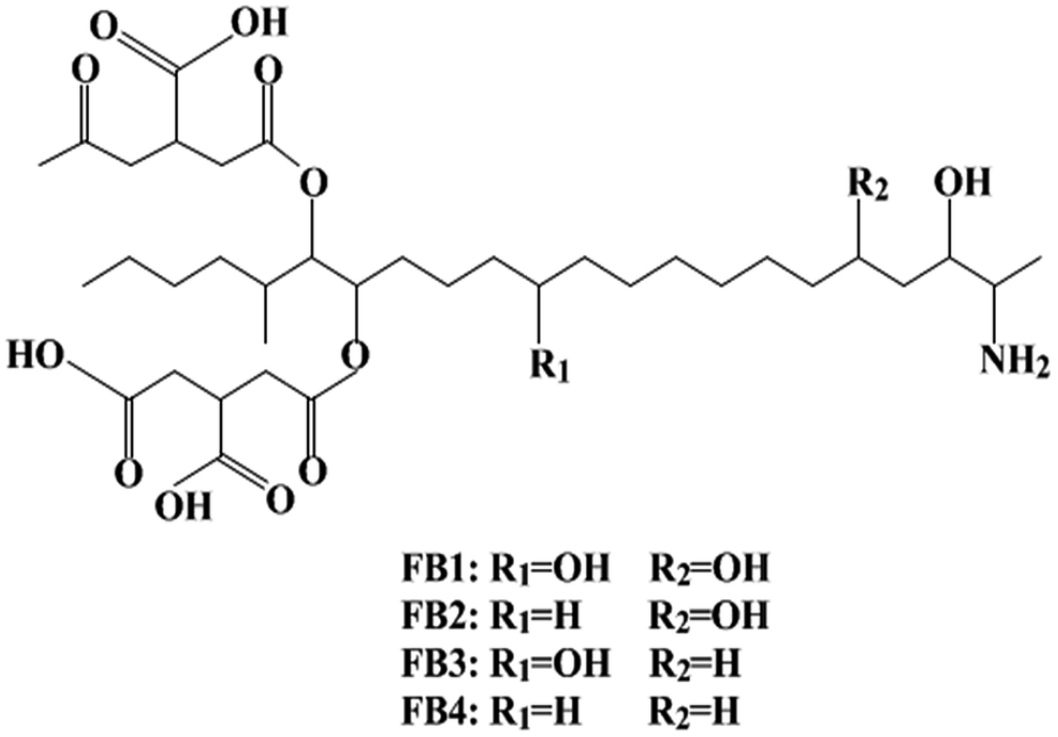


Figure 2.4: Chemical structure of fumonisin B

2.3.3 Zearalenone and its metabolites (mycoestrogens)

Zearalenone (ZON) and its metabolites are termed mycoestrogens, a subgroup of naturally occurring estrogenic compounds. Zearalenone being the principal representative of this group of non-steroidal myco-estrogens is a 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid lactone (Fig.2.4). It is biosynthesized through a polyketide pathway. The keto group at C-8 is reduced to α- and β-isomers in mammalian tissues, and these metabolites can be produced at low concentrations by the fungi. Main production by *F. graminearum*, *F. crookwellense*, *F. sporotrichioides* and *F. culmorum* is described, consequently co-occurrence with DON and other trichothecenes is contingent (Pittet, 1998). In addition, following oral exposure, zearalenone is metabolised in diverse tissues, predominantly in the liver, the main metabolites being α-zearalenol and β-zearalenol (Zinedine *et al*., 2007b)

Zearalenone also known as F-2, commonly contaminates maize but can also occur in other crops worldwide (CAST, 2003; Richard, 2007; EFSA, 2011). Several authors have published on ZON occurrence in foods as well as in animal feeds from various African countries, for example South Africa (Mwanza, 2007), Nigeria (Makun *et al*., 2011). Being classified as endocrine disruptor chemicals, they are usually suspected of reducing fertility in males amongst human and wildlife populations and possibly involved in numerous types of cancer development (Bailly and Guerre 2016). There has been claims that the high rate of premature menarche in Puerto Rico could be as a result of ZON and related compounds in diet (Bennett and Klich, 2003). Due to its high estrogenic action, zearalenone was used for the treatment of post-menopausal symptoms in women. Despite that, research has revealed the potential for ZON to aggravate the development of human breast cancer cells having oestrogen response receptors (Ahamed *et al*., 2001). Nevertheless, the biological potency of this compound is high, but its real toxicity is low (Bennett and Klich, 2003) and was placed in Group III, meaning not classifiable with regard to their carcinogenicity to humans (IARC, 1993). Since ZON seems to bind to oestrogen receptors (Richard, 2007), it has been implicated in a number of mycotoxicoses in farm animals. The consumption of contaminated grains by farm animals can lead to the manifestation of female features in males, early sexual development of young females, infertility in adults, abortion, stillbirth and birth of malformed offsprings. It can also cause atrophy of the testes and weakened libido in males. Cattle might have reddened, inflamed, or swollen vulvas and enlarged nipples, whereas vaginal and rectal prolapse may possibly follow. It also causes reduced feed intake and possibly feed refusal. Its effects are most noticeably seen in pigs, in fact pigs seem to be the most susceptible species to ZON (Richard,

2007; EFSA, 2011). Recommended safe daily human consumption of ZON is estimated to be 0.05 microgram per kilogram body weight (Bennett and Klich, 2003). Less common or emerging *Fusarium* mycotoxins include moniliformin, fusarin C, enniantins, beauvericin, fusaproliferin (section 2.3.4).

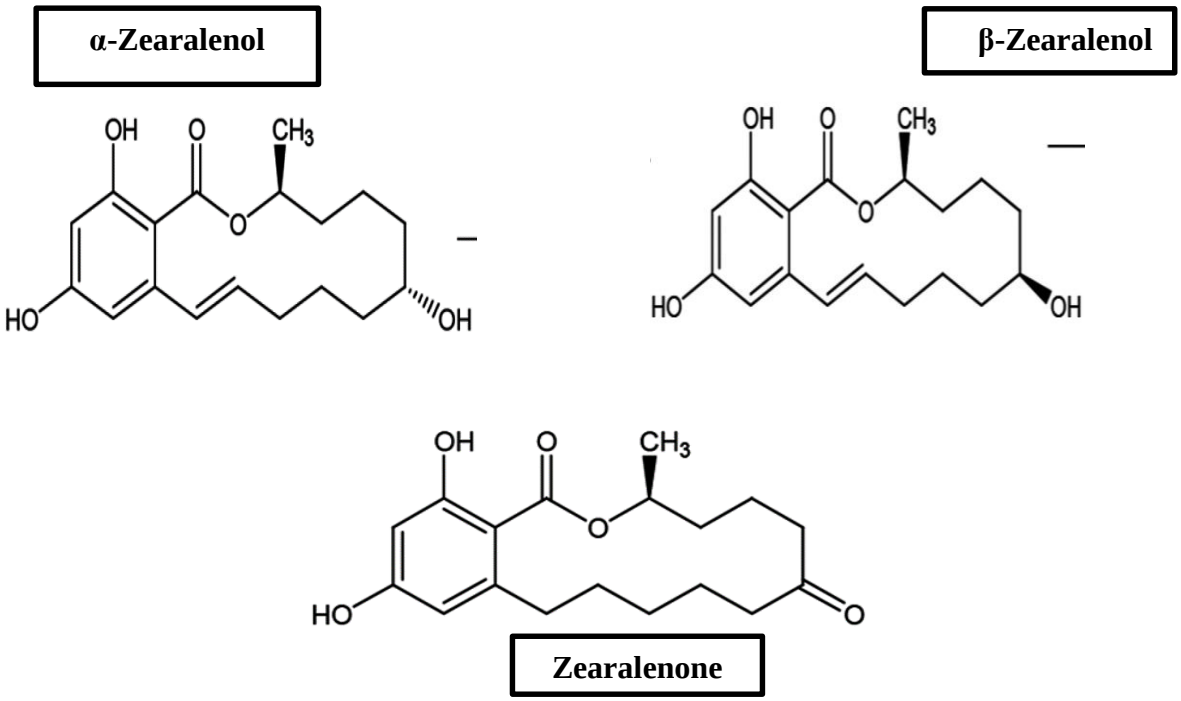


Figure 2.5: Chemical structure of zearalenone, α-zearalenol and β-zearalenol

2.3.4 Emerging *Fusarium* toxins

"Emerging toxins" are a group of chemically different mycotoxins for which no regulations exist even up to the present. Current studies using cutting-edge LC–MS/MS for structure elucidation, offer insights regarding newly discovered metabolites as do plant breeding efforts adjusting to climate change (Krska, 2015). Risk assessment studies are ongoing in preparation for legislation when considered decisive (Vaclavikova *et al*., 2013).

Normally referred to in this group are moniliformin, fusarin C, beauvericin, enniantins, fusaproliferin

2.3.4.1 Moniliformin

Moniliformin is known to be a worldwide natural contaminant of cereals such as rice, wheat, oats, barley, rye and maize (Sanhueza and Degrossi, 2004). It is produced by different species of *Fusarium* mostly by *Fusarium proliferatum* (Zain, 2011). Its natural occurrence was firstly reported in 1982 from mouldy maize obtained from the Transkei region of South Africa at levels ranging from 16-25ppm (Thiel *et al*., 1982). Moniliformin usually co-occur with other *fusarium* mycotoxins such as FB_1, ZON, trichothescenes, fusarin C, and beauvericin. In humans, MON consumption has been associated with some disease outbreaks for example, the case of Keshan disease, which is a myocardia disease that occurred in the rural areas of China and South Africa (Bottalico, 1998).

2.3.4.2 Enniatins

Enniatins have antimicrobial, insecticide and antifungal properties and as well might also have herbicide properties. Their mechanism of action is in the direction of cellular membrane transport proteins which are inhibited by the toxin. The toxicity of enniatins is in particular profound towards mitochondria. These organelles are vital constituents of living beings, are responsible for respiration in the cell, producing most of the adenosine triphosphate (ATP) necessary for energy transfer. Enniatins do seem to be efficiently degraded in animal intestinal systems but more research is necessary (Biomin.net, 2016).

2.3.4.3 Beauvericin

Beauvericin shows strong antimicrobial activity towards a broad spectrum of bacteria, with no distinction between Gram-positive and Gram-negative. This toxin also shows cytotoxic, apoptotic and immunosuppressive activity. Beauvericin acts on the cellular membranes increasing the permeability and disrupting the cellular homeostasis. Although moniliformin shows a relatively lower toxicity compared to enniatins and beauvericin, it has been reported to be toxic towards lymphocytes, skeletomyocytes and cardiomyocytes, with birds and minks being the most sensitive species. The mechanism of action has not been fully elucidated yet, but toxicity towards mitochondria, with a mechanism similar to enniatins, is suspected (Biomin.net, 2016).

2.3.4.4 Fusaproliferin

Fusaproliferin has demonstrated toxicity towards human B-lymphocytes and some insect cell lines. This emerging mycotoxin also showed teratogenic and pathogenic effects on chicken embryos. In recent years, some studies were conducted using brine shrimp (Artemia salina) as a model organism. The toxin often co-occurs with deacetylfusaproliferin, although the toxicity of the latter is much lower when compared to fusaproliferin (Biomin.net, 2016).

Studies on the synergistic effects between the two toxins have not be reported so far (Biomin.net, 2016).

2.3.5 Masked Mycotoxins

2.3.5.1 Introduction

In the past decades, it has become clear that in mycotoxin-contaminated commodities, many structurally related compounds generated by plant metabolism, fungi or food processing coexist with their parent mycotoxins. In the mid 1980s, the topic of masked mycotoxins received attention because in some cases of mycotoxicosis clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed (Gareis *et al.*, 1990). The unexpected high toxicity has been attributed to undetected conjugated forms of mycotoxins that possibly were hydrolysed into the parent toxins in the digestive tract of animals.

Young *et al.* (1984) showed that the DON content of yeast-fermented food products was higher than that of the contaminated flour used for their production, so it has been speculated that a DON conjugate of some form arising from plant metabolism might exist. Savard (1991) was the first to chemically synthesize glucose and fatty-acid conjugates of DON, while Sewald *et al*. (1992) could identify deoxynivalenol-3-glucoside (DON-3G) as a DON metabolite in maize cell suspension cultures. The glycosylated form of ZON, zearalenone-14-glucoside (ZON-14G) is certainly the best studied conjugated form. It was originally found as a fungal metabolite (Kamimura, 1986) but Engelhardt *et al.* (1988) reported that ZON was also transformed to ZON-14G by plant cultures. Gareis *et al.* (1990) hypothesized that ZON-14G was cleft during digestion in swine, releasing the eostrogenic aglucone, ZON. Sulphate and glucuronide conjugates of ZON were shown to occur in urine of ZON-fed animals (Mirocha, 1981), although their identity could not be confirmed. Zearalenone-14-sulfate (ZON-14S) was isolated by Plasencia and Mirocha (1991) as a fungal metabolite from *Fusarium*.

Two types of masked mycotoxins can be distinguished: extractable, conjugated and non-extractable, bound varieties. Extractable, conjugated mycotoxins can be detected by appropriate analytical methods when their structure is known and analytical standards are available. Non-extractable, bound mycotoxins are covalently or non-covalently attached to polymeric carbohydrate or protein matrices. Bound mycotoxins are not directly accessible and have to be liberated from the matrix by chemical or enzymatic treatment prior to chemical analysis.

2.3.5.2 Formation and occurrence of masked mycotoxins

Mycotoxins can be subjected to biological modification through conjugation by plants or through chemical modification, either thermally or non-thermally e.g.by food processing. As indiceted earlier, these modified mycotoxins can contribute to the degree of contamination and may escape detection methods, causing an underestimation of the mycotoxin exposure and risk (Broekaert *et al*., 2015). Different mechanisms include:

i) Plant conjugates

It has been shown that analogously to animals, plants as living organisms, have the capability to defend themselves against the potentially toxic effects of xenobiotic compounds including mycotoxins. The defence mechanisms of plants include three phases of biosynthetic pathways; phase I is the transformation or activation phase, phase II is the solubilisation or conjugation phase while phase III is known as compartmentalization phase (Figure 2.6) (Broekaert *et al*., 2015).

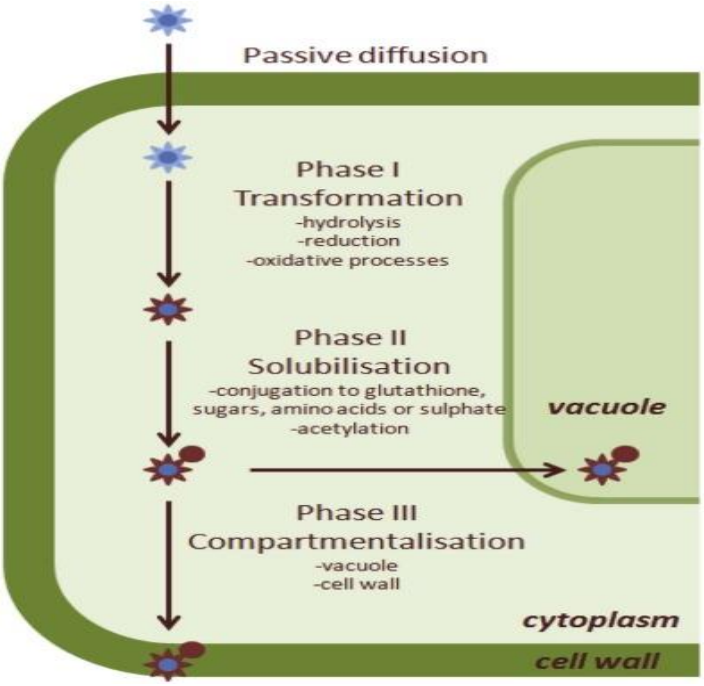


Figure 2.6: Stages of plant biosynthesis pathway: transformation, solubilisation and compartmentalisation. Adapted from Coleman *et al.*, (1997).

After the infection of crop plants, mycotoxins are modified or transformed by introducing reactive groups into the toxic molecules, using plant enzyme (esterases, amidases and the cytochrome P-450 system). The biotransformed molecules are then conjugated to more polar substances (often with sugars, amino acids or sulphates), by the help of plant enzymes i.e. glucosyltransferases attaching a glucose molecule to the toxin and increasing its water solubility. Conjugated forms are compartmentalized in specific organelles (e.g. vacuoles, chloroplasts) or in extracytoplasmic space (e.g. cell wall) of plant cells (Conn, 1985; Sandermann, 1992; Engelhardt *et al*., 1999; Bryla *et al*., 2018). These conjugated mycotoxins although, unable to exert harmful effects to the plant, because they have been compartmentalized, the residues may persist for considerable periods of time in plants and can have important toxicological consequences for their consumers.

In planta transformation of mycotoxins has been predominantly described for *Fusarium* toxins (Diana di Mavungu, 2011). And since *Fusarium* infection normally occurs in the field, the *Fusarium* mycotoxins e.g. (deoxynivalenol, (DON), zearalenone, (ZON), fumonisins, (FUM), nivalenol, (NIV), fusarenon-X, (FUS-X), T-2 toxin, HT-2 toxin, fusaric acid) are the utmost target for conjugation (Berthiller *et al*., 2013). Metabolism by the host plant generally aims at detoxification of these compounds, which is often accomplished by attachment of hydrophilic

groups, thus increasing their solubility in water (Engelhardt *et al*., 1999; Karlovsky 1999). In this manner, for example, DON is converted to DON-3-glucoside (D3G) (Sewald *et al*., 1992; Berthiller *et al.*, 2005a) Figure 2.7, while ZON can be altered to ZON-4-glucoside (Z4G) (Engelhardt *et al*., 1988) by plants. Also, Nakagawa *et al*. (2011) reported glucoside forms of other trichothecenes such as and nivalenol (NIV) in artificially contaminated wheat.

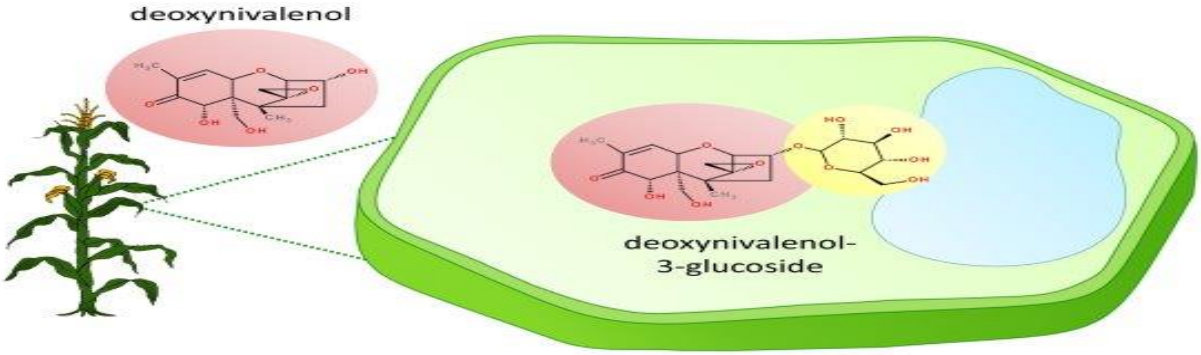


Figure 2.7: How plants metabolise free mycotoxins to form masked mycotoxins e.g. DON forms DON-3-glucoside. Adapted from (Berthiller, 2016).

ii) Food processing conjugates

Besides plant metabolism, processing of food commodities is another source of mycotoxin conjugate formation. The (thermo) stability of the *Fusarium* mycotoxins, trichothecenes allows them to withstand most food and feed processes (Malachova *et al*., 2011). The technological process also has an important role in the masking process, particularly in cereal derived products. Indeed, mechanical or thermal energy during the transformation process may cause significant modification, for instance the induction of reactions with macromolecular components such as polysaccharides, proteins or lipids, or the release of the native toxins through decomposition of the masked derivatives. In the case of fumonisins, the phenomenon has been described as 'the fumonisin paradox' because apparently low contaminated commodities have also been found to induce toxic effects. This highlights the problem of 'bound' or 'hidden' fumonisins, which may be released upon alkaline hydrolysis (Dall'Asta *et al*., 2009a; Galaverna *et al*., 2009). The effect of food being processed thermally on chemical structure as well as on the toxicity of fumonisins has been extensively reviewed (Humpf and Voss, 2004). A major reaction occurring in heat-treated food involves fumonisin B_1 (FB_1) and reducing sugars to form N-carboxymethyl-FB_1 (Howard *et al*., 1998) and N-1-deoxy-D-fructos-1-yl-FB_1 (Poling *et al*., 2002). The occurrence of these fumonisin derivatives in corn

products, has been described (Seefelder, *et al*., 2001). On the other hand, low recoveries of FB_1 have been observed in different matrices such as rice flour, cornstarch, cornmeal and glucose after thermal treatment (Kim *et al*., 2002). It is believed that FB_1 interacted with food macro constituents such as protein or starch. In order to yield evidence that FB_1 might bind to matrix components in thermally treated food, model experiments have been were performed (Kim *et al*., 2003; Park *et al*., 2004; Seefelder *et al*., 2003). Results indicated that fumonisins can bind to polysaccharides and proteins via their two tricarboxylic acid groups and that binding to starch occurs to a greater extent than in proteins. The occurrence of protein-bound fumonisins in commercial corn flakes has also been shown (Kim *et al*., 2003; Park *et al*., 2004; Dall'Asta *et al*., 2008).

Similarly, microorganisms used in the fermentation process (introduction of yeast) or a malting process may transform mycotoxins into products that are also not detected by analytical methods. These derivatives are formed from enzymatic activities of microbial cultures used for fermentation, such as in manufacturing beer, wine, fermented sausages or mixed pickles. Another food commodity frequently contaminated with modified mycotoxins is bread. During the processing of wheat to bread, it has been described that milling had little influence on the ratio of DON3G to DON. Due to fractionation, milling decreased the DON3G and DON content in white flour compared to initial unprocessed wheat (Kostelanska *et al.*, 2011). These findings are supported by a previous study in which the fractionation of *Fusarium* mycotoxins during dry milling of maize was investigated. It was observed that bran, the hard outer layers of cereal grain which are discarded for the production of white flour, contained the highest concentrations for all tested mycotoxins (Schollenberger *et al*., 2008). During kneading, fermenting and proofing, no significant changes occurred for DON, DON3G and 3ADON. However, when bakery improvers, such as enzyme mixtures, were employed as a dough ingredient, a distinct increase in DON3G was noticed in fermented dough (Lancova *et al*., 2008a; Kostelanska *et al.*, 2011). It is assumed that this increase in DON3G is due to a release from starch-based, matrix-bounded forms. The effect of food-processing on T2-G, HT2-G and FUS-X has also been investigated although, to a lesser extent (Lancova *et al*.,2008a; De Angelis *et al*., 2014). Then for α-ZOL, β-ZOL, ZON14G, α-ZOL4G, β-ZOL4G and ZON14, reported data regarding their fate during food processing are available (Broekaert *et al*., 2015).

