Diversity of fumonisin-producing *Aspergillus* and *Fusarium* species in maize collected in South Africa

By

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Declaration

I declare that this dissertation submitted to the University of Pretoria for the MSc degree is my original work and has not been previously submitted by me in other Universities. Where other people's work has been used, this has been properly acknowledged and referenced.

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Dedication…

This dissertation is dedicated to my late father Makhofi Biya a Chairman of Lijahasisu small-scale farmers who passed on six months after registering for an MSc degree on 19 December 2020. He did not only raise and nurture me but also contributed for years to my education and overall development.

PREFACE

Fusarium and *Aspergillus* species are fungal pathogens associated with maize ear rot. These fungal pathogens affect successful maize (Zea mays) production by contaminating maize grains with mycotoxins, which has adverse health effects on humans and animals. *Fusarium* and *Aspergillus* produce diverse mycotoxins namely fumonisins, aflatoxins and trichothecenes on maize. The aim of study was to identify fumonisin-producing *Aspergillus* and *Fusarium* species in maize collected from farmers in South Africa.

Chapter 1 provide the general background and the motivation for the study where the economic importance of maize is highlighted. Maize production limitation caused by plant diseases, and the mycotoxins produced by *Fusarium* and *Aspergillus* are discussed based on literature. Chapter 1 also covers morphological and molecular identification of *Fusarium* and *Aspergillus*. Fumonisin detection methods are also briefly discussed. Lastly, the chapter discusses the impact of mycotoxin contamination caused by *Fusarium* and *Aspergillus*.

Chapter 2 is the extensive literature review on *Fusarium* and *Aspergillus* species associated with maize, mycotoxins production and the epidemiology of *Fusarium* and *Aspergillus* in maize as well as maize ear rot control strategies. Chapter 2 also provide reviews on the detection methods used in this study

Chapter 3 is a research chapter that presents the incidence of *Fusarium* species and fumonisins in maize collected in South Africa. The *Fusarium* species were identified by morphological identification through isolation on a selective growth medium. Molecular identification of *Fusarium* isolates using the *TEF-1α* gene region was performed. DNA sequencing and phylogenetic analysis were done to confirm strains and group isolates to species level.

Chapter 4 is a research chapter that determines fumonisin production by *Aspergillus* species isolated from maize kernels collected in South Africa. The morphological, molecular, and phylogenetic analysis of the *Aspergillus* species is presented in this chapter.

Chapter 5 is a concluding chapter that summarizes the findings of this study and highlights future work to address questions raised by current findings.

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Abbreviations

Afs	Aflatoxins
ARC	Agricultural Research Council
BE	Elution buffer
bp	Base pair
CaM	Calmodulin
CLA	Carination Leaf Agar
CYA	Czapek Yeast Autolysate Agar
DAS	Diacetoxyscirpenol
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
EDTA	Ethylene Diamine Tetraacetic Acid
ELISA	Enzyme-linked immunosorbent assay
FA	Formic acid
FB_1	Fumonisin B1
FB_2	Fumonisin B2
FB_3	Fumonisin B3
FB_4	Fumonisin B4
FER	Fusarium Ear Rot
FFSC	*Fusarium Fujikuroi* Species Complex
FUM	Fumonisin
g	Gram
GRAS	Generally Regarded as Safe
H_2O	Water
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
ITS	Internal Transcribed Spacer
kg	Kilogram
LC	Liquid Chromatography
MEA	Malt Extract Agar
MEGA7	Molecular Evolutionary Genetics Analysis software v7
mg	Milligram
mL	Millilitre
MLST	Multilocus DNA Sequence Typing
mm	Millimetre
MUSCLE	Multiple Sequence Comparison by Log- Expectation
NaOCl	Sodium Hypochlorite
nBLAST	Nucleotide Basic Local Alignment Search Tool
NCBI	National Centre for Biotechnology Information
NIV	Nivalenol
nm	Nanometre
ntc	Negative control
PCNB	Pentachloronitrobenzene

OTA	Ochratoxins
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
ppb	Parts per billion
QTOF	Quadrupole Time-of-Flight
RNA	Ribonucleic acid
rpm	Revolutions per minute
s	Seconds
SA	South Africa
spp.	Several species
STC	Sterigmatocystin
TBE	Tris-borate- EDTA
TC	Trichothecenes
TEF-1α	Translation Elongation Factor alpha 1
UHPLC	Ultra High Performance Liquid Chromatography
USA	United States of America
UV	Ultra Violet
WA	Water agar
YES	Yeast Extract Sucrose Agar
ZEA	Zearalenone
µL	Microlitre
µm	Micrometre

Abstract

Maize(*Zea mays* L.) is a major crop grown all over the world, and it is a staple in the diets of millions of people, particularly in Africa. *Fusarium* and *Aspergillus* fungal species are reported to be associated with maize ear rot in South Africa. The most common and frequently isolated maize ear rot pathogen is *Fusarium verticillioides,* which can produce fumonisins that can be harmful to humans and animals. *Aspergillus niger* has recently been reported to cause maize ear rot and it can produce fumonisin B_2 which can have adverse health implications on humans and animals. Maize samples were collected from subsistence farmers in five provinces namely Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga, and North-West in South Africa. The morphological characterization, Sanger DNA sequencing, and phylogenetic analysis of *Fusarium* and *Aspergillus* were investigated in this study. Fumonisin levels were quantified using Enzyme-Linked Immunosorbent Assay (ELISA). The production of fumonisin B_2 by *Aspergillus* spp. Isolates was analyse using Ultra-High-Performance Liquid Chromatography (UHPLC) and Quadrupole Time-Of-Flight (QTOF) -High Resolution Mass Spectrometry (HRMS). The results indicate *F. verticillioides* is major species isolated in maize. Based on morphological and molecular identification, Six *Aspergillus* species were identified belonging to section *Nigri* (*A. niger*; *A. tubingensis*; *A. brasiliensis* and *A. welwitschiae*) and section *Flavi* (*A. flavus* and *A. parasiticus*). Sample from KwaZulu-natal had high concentration of fumonisin that exceeded maximum allowable of 2000 µg/kg set by the department of health in South Africa. Five strains of *A. niger* appear to produce fumonisin B_2 in Yeast Extract Sucrose Agar. The production of fumonisins by *Fusarium* and *Aspergillus* spp., serious health implications on small-scale farmers and their community because of the high-level exposure to fumonisins. The study has addressed understanding of maize ear rot associated with *Fusarium* and *Aspergillus* spp., and fumonisin contamination levels from subsistence farmers in South Africa.

CHAPTER 1

GENERAL INTRODUCTION

Maize (*Zea mays* L.) originated from wild grass (teosinte) in central Mexico about 7000 years ago (Doebley, 2004, Ranum *et al.*, 2014). Maize rapidly spread from Mexico to other parts of the world as a cultivated crop and food product. Some authors believed that maize originated from the formation of a hybrid of two wild types of grass (teosinte and Tripsacum), after which Native Americans modified the maize into a better source of food (Ranum *et al.*, 2014).

Traders from Europe introduced maize to Asia and Africa (Ranum *et al.*, 2014). In 1655, maize was first introduced to South Africa (McCann, 2001). In South Africa, maize is produced throughout all the provinces under different climatic conditions and the country is among the top 10 maize-producing countries in the world (Du Plessis, 2003, Nag, 2017). The top three maize-producing countries in the world are the United States of America, China, and Brazil. The global maize production was estimated to be 1 080.5 million tons as of 2019 (Maluleka, 2019).

Maize production is limited by pests and diseases, which have a direct impact on yield loss and grain quality reduction (Beukes *et al.*, 2017). As an important crop worldwide, maize plays a crucial role in the diet of millions of people particularly in Africa, due to its ability to produce high yields per hectare. In South Africa, maize is a staple food with an average daily intake of more than 300 g per person (Shephard *et al.*, 2007). Thus, the quality of maize consumed determines the quality of life (Ncube *et al.*, 2011). In Africa, maize is predominantly produced by smallholder farmers (Guilpart *et al.*, 2017).