2.3.5.3 Health effects of masked mycotoxins

i) Zearalenone and its modified derivatives

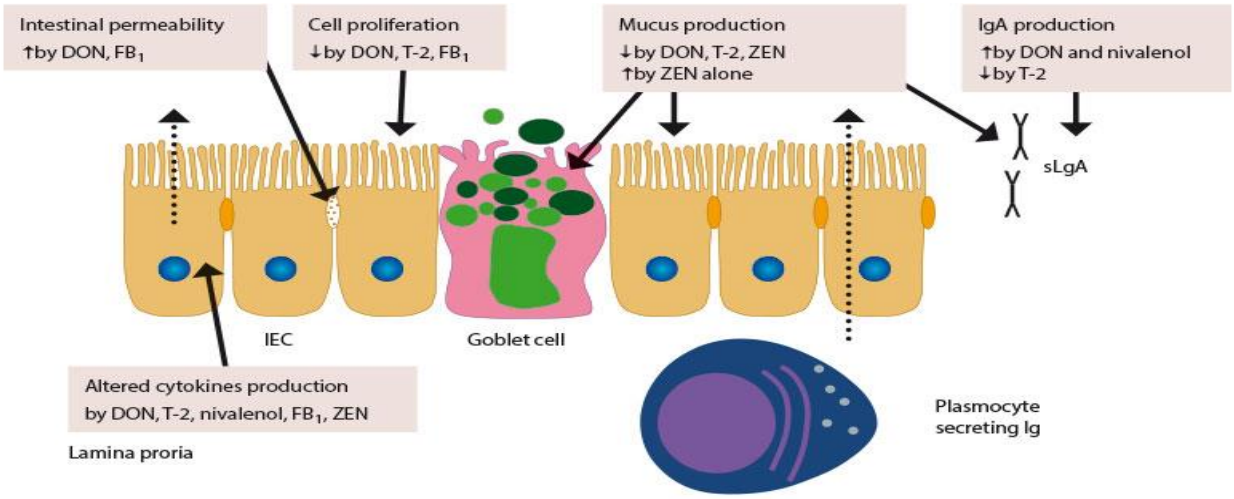
The toxicity of the zearalenone-modified forms,α- and β-ZOL has been widely described, with the majority of the studies focusing on oestrogenicity and reproductive disorders. Despite the pronounced oestrogenic effects of ZON and its metabolites, there have also been reports of their cytotoxicity. For example, a study on cell viability was carried out using Caco-2 cell lines in MTT assay after 48 h incubation with ZON, α-ZOL, or β-ZOL, as well as the oxidative stress induction by measuring malondialdehyde generation. Result showed that both metabolites demonstrated a decreased toxicity compared to ZON, which can be ranked as follows: α-ZOL < β-ZOL < ZON. The measurement of DNA-lesions due to oxidative stress also resulted in similar relative ranking of the toxicity (Abid-Essefi *et al*., 2009).

ii) Deoxynivalenol (DON) and its modified derivatives

Toxicity of DON is mainly by inhibiting protein synthesis and it is known for its irritation of the gastrointestinal (GI) tract (Figure 2.8). In fact, it can be argued that most mycotoxins do not cross the intestinal barrier; so, not all mycotoxins will cause mycotoxicoses. Still, the first cells to be exposed to mycotoxins are the gastrointestinal cells and at greater concentrations than in other tissues. Therefore, non-absorbed toxins can affect the intestinal epithelium and its whole extension before absorption can even begin. Hence, a provisional tolerable dietary intake (TDI) for DON was set at one μg/kg body weight (Scientific Committee on Food, 2002).

Generally, the majority of the modified mycotoxin toxicity research is dedicated to human in vitro toxicity while less has been done using animals and plants. Several studies have been carried out to investigate the toxicity of DON and its modified derivatives using human cell lines. For example, Alassane-Kpembi *et al*., (2015) investigated the influence of DON, 3ADON and15ADON on human epithelial colorectal adenocarcinoma cell line (Caco-2) and the induced cytotoxicity was measured by means of MTT assay and found out that 15ADON was more toxic than DON and 3ADON, as it exhibited higher inhibition of epithelial cell viability. Another study by Kadota *et al*. compared the toxicity of these compounds by measuring their effect on interleukin-8 (IL-8) secretion and intestinal transport in Caco-2 cells. A significantly higher absorption of 15ADON compared to DON and 3ADON was demonstrated and 15ADON had a more profound effect on intestinal integrity, facilitating

absorption through passive diffusion. Furthermore, the production of IL-8 was the lowest after 3ADON exposure, followed by DON and 15ADON respectively (Kadota *et al*., 2013).



(IEC-intestinal epithelia cell, Ig-immunoglobulins, sIgA- Segregated immunoglobulin A)

Figure 2.8: The effect of *Fusarium* mycotoxins on the intestinal epithelium. Adapted from (Biomin.net, 2015).

Generally, one can possibly argue that metabolism of *Fusarium* mycotoxins by the plant are a detoxification pathway usually resulting in less toxic or inactive compounds. Normally, this should cause an increased water solubility of mycotoxins, facilitating their excretion, and hence, a decreased toxicity (Yiannikouris and Jouany, 2002). Contrary to this, most recent studies have shown that most masked mycotoxins are not functionally uniform and that their toxicity greatly depends on the chemical properties of the mycotoxin in question and the exposure (Dellafiora *et al*., 2017). However, many researchers have carried out studies on the occurrence of free and masked *Fusarium* mycotoxins in cereal-based food and feed commodities (Table 2.2).

Table 2.3: Occurrence of free and masked *Fusarium* mycotoxins in cereal-based food and feed commodities.

Mycotoxin	Food/feed commodity	% positive Samples	Range(ug/kg)	References
ZON	Maize	83	59-1071	(DeBoevre *et al.*, 2012)
	Maize	-	<LOQ-15,700	(DeBoevre *et al.*, 2014)
	Maize	-	56	(Ngoko *et al*., 2008)
	Maize	37%	<50-196	(Afolabi *et al.*, 2013)
	Maize	90	0-135	(Chilaka *et al*., 2012a)
	Maize	-	14	(Zinedine *et al*., 2006)
	Maize	-	10.2	(Mohale *et al*., 2013)
	Maize	92.5	247.8	(Mwanza *et al.*, 2013)
	Maize silage(feed)	79.4	20-11	(Kovalsky *et al*., 2016)
	Barley	5.9	<LOQ-17	(Nathanail *et al.*, 2015)
	Barley	67	2-31	(Bryła *et al*., 2016)
	Beans	90	185.2	(Mwanza *et al*., 2013)
	Wheat	46.7	<LOQ-234	(Nathanail *et al.*, 2015)
	Wheat	83.3	12-109	(DeBoevre *et al.*, 2012)
	Wheat	47.5	1-100	(Bryła *et al*., 2016)
	Peanut	57	70	(Njobeh *et al.*, 2010)
	Oat	41.9	<LOQ-675	(Wu *et al*., 2017)
	Oat	66.7	13-85	(DeBoevre *et al.*, 2012)
	Oat	100	5-15	(Bryła *et al*., 2016)
	Cocoa seeds	23.5	24.2-83.6	(Egbuta *et al*., 2013)
	Bread	83.3	19-53	(DeBoevre *et al.*, 2012)
	Breakfast cereals	-	4	(Mashinini and Dutton, 2006)
	Corn flakes	83.3	34-90	(DeBoevre *et al.*, 2012)
	Malt	56	102-2213	(Nkwe *et al.*, 2005)
	Traditional brewed beer	45	2.6-426	(Odhav and Naicker, 2002)
ZON-14G	Maize	17	274	(DeBoevre *et al.*, 2012)
	Maize	-	<LOQ-9750(total)	(DeBoevre *et al.*, 2012)
	Barley	17.6	<LOQ-9.6	(Nathanail *et al.*, 2015)

	Wheat	6.7	<LOQ-0.6	(Nathanail *et al.*, 2015)
	Wheat	0	-	(DeBoevre *et al.*, 2012)
	Oats	3.2	<LOQ	(Nathanail *et al.*, 2015)
	Oats	0	-	(DeBoevre *et al.*, 2012)
	Bread	33.3	20-20	(DeBoevre *et al.*, 2012)
	Corn flakes	0	-	(DeBoevre *et al.*, 2012)
ZON-16G	Barley	23.5	<LOQ	(Nathanail *et al.*, 2015)
	Wheat	6.7	<LOQ-2.8	(Nathanail *et al.*, 2015)
	Oat	58.1	<LOQ-7.9	(Nathanail *et al.*, 2015)
ZON-14S	Maize	17	51	(DeBoevre *et al.*, 2012)
	Maize	-	<LOQ-9750 (total)	(DeBoevre *et al.*, 2014)
	Maize silage(feed)	42.3	2-4318	(Kovalsky *et al*., 2016)
	Barley	8.8	<LOQ-26.1	(Nathanail *et al.*, 2015)
	Wheat	33.3	11	(DeBoevre *et al.*, 2012)
	Wheat	40.0	<LOQ-22.5	(Nathanail *et al.*, 2015)
	Oats	16.7	12	(DeBoevre *et al.*, 2012)
	Oats	29.0	<LOQ-220	(Nathanail *et al.*, 2015)
	Bread	16.7	24	(DeBoevre *et al.*, 2012)
	Corn flakes	0	-	(DeBoevre *et al.*, 2012)
DON	Maize	100	255-5245	(DeBoevre *et al.*, 2012)
	Maize	100	90-680	(Berthiller *et al*., 2005a)
	Maize	100	32-2246	(Rasmussen *et al*., 2012)
	Maize	90	74-1382	(Zhao *et al*., 2014)
	Maize	22	9.6-745.1	(Adejumo *et al.*, 2007)
	Maize	100	1,469	(Mohale *et al*., 2013)
	Maize	100	0.3-4374	(Rasmussen *et al*., 2012)
	Maize	-	380	(Ngoko *et al.*, 2008)
	Maize silage (feed)	71.8	1.5-13,488	(Kovalsky *et al*., 2016)
	Barley	82.4	LOQ-1180	(Nathanail *et al.*, 2015)
	Barley	100	<60	(Rasmussen *et al*., 2012)
	Barley	83	54-1602	(Bryła *et al*., 2016)
	Whole wheat	-	8000	(Mashinini and Dutton, 2006)

	Wheat	83	11-1265	(Bryła *et al*., 2018)
	Wheat	97.6	LOQ-5510	(Nathanail *et al.*, 2015)
	Wheat	66.7	16-150	(DeBoevre *et al.*, 2012)
	Wheat	100	540-5080	(Berthiller *et al*., 2005a)
	Wheat	100	46-2683	(Rasmussen *et al*., 2012)
	Wheat	46.5	25-2975	(Bryła *et al*., 2016)
	Wheat	46.1	LOQ-297	(Trombete *et al.*, 2016)
	Wheat	75	LOQ-10,130	(Jin *et al*., 2018)
	Wheat	-	40-490	(Ayalew *et al*., 2006)
	Wheat	68	up to 302	(Muthomi *et al*., 2008)
	Rice	23.8	107.9	(Makun *et al*., 2011)
	Durum wheat	100	1750	(Jin *et al*., 2018)
	Rye	100	<50	(Rasmussen *et al*., 2012)
	Oat	100	2690	(Nathanail *et al.*, 2015)
	Oat	16.7	46	(DeBoevre *et al.*, 2012)
	Oat	81.8	62-2216	(Rasmussen *et al*., 2012)
	Oat	100	67-149	(Bryła *et al*., 2016)
	Bread	66.7	20-102	(DeBoevre *et al.*, 2012)
	Corn flakes	16.7	207	(DeBoevre *et al.*, 2012)
	Breakfast cereals	28.6	28.6	(Malachova *et al.*, 2011)
	Snacks	61.8	61.8	(Malachova *et al.*, 2011)
	Flours	72.3	72.3	(Malachova *et al.*, 2011)
	Wheat flour	97.2	1.3-825.9	(Li *et al*., 2016)
	Wheat flour	100	78.9-325.8	(Trombete *et al.*, 2016)
	Swine feed	93.8	50-931	(Zhao *et al*., 2014)
	Poultry feed	93.3	157-1231	(Zhao *et al*., 2014)
	Feed	99	124-2352	(Njobeh *et al.*, 2012)
	Lager beer	100	1.6-6.4	(Mbugua and Gathumbi, 2004)
	Beer	90	LOQ-35.9	(Zachariasova *et al.*, 2008)
DON-3G	Maize	100	36-1003	(DeBoevre *et al.*, 2012)
	Maize	67	<LOQ-70	(Berthiller *et al*., 2005a)
	Maize	100	<35	(Rasmussen *et al*., 2012)

	Maize	80	14-121	(Zhao *et al*., 2014)
	Maize	-	<LOQ-1100	(DeBoevre *et al.*, 2014)
	Maize silage(feed)	63.0	1.0-3159	(Kovalsky *et al*., 2016)
	Barley	73.5	<LOQ-1300	(Nathanail *et al.*, 2015)
	Barley	0	-	(Rasmussen *et al*., 2012)
	Barley	29	43-277	(Bryła *et al*., 2016)
	Wheat	83.3	<LOQ-922	(Nathanail *et al.*, 2015)
	Wheat	16.7	18	(DeBoevre *et al.*, 2012)
	Wheat	100	59-200	(Berthiller *et al*., 2005a)
	Wheat	83.3	43-737	(Rasmussen *et al*., 2012)
	Wheat	27.3	40-356	(Bryła *et al*., 2016)
	Wheat	27	16-138	(Bryła *et al*., 2018)
	Wheat	31	<LOQ	(Trombete *et al.*, 2016)
	Wheat	75	100-1230	(Jin *et al*., 2018)
	Durum wheat	94	LOQ-850	(Palacios *et al*., 2017)
	Rye	0	-	(Rasmussen *et al*., 2012)
	Oat	87.1	<LOQ-6600	(Nathanail *et al.*, 2015)
	Oat	100	28-97	(DeBoevre *et al.*, 2012)
	Oat	45.5	162-287	(Rasmussen *et al*., 2012)
	Oat	0	-	(Bryła *et al*., 2016)
	Bread	83.3	26-29	(DeBoevre *et al.*, 2012)
	Corn flakes	50.0	24-28	(DeBoevre *et al.*, 2012)
	Breakfast cereals	85.7	19-66	(Malachova *et al*., 2011)
	Snacks	82.4	11-94	(Malachova *et al*., 2011)
	Flours	68.2	5-72	(Malachova *et al*., 2011)
	Wheat flour	33.4	0.2-15.7	(Li *et al*., 2016)
	Swine feed	93.8	6-80	(Zhao *et al*., 2014)
	Poultry feed	93.3	30-107	(Zhao *et al*., 2014)
	Beer	95	LOQ-27.5	(Zachariasova *et al.*, 2008)
NIV	Barley	73.5	<LOQ-302	(Nathanail *et al.*, 2015)
	Wheat	43.3	<LOQ-74.0	(Nathanail *et al.*, 2015)
	Oat	71.0	<LOQ-4940	(Nathanail *et al.*, 2015)

	Rice	-	97	(Serrano *et al*., 2012)
	Wheat flour	100	LOQ-140.6	(Trombete *et al.*, 2016)
NIV-3G	Barley	61.8	<LOQ- 65.3	(Nathanail *et al.*, 2015)
	Wheat	43.3	<LOQ-33.6	(Nathanail *et al.*, 2015)
	Oat	16.1	<LOQ-58.3	(Nathanail *et al.*, 2015)
HT-2	Barley	35.3	<LOQ-39.5	(Nathanail *et al.*, 2015)
	Barley	100	23-233	(Veprikova *et al*., 2012)
	Barley	100	3-213	(Lattanzio *et al*., 2015)
	Wheat	100	19-96	(Veprikova *et al*., 2012)
	Wheat	63.3	<LOQ-39.5	(Nathanail *et al.*, 2015)
	Wheat	77.8	26-85	(Lattanzio *et al.*,2012)
	Oat	100	11-187	(Veprikova *et al*., 2012)
	Oat	74.2	<LOQ-1830	(Nathanail *et al.*, 2015)
	Oat	88.9	21-851	(Lattanzio *et al.*,2012)
HT-2G	Barley	52.9	<LOQ-38.5	(Nathanail *et al.*, 2015)
	Barley	94.4	0.6-162.8	(Lattanzio *et al*., 2015)
	Wheat	53.3	<LOQ-15.9	(Nathanail *et al.*, 2015)
	Oat	16.1	<LOQ-300	(Nathanail *et al.*, 2015)
T-2	Barley	100	41-160	(Veprikova *et al*., 2012)
	Barley	100	1-154	(Lattanzio *et al*., 2015)
	Wheat	100	17-76	(Veprikova *et al*., 2012)
	Wheat	55.6	11-23	(Lattanzio *et al.*,2012)
	Oat	100	31-165	(Veprikova *et al*., 2012)
	Oat	88.9	10-377	(Lattanzio *et al.*,2012)
	Maize	36	7.5-29	(Afolabi *et al*., 2013)
T-2G	Barley	50	0.1-14.5	(Lattanzio *et al*., 2015)

Data in Table 2.3 describes only glucoside and sulphate conjugates (masked mycotoxins) and their respective free forms identified in cereal grains and food/feed products.

2.4 Mycotoxin research in South Africa

South Africa has a long and reputable history of mycotoxins research. Over 100 mycotoxins have been documented, which makes South Africa a world leader in mycotoxins research (Gelderblom *et al*., 1988; Dutton, 2003). The research has primarily focused on detection of different mycotoxins in food and feed commodities. Another important focus area was determining the effects of these mycotoxins on animal and human health (Dutton, 2003). Previous studies included work on such mycotoxins as the fumonisins and aflatoxins (Marasas *et al*., 1979). These toxins are highly toxic and carcinogenic to farm and laboratory animals and have been associated with human oesophageal cancer and birth defects (Leslie and Summerell, 2006). Due to the predominance of many chronic diseases of humans and animals in South Africa, research began to focus on the interaction between such diseases and the consumption of mycotoxin-contaminated foods and feed (Marasas *et al*., 1979; Marasas *et al*., 1981; Marasas, 2001; Dutton, 2003). This triggered suggestions of a possible link between the consumption of *Fusarium*-contaminated maize and/or home-brewed beer made from highly infected grains (and in particular fumonisins contaminated grain) and oesophageal cancer in rural Eastern Cape Province of South Africa. Following such findings, there were more publications as the fumonisins were frequently found in contaminated maize based meals and beverages consumed by residents of this area (Gqaleni *et al*., 1997).

By the onset of the 1980's, the South African Maize Board had begun annual mycological surveys of commercial maize. Research was able to reveal that the mycotoxin concern in South African cereal grain was similar to that of other countries. Despite that, most of the research concentrated on the parent mycotoxins, whereas very limited data are available on the occurrence and toxicity of mycotoxin metabolites known as 'masked mycotoxins' in food and feed.

2.5 Maize (Zea mays L.) production in South Africa

South Africa is suitable for growing a large assortment of crops. The primary crops cultivated in South Africa include maize, wheat, soybeans, sorghum and sugarcane. Oats, groundnut, sunflowers, tobacco and dry beans are some of the minor crops cultivated in the country (Gbetibouo and Hassan, 2004). In terms of production, maize is the third most important crop grown worldwide and is an important component of the diet of millions of people due to relatively good yields, easy to cultivate, able to adapt to different agro-climatic areas, versatile

food uses and storage characteristics (Fandohan *et al*., 2003). World production covers an area of 110 million ha that yields approximately 230 million tons of grain per annum. More than half of this is produced in the USA, i.e. 144 million tons on an area of 25 million ha with a market value of approximately US $20 trillion. The American economy (as in the South Africa) is highly dependent on maize production (Du Toit, 1999). Approximately 8 million tons of maize grain is produced in South Africa annually on approximately 3.1 million ha. of land under diverse environments. Half of the production consists of white maize, for human food consumption (FAOSTAT data, 2009). In developed countries, maize is consumed mainly as a second-cycle product, in the form of meat, eggs and dairy products. In developing countries, maize is consumed directly as food and is processed into different food products. Besides, it also serves as a staple diet for some 200 million people. In South Africa, most people regard maize as a breakfast cereal, cornflakes, grits and snacks. However, in a processed form it is also found as fuel (ethanol), starch, oil (corn oil), corn syrup and maize gluten (Sydenham *et al.,* 1991). Starch in turn involves enzymatic conversion into products such as sorbitol, dextrine, sorbic and lactic acid. It is also a potential raw material for the manufacture of medicines, chemicals as well as biofuel (Abassian, 2006). Starch also appears in household items such as beer, ice cream, syrup, shoe polish, glue, fireworks, ink, batteries, mustard, cosmetics, aspirin and paint (Du Toit, 1999). Maize is one of the highest energy grains and its use as animal feed is because it can supply energy primarily as digestible carbohydrate plus a small amount of energy as natural oil. It can produce more energy per unit of land than any other cereal because it has a carbon-4 photosynthetic pathway that utilises solar energy more efficiently than carbon-3 plants (Kellems and Church, 2010). Maize has the highest metabolisable energy (ME) in Mcal/Kg [89 % dry matter (DM)] compared to all other grains with values that range from 3.03 and 3.38 for swine and poultry respectively. The main energy yielding fraction is the starchy endosperm consisting of amylose (about 23 %) and the remaining 77 % of starch is amylopectin (Kellems and Church, 2010)

Maize is a warm weather crop and is not grown in areas where mean daily temperature is below 19 °C. Although the minimum temperature requirement for germination is 10 °C, germination and emergence will be faster and less variable at soil temperatures of 16 to 18 °C. Development of maize early in the season increases linearly with an increase in soil temperature from 15 to 17 °C. Exceptionally high temperatures and low humidity during flowering have an adverse effect on pollination and fertilization, resulting in poor seed set. The critical supra-maximal temperature affecting yield is approximately 32 °C. Frost can damage maize at all growth

stages and a frost-free period of 120 to 140 days is required to prevent damage. Leaves of mature plants are easily frosted and grain fill can be adversely affected (Du Toit, 1999).

2.6 The climate of South Africa

South Africa has a warm and dry climate and is situated in a subtropical high-pressure zone, predominated by dry descending winds. Rainfall patterns in South Africa are not uniformly distributed, with an average annual rainfall being less than 500 mm, which is about 60 % of the world average (Preston-Whyte and Tyson, 1993; Durand, 2006; DoA, 2007; Ziervogel *et al.*, 2010). Rainfall patterns are extremely variable and sometimes in short supply (Cook *et al.*, 2004).

The study was conducted in two climatically and geographical distinct maize production areas in South Africa; namely, western Region which is situated in the drier and warmer areas and the eastern Region which is situated in the higher rainfall and cooler areas.

The main maize producing provinces (North west, Free State and Gauteng) falls within two different climate zones (Figure 3.1) based on spatial variance in temperature and rainfall. These zones are (1) the dry and warm western region and (2) the wet and cool eastern region (ARC-GCI, 2008). A majority of the interior and western part of the country is arid or semi-arid. Western regions of the country experience dry, desert like weather patterns, while eastern regions experience subtropical conditions and are humid (DoA, 2007). Rainfall decreases during summer in an east to west direction (Preston-Whyte and Tyson, 1993; Durand, 2006). A large portion of the country receives summer rainfall that is poorly distributed and droughts are common within these areas (Bennie and Hensley, 2000).