Maize is produced in both commercial and resource-poor subsistence farming systems in South Africa (Ncube *et al.*, 2011), with a yield of 16 million tons for 2020/2021 season (USDA, 2021, Crop Estimates Committe, 2021). In South Africa, more than four million people depend on subsistence farming (Aliber and Hart, 2009). However, these farmers lack the required resources to produce mycotoxin-free grains

that are safe for animal and human consumption. Subsistence farming in South Africa play important role in reducing food-insecure household in rural and urban areas. Maize is grown for personal use, informal market trading, and in some circumstances, the formation of farmer cooperative programs that sell the majority of their grain to commercial markets (Alberts *et al.*, 2019).

Mycotoxins are toxic metabolites produced by fungal genera, such as *Fusarium* and *Aspergillus* (van Egmond *et al.*, 2007). The *Fusarium* and *Aspergillus* species produce mycotoxins in maize before harvest and during storage (García-Díaz *et al.*, 2020, Phokane *et al.*, 2019). Fumonisins (FUM) were discovered in 1988 in South Africa (Gelderblom *et al.*, 1988) and maize contaminated with FUM have been associated with human oesophageal cancer in the Eastern Cape Province of South Africa (Rheeder *et al.*, 1992) and Chinese counties of Cixian, Linxian, and Shangqiu (Chu and Li, 1994). In Mexico, infants whose mothers ingested FUM-contaminated grains during pregnancy have been reported to have a high incidence of neural tube defects (Missmer *et al.*, 2006).

Extensive research has been conducted in South Africa on *Fusarium verticillioides* (Sacc) Nirenberg as the major FUM producer (Chilaka *et al.*, 2012, Gelderblom *et al.*, 1988, Ncube *et al.*, 2011, Rheeder *et al.*, 2002, van Rensburg *et al.*, 2015), however, *Aspergillus niger* Tiegh has also been found to produce FUM (Logrieco *et al.*, 2014). A study by Ncube *et al* (2020) indicated that *Busseola fusca* (maize stem borer) frass is a reservoir of different fungal species where *A. niger* and *F. verticillioides* were found to colonize the same maize tissue in South Africa. *Aspergillus niger* has been reported to often occur on maize kernels as an opportunistic pathogen, but to this date, little research has been done on *A. niger* isolates from maize (Logrieco *et al.*, 2014) and concomitant fumonisin B_2 (FB_2) production by *A. niger* (Frisvad *et al.*, 2007).

Fusarium and *Aspergillus* species can produce diverse mycotoxins such as FUM, aflatoxins and trichothecene (TC) on maize in South Africa (Alberts *et al.*, 2019, Probst *et al.*, 2014). Fumonisin producing *Fusarium* species, such as *F. verticillioides* and *F. proliferatum* were associated with maize and sorghum (*Sorghum bicolor*) in South Africa. *Fusarium graminearum* (Schwabe) produce type B TC (Beukes *et al.*, 2017). Aflatoxins are produced by *Aspergillus flavus*, *A*. *parasiticus* and A. *nomius* (Varga *et*

al., 2004, Zulkifli and Zakaria, 2017) whereas ochratoxins are produced by *A. ochraceus* (Frisvad *et al.*, 2004).

For *Fusarium* species, identification of morphological characters coupled with recent molecular and phylogenetic approaches should be used (Kee *et al.*, 2020). Morphological identification of *Aspergillus* is commonly used and identification is based on the spores, vesicle and conidia (Diba *et al.*, 2007).

DNA sequence characters provide the best means to indicate a relationship among organisms. Molecular phylogenetic analysis has uncovered confusing speciation in several taxa, proposing that morphology characters provide wide species concepts (Geiser *et al.*, 2007). A DNA sequence should meet certain requirements to be used for identification. These requirements include; the target locus should be orthologous, have a high level of inter-species variation with a low level of intra-species, and should be easy to amplify (Balajee *et al.*, 2007, Geiser *et al.*, 2007).

The internal transcribed spacer (ITS) region is the official DNA barcode of fungi as it is the often sequenced marker in fungi and its primers work universally (Samson *et al.*, 2014). ITS is part of the nuclear ribosome with two segments (ITS1 and ITS2) divided by 5.8S rDNA located near nuclear small and large subunit rRNA genes (Balajee *et al.*, 2007).

Contamination of maize with mycotoxins remains a serious problem, particularly in subsistence farming (Ncube *et al.*, 2011, Phokane *et al.*, 2019). *Fusarium* and *Aspergillus* are common fungal contaminants of maize (Hussain *et al.*, 2013). *Fusarium verticillioides* is commonly known as a FUM producer (van Rensburg *et al.*, 2015); whereas *A. niger* is commonly known as an ochratoxin producer (Taniwaki *et al.*, 2018). There is a lack of research on *A. niger* as a potential FUM producer in maize. Thus, this study investigates the infection of maize collected from subsistence farmers in South Africa with *F. verticillioides* and *A. niger* as FUM producers

It is also of vital importance to quantify the levels of FUM produced by these fungi using chromatographic techniques. This will ensure adoption of proper mitigating strategies to reduce the infection of maize by *F. verticillioides* and *A. niger* and subsequent FUM contamination. This research is a follow-up study from the work done by Ncube *et al.* (2011) that identified hot spots for *F. verticillioides* and *A. flavus*

infection. Limpopo, Mpumalanga, KwaZulu-Natal, Eastern Cape, and North West Provinces were amongst the Provinces under investigation by Ncube *et al.* (2011). Despite the lack of research on *A*. *niger* as a potential maize FUM producer. Grapes (*Vitis vinifera*), coffee (*Coffea Arabica***)**, onions (*Allium cepa*), and mango (*Mangifera indica*) have all been found to be infected with *A*. *niger*, which can produce ochratoxins A and fumonisin B_2 (Frisvad *et al.*, 2007; 2011). *Aspergillus niger* has been found to produce fumonisin B_2 in maize, wheat (*Triticum*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), millet (*Pennisetum glaucum*), oats (*Avena sativa*), coffee, and grapes (Ráduly *et al.*, 2020; Soares *et al.*, 2013). However, the current study aims to provide in-depth knowledge about *Fusarium* and *Aspergillus* spp., occurring in these particular provinces, as a FUM producer.

The aims of the study are to determine:

- The incidence of *Aspergillus* species in maize kernels in South Africa and do these *Aspergillus* species produce FUM.
- *Fusarium* species found associated with maize in South Africa.
- The FUM levels in maize grain collected from smallholder farmers.

CHAPTER 2

LITERATURE REVIEW

1.1 *Fusarium* species in maize

Fusarium species are filamentous fungi and produce secondary metabolites called mycotoxins that are harmful and carcinogenic to humans and animals (van Egmond et al., 2007). The genus *Fusarium* belongs to the kingdom Fungi, phylum Ascomycota, class Sordariomycetes, sub-class Ascomycetes, order Hypocreales and family Nectriaceae (Michielse and Rep, 2009). The genus consists of approximately 300 species, amongst them 23 different Fusarium species complexes (Atanasoff-Kardjalieff and Studt, 2022). *Fusarium* species are fungal pathogens found in cereal crops such as, oats (*Avena sativa* L), barley (*Hordeum vulgare*), wheat (*Triticum*), as well as maize (*Zea mays* L.) and can also infect other crops such as asparagus (*Asparagus officinalis*), figs (*Ficus carica*), soybean (*Glycine max*), cumin (*Cuminum cyminum*), medicinal plants (Lamiaceae and Asteraceae) and some nuts crops (*Anacardium occidentale* and *Arachis hypogaea*) (Munkvold, 2017). Furthermore, *Fusarium* species can live as saprophytes in soil and plants worldwide (Kamle *et al.*, 2019, Moretti, 2009). *Fusarium* species are ubiquitous in soils; they can infect up to 50 % of the maize fields before harvest (Fandohan *et al.*, 2003).