During December, the eastern maize region receives its maximum rainfall and is thus designated as an early summer rainfall area. The western maize region is a late-summer rainfall area, only receiving the maximum rain during February (Schulze, 1997).

Agriculture in South Africa is highly dependent on environmental temperature and rainfall patterns (Behnin, 2008). It is estimated that approximately 5 % of white maize and 11 % of yellow maize is cultivated under irrigation while the remainder of the maize is produced through dryland cropping (Durand, 2006). The production of maize as a staple grain requires rain as a critical input in rain-fed agricultural systems (Nicholson *et al.*, 2000).

Particular sensitivity to climate variability has been shown by the average maize yields in the western part of the maize region. The maize yield in this area is highly dependent on farming practices and rainfall received during the growing season (Du Toit *et al.*, 2000). Temperatures that exceed 32 °C combined with water stress resulting from higher evaporation rates may

severely affect maize cultivation (Du Toit, 1997; Du Plessis, 2003). South Africa has a warm climate with most regions experiencing an annual temperature above 17 °C. Monthly temperature variations seem to occur gradually throughout the country with little to no sudden changes occurring frequently. Monthly averages of daily temperatures in the summer months range from 26-30 °C for both eastern and western regions. The minimum temperatures during the summer months range between 12 and 16 °C (Schulze, 1997; Tadross *et al.*, 2011; Luhunga and Mutayoba, 2013).

It is estimated that maize in South Africa requires between 450 mm and 600 mm of water per growing season, depending on the local environment (Du Plessis, 2003) while the mean annual rainfall in South Africa is less than 500 mm (Ziervogel *et al.*, 2010). The mean annual rainfall of the main maize producing areas range between 400-600 mm, and is mostly received in the summer months when maize is cultivated (Bennie and Hensley, 2000).

2.7 Factors influencing fungal and mycotoxin contamination of agricultural commodities

Several factors do influence fungal and mycotoxins contamination of agricultural commodities. Among them, chemical and physical environmental factors (non-living components) in ecosystems, abiotic factors such as physiographic factors (location and topography), climatic factors (temperature, light, air pressure and wind, rainfall, humidity), edaphic factors: soil composition (such as clay, loam or sand, pH of soil, mineral salts and trace elements, water-holding capacity) and gases (vapour, oxygen, carbon dioxide and nitrogen) (Ingram, 1999). Temperature, humidity, and rainfall (together with sea levels) are those climatic factors that are most likely to be affected widely by future global change, and variations in these are expected to have a wide range of effects on plants and their pathogens (Ingram, 1999), as well as mycotoxin concentrations in these commodities (Miraglia *et al.*, 2009). Global warming will not only act on pathological systems already present in certain regions, but progress of new diseases will be favoured, because the distributional range, temporal activity and community structure of pathogens will be altered (Desprez- Loustau *et al*., 2007b ; Shaw *et al*., 2008). Nevertheless, some changes in pathosystems are not related to climate change (Rogers and Randolph, 2006) but the evidence that climate change can greatly influence host–pathogen dynamics is growing (Purse *et al*., 2005; Haines, *et al*., 2006).

However, field fungi such as *Fusarium* species and their mycotoxins are regularly associated with South African maize kernels (Ncube *et al*., 2011). They have been implicated in a number of disorders and cancers in humans and animals, which includes the human oesophageal cancer

in Transkei region of South Africa (Sydenham *et al*., 1990). They also cause equine leukoencephalomalacia, ELEM (hole in the head syndrome), which is a necrosis of the brain, in horses, mules and donkeys (Marasas *et al*., 1988). These *Fusarium* mycotoxins are produced in the field during the fungal infection of maize plant, but they can also continue during postharvest storage if environmental conditions are favourable. The occurrence of these types of fungi in maize kernels is mainly attributed to high humidity and moisture during the harvesting and storage process. Most field fungi are metabolically inactive at moisture content lower than 20 % while storage fungi thrive between moisture contents between 13 and 19 %. Therefore, there is need to investigate the occurrence and variation of *Fusarium* fungal specie and their mycotoxins (free and masked forms) in maize from different maize producing regions of South Africa.

CHAPTER THREE

RESEARCH METHODS

3.1 MATERIALS

The reagents and chemicals used were of analytical grade and were obtained from Merck and Co, Sigma or Microsep, Pretoria, South Africa except others from Sigma-Aldrich (Vienna, Austria). Water was purified successfully by reverse osmosis using Purite™ water purification technology Suez, UK from LASEC, South Africa.

3.2 Sample Collection

3.2.1 Site Description: Maize grains were obtained from differerent silo sites in the two climatically and geographical distinct maize production regions in South Africa. The agriculture regions are eastern region (ER), which is situated in the higher rainfall and cooler areas and western region (WR), which is located in the drier and warmer areas. The ER consists of Gauteng province and the eastern Free State where the mean maximum temperatures ranged from 24 °C to 27 °C while the WR consists of the Northwest province and western Free State, where the average temperature ranged from 29 °C to 32 °C. The geography and climatic conditions of the regions have previously been documented (Boutigny *et al.*, 2012). Individual silos constitute a local grain storage market for a radius of 60 kilometres surrounding it. SENWES silos have 90 % of the storage capacity in 45 of the silos areas (SENWES, 2013). They retained their central location in the grain planting areas (Figure 3.1). The grain market comprises several produces that have different seasons. The products are white and yellow maize, wheat, soya and sunflower seeds. The largest crop is maize, comprising 80 % of the grain crop and hence its season is the one that has occupied most of the attention. Usually, a season starts with planting from October to December (at times late January) and the majority of the crop is then harvested from June to August (again a very small quantity in September) of the subsequent year (SENWES decision, 2009). When the farmer transports grain to a silo it is graded and then stored in a silo according to its grade e.g. white maize grade 1. Therefore, samples were collected as the different farmers bring their maize to the silos.

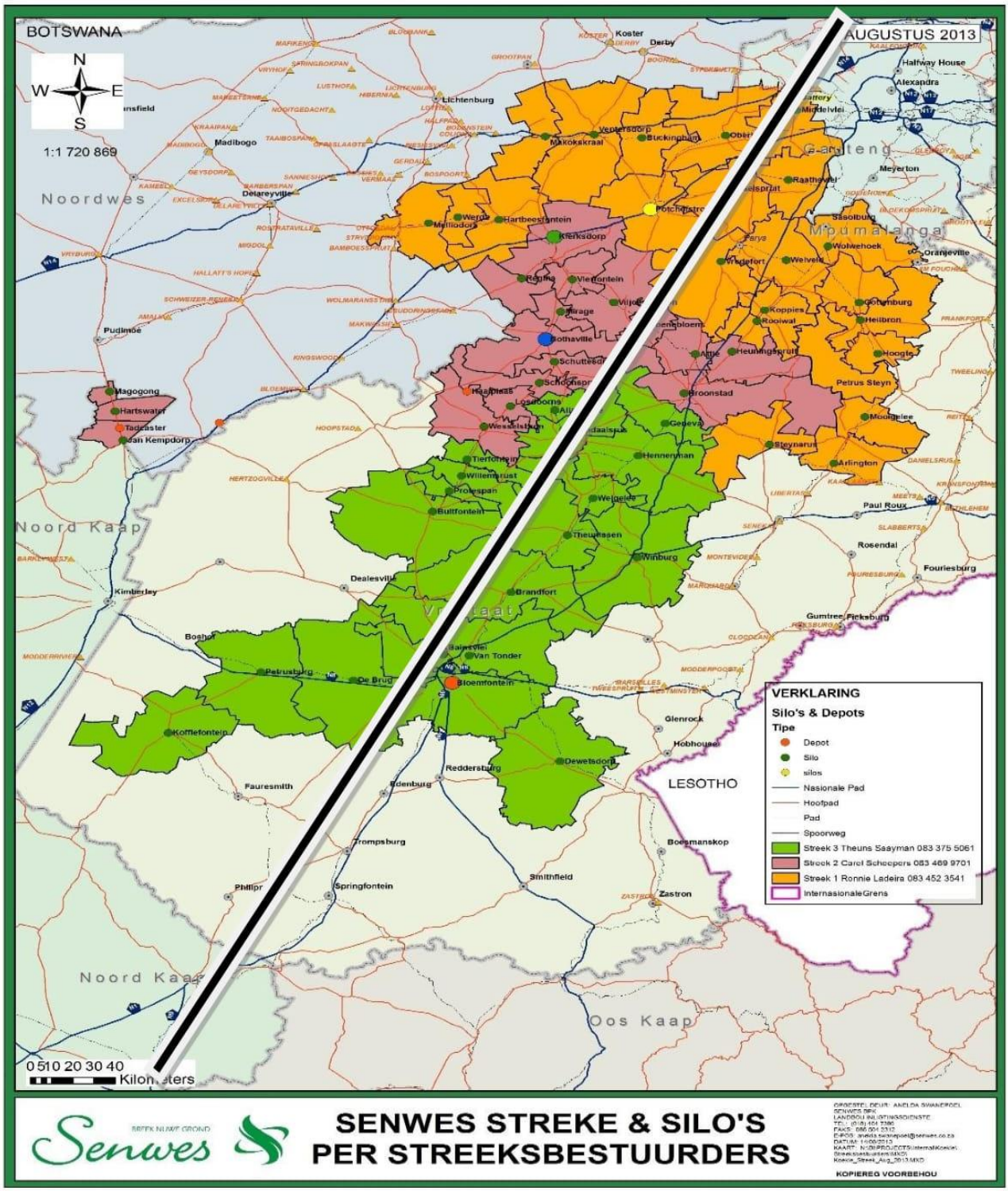


Figure 3.1 Map of the sampled areas. SENWES AREA localities in South Africa where maize was sampled for the detection of *Fusarium* species and their mycotoxins. The black line divides the maize production area in the country into western (left) and eastern (right) localities.

3.2.2 Sampling and sample preparation

A total of 123 maize samples harvested during 2015/2016 season were collected from randomly selected silo sites in the North-West, Gauteng and Free state province in the two agriculture regions. From the silos located at the western region (WR), 65 composite samples were collected, comprising of 44 white maize and 21 yellow maize. From the eastern region (ER) silo locations, 58 composite samples were collected, comprising of 30 white maize and 28 yellow maize. The eastern region (ER), is located in the higher rainfall and cooler areas and western region (WR) is located in the drier and warmer areas. For each maize sample, at least 10 incremental samples of 100 g each were taken and combined, making approximately 1kg according to EC 401/2006, in sterile zip lock polythene bags, well-labelled and transported to the laboratory. The samples were thoroughly mixed and milled using a sterile high-speed laboratory blender (IKA M20, Merck, Germany) and packaged in sealed sterile plastic bags to avoid contamination. A cleaning and decontamination routine of the equipment was performed using 70 % methanol, after each milling practice. Samples were stored prior to analysis at 4 °C in the freezer. The milled sample material was vigorously homogenised with a spatula before weighing.

3.3 Isolation and identification of *Fusarium*

3.3.1 Fungal culture

Fungi were cultured aseptically with the plate dilution technique as previously described by Rabie *et al*., (1997) with slight modifications. Briefly, one gram of the milled sample was weighed into a sterile test tube, suspended in 9 ml of sterile Ringer's solution (Sigma-Aldrich) and vortexed with whirl mixer. The suspension (1 ml) was decimally diluted and one millilitre aliquot of each dilution was inoculated onto a selective culture medium, malachite green agar 2.5 ppm (MGA 2.5) supplemented with 80 mgl^{-1} each of chloramphenicol and streptomycin and incubated at 25 ± 2 °C for 4–7 days. Fungal growth and sporulation was observed using compound microscope. The representative isolates of different *Fusarium* species were transferred onto potato dextrose agar (PDA) and were further incubated at 25 ˚C for 7 days. Macro and microscopic identification of the *Fusarium* species isolated from the samples was done according to their morphological characteristics between the 5^{th} and 7^{th} day of incubation using the identification keys as described by Pitt and Hocking (1997) and Klich (2002).

3.3.2 Storage of the isolates

The mycelia of the fungal isolates were harvested and stored in 10 % (v/v) glycerol at – 80 ºC for long-term preservation (Oliver *et al*., 2013). Working cultures were continuously sub-cultured and maintained on PDA plates, incubated at 28 ºC for 2-4 days where after it was stored at 4 ºC.

3.3.3 Molecular Analysis

3.3.3.1 *Fusarium* DNA extraction

The genomic DNA of fungi was isolated using Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, Southern California, USA) according to the manufacturer's instructions. The fungal isolates were grown on half strength Potato Dextrose Agar (PDA) (Biolab Diagnostics, South Africa) supplemented with 80 mgl^{-1} of chloramphenicol (Sigma-Aldrich, Germany) and 7-day old cultures were used for DNA extraction. The mycelium was harvested by scraping the agar surface and about 200 mg of the mycelia was suspended in 750 µl of lysis solution contained in a 1.5 ml ZR Bashing Bead™ lysis tube. The lysis tube was placed in disruptor genie bead beater fitted with a 2 ml tube holder assembly (Scientific industries Inc., USA) and processed at maximum speed for 5 minutes, then followed by centrifugation of lysed samples at 10,000 x g for 1 minute.

The supernatant was transferred to a Zymo-Spin™ IV spin filter in a 1.5 ml eppendorf tube and again centrifuged at 7000 x g for 1 minute. The content was then filtered into a collection tube and 1,200 µl of fungal/bacterial DNA binding buffer added and vortexed. The extracted mixture (800 µl) was transferred to a Zymo spin™ IIC column in the collection tube and again centrifuged at 10,000 x g for 1 minute and the supernatant discarded. An aliquot (200 µl) of DNA pre-wash buffer was then added to Zymo spin™ IIC column in a new collection tube and centrifuged at 1000 x g for 1 minute. The filtrate was discarded while retaining the column, which was placed into a new tube. A 500 µl aliquot of the DNA wash buffer was added to Zymo spin™ IIC column and again centrifuged at 10,000 x g for 1 minute. The Zymo spin™ IIC column was transferred into a sterile 1.5 ml eppendorf tube and 100 µl DNA elution buffer was added directly to the column matrix, and was centrifuged at 10,000 x g for 30 seconds to elute the DNA. The eluted purified DNA was stored at -20 °C until further analysis.

3.3.3.2. Polymerase chain reaction (PCR)

Subsequent to DNA extraction, the quality and quantity of the extracted genomic DNA was determined by spectroscopy using a Nanodrop 100™ spectrophotometer. The internal transcribed spacer (ITS) region of the fungal DNA was amplified by PCR using the primer set: ITS1; 5'-CTTGGT CATTTAGAGGAAGTA A-3' (forward) and ITS4; 5'-TCCTCCGCTTATTGATATGC -3' (reverse) as described by O'Donnell & Cigelnik, (1997). The primer set were procured from Inqaba Biotec, South Africa. Individual reactions were made up to 5 µl of the DNA sample mixed with 12.5 µl master mix (Taq DNA polymerase (Fermentas Life Science, Lithuania), dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR), 1 µl of the primers i.e. ITS1 (0.5 µl), ITS2 (0.5 µl) each and 6.5 µl of nuclease free water to make up a reaction volume of 25 µl. A negative control containing all the reagents except the DNA was also prepared. The PCR was performed in a C1000 Touch™ thermal cycler (Bio-Rad, USA). The conditions for PCR were as follows: initial denaturation of DNA at 95 °C for 3 minutes and then 35 cycles of denaturation at 94 °C for 1 minute, primer reannealing at 58 °C for 45 seconds and a further extension at 72 °C for 1 minute, 30 seconds. The PCR was further extended for 10 minutes at 72 °C and held at 4 °C until samples were retrieved. The amplicons were then separated on a 2 % agarose gel electrophoresis to confirm if the DNA has been successfully amplified.

3.3.3.3 Gel electrophoresis

Agarose gel DNA electrophoresis was performed according to the method previously described (Saghai-Maroof *et al.*, 1984 and Ekwomadu *et al*., 2018). The gel solution was allowed to cool to 60 °C prior to the addition of 3µl ethidium bromide (Sigma-Aldrish, ST Louis, MO, USA) and thoroughly mixed. The gel was poured into the casting chamber (Bio-Rad laboratories, California, USA) and the combs of desired sizes were inserted in such a way that no bubbles were caught under the teeth. After the gel was set, the combs were gently removed and the gel was placed in the electrophoresis tank. The PCR product (10 µl) was mixed with 6 µl of 6x loading buffer and 16 µl slowly loaded into each of the wells in the gel with sterile micro pipette. Care was also taken not to cross-contaminate the wells. A 6 µl of molecular marker (1-kilo base (kb)) DNA ladder (Fermentas Life Science, Lithuania) was loaded in the first well and the negative control (water) was loaded in the last well of each comb. The chamber was closed and run at 450 V and 70 mA for 30 minutes and DNA fragments (Figures 4.1, 4.2 and 4.3) were viewed by removing the gel slab from the tray and placing it on a UV

transilluminator, the gel was photographed with the Chemidoc™ MP imaging system (Bio-Rad Laboratories, California, USA). The PCR products obtained were sent to Inqaba Biotechnological Industries (Pty) Ltd, South Africa for sequencing.

3.3.3.4 Sequencing

PCR products were purified to remove excess primer using shrimp alkaline phosphatase and E. coli exonucleous 1. The purified products were sequenced in both directions (forward and reverse) with the primer set FF2 and FR1. Sequencing of the amplified ITS region was accomplished using the ABI PRISM® 3700XL automated DNA Sequencer (Applied Biosystems, USA) at the Inqaba Biotechnological Industries (Pty) Ltd, Pretoria, South Africa.

3.3.3.5 Species identification using DNA sequence of the amplified ITS region

The resulting ITS region chromatograms of forward and reverse sequences of fungal DNA obtained in this study were cleaned, combined, analysed and edited using Chromas Lite version 2.4 software (Technelysium Pty Ltd 2012). Nucleotide sequences were analysed and edited using the BioEdit software (Hall, 1999) to form consensus sequences. The consensus sequences were imported into GenBank (www.ncbi.nlm.nih.gov) and sequence BLAST search was done. Partial nucleotide sequences of the fungal isolates were compared with entries in nucleotide database of the NCBI web server and used to identify the specific fungi. The criteria for species identification were ≥97 % identity to the database sequence. Sequences generated in this study were submitted to the GenBank for accession numbers.

3.3.4 Uniplex PCR analysis for mycotoxin biosynthetic potential of *Fusaria* isolates

The PCR was carried out in a 25 µl reaction mixture containing the following: 5 µl of DNA, 12.5 µl master mix (Taq DNA polymerase (Fermentas Life Science, Lithuania), dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR, 0.5 µl, 25 µm of each primer (Table 2) and 6.5 µl of nuclease free water. To each reaction mixture, 3 µl of Q-Solution® was added to avoid primer dimerization. Amplifications were performed in C1000 Touch™ thermal cycler (Bio-Rad, USA), under the following conditions: For *fum13* gene, initial denaturation was at 94 °C for 4 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute and extension at 72 °C for 1 minute with a final extension of 72 °C for 8 minutes. For *tri6* gene, initial denaturation was

at 94 °C for 4 minutes, followed by 30 cycles of denaturation at 94 °C for 1 minute, 58 °C for 1 minute and 72 °C for 1.5 minutes, with a final extension of 72 °C for 8 minutes. For *zea13*, initial denaturation was at 94 °C for 4 minutes, followed by 30 cycles of denaturation at 94 °C for 30s, annealing at 57 °C for 1 minute and extension at 72 °C for 1 minute with a final extension of 72 °C for 8 minutes. Amplification products were separated on 2 % agarose gel (Invitrogen), placed under ultraviolet (UV) light and photographed with the Chemidoc™ MP imaging system (Bio-Rad Laboratories, California, USA), as described in section 3.2.3.3 above. A 100-bp DNA LadderPlus (Fermentas) was used as a size standard. The following primer sets (Table 3.1) were used.

Table 3.1: The primers that were used to detect the genes essential for mycotoxin biosynthesis

Primer Pair	**Primer sequence (5'-3')**	**Amplicon size (bp)**	**Annealing temp. °C**	**Reference**
FUM 13F	GAGCTTGTCCTTCTCACTGG	982	58 °C	Rashini *et al*., 2012
FUM 13R	GAGCCGACATCATAATCAGT			Rashini *et al*., 2012
TRI6F	GATCTAAACGACTATGAATCACCCT	541	58 °C	Venkatarama *et al*., 2013
TRI6R	GCCTATAGTGATCTCGCATGT			Venkatarama *et al*., 2013
ZEA13F	CATTCTTGGTCTTGTGAGGA	351	58 °C	Priyanka *et al.*,2015
ZEA13R	CCTTATGCTCATCGACATG			Priyanka *et al.*,2015

3.4 Free and masked *Fusarium* mycotoxins analysis

3.4.1 Sample Preparation and Cleanup for LC-MS/MS multimycotoxin analyses

This was done according to Sulyok *et al*. (2007), sample preparation was rather simple and eludes any clean-up. Briefly, 5 g representative amount of ground maize kernels (Section 3.1.2**)** was weighed into a 50 ml polypropylene tube (Sarstedt, Nümbrecht, Germany) and extracted for 90 minutes at 180 rpm on on a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) with 20 ml of extraction solvent, (acetonitrile/water/acetic acid 79:20:1, v/v/v). Subsequently, the extracts were centrifuged for 2 minutes at 3,000 rpm (radius 15 cm) on a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA). The raw extracts were transferred into glass vials using Pasteur pipettes, and 350 µl aliquots were diluted in same volume (1/1) with dilution solvent, (acetonitrile/water/acetic acid 20:79:1, v/v/v) to adjust the solvent strength. After appropriate mixing, 5 µl of the diluted extract was analysed by LC-MS/MS without further pre-treatment.

3.4.2 LC-MS/MS multimycotoxin measurement parameters

Analyses for the *Fusarium* toxins was performed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) multi-mycotoxin method, at the Centre for Analytical Chemistry, Department of Agro biotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna, Austria. The analysis was performed according to the methods described by Sulyok *et al*. (2007) and Malachová *et al*., (2014), with slight modifications.

Briefly, a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a Turbo Ion Spray electrospray ionization (ESI) source and a 1100 Series HPLC System (Agilent, Waldbronn, Germany), including a 1100 Series diode array detector. Chromatographic separation of the conjugates was performed at 25 °C on a Gemini® C_{18} column, 150 × 4.6 mm i.d., 5µm particle size, equipped with a C_{18} 4 × 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, US), using (eluent A) methanol/water/acetic acid 10:89:1 (v/v/v) or (eluent B), methanol/water/acetic acid 97:2:1 on a C_{18} 4 × 3 mm i. d security guard cartridge. Both eluents contained 5 mM ammonium acetate. After an initial time of 2 minutes at 100 % A, the proportion of B was increased linearly to 100 % within 12 minutes, followed by a hold-time of 3 minutes at 100 % B and 4 minutes column re-equilibration at 100 % A. The injection volume of 50 µl and flow rate of 1 ml min^{-1} was used.

The ESI-MS/MS source temperature operated at 550 °C, in the multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. Further MS parameters were as follows: curtain gas 10 psi (69 kPa of max. 99.5 % nitrogen); ion source gas 1 (sheath gas) 50 psi (345 kPa of nitrogen); ion source gas 2 (drying gas) 50 psi (345 kPa of nitrogen); ion spray voltage −4,000 V and +4000 V respectively, collision-activated dissociation gas (nitrogen) high.