There are two distinct diseases caused by *Fusarium* species on maize kernels, namely *Fusarium* ear rot (FER, also called pink ear rot) and Gibberella ear rot (also called red ear rot) (Folcher *et al.*, 2009, Munkvold, 2003). Fusarium ear rot is caused by *F. verticillioides*, *F. proliferatum* and *F. subglutinans* which are all fumonisin (FUM)-producers (Mukanga *et al.*, 2010, Presello *et al.*, 2007, Small *et al.*, 2012); whereas Gibberella ear rot is caused by *F. graminearum* (Munkvold, 2003) and it produces mycotoxins such as zearalenone (ZEA) and deoxynivalenol (DON) (Logrieco *et al.*, 2002). Both maize ear rots can co-exist on the same maize plant as a result of climatic changes during the growing season (Logrieco *et al.*, 2007). These ear rots can result in mycotoxin contamination of maize grain (Logrieco *et al.*, 2007, Munkvold, 2003).

Fusarium verticillioides infects the maize crop by colonizing the plant roots, stems, ears and seed as an endophyte (Venturini *et al.*, 2011). Moretti (2009) indicated that

Fusarium spp. produce three types of spores, namely; macroconidia, microconidia and chlamydospores. The macroconidia are long, multicellular and banana-shaped structures produced on monophialides and/or polyphialides. Many species have small, single-celled and oval to spherical microconidia produced in the aerial mycelium in clumps or chains on the monophialides and polyphialides (Glenn, 2007). Some species produce thick-walled resistant chlamydospores formed in the middle of hyphae or at their termini, which are essential for long-term survival. Macroconidia and microconidia play a role in the dispersal of the fungi by wind and rain splash (Glenn, 2007, Moretti, 2009).

1.2 *Fusarium* mycotoxins in maize

The *Fusarium* genus consists of several toxigenic species that cause diseases to plants, humans and animals and are adaptable to a variety of host plants. The three most important groups of mycotoxins are the FUM, trichothecenes (TC) and (ZEA) (Agrios, 2005, Jimenez-Garcia *et al.*, 2018). *Fusarium verticillioides* and *F. proliferatum* are the primary producers of FUM, whereas *F. graminearum* produce TC and ZEA (Beukes *et al.*, 2017, Boutigny *et al.*, 2012). *Fusarium verticillioides* is associated with production of three mycotoxins namely FUM, moniliformin and fusarin C. In maize, *F. verticillioides* and *F. proliferatum* were linked with fumonisin B_1 (FB_1) and other two mycotoxins, fusaproliferin and beavericin. In addition, moniliformin is also synthesised by *F. oxysporum* (Placinta *et al.*, 1999).

Fusarium species can produce a wide range of mycotoxins of diverse structures and action (Chilaka *et al.*, 2017). Fusarium mycotoxins, including FUM, ZEA and TC, are of great economic importance (Munkvold, 2017). Mycotoxins can become life threatening on human and animal biological systems (Ji *et al.*, 2019, Perincherry *et al.*, 2019). Pre-harvest practices such as cultivars selection, mono cropping and crop residue management can result in contamination of maize by *Fusarium* mycotoxins (Tran *et al*., 2021). Improper harvesting practices, poor drying, packaging, transport and storage conditions promote fungal growth and increase the risk of mycotoxin production (Bhat *et al.*, 2010, Phokane *et al.*, 2019).

1.2.1 Fumonisin

Fumonisin incidence in South Africa was first discovered in 1988 (Gelderblom *et al.*, 1988). The incidences of FUM were more prevalent in home-grown decayed maize kernels in the Transkei region of South Africa (Marasas, 2001). The FUM standard set by Food and Drug Administration (FDA) is 2 mg total fumonisin (TFB) per kilogram (kg) maize in the United States and 1 mg TFB/ kg by the European Commission for direct human consumption of maize (EC, 2007; FDA, 2001). The National Department of Health in South Africa has adopted the maximum levels (MLs) for total fumonisin (TFB) as set by the Codex Alimentarius Committee, with 4000 µg/kg in raw maize and 2000 µg/kg for maize flour and mealie-meal (Shephard *et al.*, 2019). The set regulation method cannot address FUM exposure in rural farming areas of South Africa, because maize is produced for direct human consumption and it is consumed in high quantities (Alberts *et al.*, 2019).

More than 15 FUM homologs are known and characterized as FUM A, B, C, and P. Fumonisin B (FB) analogs consists of B_1, B_2 and B_3 forms (Figure 2.1) are the most abundant and main food contaminants with FB_1 classified as the most toxic FUM and can co-exist with other forms of FUM (Kamle *et al.*, 2019, Rheeder *et al.*, 2002). Fumonisin modifies sphingolipid biosynthesis, induces hepatotoxicity and increases serum cholesterol concentration in all species studied (Haschek *et al.*, 2001). Fumonisin is known to possess high cancer-inducing properties (Bhat *et al.*, 2010). Moreover, *A. niger* has been discovered as a producer of FUM in maize, peanut (*Arachis hypogaea*) and grapes (*Vitis vinifera*) (Kamle *et al.*, 2019). Fumonisin B_1 is categorized as Group 2B by the International Agency for Research on Cancer because of its strong toxicity (Munawar *et al.*, 2019, Pestka *et al.*, 1994). Animal ingestion of maize contaminated with FUM causes diseases such as equine leukoencephalamacia (LEM) and porcine pulmonary edema (PPE), LEM is a syndrome of acute illness and death in horses associated with FB_1 and FB_2 (Turner *et al*., 1999) and PPE is acute, fatal disease in pigs appear to be caused by pulmonary hypertension (Chen *et al*., 2021).

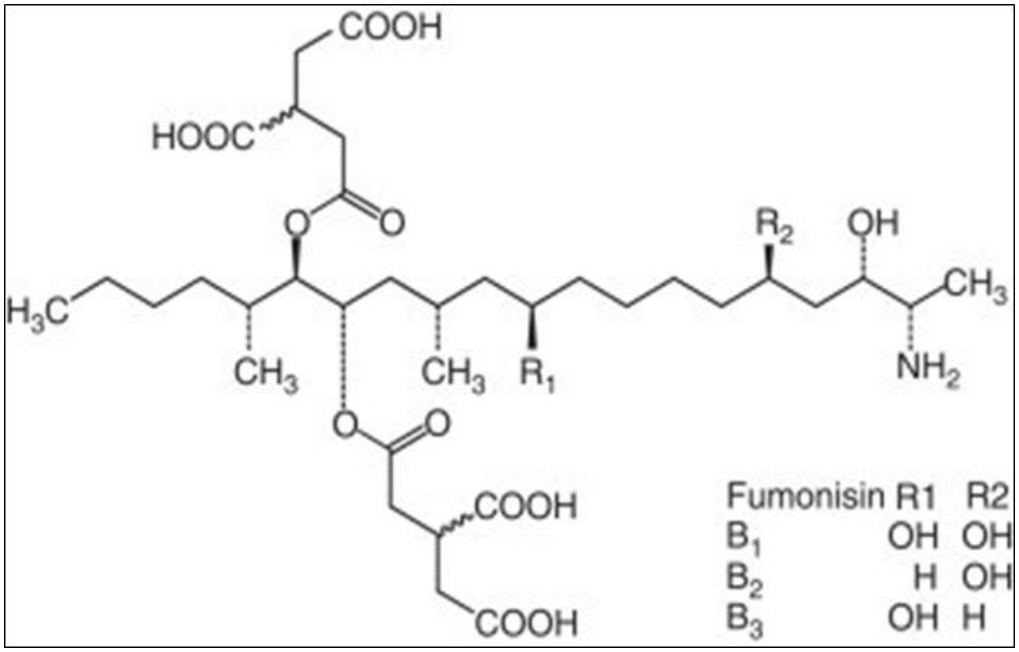


Figure 2. 1: Chemical structures of fumonisins B_1, B_2, and B_3 (Trucksess and Diaz-Amigo, 2011).