The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (diluted in a 1:1 mixture of eluent A and B) into the MS. It was done using eleven plus syringe pump (Harvard Apparatus, US) at flow rate of ten microlitre per minute. Table 3.2 shows the corresponding values; parameters were set to the optimal values that had been determined for the respective analytes.

Table 3.2

ESI-MS/MS parameters used for the LC-MS/MS multimycotoxin analyses of some of the analytes

Mycotoxin	**Retention time[a](min)**	**Precursor ion m/z**	**DP[b] (V)**	**Product ions[c] (m/z)**	**Relative intensity[d]**	**Collision energy CE[e](V)**
Fumonisin B_1	12.91	722.5 $[M+H]^+$	91	334.4/352.3	0.78	57/55
Fumonisin B_2	14.12	706.3 $[M+H]^+$	96	336.3/318.5	0.47	59/51
Fumonisin B_3	13.53	706.3 $[M+H]^+$	96	336.3/318.5	0.40	
Deoxynivalenol	7.59	355.1 $[M+Ac]^-$	−40	265.2/59.2	4.72	−22/−40
Nivalenol	5.48	371.1 $[M+Ac]^-$	−45	281.1/59.1	1.55	−22/−42
Zearalenone	14.62	317.1 $[M-H]^-$	−80	131.1/175.0	0.98	−42/−34
HT-2 Toxin	13.08	442.2 [M+NH4]+	46	263.1	5.18	21/27
T-2 Toxin	13.67	484.3[M+NH4]+	56	215.2/185.1	29/31,8/11	
DON-3-glucoside	7.61	517.3 $[M+Ac]^-$	−50	427.1/59.1	1.37	−30/−85
Zearalenone-4-sulphate	14.32	397.1 $[M-H]^-$	−75	317.1/175.0	0.17	−32/−48
HydrolysedFumonisinB_1	12.03	406.3 $[M+H]^+$	86	370.3/388.3	0.88	29/27
Fusarenon-X	8.92	413.3 $[M+Ac]^-$	−40	262.2/59.1	3.93	−22/−44
Monoacetoxyscirpe	11.06	342.2 [M+NH4]+	41	265.0/307.0	0.42	13/13
Diacetoxyscirpenol	11.94	384.2 [M+NH4]+	51	307.2/105.1	0.54	21/27
Neosolaniol	9.24	400.20$(M+NH_4)^+$	46	215.1/185.0	0.90	25/29
Enniatin B	15.80	657.5 $[M+NH_4]^+$	51	196.3/214.1	0.52	45/47
Enniatin B_1	15.97	671.4 $[M+NH_4]^+$	81	196.0/210.0	0.73	43/41
Enniatin A_1	16.12	685.4 $[M+NH_4]^+$	66	210.1/228.2	0.67	41/49
Enniatin A	16.26	699.4 $[M+NH_4]^+$	76	210.1/228.0	0.31	43/47
Beauvericin	16.03	801.5 $[M+NH_4]^+$	86	244.2	2.29	47/73
Moniliformin	3.38	96.9 $[M-H]^-$	−70	41.2	−	-

a Retention time
b Declustering potential
cValues are given in the order quantifier ion/qualifier ion
d Intensity of the qualifier transition/intensity of the quantifier transition
e In-source fragment obtained from cleavage of the corresponding peptide bond

3.4.3 Determination of fumonisin B_1, B_2 and zearalenone using Immuno-affinity column (IAC) mycotoxin extraction and clean-up for HPLC analysis.

Samples were also analysed using the HPLC for some of the major *Fusarium* mycotoxins. Fumonisins were extracted from the sample using Immuno Affinity Column (IAC) according to the manufacturer's instructions (Biopharm, SA). About 25 g of milled sample was mixed with 5 g of NaCl and 125 ml of acetonitrile: methanol: water (25:25:50 v/v/v) in a blender and blended at high speed for 2 minutes. The mixture was filtered through Whatman number 113 filter paper (Whatman International Ltd, Maidstone England) into a clean vessel. A 10 ml aliquot of filtrate was diluted with 40 ml of phosphate buffered saline (1xPBS) solution (8.0 g NaCI, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCI, dissolved in 990 ml distilled water) with pH adjusted to 7.0 with HCI. The diluted extract was filtered through glass microfiber filter paper. A 10 ml of the diluted filtrate, which is equivalent to 0.4 g of sample was passed through the Fumonitest™ IAC at a flow rate of 2 ml per minute to enable the mycotoxins to be captured by the antibodies present in the column. After that, the column was washed with 10 ml of 1x PBS at a flow rate of 5 ml per minute in order to remove the unbound materials, until air passed through the column. Fumonisins were released from the column following elution with 1.5 ml of 100 % methanol at a flow rate of 1 drop per second. A 1.5 ml of water was also passed through the column and collected in the same vial to give a total of 3 ml. The eluate was collected in an amber vial, evaporated to dryness with stream of nitrogen gas at 60 °C and stored at 4 ˚C.

Zearalenone was also extracted from the sample using Immuno Affinity Column (IAC) according to the manufacturer's instruction (Biopharm, SA). About 25 g of milled sample was mixed with 5 g of NaCl and 125 ml of acetonitrile: water (75: 25 v/v) in a blender and blended at high speed for 2 minutes. The mixture was filtered through Whatman number 113 filter paper (Whatman International Ltd, Maidstone England) into a clean vessel. The filtrate, (20 ml) was diluted with 80 ml of phosphate buffered saline (1x PBS) solution (8.0 g NaCI, 1.2 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCI, dissolved in 990 ml purified water). The pH was adjusted to 7.4 using 2M sodium hydroxide. The diluted filtrate, (25 ml) which is equivalent to 1 g of sample was passed through the IAC at a flow rate of 2 ml per minute to enable the mycotoxins to be captured by the antibodies present in the column. After that, the column was washed with 20 ml of 1x PBS at a flow rate of 5 ml per minute in order to remove the unbound materials, until air passed through the column. Zearalenone was released from the column following elution with 1.5 ml of 100 % acetonitrile at a flow rate of 1 drop per second and 1.5 ml of water passed through the column and collected in the same vial to give a total of 3 ml. The eluate was

collected in an amber vial, evaporated to dryness with stream of nitrogen gas at 60 °C and stored at 4 ˚C.

3.4.4 Quantification of fumonisin B_1, B_2 and zearalenone on High Performance Liquid Chromatography (HPLC).

Analysis on HPLC was performed to determine the exact concentrations of the extracted mycotoxins according to the method previously described (Mwanza, 2011) with modifications. The Shimadzu Prominence UFLC Liquid chromatography system (Kyoto, Japan) was used for the HPLC determination. It consists of a Liquid Chromatography, LC-20AD which is fitted to a degasser, DGU $20A_{5R}$, auto sampler (injection) SIL 20A, communication bus module CBM 20A, column oven CTO 20A, photodiode array detector SPD M20A and fluorescent detector RF 20A XL, connected to a gigabyte computer with Intel Core DUO and Microsoft XP operating system.

Fumonisin Bs and zearalenone were analysed using fluorescent detector at specific excitation and emission wavelengths also referred to as the compound's fluorescence signature. The excitation and emission wavelengths for fumonisin Bs are 335 nm/440 nm and those of zearalenone are 274 nm/418 nm. Extracts from IAC were dissolved in 500 µl of HPLC grade acetonitrile. The extracts for fumonisin B analysis were dissolved in 500 µl of HPLC grade methanol according to the method described by Shepherd *et al*. (2005) and an aliquot (25 µl) was derivatised with (250 µl) of O-phthaldialdehyde solution (OPA) (Sigma). The derivatised sample was injected into the HPLC system within minutes due to the instability of OPA. The derivatised analogues become highly unstable within a short time and rapidly break down into non-fluorescent substances. Samples were run at 20 µl injection volume at a flow-rate of 1 ml per minute (min^{-1}) retention times. Analysis of zearalenone was done according to the method previously prescribed, with some modifications (Abdulkadar *et al.*, 2004; Njobeh *et al.*, 2009). The different mobile phases for mycotoxins are as follows: Fumonisins mobile phase-methanol/sodium dihydrogen phosphate (80/20, v/v) and zearalenone mobile phase-Acetonitrile/Water (15/55, v/v). Standard were 10, 20, 40 ng/ml

3.4.5 Zearalenone analysis using Enzyme linked immunosorbent assay (ELISA)

Five grams of milled sample was weighed into a suitable container, followed by the addition of 25 ml of methanol/water (70/30; v/v) and shaken vigorously for 3 minutes using a Stuart® Orbital Shaker (Karlsruhe, Germany). The extract was filtered with Whatman No.1 filter paper and the filtrate diluted in the ratio of 1:7 with sample dilution buffer (buffer 1) (contained in the ELISA kit). Sufficient numbers of microtitre wells were inserted into the microwell holder with the standard and sample positions recorded. Fifty microliter of the standard solutions or prepared samples was added into the wells followed by addition of 50 µl of the diluted enzyme conjugate. The content was mixed gently by shaking the plate manually and incubated for 2 hours at room temperature (20-25 °C) in the dark. Thereafter, the liquid was poured out of the wells and the microwell holder tapped upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. The wells were washed by filling each of them with 250 µl of distilled water and the liquid was again poured out. The washing process was repeated twice. Then 50 µl of chromogen and 50 µl of substrate was added to each well, mixed gently by shaking the plate manually and incubated at room temperature (20-25 °C) for 30 minutes in the dark. Finally, 100 µl of stop solution was added to each well and mixed gently by shaking the plate manually. The absorbance was read at 450 nm wavelength within 30 minutes of addition of stop solution using an automatic micro plate reader (Heales Model MB-580, (Wellkang Ltd, London)).

3.5. Estimation of the exposure risk assessment to South African maize consumers (PDI)

The risk assessment, which is the magnitude and probability of harmful effect of consumption of the contaminated maize grains, was based on a deterministic approach involving the estimation of the probable daily intake (PDI) of mycotoxins. To estimate the PDI (µg/kgbw/day) for each mycotoxin, the quantity of mycotoxin in the maize samples was multiplied by the average maize consumption in South Africa. The exposure risk of maize consumers in South Africa to the three South African regulated *Fusarium* mycotoxins (DON, FB_1 and FB_2) found to be predominant in the grains was determined according to the method of Liu and Wu, (2010). The average maize consumed (248g/person/day) in South Africa as amended by GEMS/food (WHO, 2008) was multiplied with the mean concentration of the toxins in the grains in the various maize regions. The product was divided with the average body weight of the individuals, which we assumed to be 60 kg as illustrated below:

$$PDI_m = (\mu_X C \times C_c)/Bw$$

where

PDI_m: Probable daily intake for each mycotoxin (µg/kg.bw/day)

$\mu_X C$: Mean of mycotoxin concentration/AR

C*c*: Average consumption of maize in South Africa

B*w*: Body weight for the population group

3.6 Method validation

i) Method validation for LC-MS/MS

Based on the European Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs, the method was validated in terms of linearity, apparent recovery (AP), limit of detection (LOD), limit of quantification (LOQ), using blank matrices of maize, (401/2006/EC, 2006). Apparent recoveries of the analytes were taken by spiking five different samples that were not contaminated with mycotoxins with a multi-analyte standard. The spiked samples were left overnight in the dark at room temperature for evaporation of the solvent to establish equilibrium between the analytes and the sample was then extracted with 1 ml of extraction solvent as described above. The corresponding peak areas of the spiked samples were used for estimation of the apparent recovery by comparison with a standard of the same concentration prepared by dilution in pure solvent:

$$RA\% = 100 \text{ X } \frac{Peak\ area\ spiked\ samples}{Peak\ area\ liquid\ standards}$$

Limits of detection (LOD) and limit of quantification (LOQ) were calculated from the signal to noise ratios (S/N) of the respective multiple reaction monitoring (MRM) chromatograms deriving from analysis of the spiked samples: LOD =3×S/N and LOD =10 ×S/N, respectively.

ii) Method validation for HPLC

Linearity was determined by injecting mycotoxin standards at three different concentrations into the HPLC column. Calibration curves between the different concentrations and correlation

coefficient (R^2) were used to evaluate the linearity of the HPLC method. See appendix II for the calibration curves of different mycotoxin standards. The results indicated good linearity with R^2 values ranging from 0.9935-0.9999 for the different mycotoxin standards (See appendices).

3.7 Statistical analysis

The data collected were subjected to analyses of variance (ANOVA), as outlined for factorial arrangement in a completely randomised design (CRD) using GenStat Release 10.3DE (2012) statistical software. The means were compared using Fisher's least significant difference (F-LSD) at 5 % probability level. The data were not log-transformed, only the geometric means were taken. Microsoft Office Excel software version 2016 was used to determine the frequencies, range and percentages.

CHAPTER FOUR

RESULTS

4.1 Isolation and identification of *Fusarium* species.

Isolation and identification of *Fusarium* species. from the 123 maize samples collected from randomly selected silos in the two agriculture regions, eastern (58 samples) and western regions (65 samples) of South Africa revealed that different F*usarium* fungal species contaminated the maize samples (Table 4.1 and Figure 4.1).

i) Morphological Identification

The *Fusarium* colonies were recovered from different serial decimal dilution petri dishes of a selective culture medium, malachite green agar 2.5 ppm (MGA 2.5). The representative isolates of different *Fusarium* species were sub cultured onto potato dextrose agar (PDA) and identified to species level. Macro and microscopic identification using the identification keys as described by Pitt and Hocking (1997) and Klich (2002), showed morphological characteristics, which strongly suggested that isolates belonged to *Fusarium* genius as shown in Figure 4.1

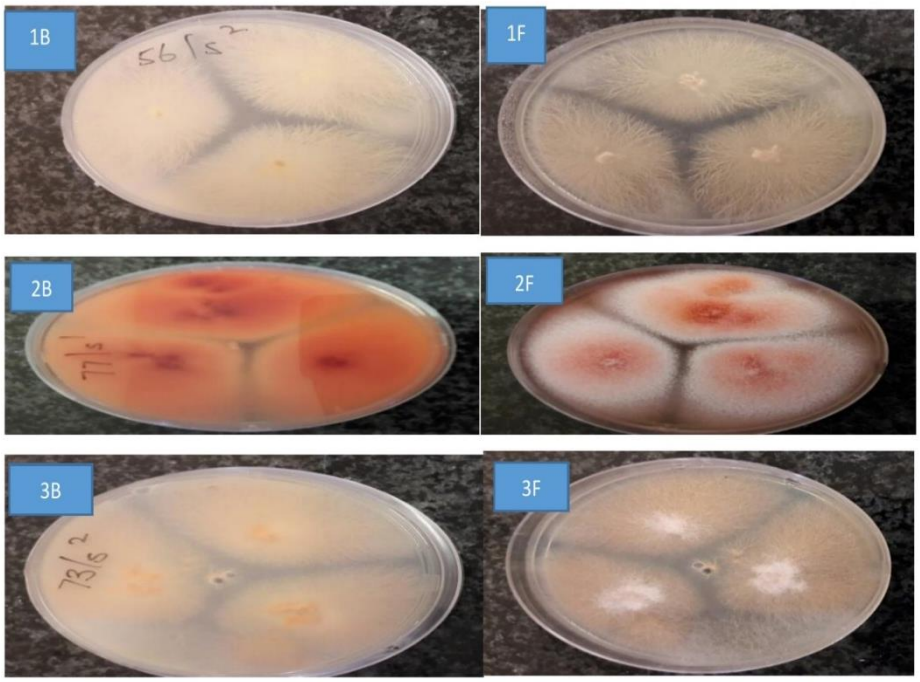


Figure 4.1: Some of the isolated *Fusarium* species from the maize samples on culture medium: 1-*F. oxysporum*, 2- *F. graminearum*, 3- *F. verticiloides* (Bs and Fs) are back and front of the same isolate.

i) Molecular identification

The BLAST search inferred that the isolates were members of the *Fusarium* genus. The ITS gene sequence of different *species* was obtained by Megablast search; however, seven species of *Fusarium* were identified based on high identity (%) with good E value. Table 4.1 results shows that the query sequences were pairwise aligned with ITS gene sequence of *Fusarium* species with sequence identification similarities ranged between 99-100 %, with reference species from the GenBank database and E value of 0.0.

Table 4.1:ITS gene sequence similarities of isolated *Fusarium* species and GenBank sequence identification numbers using Megablast Algorithm**.**

***Fusarium* Isolates**	**Sequence length**	**Sequence Id**	**Similarity %**	**E.value**
Fusarium verticiloides	1612	MG274315.1	99 %	0.0
Fusarium oxysporum	1164	FJ867936.1	99 %	0.0
Fusarium subglitans	1612	MG274315.1	99 %	0.0
Fusarium proliferatum	549	KU204757.1	100 %	0.0
Fusarium napiforme	1374	MH862670.1	100 %	0.0
Fusarium fujikoroi	810	MH084746.1	99 %	0.0
Fusarium graminearum	515	HQ647306.1	100 %	0.0

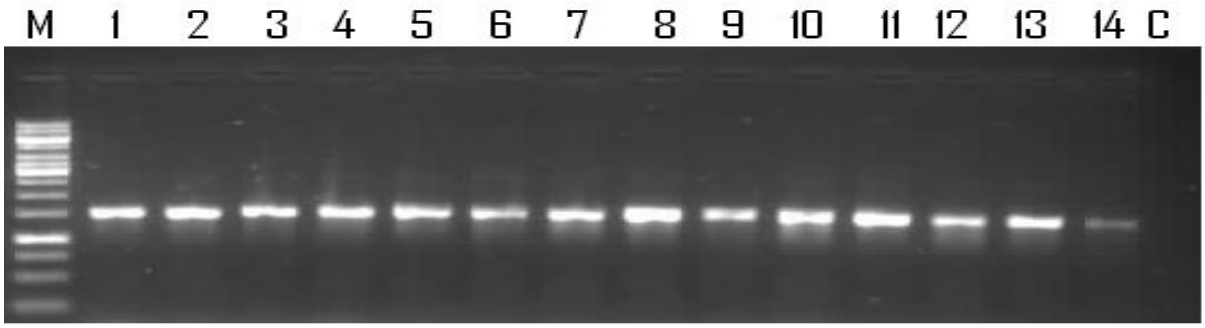


Figure 4.2: Image of agarose gel showing DNA bands of isolated *Fusarium* fungal species (1-14). **M**= 1kb DNA marker, **C**= negative control and lanes **1**-**14**= PCR amplification of isolates

4.2 Contamination of maize samples with *Fusarium* species in Eastern and Western maize regions of South Africa

Mycological analyses of the maize samples in this study revealed that different F*usarium* species contaminated the maize samples. The seven *Fusarium* species that contaminated the maize in both the western and eastern regions were *Fusarium verticiloides, Fusarium oxysporum, Fusarium subglitans, Fusarium proliferatum, Fusarium napiforme, Fusarium fujikuroi* and *Fusarium graminearum* with total incidence rate of 96 %, 84 %, 66 %, 83 %, 25 %, 24 % and 34 % respectively. *Fusarium verticiloides* was the predominant *Fusarium* species irrespective of the agricultural regions as summarised in Table 4.2**.** Mycological analyses also showed that most of the samples were contaminated with at least one fungal species, whereas co-contamination with different *Fusarium* fungi species occurred in most of the samples. *Fusarium verticiloides* occurred in 96 % (118) of all the samples with 51 % (63 samples) from western region (WR) and 45 % (55 samples) from eastern regions (ER) being contaminated. Samples from western region had the least contamination with *Fusarium napiforme* having only 16 samples contaminated. Likewise, samples from the eastern regions had the least contamination with 11 samples being contaminated with *Fusarium fujikuroi.* Finally, of all the *Fusarium* species isolated, *Fusarium subglitans* and *Fusarium graminearum* occurred predominantly in the eastern region while the rest of the contaminants occurred higher in the western region.

Table 4.2: Summary of the frequency of *Fusarium* species isolated from agriculture regions of South Africa (Frequency=n/total samples x100)

Dominant *Fusarium* spp.	Eastern Region(% incidence)	Western Region(% incidence)	Total incidence% out of 123 samples
Fusarium verticiloides	45 (55)	51 (63)	96 (118)
Fusarium oxysporum	41 (51)	43 (53)	84 (104)
Fusarium subglitans	41 (50)	26 (32)	66 (82)
Fusarium proliferatum	41 (50)	42 (52)	83 (102)
Fusarium napiforme	12 (15)	13 (16)	25 (31)
Fusarium fujikuroi	11(13)	14 (17)	24(30)
Fusarium graminearum	17 (22)	16 (20)	34 (42)

4.3 Screening of *Fusarium* isolates for the presence of mycotoxin biosynthetic genes.

Screening for the presence of *Fum13, Tri 6* and *Zea13* genes in the *Fusarium* isolates, which underlie *Fusarium* mycotoxins production showed that the isolates have biosynthetic genes as shown in Figures 4.3-4.5.

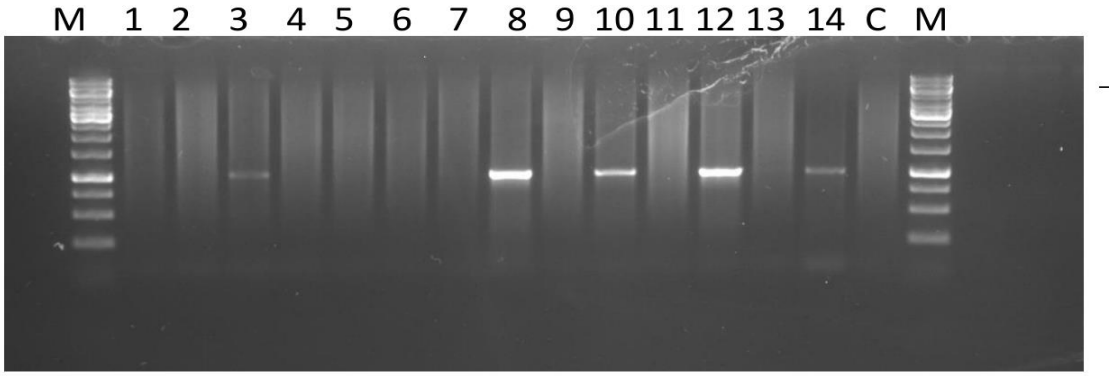


Figure 4.3: Image of agarose gel showing bands of amplified genes for *Fum13* gene cluster (**982bp) M**= 2kb DNA marker, **C**= negative control and lanes **3, 8, 10.12, 14**= PCR amplification of *Fum13* gene cluster.

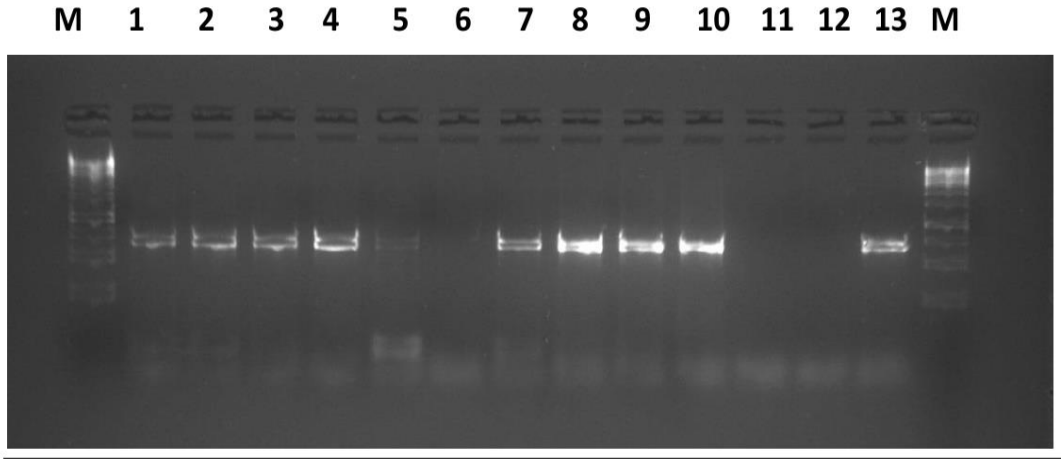


Figure 4.4: Image of agarose gel showing bands of amplified genes for *Tri6* gene cluster (541bp) M= 1.5kb DNA marker, lanes 1, 2, 3, 4, 7, 8, 9, 10, 13= PCR amplification of *Tri6* gene cluster.

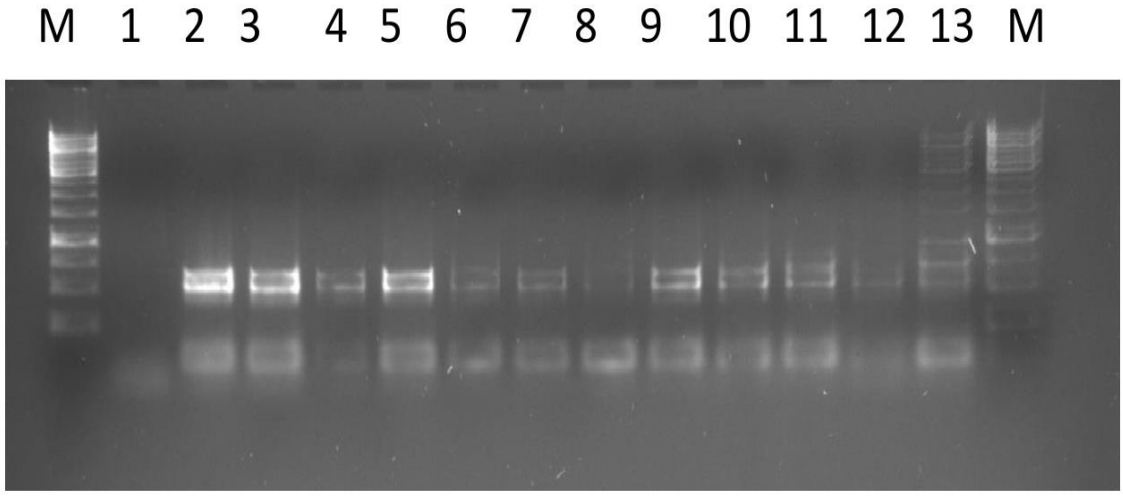


Figure 4.5: Image of agarose gel showing bands of amplified genes for *Zea 13* gene cluster (351bp) M= 1kb DNA marker and lanes 2, 3, 5 = PCR amplification of *Zea 13* gene cluster.

4.4 MYCOTOXIN CONTAMINATION

4.4.1 General mycotoxin contamination pattern

Results of regulated mycotoxins, masked and emerging toxin concentrations from 123 maize samples from agriculture regions of South Africa are presented. Table 4.3 provides a description and basic statistical information of the data set investigated. All the maize samples analysed were contaminated with an average of 5 and up to 24 out of 42 mycotoxins, including 0 to 3 masked forms at the same time. Summary data in Table 4.3 highlights the relevance of fumonisin B_1, B_2, B_3, B_4 and A_1 Vorstufe in the samples with 98 %, 91 %, 80 %, 82 % and 54 % of 123 samples contaminated with maximum contamination levels 8907.7, 3383.3, 990.4, 1014.4 and 51.5 µg/kg respectively. The mean total fumonisin, which is the summation of all the mean fumonisin values, was 990.1 µg/kg. Deoxynivalenol occurred in 50 % of the samples with mean concentration of 152 µg/kg (max 1380 µg/kg). Thirty-three % of the samples were contaminated with zearalenone at mean concentration of 13.6 µg/kg (max 145.6 µg/kg). Occurrence of HT-2 and T-2 in the samples was at a very low level at 0.8 % each and at maximum concentration of 40.2 µg/kg and 148.0 µg/kg respectively, while nivalenol occurred in 11 %. Of the masked mycotoxins, DON-3-glucoside occurred at high incidence rate of 53 % than the others. Among emerging toxins, moniliformin, fusarinolic acid and beauvericin showed high occurrence in 98 %, 98 % and 83 % of samples, respectively.

Table 4.3: Summary statistics of occurrence of investigated 42 *Fusarium* regulated mycotoxins and mycotoxins with guidance levels, masked and emerging mycotoxin metabolites in 123 maize samples on LC-MS/MS.

	Concentration(µg/kg)			**Number of positive samples across ARs[a]**		**N[b] (123)**	**P[c] %**	**LOQ[d]**	**LOD[e]**	**Rec[f] (%)**
Metabolite Group	Minimum	Maximum	Mean	ER (n=58	WR (n=65)					
Regulated *Fusarium* mycotoxins and toxins with guidance levels										
Fumonisin B_1	12.6	8907.7	596.2	58	63	121	98	8	2.4	75.0
Fumonisin B_2	7.9	3383.3	220.6	55	57	112	91	7	2.1	79.0
Fumonisin B_3	<LOQ	990.4	85.6	44	54	98	80	7	2.1	75.0
Fumonisin B_4	<LOQ	1014.4	81.6	45	56	101	82	7	2.1	75.0
Fumonisin $A_{1Vorstufe}$	<LOQ	51.5	6.1	28	38	66	54	2.1	0.6	75.0
Fumonisin Total	**20.5**	**14347.3**	**990.1**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**	**n/a**
Zearalenone	<LOQ	145.6	13.6	19	22	41	33	0.6	0.2	54.0

HT-2 toxin	40.2	40.2	40.2	0	1	1	0.8	6.4	1.9	96.8
T-2 toxin	148.0	148.0	148.0	0	1	1	0.8	2.4	0.8	102.0
Deoxynivalenol	8.2	1380.0	152.0	35	26	61	50	–	1.2	85.0
Nivalenol	7.7	35.7	14.2	4	10	14	11	3.8	1.1	80.0
Masked *Fusarium* mycotoxins										
DON-3Glucoside	2.43	111.6	15.0	31	34	65	53	–	0,8	92.0
ZON-sulphate	11	146	14.0	9	13	22	18	n.a	–	75.0
Hydrolysed FB_1	0.7	28.0	3.9	12	19	31	25	0.7	0.2	100.0
HT-2 Glucoside	31.2	31.2	31.2	0	1	1	0.8	5.7	1.7	71.9
Emerging mycotoxins and other less well-known *Fusarium* metabolites										
MAS	20.9	20.9	20.9	0	1	1	0.8	5.2	1.6	89.0
DAS	4.4	5.0	4.7	0	2	2	1.7	0.5	0.2	81.8
Neosolaniol	4.5	4.5	4.5	0	1	1	0.8	4.6	1.4	83.3
Culmorin	13.3	465.4	90.0	7	11	18	15	5.5	1.6	76.0
15Hydroxyculmorin	<LOQ	2022.4	180.8	24	25	49	40	20.8	6.2	55.7
5Hydroxyculmorin	<LOQ	578.0	167.0	12	6	18	15	50	15	102.0
Moniliformin	<LOQ	1129.8	218.8	57	63	120	98	5	1.5	100.0
Beauvericin	<LOQ	141.8	7.2	56	51	107	87	0.03	0.01	100.0
Enniatin B	0.1	4.9	2.5	0	2	2	1.7	0.04	0.01	81.0
Enniatin B_1	0.13	3.0	1.6	0	2	2	1.7	0.11	0.03	100.0
Enniatin B_2	<LOQ	<LOQ	<LOQ	0	1	1	0.8	0.57	0.2	91.2
Aurofusarin	<LOQ	5470.2	295.8	46	43	89	72	3.7	1.1	79.8
Bikaverin	<LOQ	651.4	72.0	38	44	82	67	55	16	79.6
Butenolid	<LOQ	214.4	48.9	22	13	35	28	10.8	3.2	61.0
Epiequisetin	<LOQ	18.9	5.0	7	12	19	15	2	0.6	70.3
Equisetin	<LOQ	129.0	19.4	14	16	30	24	2.3	0.7	79.9
Apicidin	2.9	15.4	9.1	1	1	2	1.6	0.65	0.2	108.0
Deoxyfusapyron	<LOQ	53.0	11.1	7	16	23	19	2.7	0.8	103.0
Fusapyron	<LOQ	18.0	11.1	14	24	38	31	2.4	0.7	106.0
Fusaric acid	57.9	195.0	85.2	14	10	24	20	30	10	91.4
Fusarinolic acid	<LOQ	3422.1	506.4	57	63	120	98	30	10	89.5
7Hydroxypestalotin	<LOQ	16.5	7.5	8	17	25	20	2.9	0.9	100.0
Acuminatum B	<LOQ	218.9	33.0	4	8	12	9.8	30	10	105.0
Acuminatum C	26.5	204.3	98.8	4	3	7	5.7	30	10	62.4

Chlamydospordiol	2.1	5.1	7.3	1	1	2	1.7	0.3	0.1	96.5
Chlamydosporol	27.0	26.9	26.9	0	1	1	0.8	1.1	0.3	94.0
Chrysogin	<LOQ	7.7	4.4	6	42	48	30	1.9	0.6	99.2
Siccanol	34.6	251.5	64.3	43	48	91	74	—	—	86.9

a Contamination across agricultural regions(ARs)-Eastern region (ER), Western region (WR)
b Total number of contaminated samples (N)
c Percentage of total number of samples contaminated(P)
d LOQ:limit of quantification, <LOQ: less than LOQ
e LOD: limit of detection [s/n= 3:1] expressed as µg/kg sample.
f Recovery: calculated from spiking experiment of maize samples.
n/a-Not applicable

4.4.2 Percentage distribution of *Fusarium* (free and masked) mycotoxins across the Agriculture Regions of South Africa

The main effect of Agriculture Regions (AR) on the distribution of *Fusarium* free and masked mycotoxins in the maize samples is presented in Table 4.4.

All the 42 *Fusarium* toxins and metabolites investigated in the maize samples across the agricultural regions were detected and quantified except for the emerging toxin, enniantin B_2 which was only detected in 2 % of the samples from the western region but was not quantified because the values were below the limit of quantification. Of the major mycotoxins, HT-2 was not detected at all in the eastern region but was quantified only in 2 % of the maize from the western region. Of the fumonisin Bs, FB_1 occurred at a most frequent rate than FB_2, FB_3 and FB_4. Fumonisin B_1 was the most contaminant mycotoxin, occurring at mean concentrations of 752.46±1469µg/kg from the warm western region and of 439.88±514 µg/kg in the cold eastern region with only 3% (2 samples) not contaminated. Fumonisin B_2 was the second most contaminant, occurring in 95 % and 88 % of the samples from the eastern and western regions respectively. Generally, Fumonisin A_1Vorstufe was quantified in 32 % of all the maize samples analysed and in 22 % of the samples, it was detected but below the limit of quantification. Deoxynivalenol was more prevalent in the eastern region than in the western having 60% and 40 % of the samples all detected and quantified respectively. Zearalenone, nivalenol and T-2 were also among the major *Fusarium* mycotoxins, detected and quantified in the two regions. Masked mycotoxins detected in the samples include DON-3-glucoside, zearalenone-sulphate, hydrolysed fumonisin B_1 and HT-2-glucoside, with DON-3-glucoside and HT-2-glucoside having the highest and least occurrence respectively across the two regions. Other detected metabolites included emerging mycotoxins and other less well-known *Fusarium* metabolites such as beauvericin, enniatins (B, B_1, and B_2), moniliformin, monoacetoxyscirpenol, diacetoxyscirpenol, equisetin, epi-equisetin, aurofusarin, apicidin, neosolaniol, culmorin, 5- & 15-hydroxyculmorin, bikerverin, butenolide, chlamydospordiol, chlamydosporol, chrysogin,

fusaric acid, fusapyron, deoxyfusapyron, 7-hydroxypestalotin, acuminatum (B and C) fusarinolic acid and fusarin C.

Monoacetoxyscirpenol, diacetoxyscirpenol, neosolaniol, enniatins (B and B_1,) were not detected in maize samples from the eastern regions but had low incidences of 2 %,3 %,2 %,3 % and 3 % respectively in the western region.

Table 4.4 Occurrence of mycotoxins according to agriculture regions

Regulated mycotoxins and toxins with guidance levels	**ER**			**WR**		
	Qty	**LOD**	**LOQ**	**Qty**	**LOD**	**LOQ**
Fumonisin B_1	100(58)	0(0)	0(0)	97(63)	3(2)	0(0)
Fumonisin B_2	95(55)	5(3)	0(0)	88(57)	12(8)	0(0)
Fumonisin B_3	76(44)	24(14)	0(0)	82(53)	17(11)	2(1)
Fumonisin B_4	67(39)	22(13)	10(6)	77(50)	14(9)	9(6)
Fumonisin A_1Vorstufe	22(13)	52(30)	26(15)	40(26)	42(27)	18(12)
Zearalenone	24(14)	67(39)	9(5)	29(19)	66(43)	5(3)
Deoxynivalenol	60(35)	40(23)	0(0)	40(26)	60(39)	0(0)
Nivalenol	7(4)	93(54)	0(0)	15(10)	85(55)	0(0)
T-2	2(1)	98(57)	0(0)	2(1)	98(64)	0(0)
HT-2	0(0)	100(58)	0(0)	2(1)	98(64)	0(0)
Masked Toxins						
DON-3-glucoside	38(22)	47(27)	16(9)	37(24)	48(31)	15(10)
Zearalenone-sulphate	16(9)	84(49)	0(0)	20(13)	80(52)	0(0)
Hydrolysed Fumonisin B_1	21(12)	79(46)	0(0)	29(19)	71(46)	0(0)
HT-2-glucoside	0(0)	100(58)	0(0)	2(1)	98(64)	0(0)
Emerging mycotoxins and other less well-known *Fusarium* metabolites						
Monoacetoxyscirpenol	0(0)	100(58)	0(0)	2(1)	98(64)	0(0)
Diacetoxyscirpenol	0(0)	100(58)	0(0)	3(2)	97(63)	0(0)
Neosolaniol	0(0)	100(58)	0(0)	2(1)	98(64)	0(0)
Culmorin	12(7)	88(51)	0(0)	17(11)	83(54)	0(0)
15Hydroxyculmorin	40(23)	59(34)	2(1)	32(21)	62(40)	6(4)
5Hydroxyculmorin	7(4)	79(46)	14(8)	3(2)	91(59)	6(4)
Moniliformin	97(56)	2(1)	2(1)	97(63)	3(2)	0(0)
Beauvericin	91(53)	3(2)	5(3)	74(48)	22(14)	5(3)
Enniatin B	0(0)	100(58)	0(0)	3(2)	97(63)	0(0)
Enniatin B_1	0(0)	100(58)	0(0)	3(2)	97(63)	0(0)
Enniatin B_2	0(0)	100(58)	0(0)	0(0)	98(64)	2(1)
Aurofusarin	78(45)	21(12)	2(1)	66(43)	34(22)	0(0)

Bikerverin	28(16)	34(20)	38(22)	35(23)	32(21)	32(21)
Butenolid	28(16)	62(36)	10(6)	11(7)	80(52)	9(6)
Epiquisetin	5(3)	88(51)	7(4)	12(8)	82(53)	6(4)
Equisetin	12(7)	76(44)	12(7)	17(11)	75(49)	8(5)
Apicidin	2(1)	98(57)	0(0)	2(1)	98(64)	0(0)
Deoxyfusapyron	7(4)	88(51)	5(3)	6(4)	75(49)	18(12)
Fusapyron	5(3)	76(44)	19(11)	20(13)	63(41)	17(11)
Fusaric acid	24(14)	76(44)	0(0)	15(10)	85(55)	0(0)
Fusarinolic acid	98(57)	2(1)	0(0)	97(63)	3(2)	0(0)
7-hydroxypestalotin	14(8)	86(50)	0(0)	25(16)	74(48)	2(1)
Acuminatum B	0(0)	93(54)	7(4)	8(5)	88(57)	5(3)
Acuminatum C	7(4)	93(54)	0(0)	5(3)	95(62)	0(0)

	ER			**WR**		
	Qty	**LOD**	**LOQ**	**Qty**	**LOD**	**LOQ**
Chlamydospordiol	2(1)	98(57)	0(0)	2(1)	98(64)	0(0)
Chlamydosporol	2(1)	98(57)	0(0)	2(1)	98(64)	0(0)
Chrysogin	9(5)	90(52)	2(1)	63(41)	35(23)	2(1)
Siccanol	74(43)	26(15)	0(0)	74(48)	26(17)	0(0)

AR-Agriculture region, ER- Eastern region, WR-Western region, Qty-percentage quantified, < LOD-percentage below limit of detection, <LOQ- percentage below limit of quantification, YM-Yellow maize, WM-White maize, LSD-Least significant difference

4.4.3 Percentage interaction between the agricultural region and maize type on the distribution of *Fusarium* mycotoxins on maize samples

The interaction between the agricultural region and maize type on the distribution of *Fusarium* mycotoxins on maize samples is presented in Table 4.5.

From the LC/MS-MS analyses, all (100 %) of the white maize (WM) samples from the eastern region (ER) were contaminated with the major mycotoxins, fumonisin B_1 and B_2 and also with the emerging mycotoxin, fusarinolic acid, while none (0 %) of the samples had the following toxins; HT-2, HT-2-glucoside, fusaric acid, and chlamydosporol. On the other hand, 100 % of the yellow maize samples from the same region also had fumonisin B_1. None of the yellow maize samples was contaminated with T-2, HT-2, HT-2-glucoside, apicidin, chlamydospordiol or chlamydosporal. Moreover, as presented earlier in section 4.4.1.1, none of the maize samples from the eastern region were contaminated with the metabolites; monoacetoxyscirpenol, diacetoxyscirpenol, neosolaniol and enniatins (B, B_1 and B_2), irrespective of the maize type.

The least contaminating mycotoxins of white maize samples from the eastern region was acuminatum B which was detected in 3 % of the samples but was not quantified because the values were below the limit of quantification. Other metabolites that occurred at low incident rates on the white maize samples from the eastern region include; nivalenol, T-2, apicidin, deoxyfusapyron and chlamydospordiol which occurred at 3 % each.

In addition, acuminatum B is the least contaminant mycotoxin on the yellow maize (YM) samples from the eastern region (ER) at 11 % but below the limit of quantification. Zearalenone, deoxynivalenol and nivalenol were detected in 30 %, 57 % and 3 % respectively of the white maize samples from the eastern region while they occurred in yellow maize of same region at a rate of 35 %, 64 % and 11 % respectively. The masked mycotoxin DON-3-glucoside was detected in 53 % and 54 % of the white and yellow maize samples from the eastern region.

In the western region, the white maize samples had high incidence rate of these mycotoxins; fumonisin (B_1 and B2) at 98 % and 93 % respectively while they were at 95 % and 76 % in the yellow maize. Zearalenone, deoxynivalenol and nivalenol were detected only in 39 %, 36 % and 16 % respectively on the white maize samples from the western region (WR) while they occurred in yellow maize of same region at the incidence rate of 24 %, 48 % and 14 % respectively. In the western region, the masked mycotoxin DON-3-glucoside occurred at a rate of 54 % and 48 % respectively of the white and yellow maize samples.

Furthermore, fusarinolic acid and moniliformin are the most dominant emerging toxins in the maize samples from the western region with incidence rate of 100 and 95 % on the white, 90 and 100 %, on the yellow maize samples respectively.