1.2.2 Trichothecenes

There are more than 40 naturally occurring TC produced by *Fusarium* species with a combined total of 150 TC analogs produced by species of *Fusarium*, *Cephalosporium*, *Cylindrocarpon, Myriothecium*, *Stachybotrys* and *Trichoderma* (Glenn, 2007).Trichothecenes are economically important mycotoxins produced by *Fusarium* species and other fungal pathogens like *Trichoderma*, *Trichotecium*, *Myrothecium* and *Stachybotrys* (Glenn, 2007, Larsen *et al.*, 2004). The health impact of TC are associated with livestock emesis, diarrhoea, feed refusal, skin irritation, haemorrhaging, reproductive problems and hematological abnormalities (Abramson *et al*., 1993, Mudili *et al.*, 2014). Trichothecenes are sesquiterpenoid mycotoxins that accumulate in maize kernels, thus rendering the grain unsuitable for humans and animals (Bhat *et al.*, 2010, Munkvold, 2017). Trichothecenes are classified into four groups (A, B, C, and D) and TC produced by *Fusarium* spp. are type A which have a hydroxyl or no oxygen substitution at C-8, or type B, which has a C-8 keto (carbonyl) group. Type B trichothecenes are formed in greater quantities but are less poisonous than those of type A. Type A, includes diacetoxyscirpenol (DAS), HT-2, T-2 and neosolaniol with T-2 as the most acutely toxic in animals. (Munkvold, 2017, Omurtag, 2008). *Fusarium graminearum* species complex (FGSC) pathogens infect maize causing disease such as Gibberella ear rot and Fusarium head blight of maize and are

associated with TC mycotoxins in maize in South Africa. Trichothecenes, which consist of DON, nivalenol (NIV), DAS and T-2 toxin, occur in animal feed and cereals in the KwaZulu-Natal Province (Placinta *et al.*, 1999).

1.2.3 Zearalenone

Fusarium graminearum produces ZEA (Boutigny *et al.*, 2011). The ZEA are estrogenic mycotoxins with less acute toxicity and do not cause fatal toxicoses (Kosawang *et al.*, 2014). The European commission has set the maximum allowable levels for ZEA at 100 µg/kg in unprocessed cereals and 350 µg/kg in unprocessed maize (Atoui *et al*., 2012). Zearalenone is more prevalent during the cold storage of infected maize with high moisture content (Velluti *et al.*, 2000). In swine, ZEA is associated with reproductive abnormalities (Kosawang *et al.*, 2014, Munkvold, 2017, Velluti *et al.*, 2000). Zearalenone affects the reproductive tract and mammary glands in swine, inducing clinical signs such as hyperemia, edematus swelling of the vulva, at times vaginal and rectal prolapse (Minervini and Dell'Aquila, 2008). Children are most affected by the consumption of ZEA contaminated cereals and cereals-based food products. This toxin is implicated in the incident pubertal changes (Bhat *et al.*, 2010).

1.3 Fusarium ear rot

Fusarium verticillioides is the main causal agent for FER, however, *F. proliferatum* and *F. subglutinans* are also economically important FER pathogens (Mukanga *et al.*, 2010, Presello *et al.*, 2007, Small *et al.*, 2012). *Fusarium verticillioides* also causes kernel, stalk, root rot and seedling blight of maize (Baldwin *et al.*, 2014, Murillo-Williams and Munkvold, 2008). *Fusarium verticillioides* is a member of the *F. fujikuroi* species complex (FFSC), which is divided into the American, Asian and African clades, with *F. verticillioides* falling within the African clade (Blacutt *et al.*, 2018). The combination of numerous biotic and abiotic variables leads to *Fusarium* infection and fumonisin production. Water availability, temperature, pH, and nutrients, as well as plant natural defenses, are major elements that influence fungal colonisation by *F. verticillioides* (Peter Mshelia *et al*., 2020, Marín *et al*., 2010). Temperature and water availability are the two most important abiotic parameters that influence fumonisin biosynthesis by *F. verticillioides*; more water availability leads to greater fungal growth and fumonisin production, with an ideal temperature of 20 °C to 30 °C. (Picot *et al*., 2010, Samapundo *et al*., 2005). The carbon:nitrogen ratio or the pH of the maize kernel

during ripening are major regulators of fumonisin synthesis. The combined effects of sugar and nitrogen metabolism, as well as pH changes, play a key role in activating the fumonisin biosynthetic pathway during kernel colonization (Picot *et al*., 2010). According to Picot *et al*. (2010), the maize-fungus interaction causes physiological and molecular changes in the plant cell, triggering plant defence mechanisms that can either promote or inhibit fungal growth and toxin generation.

1.3.1 Environmental conditions

Conditions that favour FER are warmer, drier areas with optimal temperatures of 30 ^{o}C, and drought stress results in elevated *F. verticillioides* infection (Munkvold, 2003). Fandohan *et al.* (2003) mentioned that temperature and moisture conditions during growing seasons and storage affect maize infection. *Fusarium verticillioides* proliferates in grain with water content of between 18-20 % (Munkvold and Desjardins, 1997).

1.3.2 Symptoms

Fusarium ear rot (Figure. 2.2) occurs on individual or group of kernels on mechanically injured kernels and it is characterized by white or light pink mould (Venturini *et al.*, 2011). The fungus can be found in all maize production areas and can be isolated from maize that is symptomless (Brown *et al.*, 2012). *Fusarium verticillioides* infects maize mostly under hot dry climatic conditions after flowering (Munkvold, 2003). Duncan and Howard (2010) highlighted that infected kernels could have classic starbursts symptoms consisting of streaks on the pericarp radiating from the silk scarf region of the kernels. The white streak is caused by the loss of transparency due to the disintegration of the pericarp cells.

Figure 2. 2: Fusarium ear rot with a group of infected kernels (A) and Fusarium ear rot with starburst symptoms (B) (Janse van Rensburg, unpublished data).

1.3.3 Disease cycle

Fusarium verticillioides constitute 10 % or less of the *Fusarium* soil-borne species. The fungus has been known to infect maize residues (Figure. 2.3) and can survive aboveground for over a year in buried stalks (Blacutt *et al.*, 2018). The soil-borne hyphae infect the germinating seed and root, and is taken up the plant through systematic growth (Oren *et al.*, 2003). The host-pathogen interaction does not happen in isolation and maize-associated insect vectors play an important role in the disease cycle of *F. verticillioides* (Duncan and Howard, 2010). The feeding of the lepidopteran larvae on stalk, ears, and collar tissues, plays a significant role in the infection development of *F. verticillioides* on the stalk and ear in maize (Blacutt *et al.*, 2018). *Fusarium verticillioides* can infect through systemic infection and through the silk channel by windborne spores (Oren *et al.*, 2003, Venturini *et al.*, 2011). The infection through the silk starts from the tip of the ear and channels downward. Infection through silks is promoted by late-season rainfall and the physiological condition of the silk after pollination (Bush *et al.*, 2004).

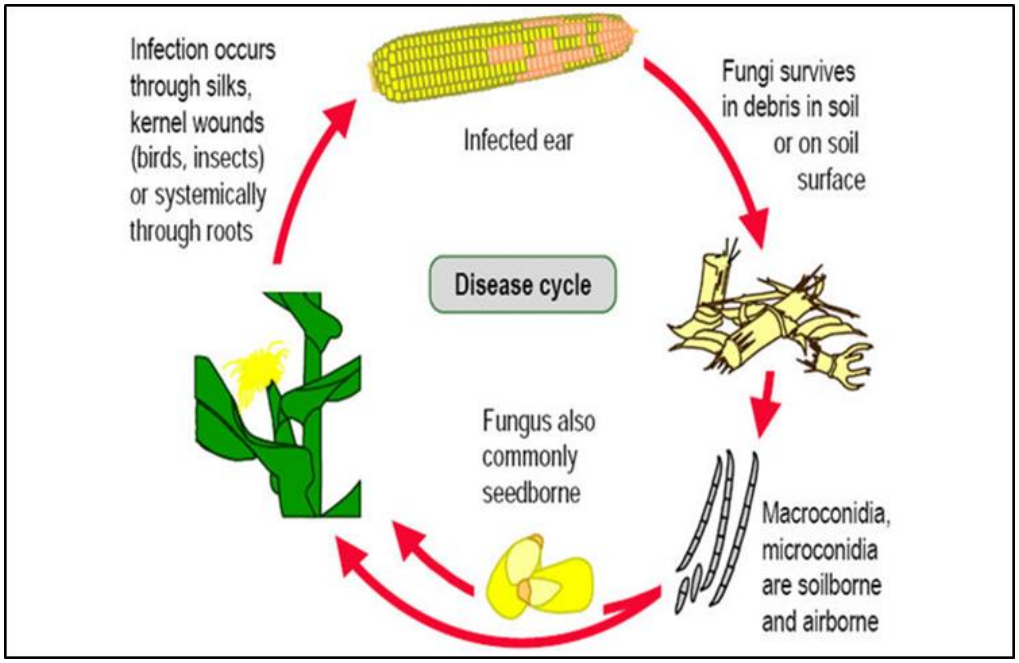


Figure 2. 3: Fusarium ear rot disease cycle (Samsudin, 2015).

1.3.4 Control measures

Crop residues from previous diseased crops are the main inoculum source for FER and Gibberella ear rot of maize (Munkvold, 2003). The risk of FER increases if maize is grown in monoculture or after wheat (*Triticum* spp) (Pfordt *et al.*, 2020).

Pest and disease cycles are disrupted by crop rotation, consequently reducing fungal inoculum, infestation and subsequent mycotoxin contamination in the field (Phokane *et al.*, 2019). Control measures include planting of cultivars well-adapted to local environmental conditions, avoiding planting late, control of ear damage by insects, avoiding high-density plant population, ensuring adequate fertilization, and avoiding late harvesting (Reyneri, 2006).

Good maize storage procedures need to be taken into consideration to prevent the development of FUM contamination (Phokane, 2018, Phokane *et al.*, 2019). These include drying maize kernels to moisture levels below 16 % post-harvest and storing grain in a well-aerated place. Mechanically damaged kernels should be minimized during harvesting to reduce mycotoxin contamination (Magan and Aldred, 2007).

1.3.5 Mycotoxicoses in humans and animals

According to Agrios (2005) the induction of animal and human diseases such as necrosis of the skin, haemorrhage, liver and renal failure in both humans and animals can result in death (Pietri *et al.*, 2004, Sampietro *et al.*, 2013). The root cause is the consumption of food and feed infected with toxic substances called mycotoxins. Fumonisin causes blind staggers (equine leukoencephalomalacia) in horses (*Equus caballus*), donkeys (*Equus africanus asinus*), and mules (*Equus mulus*), pulmonary edema in pigs (*Sus scrofa domesticus*), and cancer in humans (Agrios, 2005). Fumonisin was reported to be associated with human oesophageal cancer in the Eastern Cape Province of South Africa (Missmer *et al.*, 2006, Rheeder *et al.*, 1992).

1.4 *Aspergillus* species in maize

Micheli first described *Aspergillus* in 1729 as fungi with long stalks and spore heads radiating in long chains from a central structure resembling an aspergillum (Plascencia-Jatomea *et al.*, 2014). *Aspergillus* belongs to the family Trichocomaceae, of the order Eurotiales, in the class Plectinomycetes and phylum Ascomycota (Gugnani, 2003). The genus *Aspergillus* contains about 250 species (Meyer *et al.*, 2011) which are grouped into six subgenera that are further subdivided into several species complexes (Visagie and Houbraken, 2020). Aspergillosis infection that affect the respiratory system and most commonly caused by *A. fumigatus,* followed by *A. flavus*, *A. terreus,* and *A. niger*, however, many other species have been discovered in human infections (Alastruey-Izquierdo *et al.*, 2012).

Aspergillus has a tremendous impact on various fields of research. For instance, many *Aspergillus* species are important human and animal pathogens, spoilage agents in food, and producers of poisonous toxins (Samson and Varga, 2009). On the contrary, other *Aspergillus* species are useful microorganisms in food fermentation and the biotechnology industry (Samson and Varga, 2009, Souza Guimaraes and da Costa Souza, 2017).

The black spored *Aspergillus* are part of the subgenus *Circumdati*, and in the section *Nigri* (*A. niger* group). *Aspergillus niger* produces rots in grapes (*Vitis vinifera*), maize, and many fruits, including apples (*Malus domestica*), pears (*Pyrus* spp.), peaches

(*Prunus persica*), figs (*Ficus carica*), strawberries (*Fragaria* × *ananassa*), and melons (*Citrullus* spp.), and they are mainly regarded as post-harvest pathogens (Perrone and Gallo, 2017). The black Aspergilli are producers of mycotoxins such as FUM, which are carcinogenic and are commonly known to be produced by *Fusarium* species. Mycotoxins produced by *Aspergillus* are reported to be toxic to livestock, poultry, fish, and humans (Palencia *et al.*, 2010).

Aspergillus niger is often found in soil from decaying plant residues and infects several crops (including grapes, onions (*Allium cepa*), maize, and peanuts (*Arachis hypogaea*) causing diseases such as maize seedling blight and maize ear rot (Palencia *et al.*, 2010).

1.4.1 Mycotoxins by *Aspergillus* spp.

Aspergillus species produce several mycotoxins, which have considerable agricultural, epidemiological, and economic impacts (Perrone and Gallo, 2017). When the spores come into contact with necessary nutrients and optimal climatic conditions, they colonize quickly and produce poisonous toxins (Plascencia-Jatomea *et al.*, 2014). *Aspergillus flavus* and *A. parasiticus* are the main aflatoxin producers. These fungi also produce FUM, sterigmatocystin, cyclopiazonic acid, and patulin (Plascencia-Jatomea *et al.*, 2014).

1.4.1.1 Aflatoxins

There are more than 20 types of AFs, however only four are harmful to humans and livestock, these are AF B_1, B_2, G_1 and G_2 (Abbas *et al.*, 2006, de Oliveira and Corassin, 2014). Aflatoxins can be produced by other strains of *Aspergillus* such, *A. nomius* Kurtzman, Horn & Hesseltine, and *A. pseudotamarria* Kita (Liang *et al.*, 2006). The carcinogenic, immunotoxic, and mutagenic effects are contributed by the presence of a lactone ring and the difuran ring. Aflatoxin B_1 is the most toxic mycotoxin and it has been classified as the class 1 human carcinogen by the International Agency for Research on Cancer (IARC) (Ráduly *et al.*, 2020, IARC, 2002). Agrios (2005) highlighted that during some years, a high percentage of 30 % or more of the maize harvest over large areas contained more than 100 parts per billion (ppb) aflatoxin, which is five times more than the maximum allowed limit in human food and animal feed. Some of the AFs consumed by dairy cattle are excreted in milk in the toxic form

of AFM_1, which poses a serious health risk to humans (Cavallarin *et al.*, 2011). The IARC of the World Health Organization (WHO) and European Commissions set a limit of 2 µg/kg for AF B_1 in foods for human consumption and 10 µg/kg total AFs in maize (EC, 2010; IARC, 2002). Aflatoxins limit in South Africa for human consumption must be less than 10 ppb maximum or 50 ppb in animal feed (Mwanza, 2012).