Table 4.5 Occurrence of mycotoxins in white and yellow maize from the agriculture regions

AR	**ER**						**WR**					
Maize type	**WM**			**YM**			**WM**			**YM**		
Regulated mycotoxins and toxins with guidance levels	**Qty (%)**	**<LOD (%)**	**<LOQ (%)**	**Qty (%)**	**<LOD (%)**	**<LOQ (%)**	**Qty (%)**	**<LOD (%)**	**<LOQ (%)**	**Qty (%)**	**<LOD (%)**	**<LOQ (%)**
Fumonisin B_1	100(30)	0 (0)	0 (0)	100(28)	0 (0)	0 (0)	98(43)	2 (1)	0 (0)	95(20)	5 (1)	0 (0)
Fumonisin B_2	100(30)	0 (0)	0 (0)	89(25)	11 (3)	0 (0)	93(41)	7 (3)	0 (0)	76(16)	24 (5)	0 (0)
Fumonisin B_3	83(25)	17 (5)	0 (0)	68(19)	32 (9)	0 (0)	86(38)	11(5)	2 (1)	71(15)	29 (6)	0 (0)

Fumonisin B_4	80(24)	10 (3)	10 (3)	54(15)	36 (10)	11(3)	82(36)	9 (4)	9 (4)	67(14)	24 (5)	10 (2)
Fumonisin A_1Vorstufe	33(10)	37(11)	30 (9)	11(3)	68 (19)	21(6)	41(18)	39(17)	20(9)	38 (8)	48 (10)	14 (3)
Zearalenone	27 (8)	70(21)	3 (1)	21 (6)	64 (18)	14(4)	32(14)	61(27)	7 (3)	24 (5)	76 (16)	0 (0)
Deoxynivalenol	57(17)	43 (13)	0 (0)	64(18)	36 (10)	0 (0)	36(16)	64(28)	0 (0)	48(10)	52 (11)	0 (0)
Nivalenol	3 (1)	97 (29)	0 (0)	11 (3)	89 (25)	0 (0)	16 (7)	84(37)	0 (0)	14 (3)	86 (18)	0 (0)
T-2	3 (1)	97 (29)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
HT-2	0 (0)	100(30)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
Masked Toxins												
DON-3-glucoside	33(10)	47(14)	20(6)	43(12)	46(13)	11(3)	34(19)	45(20)	20(9	43 (9)	52(11)	5 (1)
Zearalenone-Sulphate	23 (7)	77(23)	0 (0)	7(2)	93(26)	0(0)	16(7)	84(37)	0(0)	29(6)	71(15)	0(0)
Hydrolysed Fumonisin B_1	23 (7)	77 (23)	0 (0)	18 (5)	82 (23)	0 (0)	36(16)	64(28)	0 (0)	14 (3)	86 (18)	0 (0)
HT-2-glucoside	0 (0)	100(30)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
Emerging mycotoxins and other less well-known *Fusarium* metabolites												
Monoacetoxyscirpenol	0 (0)	100(30)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
Diacetoxyscirpenol	0 (0)	100(30)	0 (0)	0 (0)	100(28)	0 (0)	5 (2)	95(42)	0 (0)	0 (0)	100(21)	0 (0)
Neosolaniol	0 (0)	100(30)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
Culmorin	10 (3)	90(27)	0 (0)	14(4)	86(24)	0 (0)	9 (4)	91(40)	0 (0)	33 (7)	67 (14)	0 (0)
15Hydroxyculmorin	27 (8)	70 (21)	3 (1)	54(15)	46 13)	0 (0)	25(11)	68(30)	7 (3)	48(10)	48(10)	5 (1)
5Hydroxyculmorin	10 (3)	80 (24)	10(3)	4 (1)	79(22)	18(5)	0 (0)	93(41)	7 (3)	10 (2)	86 (18)	5 (1)
Moniliformin	97(29)	0 (0)	3 (1)	96(27)	4 (1)	0 (0)	95(42)	5 (2)	0 (0)	100(21)	0 (0)	0 (0)
Beauvericin	87(26)	3 (1)	10 (3)	96(27)	4 (1)	0 (0)	66(29)	30(13)	5 (2)	90(19)	5 (1)	5 (1)
Enniatin B	0 (0)	100 (30)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	5 (1)	95 (20)	0(0)
Enniatin B_1	0 (0)	100 (30)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	5 (1)	95 (20)	0 (0)
Enniatin B_2	0 (0)	100 (30)	0 (0)	0 (0)	100(28)	0 (0)	0 (0)	98(43)	2 (1)	0 (0)	100(21)	0 (0)
Aurofusarin	77(23)	23 (7)	0 (0)	79(22)	18 (5)	4 (1)	61(27)	39(17)	0 (0)	76(16)	24 (5)	0 (0)
Bikerverin	23 (7)	37 (11)	40(12)	32 (9)	32 (9)	36(10)	34(15)	36(16)	30(13)	38 (8)	24 (5)	38(8)
Butenolid	17 (5)	77 (23)	7 (2)	39(11)	46 (13)	14(4)	11 (5)	77(34)	11(5)	10(2)	86 (18)	5 (1)
Epiquisetin	3 (1)	87 (26)	10 (3)	7 (2)	89 (25)	4 (1)	16 (7)	80(35)	5 (2)	5 (1)	86 (18)	10 (2)
Equisetin	13 (4)	77 (23)	10 (3)	11 (3)	75 (21)	14(4)	20 (9)	73(32)	7 (3)	10 (2)	81 (17)	10 (2)
Apicidin	3 (1)	97 (29)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
Deoxyfusapyron	3 (1)	97 (29)	0 (0)	11 (3)	79 (22)	11(3)	5 (2)	75(33)	20(9)	10 (2)	76 (16)	14 (3)
Fusapyron	3 (1)	80 (24)	17 (5)	7 (2)	71 (20)	21(6)	18 (8)	64(28)	18(8)	24 (5)	62 (13)	14 (3)
Fusaric acid	0 (0)	100 (30)	0 (0)	50(14)	50 (14)	0 (0)	5 (2)	95(42)	0 (0)	38 (8)	62 (13)	0 (0)
Fusarinolic acid	100(30)	0 (0)	0 0)	96(27)	4 (1)	0 (0)	100(44)	0 (0)	0 (0)	90(19)	10 (2)	0 (0)
Hydrolysed Fumonisin B_1	23 (7)	77 (23)	0 (0)	18 (5)	82 (23)	0 (0)	36(16)	64(28)	0 (0)	14 (3)	86 (18)	0 (0)
7-hydroxypestalotin	20 (6)	80 (24)	0 (0)	7 (2)	93 (26)	0 (0)	20 (9)	80(35)	0 (0)	33 (7)	62 (13)	5 (1)
AcuminatumB	0 (0)	97 (29)	3 (1)	0 (0)	89 (25)	11(3)	5 (2)	89(39)	7 (3)	14 (3)	86 (18)	0 (0)
AcuminatumC	7 (2)	93 (28)	0 (0)	7 (2)	93 (26)	0 (0)	2 (1)	98(43)	0 (0)	10 (2)	90 (19)	0 (0)
Chlamydospordiol	3 (1)	97 (29)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
Chlamydosporal	0 (0)	100(30)	0 (0)	0 (0)	100(28)	0 (0)	2 (1)	98(43)	0 (0)	0 (0)	100(21)	0 (0)
Chrysogin	10 (3)	87 (26)	3 (1)	7 (2)	93 (26)	0 (0)	84(37)	14 (6)	2 (1)	19 (4)	81 (17)	0 (0)
Siccanol	73(22)	27 (8)	0 (0)	75(21)	25 (7)	0 (0)	77(34)	23(10)	0 (0)	67(14)	33 (7)	0 (0)

AR-Agriculture region, ER- Eastern region, WR-Western region, Qty-percentage quantified, < LOD-percentage below limit of detection, <LOQ- percentage below limit of quantification, YM-Yellow maize, WM-White maize, LSD-Least significant difference

4.4.4 Interaction between agricultural region and maize type on *Fusarium* mycotoxin accumulation on maize.

Table 4.6 shows the main influences of agricultural region on the concentration of *Fusarium* mycotoxins on maize samples; Table 4.7 shows the influence of maize type on the concentration of *Fusarium* mycotoxins on maize samples while the interaction between agricultural region and maize type on the concentration of *Fusarium* mycotoxins on maize samples is shown in Table 4.8

From the statistical analyses, the concentration of the major mycotoxins; fumonisin Bs (B_1, B_2, B_3, B_4) and fumonisin A_1vortuse in white maize samples collected from the western region(WR) was significantly higher ($p<0.05$) than that of yellow maize from the same region, white and yellow maize from the eastern region (ER). Deoxynivalenol concentration in yellow maize samples from the western region is significantly higher ($p<0.05$) than that of the white maize samples from the same region and with the white and yellow maize samples from the eastern region.

Zearalenone and nivalenol concentrations had no significant difference($p>0.05$) amongst white and yellow maize samples collected from the western region, as well as white and yellow maize from the eastern region.

The concentration of the masked mycotoxins DON-3-glucoside in yellow maize samples from the western region was significantly higher ($p<0.05$) than that of the white maize samples from the same region and also with the yellow maize samples from the eastern region. White maize samples from the eastern region have no significant difference ($p>0.05$) of DON-3-glucoside concentration with the yellow maize samples from the same region and with white maize samples from the western region.

There were no significant differences ($p>0.05$) of zearalenone sulphate concentrations amongst white and yellow maize samples collected from the western region, white and yellow maize from the eastern region. The concentration of hydrolysed fumonisin B_1 in white maize samples collected from the western region was significantly higher ($p<0.05$) than that of yellow maize from eastern region. Hydrolysed fumonisin B_1 in white maize samples collected from the

western region had no significant difference ($p>0.05$) with that of the yellow maize samples from the same region and also with the white maize samples from the eastern region.

On the emerging toxins, the concentration of 15-Hydroxyculmorin, moniliformin, aurofusarin and butenolid in yellow maize from the western region was significantly higher ($p<0.05$) than that of the white maize samples from the same region, and higher than that on white and yellow maize from the eastern region. Bikaverin, epiquisetin and equisetin concentrations in yellow maize samples from the eastern region was significantly higher ($p<0.05$) than that of the white maize samples from the same region and with white and yellow maize samples from the western region. The concentrations of deoxyfusapyron and fusaric acid in white maize samples collected from the western region (WR) was significantly higher ($p<0.05$) than that of yellow maize from the same region, as well as its concentrations white and yellow maize from the eastern region. Fusarinolic acid and chrysogin concentrations had no significant difference($p>0.05$) amongst white and yellow maize samples collected from the western region and white and yellow maize from the eastern region. Then, beauvericin, acuminatum B and C concentrations in yellow maize samples collected from the western region were significantly higher ($p<0.05$) than that of yellow and white maize from eastern region and had no significant difference($p>0.05$) with white maize samples from the western region.

Table 4.6 Occurrence of *Fusarium* mycotoxins on maize samples across the agriculture region

Regulated mycotoxins and toxins with guidance levels	**AR**		**LSD (<0.05)**
	ER(mean±SD)	**WR(mean±SD)**	
	Concentration (µg/kg)		
Fumonisin B_1	439.88±514	752.46±1469	287.98
Fumonisin B_2	151.16±513	290.08±188	110.35
Fumonisin B_3	62.17±161	108.93±173	34.55
Fumonisin B_4	55.63±49	107.58±181	37.2
Fumonisin A_1 Vorstufe	3.95±2	8.21±11	3.09
Fumonisin Total	**712.78**	**1267.26**	**n/a**
Zearalenone	15.4±37	11.77±17	12.47
Deoxynivalenol	86.0±135	219±348	96.8
Nivalenol	12.76±4	15.63±8	4.8
T-2	0	148.02±0	---
HT-2	0	31.16±0	---
Masked Toxins			

DON-3-glucoside	12.64±18	17.27±25	9.71
Zearalenone-sulphate	19.6±43	8.4±63	1.6
Hydrolysed Fumonisin B_1	2.81±2	5.03±7	2.21
HT-2-glucoside	0	31.16±0	---
Emerging mycotoxins and other less well-known *Fusarium* metabolites			
Monoacetoxyscirpenol	0	20.86±0	---
Diacetoxyscirpenol	0	4.72±0.3	---
Neosolaniol	0	4.84±0	---
Culmorin	110.7±143	69.4±93	71.75
15Hydroxyculmorin	128.85±109	232.77±449	134.61
5Hydroxyculmorin	84.89±27	249.13±247	164.89
Moniliformin	174.78±341	262.79±522	68.03
Beauvericin	3.4±7	11.0±26	6.54
Enniatin B	0	2.5±0	---
Enniatin B_1	0	1.6±0	---
Enniatin B_2	0	<LOQ	---
Aurofusarin	206.76±432	384.81±948	248.55
Bikaverin	105.9±16	38.13±16	53.61
Butenolid	29.37±12	68.38±69	30.17
Epiquisetin	6.63±8	3.33±2	2.92
Equisetin	27.48±43	11.26±11	20.46
Apicidin	15.58±0	2.87±0	---
Deoxyfusapyron	4.13±2	18.11±2	15.37
Fusapyron	4.13±6.64	18.11±4.80	15.37
Fusaric Acid	54.24±27	116.2±46	16.05
Fusarinolic Acid	511.26±341	501.43±521	113.9
7Hyroxypestalotin	6.89±3	8±4	1.95
AcuminatumB	0	65±74	67.1
AcuminatumC	110.9±63	86.7±42	32.4
Chlamydospordiol	2.13±0	5.12±0	---
Chlamydosporal	---	26.89±0	---
Chrysogin	4.7±1.60	4.07±2	1.42
Siccanol	5.2±5.1	7.6±6	1.76

AR-Agriculture region, ER- Eastern region, WR-Western region, Qty-percentage quantified, < LOD-percentage below limit of detection, <LOQ- percentage below limit of quantification, YM-Yellow maize, WM-White maize, LSD-Least significant difference, SD-Standard deviation, n/a-Not application

Table 4.7 Occurrence of *Fusarium* mycotoxins in white and yellow maize

Regulated mycotoxins and toxins with guidance levels	**Maize Type**		
	WM(mean±SD)	**YM(mean±SD)**	**LSD (<0.05)**
	Concentration (µg/kg)		
Fumonisin B_1	814.89±1370	377.46±504	287.98
Fumonisin B_2	288.6±169	152.64±568	110.35
Fumonisin B_3	113.26±54	57.84±49	34.55
Fumonisin B_4	105.81±167	57.4±59	37.20
Fumonisin A_1 Vorstufe	7.43±10	4.73±3	3.09
Fumonisin Total	**1329.99**	**650.07**	**n/a**
Zearalenone	12.67±30	14.49±21	12.47
Deoxynivalenol	96±144	209±333	96.80
Nivalenol	12.99±9	15.39±5	4.80
T-2	148.02±0	0	---
HT-2	31.16±0	0	---
Masked Toxins			
DON-3-glucoside	13.65±18	16.26±26	9.71
Zearalenone-sulphate	1.7±36	1.05±79	1.6
Hydrolysed Fumonisin B_1	5.16±7	2.68±3	2.21
HT-2-glucoside	31.16±0	0	---
Emerging mycotoxins and other less well-known *Fusarium* metabolites			
Monoacetoxyscirpenol	20.86±0	0	----
Diacetoxyscirpenol	4.72±0.30	0	----
Neosolaniol	4.84±0	0	----
Culmorin	42.6±26.19	137.5±136	71.75
15Hydroxyculmorin	117.01±115	244.61±413	134.61
5Hydroxyculmorin	139.04±16	194.98±239	164.89
Moniliformin	189.62±518	247.95±287	68.03
Beauvericin	5.1±19.84	9.3±18	6.24
Enniatin B	4.93±2.43	0.06±0	----
Enniatin B_1	2.98±1	0.13±0	----
Enniatin B_2	0	<LOQ	----
Aurofusarin	128.52±158	463.04±1077	24.55
Bikaverin	28.04±15	115.99±0	53.61
Butenolid	25.24±13	72.51±51	30.17
Epiquisetin	2.95±2	7±7.36	2.92
Equisetin	11±11	27.74±49	20.46
Apicidin	9.13±6	0	----
Deoxyfusapyron	16.73±23	5.52±2.10	15.37
Fusapyron	16.73±4.13	5.52±6.11	15.37

Fusaric Acid	66.5±26	103.94±40	16.05
Fusarinolic Acid	543.4±518	469.3±287	113.90
7Hyroxypestalotin	7.42±3	7.48±4	1.95
Acuminatum B	14±6	51±82	67.10
Acuminatum C	93.47±39	104.13±67	32.40
Chlamydospordiol	4.69±1	----	---
Chlamydosporal	26.89±0	-----	---
Chrysogin	4.58±2	4.18±2	1.42
Siccanol	6.2±6	6.7±6	1.76

AR-Agriculture region, ER- Eastern region, WR-Western region, Qty-percentage quantified, < LOD-percentage below limit of detection, <LOQ- percentage below limit of quantification, YM-Yellow maize, WM-White maize, LSD-Least significant difference, SD-Standard deviation, n/a-Not application

Table 4.8 Occurrence of *Fusarium* mycotoxins in white and yellow maize from the agriculture regions

	ER		**WR**		
Regulated mycotoxins and toxins with guidance levels	**WM(mean±SD)**	**YM(mean±SD)**	**WM(mean±SD)**	**YM(mean±SD)**	**LSD (<0.05)**
	Concentration (µg/kg)				
Fumonisin B_1	606.6±579	273.2±362	1023.2±1698	481.7±634	407.26
Fumonisin B_2	200.2±189	102.1±121	377.0±645	203.2±248	156.06
Fumonisin B_3	80.9±60	43.5±35	145.7±197	7.2±59	4.87
Fumonisin B_4	71.6±53	39.7±32	140.1±207	75.1±74	52.61
Fumonisin A_1 Vorstufe	4.2±2	3.7±1	10.7±12	5.8±3	4.37
Zearalenone	21.0±47	9.8±13	4.4±9	19.2±27	17.64
Deoxynivalenol	121.0±186	51.0±35	71.0±71	367.0±503	136.80
Nivalenol	10.2±0	15.3±4	15.8±9	15.5±6	6.79
T-2	0.0±0	0	148.0±0	0	---
HT-2	0	0	31.2±0	0	---
Masked Toxins					
DON-3-glucoside	18.6±25	6.7±4	8.7±11	25.9±36	13.73
Zearalenone-Sulphate	2.78±48	1.14±97	7.36±54	9.5±71	22.30
Hydrolysed Fumonisin B_1	3.8±3	1.8±1	6.5±7	3.6±4	3.13
HT-2-glucoside	0	0	31.2±0	0	---
Emerging mycotoxins and other less well-					

known *Fusarium* metabolites					
Monoacetoxyscirpenol	0	0	20.9±0	0	---
Diacetoxyscirpenol	0	0	4.7±1	0	---
Neosolaniol	0	0	4.8±0	0	---
Culmorin	62.4±25	159.0±177	22.7±7	116.0±102	101.47
15Hydroxyculmorin	178.5±148	79.2±55	55.5±21	410.1±597	190.36
5Hydroxyculmorin	111.1±16	58.7±349	167.1±0	331.3±0	233.20
Moniliformin	184.3±178	165.3±144	195.0±227	330.6±327	96.21
Beauvericine	2.3±5	4.4±8	7.8±27	14.2±26	9.24
Enniatin B	0	0	5.0±0	0.1±0	---
Enniatin B_1	0	0	2.98	0.13	---
Enniatin B_2	0	0	<LOQ	<LOQ	---
Aurofusarin	130.2±188	283.3±577	126.9±127	642.8±1490	351.51
Bikaverin	56.1±0	155.72±0	0	76.3±16	75.81
Butenolid	31.2±14	27.6±11	19.3±9	117.5±97	42.67
Epiquisetin	1.5±0	11.8±7	4.4±2	2.2±0	4.13
Equisetin	4.2±2	50.7±56	17.8±10	4.7±1	28.94
Apicidin	15.4±0	0	2.9±0	0	---
Deoxyfusapyron	3.3±0	5.0±2	30.1±23	6.1±3	21.74
Fusapyron	3.3±0	5.0±8	30.1±4	6.1±5	21.74
Fusaric Acid	0	108.5±46	133.0±26	99.4±22	22.70
Fusarinolic Acid	529.6±387	493.0±281	557.3±590	445.6±293	161.10
Hydrolysed Fumonisin B_1	3.8±3	1.8±1	6.5±7	3.6±4	3.13
7Hydroxypestalotin	8.5±4	5.8±1	6.8±3	9.2±4	2.76
Acuminatum B	0	0	27±6	103±82	94.90
Acuminatum C	58.4±25	163.5±41	128.6±0	44.8±18	45.80
Chlamydospordiol	2.1±0	---	5.1±0	---	---
Chlamydosporal	---	---	26.9±0	---	---
Chrysogin	5.6±6	3.8±2	3.6 ±2	4.5±2	2.01
Siccanol	62.0±51.2	42.3±26.2	61.7±59.3	91.0±55.3	24.9

AR-Agriculture region, ER- Eastern region, WR-Western region, Qty-percentage quantified, < LOD-percentage below limit of detection, <LOQ- percentage below limit of quantification, YM-Yellow maize, WM-White maize, LSD-Least significant difference, SD- Standard deviation

4.4.5 Comparison of LC-MS/MS, HPLC and ELISA for fumonisins and zearalenone

The summary results of LC-MS/MS, HPLC and ELISA for fumonisins and zearalenone are shown in Figure 4.6 and Table 4.9

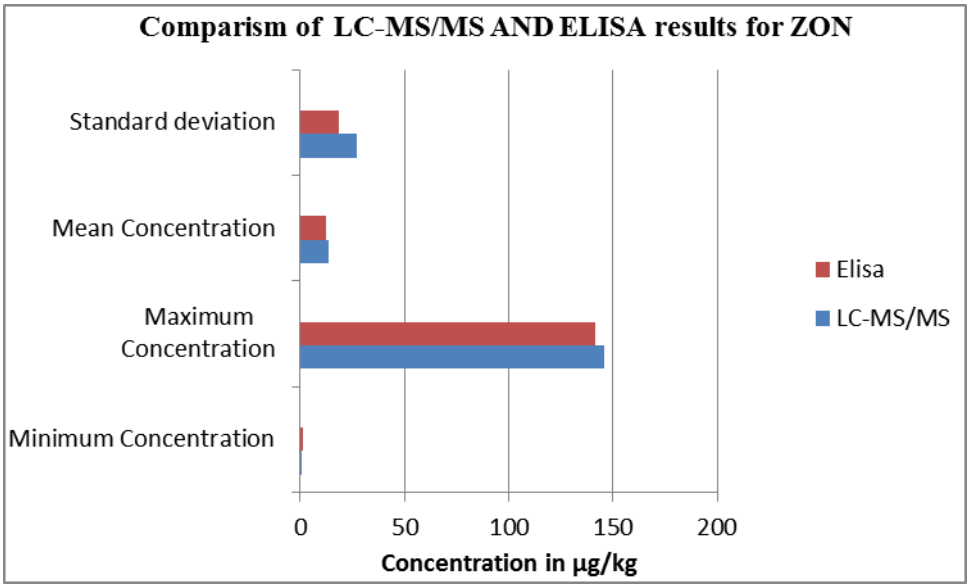


Figure 4.6 Comparison of LC-MS/MS and ELISA results for zearalenone

Table 4.9 LC-MS/MS versus HPLC for fumonisins and zearalenone analyses

PARAMETERS	**LC-MS/MS (µg/kg)**			**HPLC(µg/kg)**		
	FB_1	**FB_2**	**ZON**	**FB_1**	**FB_2**	**ZON**
Minimum Concentration	12.6	7.9	<LOQ	28.8	12.4	0.2
Maximum Concentration	8907.7	3383.3	145.6	7566.7	2639.0	90.5
Mean Concentration	596.2	220.6	13.6	572.5	186.4	12.1
Standard deviation	578.9	248.1	27.03	514.0	169.1	23.4

4.5 Dietary exposure and risk assessment for mycotoxins in adult maize consumers from agriculture regions of South Africa.

The results of the dietary exposure and risk assessment for mycotoxins in adult maize consumers from agriculture regions of South Africa are shown in Table 4.10

Only DON (2000 µg/kg), FB_1 and FB_2 (4000 µg/kg) are the regulated *Fusarium* mycotoxins in South Africa. Exposure assessments calculated through maize intake for deoxynivalenol (DON), fumonisin B_1 and B_2 across the AR showed that probable daily intake (PDI) for DON is below the maximum limit of 2000 µg/kg across the ARs. The PDI for sum of fumonisin B_1 and fumonisin B_2 in the WR was above the maximum limit of 4000 µg/kg in South African regulation while that in the ER is below the maximum limit.

Table 4.10: Risk assessment of dietary exposure to mycotoxins in adult maize consumers from agriculture regions of South Africa This was calculated as described in section 3.5 above.

AR	DON (µg/kg)	PDI(µg/kg.bw/day)	FB_1 +FB_2 (µg/kg)	PDI (µg/kg)
ER	86	355.47	439.88+151.16	2442.96
WR	219	905.20	752.46+290.08	4309.17
National	152	628.27	596.2+220.6	3376.11
Maximum limit	2000	2000	4000	4000

Chapter Five

General Discussion

5.1 Contamination of maize samples with *Fusarium* species in Eastern and Western maize regions of South Africa.

The *Fusarium* species has been one of the most economically important groups of fungi responsible for contaminating cereal grains and are a worldwide concern. Their significance does not only rest on their pathogenicity to both plants and animals, but on their ability to produce some toxic secondary metabolites, mycotoxins, e.g., the fumonisins, zearalenones and the trichothescences (Bottalico *et al*., 2002; Logrieco *et al*., 2003) in agricultural commodities. These *Fusarium* mycotoxins have attracted worldwide attention because of their impact on human and animal health, animal productivity and the associated economic losses (Gitu, 2006). Consequently, worldwide attention is given to investigating *Fusarium* species and elaborating means of controlling them. In South Africa, there have been reports of contamination of maize samples with *Fusarium* species (Gelderblom *et al*., 1988; Ncube *et al*., 2011; Phoku *et al*., 2012; Ekwomadu *et al*., 2018). Mycological analyses of the maize samples in this study revealed that different F*usarium* species contaminated the maize samples. The findings are in line with other studies done in South Africa (Ncube *et al*., 2011; Phoku *et al*., 2012; Janse van Rensburg, 2015) and elsewhere (Aguin *et al*., 2014 ; Goertz *et al*., 2010). Seven *Fusarium* species found to contaminate the maize in both the western and eastern regions were *Fusarium verticiloides, Fusarium oxysporum, Fusarium subglitans, Fusarium proliferatum, Fusarium napiforme, Fusarium fujikuroi* and *Fusarium graminearum* with total incidence rate of 96 %, 84 %, 66 %, 83 %, 25 %, 24 % and 34 %, respectively. All *Fusarium* species isolated from the maize samples have previously been described on maize grown in South Africa (Ncube *et al*., 2011; Phoku *et al*., 2012; Janse van Rensburg, 2012) . *Fusarium verticiloides* was the predominant *Fusarium* species irrespective of the agricultural regions as summarized in Table 4.2. This is in agreement with previous reports that *Fusarium verticillioides* is the dominant fungal species in South African maize (Marasas, 2001; Ncube *et al*., 2011; Chilaka *et al*., 2012a) and maize from other parts of Africa and the world (van der Westhuizen *et al*., 2003; Desjardins, 2006; Adetunji *et al*., 2014; Egbuta *et al.,* 2015). Growth of *Fusarium verticillioides* suppresses relatively that of *Fusarium graminearum* and *Fusarium subglutinans* (Rheeder *et al.,* 1990a; Reid *et al*., 1999). This could explain the relatively low percentage incidence of *Fusarium graminearum* (34 %) and *Fusarium subglutinans* (66 %) in the analysed samples. Mycological analyses also showed that most of the samples were contaminated with at least one fungal species, whereas co-contamination with different *Fusarium* fungal species

occurred in most of the samples. *Fusarium verticiloides* occurred in 96 % (118) of all the samples with 51 % (63 samples) from western region (WR) and 45 % (55 samples) from eastern regions (ER) being contaminated. The predominance of *Fusarium verticilloides* is generally associated with warm, dry climates (Shephard *et al*., 1996; Fandohan *et al*., 2003; Ncube *et al*., 2011) as the case is in western region (WR) which consists of the Northwest province and western Free State, where the average temperature ranged from 29 °C to 32 °C (www.agis.agric.za/climate).

In addition, the effect of the geographical location on the variability of occurrence of *Fusarium verticillioides* is important when climatic conditions vary across locations (Boutigny *et al*., 2012). Samples from the western region had the least contamination with *Fusarium napiforme* having only 16 samples contaminated. Likewise, samples from the eastern regions had the least contamination with 11 samples being contaminated with *Fusarium fujikuroi.* Finally, of all the *Fusarium* species isolated, *Fusarium subglitans* and *Fusarium graminearum* occurred predominantly in the eastern region, while the rest of the contaminants occurred higher in western region as observed by Janse van Rensburg *et al*., (2015).

Most *Fusarium* species are plant pathogens which can invade plant tissues and developing seeds in the fields (Mills, 1989; Pitt and Hocking, 1997). It was reported that late harvesting usually favours the growth of field fungi such as *Fusarium* species. Some are able to persist and grow during storage when moisture content becomes favourable (Pitt and Hocking, 1997; Mwanza *et al.,* 2009). However, the level of fungal contamination does not always correlate with the level of mycotoxin present but indicates the risk involved. Low levels of fungi normally indicate a relatively low level of mycotoxin but high contamination only increases the possibility of mycotoxin to be present, whether at low or high levels (Dawlal, 2010).

5.1.1 Screening of *Fusarium* isolates for the presence of mycotoxin biosynthetic genes.

Screening for the presence of *Fum13, Tri 6* and *Zea13* genes in the *Fusarium* isolates, which determine *Fusarium* mycotoxins production showed that the isolates have the requisite genes as shown in Figures 4.3-4.5. PCR assays for *Fum13, Tri 6* and *Zea13* genes were used to predict whether these isolates could produce fumonisins, trichothecenes and zearalenone respectively. PCR amplification of these genes is a useful tool for the fast detection of mycotoxin producing *Fusarium* isolates. The toxigenic *Fusarium* isolates could be detected for fumonisin, trichothecene and zearalenone producing *Fusarium* with sensitivity and specificity (Dawidziuk

et al. 2014). The PCR assays give qualitative, rather than functional information, thus reflecting the presence of a sequence or a gene but not necessarily its expression. (Niessen & Vogel 2010; Dawidziuk *et al*., 2014).

The genes responsible for the production of the three major mycotoxins were detected. This could serve as a simple and quick technique with potential application for on-site disease detection and field surveys. A diagnostic method that is rapid, accurate, and simple could help in the control of toxigenic fungi and reduction of mycotoxins produced by *Fusarium* species in various agricultural commodities (Abd-Elsalam *et al*., 2016). The results demonstrate a reliable detection of toxigenic potential for fumonisins, trichothecenes, and zearalenone. The species-specific PCR markers for key biosynthetic genes provide a sensitive detection of toxigenic fungal isolates. Although no quantitative assay of the mycotoxins accumulation was conducted, the genes present in the isolates are similar to the model counterparts (Dawidziuk *et al*., 2014). Thus, the method can easily be adapted as early warning against mycotoxin contamination allowing much more effective application of fungicides and can serve as a supplement to conventional mycotoxin detection techniques. It can also give support to farmers for instance in the appropriate and rational use of fungicide treatments in the field (Dawidziuk *et al*., 2014).

5.2 Mycotoxins occurrence

5.2.1General mycotoxin contamination pattern

Mycotoxins produced by *Fusarium* species are found on grains cultivated on every continent. The susceptibility of maize to *Fusarium* fungi and subsequent mycotoxin contamination is well documented (Marasas, 2001; Fandohan, 2003). All the maize samples analysed in this study were contaminated with an average of 5 to 24 out of 42 *Fusarium* mycotoxins, including 0 to 3 masked forms at the same time. The summary data in Table 4.3 highlights the relevance of fumonisin B_1, B_2, B_3, B_4 and A_1 Vorstufe in the samples with 98 %, 91 %, 80 %, 82 % and 54 % of 123 samples contaminated with maximum contamination levels of 8907.7, 3383.3, 990.4, 1014.4 and 51.5 µg/kg respectively. Fumonisin B_1 was the most common mycotoxin in the maize samples. These results are in agreement with the ones obtained in the mycological analysis, in which, *Fusarium verticilloides* were found in high contamination in the same analysed samples. *Fusarium verticilloides* is one of the known, prolific fumonisin-producing *Fusarium* species (Shephard *et al.*, 1996). The occurrence of fumonisins (FBs) has been positively correlated with *Fusarium* species contamination of maize produced in South Africa

(Waalwijk *et al*., 2008a). Jansen van Rensburg (2015) also reported that *Fusarium* species are common contaminant in South African commercial maize, especially in areas such as the western region. Fumonisins from *Fusarium* species commonly occur in maize while in the field, predominantly when they are cultivated in warmer regions (Bhat *et al*., 2010).

Also, not all the *Fusarium* species have the ability to produce fumonisin, that ability seems to be lacking from all the trichothecene producing *Fusarium* species (Desjardins, 2006). Contamination of maize by fumonisins was considered an important risk factor in human oesophageal cancer in the former Transkei region of South Africa and Santa Catarina State, Brazil (Peraica *et al*., 1999; Bennett and Klich, 2003). Fumonisin B_1 has also been implicated in the development of neural tube defects in babies of mothers consuming fumonisin-contaminated maize, especially in certain regions of South Africa, China and Italy (Bhat and Vasanthi, 2003; Marasas *et al*., 2004).

Deoxynivalenol occurred in 50 % of the samples with a mean concentration of 152 µg/kg (max 1380 µg/kg). The trichothescene, type B toxin, DON, causes different unspecific symptoms, for example vomiting and diarrhoea, also causes loss of weight and feed refusal in livestock, and hence is known as vomitoxin (Da Rocha *et al*., 2014). The presence of DON in maize has often been associated with samples originating from temperate regions such as northern Europe and North America (Perkowski *et al*., 1990). However, reports emerging from tropical countries, specifically from South Africa, continue to reveal the occurrence of DON in maize and maize products (Shephard *et al*., 2010). It has also been reported at a lower concentration than in this study on maize from Burkina Faso at a mean of 31.4 µg/kg, Mozambique at a range of 116–124 µg/ kg (Warth *et al*. 2012a) and in Nigerian maize at a range of 11–479 µg/kg (Adetunji *et al*., 2014) and at a mean of 225 µg/kg (Chilaka *et al*., 2016).

Furthermore, according to Ediage *et al*., (2013), DON was detected in the urine of toddlers (1.5−5 years) from Cameroon, which also affirms its presence in food commodities originating from sub-Saharan Africa. However, although half of the analysed samples contained DON, the maximum level at 1380 µg/kg did not exceed the maximum allowable levels for DON in unprocessed maize as was set by South African Regulation in 2016 at 2000 µg/kg.

Forty-one samples were contaminated with zearalenone at a mean concentration of 13.6 µg/kg. The occurrence of ZON in agricultural commodities has not been thoroughly investigated in sub-Saharan Africa. It was first reported on South African maize and subsequently recovered from maize and other commodities elsewhere on the continent (Placinta *et al*., 1999). The maize samples analysed in this study showed a 33 % occurrence rate for ZON, with none of the samples exceeding the maximum level of 350 µg/kg for unprocessed maize products

according to European commission (EC., 2006). Similarlarly, it was also reported by Boutigny *et al*., (2012) at 33 % occurrence in naturally infected field-grown maize samples. The detected maximum concentration from the samples was 145.6 µg/kg (Table 4.3). Compared to other reports on the occurrence of ZON in maize samples from other parts of Africa, the levels detected in this study were lower than levels earlier reported by Adejumo *et al.,* (2007) in maize samples from Nigeria. Zearalenone was also reported in maize samples from Cameroon with a mean concentration of 68 µg/kg and a maximum concentration of 309 µg/kg (Abia *et al.,* 2014). However, the relatively low level of ZON observed in this study somewhat supports the notion that ZON is perhaps a persistent yet minor contaminant of foods/ feeds in South Africa (Changwa *et al.,* 2018 ; Placinta *et al.,* 1999), but its significance is in its oestrogenic potential to mammals.

Occurrence of HT-2 and T-2 in the samples was at a very low level at 0.8 % (1 sample) at maximum concentration of 40.2 µg/kg and 148.0 µg/kg respectively. Similarly, South African Grain Laboratory (SAGL) reported detection of HT-2 and T-2 only from one maize sample, although at higher levels of 72 and 232 µg/kg respectively (SAGL maize quality report, 2013). The EU permissible levels of mycotoxins by the European Commission have recommended the limit of 15–1000 µg/kg for the sum of HT-2 and T-2 toxins in various matrices (EU, 2013). The detected maximum concentration of HT-2 and T-2 from the samples did not exceed the recommended limit. Meanwhile, HT-2 toxin and T-2 are two related compounds that may be synthesised by several *Fusarium* species. Their presence in cereal grain has been well documented, with several reports originating from the cold climate of Northern Europe (Van Der Fels-Klerx, *et al.,* 2014; Nordkvist and Häggblom, 2014). Nevertheless, few reports from Africa indicated; 1–8 % incidence rates for HT-2 in Nigerian cereals (Chilaka *et al*., 2016) and 25 % rate in Tanzanian maize for HT-2, with range of 15–25 ppb (Kamala *et al.*, 2015). Also, South African Grain Laboratory (SAGL) reported detection of HT-2 and T-2 only in one maize sample, with the levels 72 and 232 µg/kg respectively (SAGL maize quality report, 2013).
Nivalenol occurred only in 11 % of the samples at a contamination range of 7.7-35.7 µg/kg. Occurrence of NIV in maize has previously been reported in Nigerian samples, although at a higher incidence rate (54 %) (Adetunji *et al*., 2014) and higher contamination range of 163–271 µg/kg (Chilaka *et al*., 2016). Nivalenol is known to be immunosuppressive and also as a protein inhibitor.

Of the masked mycotoxins, DON-3-glucoside occurred at highest incidence rate of 53% than the other masked forms detected in this study. Among emerging toxins, moniliformin, fusarinolic acid and beauvericin showed high occurrence being found in 98 %, 98 % and 83 %

of samples, respectively. High incidences of these toxins in maize which serve as a staple food in South Africa is an important cause for concern. From available literature, little or no appreciable study has been done on the occurrence of these mycotoxins in food and food products in South Africa and neglecting them increases the risk of exposure to humans and animals. The occurrences of these emerging mycotoxins produced by *Fusarium* specie, have been reported in food crops which represents an important problem in some parts of the world (Kosiak *et al*., 2003; Jestoi *et al*., 2004)

Furthermore, the high incidence of these emerging toxins should not be taken for granted, since moniliformin is known to be cytotoxic to many mammalian systems (Jestoi, 2008). Beauvericin is also known to be genotoxic to human lymphocytes (Celik *et al.* 2010).

5.2.2 Percentage distribution of *Fusarium* (free and masked) mycotoxins across the Agriculture Regions of South Africa

Mycotoxins are among the food-borne risks that are dependent upon climatic conditions. Indeed, the ability of fungi to produce mycotoxins is largely influenced by temperature, relative humidity, insect attack, and stress conditions of the plants (Miraglia *et al.*, 2009). The main effect due to Agriculture Regions (AR) on the distribution of *Fusarium* free and masked mycotoxins in the maize samples is presented in Table 4.3.

All the 42 *Fusarium* toxins and metabolites investigated in the maize samples across the agricultural regions were detected and quantified except for the emerging toxin, enniantin B_2 which was only detected in 2 % of the samples from the western region but was not quantified because the values were below the limit of quantification (<LOQ). Of the major mycotoxins, HT-2 was not detected at all in the eastern region but was quantified only in 2 % (1 sample) of the maize from the western region at 40.2 μg/kg concentration. Detection of HT-2 in the warm western region and not the cold eastern region as would be expected is contrary as it was reported that HT-2 was previously considered a problem in colder European climates (Bryla *et al.,* 2018). Of the fumonisin Bs, FB_1 occurred at the most frequency rate than FB_2, FB_3 and FB_4. Fumonisin B_1 was the most contaminant mycotoxin, occurring at a mean concentration of 752.46±1469 μg/kg from the warm western region and of 439.88±514 μg/kg in the cold eastern region with only 3 % (2 samples) not contaminated. A similar trend was also observed when the sum of the fumonisins was considered, for instance the highest fumonisin (FB_1+FB_2+FB_3) concentrations were detected in warm western region and this is consistent with Munkvold (2003) and De La Campa *et al*. (2005) who reported optimum temperatures of 30 °C and 32 °C for fumonisin production. This observation could be correlated to the high mycotoxins

production potentials of *Fusarium* fungi in warmer climates (Shepherd *et al*., 1996). In general, fumonisin B_1 was detected in 98 % of all the samples analysed, irrespective of the maize type and AR, ranging from 12.6 to 8907.7 μg/kg with the mean concentration of 596.2 μg/kg (Table 4.3).

Fumonisin B_2 was the second most contaminant mycotoxin, across the ARs and generally, fumonisin A_1Vorstufe was quantified in 32 % of all the maize samples analysed and in 22 % of the samples, it was detected but below the limit of quantification (<LOQ).

Deoxynivalenol was more prevalent in the eastern region than in the western region having 60 % and 40 % of the samples all detected and quantified respectively (Table 4.4). This was in line with what was reported by Chilaka *et al*., (2016) who observed the highest incidence rate of DON in maize samples from DS zone in Nigeria, (an agricultural region) characterized by a lower temperature and higher average annual. Zearalenone, nivalenol, HT-2 and T-2 were also among the major *Fusarium* mycotoxins, detected and quantified in the two regions. Nivalenol, HT-2 and T-2 were detected in higher concentration in the WR while ZON occurred at lower concentration in the same region, occurring higher in the ER. This observation could be because ZON is known to be produced in somewhat cool environments compared to other mycotoxins (Krska *et al.*, 2015). The highly toxic mycotoxin, T-2 toxin, has until recently not been recorded in South Africa. T-2 toxin is most commonly produced by *F. sporotrichioides*, a fungus well-adapted to survive in colder countries (Leslie and Summerell., 2006). Some T-2-producing *Fusarium* species, have occasionally been isolated from wheat with *Fusarium* head blight (FHB) and maize with *Fusarium* ear rot (FER) symptoms in South Africa (Beukes *et al*., 2017). The presence of T-2 toxin in local maize grain, was reported by the SAGL, (SAGL, 2013), and it has been shown to have a relationship with *F. verticillioides* and *F. graminearum* (Patience, 2010; Matny, 2014).

Masked mycotoxins detected in the samples include DON-3-glucoside, zearalenone-sulphate, hydrolysed fumonisin B_1 and HT-2-glucoside. Deoxynivalenol-3-glucoside (DON-3-G) and HT-2-glucoside had the highest and least occurrence respectively across the two regions. Although there was no significant differences in their distribution across the agriculture regions, but to our knowledge, there seem to be no available data on the occurrence of some of these masked mycotoxins in South Africa. Hence, this is the first report of zearalenone-sulphate and HT-2-glucoside in South African maize. Although toxicological data are still limited, but the occurrence of masked mycotoxins is expected to add substantially to the overall mycotoxins load and toxicity. This invariably will increase the toxic health effects by these masked

mycotoxins, which may be either direct or indirect through hydrolysis, or released from the matrix during digestion into the free mycotoxins (De Boevre *et al*., 2015).

Other detected metabolites included emerging mycotoxins and other less well-known *Fusarium* metabolites such as beauvericin, enniatins (B, B_1, and B_2), moniliformin, monoacetoxyscirpenol, diacetoxyscirpenol, equisetin, epi-equisetin, aurofusarin, apicidin, neosolaniol, culmorin, 5- & 15-hydroxyculmorin, bikerverin, butenolide, chlamydospordiol, chlamydosporol, chrysogin, fusaric acid, fusapyron, deoxyfusapyron, 7-hydroxypestalotin, acuminatum (B and C) fusarinolic acid and fusarin C . The co-occurrence of these emerging mycotoxins with other major mycotoxins and many other *Fusarium* metabolites of unknown toxicity is a source of concern. Beauvericin like enniatins is a cyclodepsipeptide that has antibiotic, insecticidal, and cytotoxic properties presumably related to their ionophoric properties (Juan *et al*. 2013). Moreover, monoacetoxyscirpenol, diacetoxyscirpenol, neosolaniol, enniatins (B and B_1,) were not detected in maize samples from the eastern regions but had low incidences of 2 %,3 %,2 %,3 % and 3 % respectively in the western region. This could be explained by the agroclimatic differences in the agriculture regions that favoured the accumulation of these emerging mycotoxins in the warmer and drier climate of the western maize regions.

5.2.3 Effect of maize type on *Fusarium* mycotoxins accumulation on maize samples

Agriculture is affcted by the main climatic factors (temperature, precipitation, drought, and atmospheric carbon dioxide) that are slowly but surely significantly affecting the quality of grains produced**.** The main effect of maize type on *Fusarium* free and masked mycotoxins accumulation on the maize samples is presented in Table 4.4

Statistical analyses revealed that all the *Fusarium* major toxins and the masked forms (fumonisin B_1, B_2, B_3, B_4, A_1 Vorstufe,T-2, HT-2, ZON-S, HFB_1 and HT-2G) investigated in this study were detected in higher concentration in the white maize except for ZON, DON, NIV and DON-3G which occurred higher in the yellow maize (Table 4.4)**.** Then, on the emerging mycotoxins and other less well-known *Fusarium* metabolites, monoacetoxyscirpenol, diacetoxyscirpenol, neosolaniol, enniatin (B, B_1, B_2), apicidin, deoxyfusapyron, fusapyron, fusarinolic acid, chlamydospordiol, chlamydosporal and chrysogin occurred in higher concentration in the white maize samples. On the other hand, culmorin, 5- & 15-hydroxyculmorin, moniliformin, beauvericin, aurofusarin, bikaverin, butenolid, epiquisetin, equisetin, fusaric acid, 7Hyroxypestalotin, acuminatum (B and C) and siccanol were

predominant in the yellow maize variety. The occurrence of these *Fusarium* mycotoxins is a cause for concern as it increases the risk of exposure of humans and animals to mycotoxin toxicity because of the high incidence and concentration in cereals and cereal-based products, which serve as staple foods in South Africa.

5.2.4 Effect of agricultural region and maize type on *Fusarium* mycotoxin distribution and accumulation on maize.

Mycotoxin occurrence and distribution is influenced by different factors such as crop species, climatic, and environmental conditions of a given region. Table 4.6 shows the main effects of agricultural region on the concentration of *Fusarium* mycotoxins on maize samples; Table 4.7 shows the influence of maize type on the concentration of *Fusarium* mycotoxins on maize samples while the interaction between agricultural region and maize type on the concentration of *Fusarium* mycotoxins on maize samples is presented in Table 4.8

From the statistical analyses, the concentration of the major mycotoxins; fumonisin Bs (B_1, B_2, B_3, B_4) and fumonisin A_1vortuse in white maize samples collected from the western region(WR) was significantly higher ($p<0.05$) than that of yellow maize from the same region, as well as on white and yellow maize from the eastern region (ER). The mean values were 1023.2±1698, 377.0±645, 145.7±197, 140.1±207 and 10.7±12 µg/kg respectively. The fact that the white maize samples of the western region(WR) had significantly higher mean levels of fumonisin can be explained partly by high mycotoxins production potentials of *Fusarium* fungi in warmer climates as reported by (Shepherd *et al*., 1996). Then, Munkvold (2003) and De La Campa *et al*. (2005) also reported optimum temperatures of 30 °C and 32 °C for fumonisin production, which is what is obtaining in the western region. Furthermore, differences in plant genotype cannot be ruled out as it has been reported that white maize is a better substrate for fumonisin production than yellow maize (Rheeder *et al.*, 1995).

Deoxynivalenol concentration in yellow maize samples from the western region was significantly higher ($p<0.05$) than that of the white maize samples from the same region and on the white and yellow maize samples from the eastern region, with the mean value being; 367.0±503 µg/kg (Table 4.8). Occurrence of DON at significantly higher concentration in the western region could be explained by high mycotoxins production potentials of *Fusarium* fungi in warmer climates as reported by (Shepherd *et al*., 1996). Then, the higher DON concentration in yellow maize than the white maize could be due to variation in localization of pigments in

yellow maize kernel, which might have a prominent role in the actual degree of resistance to *Fusarium* infection/mycotoxin accumulation (Bernardi *et al*., 2018).

Zearalenone and nivalenol concentrations showed no significant difference($p>0.05$) amongst white and yellow maize samples collected from the western region, as well as white and yellow maize from the eastern region. In addition, white maize from the eastern region has the highest, mean concentration of 21.0±47 µg/kg. This is in line with the finding of Ediage *et al*., (2014), who noted no significant difference in zearalenone accumulation in maize samples from Cameroon irrespective of geographical location.

The concentration of the masked mycotoxins DON-3-glucoside in yellow maize samples from the western region was significantly higher ($p<0.05$) than that of the white maize samples from the same region and with the yellow maize samples from the eastern region. A similar trend was also observed with the parent toxin, deoxynivalenol in this study. This can also have the same explanation as is the case with DON accumulation.