1.4.1.2 Fumonisins

Aspergillus niger and *A. welwitschiae* can be producers of FB_2 and FB_4 in food products and animal feed, though *F. verticillioides* and other *Fusarium* species remain species that contaminates crops and feeds with higher amounts of FB_1 production (Ráduly *et al.*, 2020). Fumonisin limits are regulated; maximum level of 4 000 µg/kg total FUM is suggested for unprocessed maize and 2000 µg/kg maize products for direct human consumption (Shephard *et al.*, 2019). For animal feed, regulated level of less than 5 ppb for horses, 10 ppb for pigs, 50 ppb for beef, cattle, and poultry are suggested (Mashinini and Dutton, 2006).

1.4.1.3 Ochratoxins

These are important toxins in grain that cause degeneration and necrosis of the liver and kidney in domestic animals (Agrios, 2005). Ochratoxins (OTA) is mainly produced by *A. ochraceus*, *A*. *carbonarius,* and *A. niger* as well as some *Penicillium* spp. (Abarca *et al.*, 2004). Ochratoxins are classified as Group 2B carcinogen, thus possessing the potential to cause cancer in humans (IARC, 1993). Ochratoxin poisoning is dosage-dependent and its cancer-causing properties in animals are well known (Ráduly *et al.*, 2020). According to Regulations (EC) No. 1881/2006 and 105/2010, the maximum levels of OTA in foodstuffs in the European Union are 5 g/kg for unprocessed cereals and 3 g/kg for all products produced from unprocessed cereals (Duarte *et al*., 2010). Ochratoxin A was discovered in a multi-mycotoxins investigation of compound feed collected in feed mills in South Africa in 2010-2011 (Meyer *et al*., 2019), and OTA is known to be produced by *Aspergillus ochraceus* (Chilaka *et al*., 2012). Temba *et al*. (2017) found that 88.89 % of samples tested were positive for OTA, which was above the European Commission's 3 ug/kg legal limit.

1.4.1.4 Sterigmatocystin

There are more than 50 fungal species from *Aspergillus* that produce sterigmatocystin (STC), which are similar to AFs (Nieto *et al.*, 2018). The main causal agents are *A. flavus*, *A. parasiticus,* and *Aspergillus* section Nidulantes subclade Versicolores. Sterigmatocystin can induce tumors and it is also classified under Group 2B as a potential human carcinogen (IARC, 1987).

1.5 *Aspergillus niger*

Aspergillus niger is the most commonly reported fungus isolated from food and has a wide global distribution (Plascencia-Jatomea *et al.*, 2014). The fungus belongs to the group of *Aspergillus* species with conidia heads having a black shade. In this group, only 12 species are accepted and two varieties are distinguished by uniseriate (single palisade of cells that produce conidia) or biseriate (two palisades) structure of the conidiophores (Abarca *et al.*, 2004). *Aspergillus niger* is capable of producing poisonous toxins such as ochratoxin A and fumonisin B_2 and B_4 (Frisvad *et al.*, 2007, Mansson *et al.*, 2010). However, *A. niger* has been accorded generally regarded as safe status (GRAS) in 1993 (Schuster *et al.*, 2002), before the discoveries of its mycotoxin-producing abilities. Despite the GRAS status, it has been reported in various industrial processes, consequently, the potential for toxin production by *A. niger* poses a serious health hazard as its ability to produce mycotoxins was unknown when it was granted GRAS status (Frisvad *et al.*, 2011).

1.5.1 Symptoms in maize

Aspergillus niger causes ear rot in maize (Figure 2.4) and can be isolated from symptomless maize kernels (Palencia *et al.*, 2010). The common symptomatic signs of *A. niger* infection on grain are the black powdery group of spores that cover maize kernels and ears (Jeffers, 2004).

Figure 2. 4: Maize ear rot produced by *Aspergillus niger* (Allen, 2021).

1.5.2 The disease cycle of *Aspergillus niger*

Aspergillus niger produces powdery masses of spores that are dark brown in colour on colonized maize ears (Somda *et al.*, 2008). Infection of maize by *A. niger* results in diseases such as stunted growth and wilting, coupled with ear rot (Jeffers, 2004). *Aspergillus* survives in crop residue and in the soil. During hot and humid conditions, microscopic spores of *Aspergillus* are carried from the soil surface to the maize silks through air movement. When hot conditions prevail, the spores germinate and the fungus colonizes the silks (Figure 2.5). Moist, yellow-brown silks are susceptible to colonization and invasion through the silk channel. Fungal growth through dry silks is possible but limited. Growth of the fungus then happens down the silk channels and expands to the developing ear (Woloshuk and Wise, 2011). When the fungus is present under the husk, uninjured kernels may be infected if the plant is stressed, upon reach of dough stage. High temperatures and drought are common stress factors leading to pre-harvest aflatoxin contamination. Infection also occurs through kernel wounds created by insects and birds; this allows easy colonisation by the fungus (Paica *et al.*, 2013).

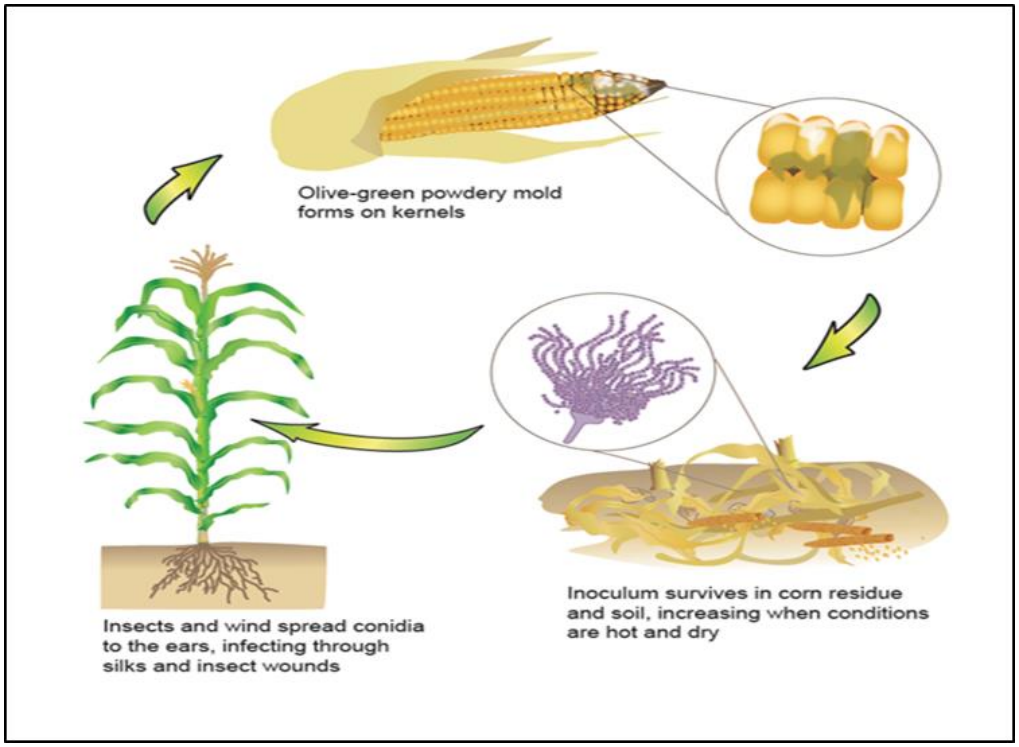


Figure 2. 5: Aspergillus ear rot disease cycle (Aspergillus Ear Rot of Corn; 2021).

1.5.3 Prevention and management of maize ear rot

Planting diverse crops with different harvest dates in smaller areas can mitigate the risk of maize ear rot (TerAvest *et al.*, 2015). This includes co-planting the “normal” crops with genetically modified crops which might change the chemical structure of mycotoxins via metabolic pathways as part of the defence against xenobiotics (Hell *et al.*, 2010, Ráduly *et al.*, 2020). Other maize ear rot management strategies include control of insect damage to reduce infection by maize ear rot causing fungi such as *A. flavus*, which causes Aspergillus ear rot (Woloshuk and Wise, 2011). Randela (2003) reported that Limpopo farmers in South Africa use ash, synthetic pesticide (Carbaryl/gamma BHC) “blue death powder” and Phostoxin tablets to protect grain from insects as a pre-harvest control strategy. Synthetic fungicides that are environmentally friendly can be applied to control Aspergillus ear rot (Plascencia-Jatomea *et al.*, 2014). When maize ear rot occurs, further growth can be reduced by ensuring proper storage conditions in a well-ventilated area to avoid the accumulation of mycotoxins (Patience *et al.*, 2010). Grain sorting of broken or damaged kernels is another management strategy as such kernels are more prone to *Aspergillus* infection (Whitlow and W. M. Hagler, 2001). Grain moisture levels during harvest must be less

than 14 % since high moisture content may lead to the production of mould (Richard, 2007).

1.6 Economic impact of maize ear rot and mycotoxins

Maize ear rot is an important global disease that results in yield loss and adverse health implications to humans and animals (Gxasheka *et al.*, 2015). Maize ear rot decreases the quality of maize kernels and the feeding value of the grain. It also results in the production of mycotoxins (Bello *et al.*, 2012). Fusarium ear rot, Aspergillus ear rot, and Gibberella ear rot are the primary ear rot diseases in maize (Xiang *et al.*, 2010). Gxasheka *et al.* (2015) indicated that under optimal conditions Gibberella ear rot can result in up to 48 % yield losses. Moreover, maize ear rot reduces the quantity and quality grain with an estimation between 7-17 % in yield reduction (Nagy *et al*., 2006). From 2012 to 2015, the economic losses caused by Aspergillus ear rot and Fusarium ear rot were $99 626 600 and $116 756 288 in the northern United States and Ontario, Canada (Mueller *et al*., 2016), respectively. Monetary loss due mycotoxins (aflatoxin, fumonisin, and deoxynivalenol) contamination were estimated to cost the United States between $418 million and $1.66 billion a year (Mitchell *et al*., 2016). AF contamination of crops generates annual losses of more than USD 750 million in Africa and almost USD 1 billion in Indonesia, Thailand, and the Philippines (Udomkun *et al*., 2017). In under-developed countries, it is difficult to measure the economic losses because the quantitative estimates are rarely accessible (Munkvold *et al*., 2019).

The potential impacts as a phyto-toxins on humans and animals includes loss of human and animal life, increased health care and veterinary care costs, and research costs on uncovering the impact and severity of the mycotoxins (Zain, 2011). In Africa, the major mycotoxins of significance concerning health and economy are aflatoxins, FUM, OTA, TC, and ZEA (Gbashi *et al.*, 2018).

1.7 Morphological identification of *Fusarium* spp.

Morphological identification is a reliable and common method used to identify fungal species for section-level identification. The initial step in the identification process is to clearly describe the pathogen, plant disease, symptoms, and climatic conditions in which the disease occurs. After evaluation of isolation and recovery methods, it is then

that morphological characters' methods can be used (Leslie and Summerell, 2006) Morphological characters of the *Fusarium* genus includes the characteristics of the macroconidia and microconidia, the mode of formation of microconidia, and the presence or absence of chlamydospores in mature *Fusarium* cultures (Laurence *et al.*, 2016).

The initial step in the identification process for *Fusarium* species is morphological identification (isolation, culture purification, morphological assessment). Although morphological observation alone is insufficient to name a species, it does aid in the morphological grouping of species for further investigation, utilizing molecular techniques (Kee *et al.*, 2020, Rahjoo *et al*., 2008). *Fusarium* morphology features can be evaluated using a stereo and light microscope and a reference to Leslie and Summerell's *Fusarium* laboratory manual (Scauflaire *et al*., 2011).

The limitation of morphological identification is that the *Fusarium* genus has a confusing and unstable taxonomic history. The lack of clear morphology characters distinguishing species leads to identification that is too broad, species variation, and mutation in culture (Geiser *et al.*, 2007).

1.8 Molecular identification of *Fusarium* species on maize

Molecular methods are a powerful and useful tool for the classification and identification of *Fusarium* species (Chandra *et al*., 2011). After morphological identification, molecular identification is necessary when morphological features overlap. Molecular identification of different gene regions is important to determine the identities and genetic diversity of *Fusarium* species (Laurence *et al.*, 2016). DNA sequence-based methods of known *Fusarium* isolates can be achieved by using the translation elongation factor (*TEF-1α*) gene region. The *TEF-1α* region appears to exist consistently as a single copy in *Fusarium* and has a high level of polymorphism among related species (Rahjoo *et al.*, 2008).

1.9 Morphological identification of *Aspergillus* species

Morphology characters assist in grouping species complex, in turn, grouping is essential for further identification by other techniques (Balajee *et al.*, 2007). Morphological characters of *Aspergillus* species used for identifying species are colony colour, size, rate of growth, texture, degree of sporulation and arrangement of

conidial heads (Diba *et al.*, 2007). Further characters include the production rate of sclerotia or cleistothecia, colour of mycelium, sporulation soluble pigments, exudates, and colony reverse (Gugnani, 2003). *Aspergillus* has both sexual and asexual reproduction and important microscopic structures (Samson *et al.*, 2014). The characteristics of the conidial head include its colour and shape. The shape of the vesicle, roughness or smoothness of the conidiophore wall, phialides as being uniseriate or biseriate are also taken into consideration. A complete description of size and shape depends on the observation of several heads in colonies (Gugnani, 2003). It is important to have a standardised culture media and incubation condition for consistent species identification. (Tsang *et al.*, 2018). However, morphological identification cannot be used in isolation, especially for closely related species (Scauflaire *et al.*, 2012).

1.10 Molecular techniques for *Aspergillus* species

Molecular methods are used to confirm morphological identity (Scauflaire et al., 2012). For *Aspergillus*, internal transcribed spacer (ITS), Beta-tubulin (*Ben A*), RNA polymerase II gene (*RPB2*), and Calmodulin (*CaM*) are used to identify species (Samson et al., 2014). The three regions, *CaM, Ben A* and *RPB2*, are recommended for *Aspergillus* species, although *RPB2* is difficult to amplify. In contrast, *Ben A* is easy to amplify however, it has been reported to vary in the number of introns and results in amplification of the paralogous gene. *CaM* is easy to amplify, easily differentiates among *Aspergillus* species and it is therefore recommended as the primary marker (Samson *et al.*, 2014).

1.11 Fumonisin detection methods

Chromatography and immunno assays were identified as standard methods for the detection of FUM (Munawar *et al.*, 2019). Fumonisin contamination in maize can be detected by various methods such as Liquid Chromatography (LC), High Performance Liquid Chromatography (HPLC), lateral-flow devices and rapid test kits Enzyme-Linked Immunosorbent Assay (ELISA) (Bowers *et al.*, 2014).

Liquid Chromatography is more sensitive and specific with a limit of detection and quantification as low as 0.001 ug/g of fumonisins however, it is laborious and requires expensive equipment (Bowers *et al.*, 2014; Deepa *et al*. 2019). The most commonly used method for fumonisin extraction has been HPLC with fluorescent detection, separation, and quantification based on fluorescence intensity (Deepa *et al*. 2019). The lateral-flow flow devices make use of strip test that are rapid and easy to use however it provides semi-quantitative results and the exact mycotoxins levels may need confirmation methods such HPLC (Maragos, 2004).

The ELISA is a common and rapid method of screening maize samples for FUM; it is less costly and easy to use in the laboratory and field, exhibiting comparable sensitivity and higher selectivity characteristics (Munawar *et al.*, 2019;).The drawback of ELISA is that although the antibodies are specific and sensitive because the target compounds are mycotoxins not the antigens, compounds with same chemical group can also interact with the antibodies (matrix effect) which can result in underestimates or overestimates in mycotoxins concentration (Zheng *et al*., 2006).