DON-3-glucoside concentration in white maize samples from the eastern region had no statistically significant difference ($p>0.05$) with the yellow maize samples from the same region and with white maize samples from the western region. In addition, yellow maize from the western region had the highest concentration with mean value of 25.9±36 µg/kg

Zearalenone sulphate concentrations had no significant differences ($p>0.05$) amongst white and yellow maize samples collected from the western region, white and yellow maize from the eastern region. A similar trend was also observed with the parent mycotoxin, zearalenone in this study.

The concentration of hydrolysed fumonisin B_1 in white maize samples collected from the western region was significantly higher ($p<0.05$) than that of yellow maize from eastern region. The same trend was observed with other fumonisin parent mycotoxins. Also, hydrolysed fumonisin B_1 in white maize samples collected from the western region had no significant difference ($p>0.05$) with that of the yellow maize samples from the same region and also with the white maize samples from the eastern region. HT-2-glucoside occurred only on white maize from the western region at mean contamination level of 31.2±0 µg/kg

Of the emerging toxins, the concentration of 15-Hydroxyculmorin, moniliformin, aurofusarin and butenolid on yellow maize from the western region was significantly higher ($p<0.05$) than that of the white maize samples from the same region, as well as white and yellow maize from

the eastern region. The mean values were 410.1±597, 330.6±327, 642.8±1490, 117.5±97 µg/kg respectively. Bikaverin, epiquisetin and equisetin concentrations in yellow maize samples from the eastern region was significantly higher ($p<0.05$) than that of the white maize samples from the same region and with white and yellow maize samples from the western region. The concentrations of deoxyfusapyron and fusaric acid in white maize samples collected from the western region (WR) is significantly higher ($p<0.05$) than that of yellow maize from the same region, white and yellow maize from the eastern region. Fusarinolic acid and chrysogin concentrations had no significant difference ($p>0.05$) amongst white and yellow maize samples collected from the western region, white and yellow maize from the eastern region. Then, beauvericin and acuminatum B concentrations in yellow maize samples collected from the western region is significantly higher ($p<0.05$) than that of yellow and white maize from eastern region and had no significant difference ($p>0.05$) with white maize samples from the western region.

5.3 Comparison of methods of analysis for selected mycotoxins (LC-MS/MS, HPLC and ELISA)

5.3.1 LC-MS/MS versus HPLC for fumonisins and zearalenone analyses

From the obtained results (Table 4.9), the multimycotoxin LC-MS/MS analyses gave higher values than the HPLC results. This shows that high throughput mycotoxin analyses could be achieved since many mycotoxins, like in this study, up to 42 *Fusarium* metabolites, could be detected simultaneously in less time. Compared to the traditional HPLC method, there are many advantages of LC-MS/MS method, such as high sensitivity, needs no derivatization, able to simultaneously analyse and confirm different metabolites (Lattanzio, *et al*., 2009). In the traditional HPLC method, a single run may be needed for each and every mycotoxin type that may take a longer time to analyse but by the use of multimycotoxin LC-MS/MS, the analyses time could be shortened. In addition, sample extraction and clean-up time could also be reduced by simultaneous treatment; even the amount of solvent needed in analysis could also be greatly decreased. According to the report by Liao *et al*., (2011), in their analyses, the LC-MS/MS gave a better detection limit since the traditional HPLC gave higher LOQ than what was obtained for the LC-MS/MS.

Additionally, the Immuno Affinity Column (IAC) sample extraction and clean-up was used for the HPLC sample preparation. This could have contributed to the lower values observed in this study, maybe due to the limited capacity since IAC has specific capacity and in the case of

highly contaminated sample, may get saturated with the antibody-binding which may lead to loss of some toxins in the sample.

Generally, the LC-MS/MS multi-method had a lot of advantages to offer and can therefore, be applied to routine mycotoxin analysis.

5.3.2 LC-MS/MS versus ELISA for zearalenone analyses

The results in Figure 4.6, showed that the methods (ELISA and LC-MS/MS) used to assess mycotoxin levels in the samples were validated and found to be sensitive for mycotoxins analyses. However, the LC-MS/MS method has higher sensitivity for mycotoxin detection than the ELISA method. In this study good agreement between the two methods was found however, the performance of ELISA depended on the skill of the analyst and the cross-reactivity of the test kits with related substances. In this study, some of the samples had higher concentrations on ELISA than on LC-MS/MS methods. This possibly will be attributed to cross-reactivity with similar compounds as well as matrix dependence that often can cause overestimation. Some researchers pointed out that aqueous extraction by the ELISA method might be the cause of overestimation when compared to LC-MS/MS using acetonitrile-water as an extraction solvent (Zachariasova *et al*. 2008). Bennet and Nielsen (1994) emphasised after a collaborative study with laboratories to analyse ZON in some cereals, that good technical skills for ELISA analysis was helpful for improving their results. A very important feature in ELISA analysis is the washing step to remove unbound antigen and co-extracted substances, and this step should be performed carefully (Bennet and Nielsen 1994)

Besides, extraction solvents may also affect ELISA results as it has been noticed that the use of organic solvents such as aqueous methanol could result in co-extraction of fatty materials in samples, which may interfere with the assay. This might have contributed to higher values in some samples since the extraction solvent was methanol.

Lastly, ELISA can be effective as a rapid screening tool for samples with high mycotoxin level. However, where there is low level of mycotoxin contamination, the ELISA results should be confirmed by LC-MS/MS.

5.4 Dietary exposure and risk assessment for mycotoxins in adult maize consumers from agriculture regions of South Africa

The results of the dietary exposure and risk assessment for mycotoxins in adult maize consumers were shown in Table 4.10

Only DON (2000 µg/kg), the sum of FB_1 and FB_2 (4000 µg/kg) are the regulated *Fusarium* mycotoxins in South Africa. Exposure assessment calculated through maize intake for deoxynivalenol (DON), sum of fumonisin B_1 and B_2 across the AR showed that probable daily intake (PDI) for DON is below the maximum limit of 2000 µg/kg across the ARs. The PDI for the eastern region, western region and national are 86, 219, 152 µg/kg respectively, with the western region having the highest dietary DON exposure. Even though DON level did not exceed the maximum limit of 2000 µg/kg of South Africa regulation, the fact is that it should not be ignored. Deoxynivalenol (DON) is known to cause different unspecific symptoms, for example vomiting and diarrhoea, also it induces loss of weight and feed refusal in livestock, and hence referred to as vomitoxin (Da Rocha *et al*., 2014). Thus, chronic exposure to low levels of DON as with other toxins, could cause various health risks coupled with the possibility of exerting synergistic or additive effects on humans and animals due to co-contamination of the different *Fusarium* toxins in the samples. This can induce a totally different symptomatology in animals as well as in human consumers as compared to exposure to a single mycotoxins (Stoev *et al*., 2010;Mwanza, 2011).

The PDI for fumonisin B_1 plus fumonisin B_2 in the WR is above the health-based maximum limit of 4000 µg/kg in South African regulations while that in the ER and national is at 2442.96 and 3376.11 µg/kg which fall below the maximum limit. This suggests high exposure of the population to these fumonisin B mycotoxins especially in the WR and which calls for public health alerts. In addition, the higher incidence of the fumonisins and most of the *Fusarium* free and masked mycotoxins in the WR may be explained by the higher susceptibility of the maize samples to mycotoxin producing *Fusarium* species or to the high mycotoxins production potentials of *Fusarium* fungi in warmer climates (Shepherd *et al*., 1996). There is therefore, a need to carry out periodic surveys and awareness campaigns in the higher-risk regions (WR) to educate farmers as well as other agricultural stakeholders on the merits of good agricultural practices (GAP) in relation to reducing mycotoxin exposure.

However, most of the *Fusarium* mycotoxin research in South Africa has mainly focused on the free mycotoxins, but the novelty of this study is that very limited data are available so far, on the impact of climatic differences on this fungi and their mycotoxins (free & masked), in the

different agriculture regions of South Africa. Futhermore, literature is also minimal on information to do with the risk assessment of maize consumers in South Africa to contaminated maize grains

Chapter Six

Conclusion and Recommendations

Maize is a main dietary staple food in South Africa and is consumed on a daily basis and in different forms. It is also an important source of carbohydrate (NDA, 2003). Moreover, the estimated daily intake of maize in South Africa can be as high as 400g per person (Shephard *et al*., 2007). Therefore, due to the high maize intake levels, dietary mycotoxin exposure can also be high. So, in determining the occurence and variation of *Fusarium* free and masked mycotoxins, through the multi-mycotoxin profiling of the maize samples;

- Mycological analyses showed that different *Fusarium* fungal species contaminated the maize samples. It also showed that most of the samples had at least one fungal species, whereas co-contamination with different *Fusarium* fungi spp. occurred in most of the samples. *Fusarium verticiloides* was the predominant *Fusarium* species irrespective of the agricultural regions
- The outcome of mycotoxin analysis showed that maize types were contaminated with a mixture of both free and masked mycotoxins across the maize producing regions of South Africa. Moreover, all the maize samples analysed had an average of 5 up to 24 out of 42 mycotoxins, including 1 to 3 masked forms at the same time.
- Fumonisin B_1 was the most common contaminant mycotoxin, occurring at amean concentrations of 752.46±1469 µg/kg from the warm western region and at 439.88±514 µg/kg in the cold eastern region with only 3 % (2 samples) not being contaminated. A similar trend was also observed when the sum of the fumonisins was considered. In general, fumonisin B_1 was detected in 98 % of all the samples analysed, irrespective of the maize type and agriculture region (AR), ranging from 12.6 to 8907.7 µg/kg with the mean concentration of 596.2 µg/kg (Table 4.3).
- The most important finding of this study was the environmental influence as well as the maize type on the occurence of the *Fusarium* and their free and masked mycotoxins. White maize samples from the western region (WR) had significantly higher mean levels of fumonisins. This can be explained partly by high mycotoxins production potentials of *Fusarium* fungi in warmer climates as reported by (Shepherd *et al*., 1996). Then, Munkvold (2003) and De La Campa *et al*. (2005) also reported optimum temperatures of 30 °C and 32 °C for fumonisin production, which is obtaining in the western agricultural region of South Africa. Furthermore, differences in plant genotype

can also not be ruled out as it has been reported that white maize are better substrates for fumonisin production than yellow maize (Rheeder *et al.*, 1995).

- Even though relatively low levels of some of these *Fusarium* mycotoxins occurred in the maize samples, it should not be overlooked. Hence, chronic exposure to low levels of mycotoxins could cause numerous health risks coupled with the possibility of exerting synergistic or additive effects on humans and animals due to co-contamination of the different toxins in the samples.
- Furthermore, the high incidence of HIV/AIDS in South Africa linked with the consumption of *Fusarium* mycotoxin-contaminated maize could negatively affect the immune system of infected persons.
- Therefore, this study has shown that there is higher risk of *Fusarium* mycotoxin exposure, especially fumonisin Bs with consumption of maize grown in the western than with the eastern agriculture regions of South Africa. It also showed that there is no significant difference in the occurrence of the masked toxins across the regions. This study also reported for the first time the occurrence of masked mycotoxins; zearalenone-sulphate and HT-2-glucoside in South African maize. Although toxicological data are still limited, the occurrence of the masked mycotoxins was extrapolated to add considerably to the overall mycotoxins load and toxicity. This invariably will increase the toxic health effects by these masked mycotoxins, which may be either direct or indirect through hydrolysis, or released from the matrix during digestion into the parent free mycotoxins (De Boevre *et al*., 2015).

- And yet, most of the *Fusarium* mycotoxin research in South Africa has mainly focused on free mycotoxins, but the novelty of this study is that very limited data are available so far, on the impact of climatic differences on these fungi and their mycotoxins (free and masked), in the different agriculture regions of South Africa. Futhermore, literature is minimal on information on the risk assessment of maize consumers in South Africa to contaminated maize grains

- Hence, the presence of *Fusarium* contaminants and their mycotoxins in the samples should warrant intervention strategies to minimize their occurrence.

However, since not much is known on the possible synergistic or additive effects between co-occurring free mycotoxins and their masked forms, that is a challenging topic for future research. So in addition to carrying out periodic surveys and awareness campaigns in the

higher-risk western region, about the good agricultural practices that can reduce mycotoxin exposure;

- There is need to buckle up in masked mycotoxin research, in order to fill the knowledge gaps. Gaps, such as the potential health effects of low-level mycotoxins chronic exposure on humans, identification of biomarkers of exposure, the synergistic effects of mycotoxins interactions and the potential effects of climate change on masked mycotoxins occurrence, since the biggest problem with incidence of these masked derivatives in food is their re-conversion into free mycotoxin along digestion.
- In order to achieve that, there is need to have access to the pure compounds of the analytical standards. This should be a prerequisite to determine masked mycotoxins in food and feed, carryout toxicological studies and evaluate food safety related risks.Conversely, most of these compounds are not commercially available yet, for routine testing laboratories to do the research needed, and this naturally hampers work on various areas of masked mycotoxins research. Only a few laboratories mostly outside of Africa, that can boast of having isolated or synthesised masked mycotoxins standards so far, which is a lot of work.

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Appendices

Appendix I: Media preparation and other reagents

1.1 Ringers solution

Preparation of Ringers solution was done by dissolving two ringers tablets (1/4 strength) in 1 litre of distilled water and autoclaving at 121 ˚C and 15psi for 15minutes.

1.2 Antibiotics

One percent, each of streptomycin and chloramphenicol were prepared by dissolving 1g of each in 100ml of sterile distilled water. The solution was filter-sterilized using 0.22µm pore sized filter before use and 8ml of each added to the culture media for suppression of bacteria growth.

1.3 Malachite green agar (MGA 2.5 ppm)

Malachite green agar (MGA 2.5 ppm) was prepared by dissolving 7.6 grams of agar, 15 grams of peptone, 1gram of KH_2PO_4, 0.5 gram of $MgSO_4 .7H_2O$, 2.5 milligrams of malachite green oxalate in 1 litre of distilled water in a 1 litre Schott bottle and autoclaved at 121 ºC and 15 psi for 15 minutes. The media was cooled to 50 ºC using water bath and supplemented with antibiotics. Approximately 20ml was aseptically poured into sterile 90mm petri dishes and allowed to set.

1.4 Potato dextrose agar

Preparation of Potato dextrose agar (PDA) was done according to the manufacturer's instruction by dissolving 39 g of PDA powder in 1 litre of distilled water in a 1 litre Schott bottle and autoclaving at 121 ºC and 15 psi for 15 minutes. The media was allowed to cool down to 50 ºC in water bath and supplemented with antibiotics. Approximately 20ml was aseptically poured into sterile 90mm petri dishes and allowed to set.

1.5 1X TAE Buffer

One time Tris/Acetate/ EDTA (1x TAE) buffer was prepared by adding 4900ml of distilled water to 100 ml of 50x TAE (375ml of Tris-Cl, 28.55 ml of acetic acid, 50 ml of EDTA and 46.45ml distilled water).

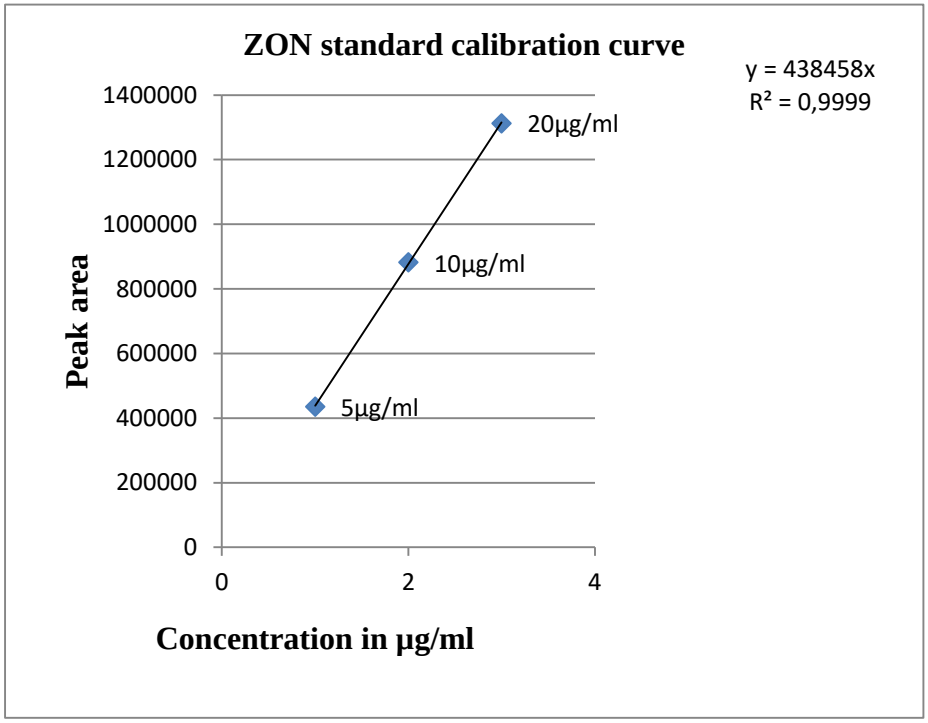
1.6 Agarose gel (2 %)

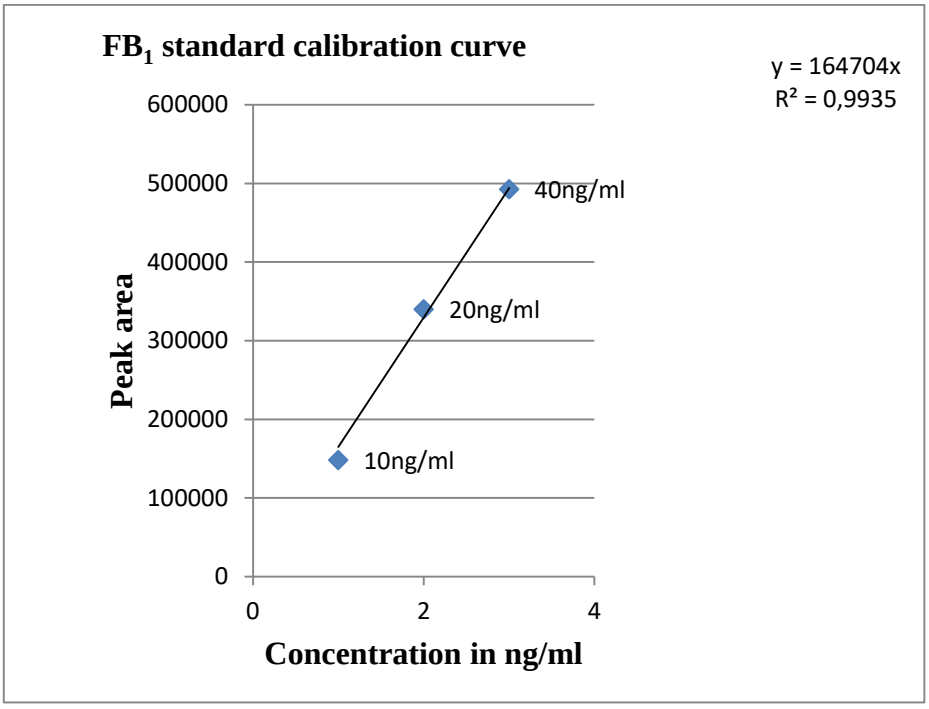
Two grams of agarose was prepared in 98 ml of 1x TAE buffer to give a 2 % solution which was melted in a microwave.

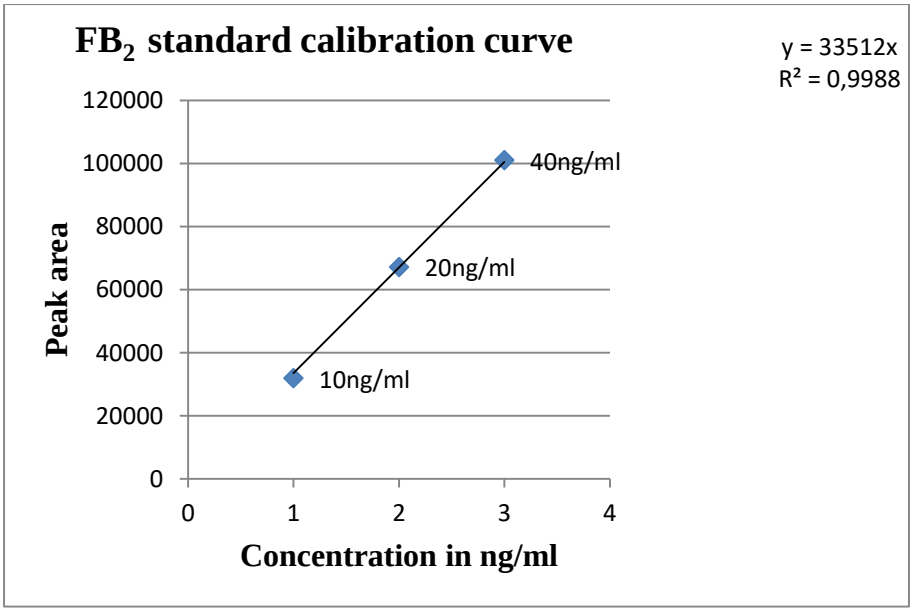
1.7 O-phthaldialdehyde reagent (OPA)

O-phthaldialdehyde reagent was prepared by dissolving 120 milligram of OPA in 3 ml of 100 % methanol and adding 15ml of 0.1M borate buffer and 150 µl of mercaptoethanol.

Appendix II: Calibration curves







Mycotoxins standards calibration curves for ZON, FB_1 and FB_2.

Appendix III: Manuscript and Conference participation during the course of the PhD

1) Manuscript in preparation: Ekwomadu TI, Gopane RE and Mwanza M (2019).Geographic difference in trichothecene occurrence in South African maize. To be submitted to Toxins Special Issue: **Fungal Growth and Mycotoxins: Challenges for developing countries** (Editors: Sarah De Saeger, J. David Miller).

2) Conference participation with poster presentations;

InterDrought - V

Hyderabad International Convention Center (HICC)

Hyderabad, India

21 - 25 February, 2017

Certificate of Participation

This certificate is presented to

Theodora Ekwomadu

for successful participation and presenting a poster / paper entitled

"Drought impacts on fungal and mycotoxin contamination of small-scale maize from the North-West province of South Africa"

in ID-V conference held during

February 21-25, 2017 at Hyderabad, India

Dr Rajeev K Varshney
(Principal Scientist-Applied Genomics)
Research Program Director-Genetic Gains
ICRISAT, Patancheru 502 324. India

Conference Organization Chair

SOT | Society of Toxicology

Creating a Safer and Healthier World by Advancing the Science and Increasing the Impact of Toxicology

March 14, 2018

Ms Theodora Ijeoma Ekwomadu
North-West University
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Mafikeng, 2735
South Africa

To Whom It May Concern:

This letter certifies that **Ms Theodora Ijeoma Ekwomadu** attended the Society of Toxicology 57th Annual Meeting in San Antonio, Texas, held on March 11–15 of 2018. The program included several keynote and other special lectures, symposia, workshops, roundtable discussions, and platform and poster presentations.

Sincerely,

E. James Dailey III

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