The black *Aspergillus* produces extrolites (Varga *et al.*, 2011) secreted into the extracellular medium that belongs to diverse chemical and structural families (Leitão and Enguita, 2014). Therefore, the production of mycotoxins by *Aspergillus* species is an efficient identification aid for allocating an *Aspergillus* isolate to a section. Important extrolites such as FUM are produced by sections *Nigri* (Samson *et al.*, 2014, Varga *et al.*, 2011). Polyphasic identification may be ideal, however, occasional secondary metabolic profiles can be sufficient to identify isolates of A*spergillus* (Samson *et al.*, 2014).

All of the discussed FUM analysis methods are available, easy to apply and are dependable to detect and quantify FUM contamination, which provide a foundation for ensuring that legislative obligations are being met (Shephard, 1998). Rapid test kits Enzyme-Linked Immunosorbent Assay is the technique used in current study to measure FUM in maize samples, which will be discussed in the next Chapter.

CHAPTER 3: The incidence of *Fusarium* species and fumonisins in maize kernels produced by smallholder farmers in South Africa

ABSTRACT

Fusarium species are important fungal pathogens associated with maize ear rot in South Africa. The most frequently isolated *Fusarium* sp. is *F. verticillioides* and is reported to produce fumonisin B_1, B_2 and B_3. The morphological characterization, Sanger DNA sequencing, and phylogenetic analysis of *Fusarium* isolates retrieved from naturally infected maize kernels were investigated in this study. Maize kernels were collected from five provinces in South Africa, namely the Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga, and North West Provinces. A total of 107 maize kernel samples were collected from which *Fusarium* spp. were isolated, purified and morphological identified into species group levels. nBLAST and phylogenetic analysis using Parsimony and Maximum Likelihood of the *TEF-1α* gene regions were performed to confirm the species identification. Fumonisin levels present in the maize kernels were quantified using ELISA. Results indicated that 41 % of samples were identified as *F. verticillioides* as a major *Fusarium* species from maize kernels and it can produce fumonisins that have adverse health effects on humans and animals. In addition, *F. temperatum* at 5.6 % was the second most common *Fusarium* spp. isolated from the maize kernels. High levels of fumonisin were detected from the KwaZulu-Natal samples that exceeded 2 000 µg/kg, which is the maximum allowable levels set by the Department of Health in South Africa for maize for direct human consumption. The results also indicate subsistence farmers and their communities are chronically exposed to high levels of fumonisins. Therefore, there is a need for more awareness and mycotoxin control measures to reduce the exposure risk of resource-poor farmers and people in the communities.

3.1 INTRODUCTION

Maize is an important field crop and staple food in South Africa and is produced in all provinces under different climatic conditions (Badu-Apraku and Fakorede, 2017, Du Plessis, 2003). The total maize production in South Africa for the 2020/2021 season was 15.3 million metric tons (Crop Estimates Committee, 2021). Successful maize production is limited by plant diseases (Sibiya *et al.*, 2013), which result in a global yield reduction of approximately 23 % (Berger *et al.*, 2020). Smallholder farming plays a role and has the potential to increase food security and reduce reliance on purchasing food in both rural and urban communities in South Africa (Baiphethi and Jacobs, 2009)

Maize ear rot is the important disease in maize because not only does it reduce yield, but it also reduces the nutritional value of the maize kernels and it can also result in contamination with secondary metabolites, called mycotoxins, such as fumonisins (FUM) that cause harm to human and animals (Lanubile *et al.*, 2017, Logrieco *et al.*, 2002). In *Fusarium*, FUM are primarily produced by *F. verticillioides* and *F. proliferatum* (Placinta *et al.*, 1999). The major *Fusarium* sp. associated with maize ear rot is *F. verticillioides.* Other *Fusarium* species that have been reported from maize ear rot include the *F. graminearum* species complex, *F. subglutinans*, and *F. proliferatum* (Beukes *et al.*, 2017, Munkvold, 2003).

Environmental conditions such as high humidity and high temperature increase the risk of fungal infection and mycotoxins production. These environmental factors impact the development, survival, distribution, and incidence of mycotoxigenic fungi and toxin accumulation (Daou *et al.*, 2021). Richard *et al.* (2003) further added that temperature and humidity also affect plant growth, strength, and health and influence the competitiveness of mycotoxigenic fungi. Agricultural practices in the field before and after planting, good harvest practices, proper drying measures, and good storage practices are important in minimizing mycotoxin accumulation (Jouany, 2007).

In South Africa, FUM were first reported from maize kernels in the Eastern Cape Province (Sydenham *et al.*, 1990). Fumonisin B_1 has been linked to human oesophageal and liver cancer (Ono *et al.*, 2001, Sun *et al.*, 2007) and the International Agency for Research on Cancer characterized the FUM as group 2B carcinogens (Gelderblom and Marasas, 2012). In animals, FUM induce several diseases such as

leukoenphalomalacia in horses (*Equus caballus* L.) and pulmonary oedema in swine (*Sus scrofa domesticus* L.) and toxic effects in broiler chicks (*Gallus gallus domesticus* L.) and turkey poults (*Meleagris gallopavo* L.) (Ono *et al.*, 2001, Weibking *et al.*, 1993). Ingested mycotoxins penetrate mammalian cells, where the mycotoxins reach the cellular genome that then results in a mutagenic defect in the genome. The defect can eventually lead to the development of cancer (Ahmed Adam *et al.*, 2017). In South Africa, high FUM levels of 104-2371 µg/kg for FB_1 and 103-673 µg/kg for FB_2, produced by natural occurring *Fusarium* spp. has been reported in high frequency from compound food that contain mixtures of products for oral feeding (Kebede *et al.*, 2020). The maximum allowable level for fumonisins (FB_1 and FB_2), as set by the South Africa Department of Health is 4 000 µg/kg in raw maize and 2 000 µg/kg maize flour and mealie meal for direct human consumption (Department of Health (DoH), 2016). Maize in the smallholder farming system of South Africa is mainly produced for direct human consumption and it can be contaminated with fumonisins, which can potentially cause cancer (Alberts *et al.*, 2019). There is currently limited information on the level of exposure by smallholder farmers and their communities who grow maize for household consumption and local trading. It is crucial to gather substantive data on the prevalence and level of fumonisins in smallholder farming in South Africa

Maize serves as a staple food for the majority of the South Africa population (Ala-Kokko *et al.*, 2021). Maize is commonly consumed fresh or processed into cooked meals (Misihairabgwi *et al.*, 2019). Maize is produced in both commercial and resource-poor subsistence farming systems in South Africa (Ncube *et al.*, 2011). The risk of consuming mycotoxins contaminated maize is high such that it could result in serious health hazards. The aim of this study was to characterize *Fusarium* species causing maize ear rot, and the quantification of FUM mycotoxins from maize kernels collected from smallholder farmers in South Africa.

3.2 MATERIALS AND METHODS

3.2.1 Origin of samples

Maize kernels were collected from randomly selected smallholder farmers in the Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga, and North West Provinces in South Africa during storage. A total of 107 samples ranging in weight from 700 g to 2 kg were collected from the five provinces (Figure 3.1; Table 3.1). The maize samples

were analyzed within a month of being received. They were shelled and packed into brown bags labelled with the location and contact information of the farmers upon receipt. The shelled maize samples were stored at 4 °C until fungal isolation was done.

Table 3. 1: Number of maize kernel samples collected from smallholder farmers per area and province

Province	**Area (District Municipality)**	**Number of samples**
Eastern Cape	Alfred Nzo	11
Eastern Cape	Amathole	2
KwaZulu-Natal	Umzinyathi	13
Limpopo	Sekhukhune	22
Limpopo	Waterberg	11
Mpumalanga	Gert Sibande	13
Mpumalanga	Nkangala	4
Mpumalanga	Enhlanzeni	16
North West	Ngaka Modiri Molema	14
North West	Kenneth Kaunda	1

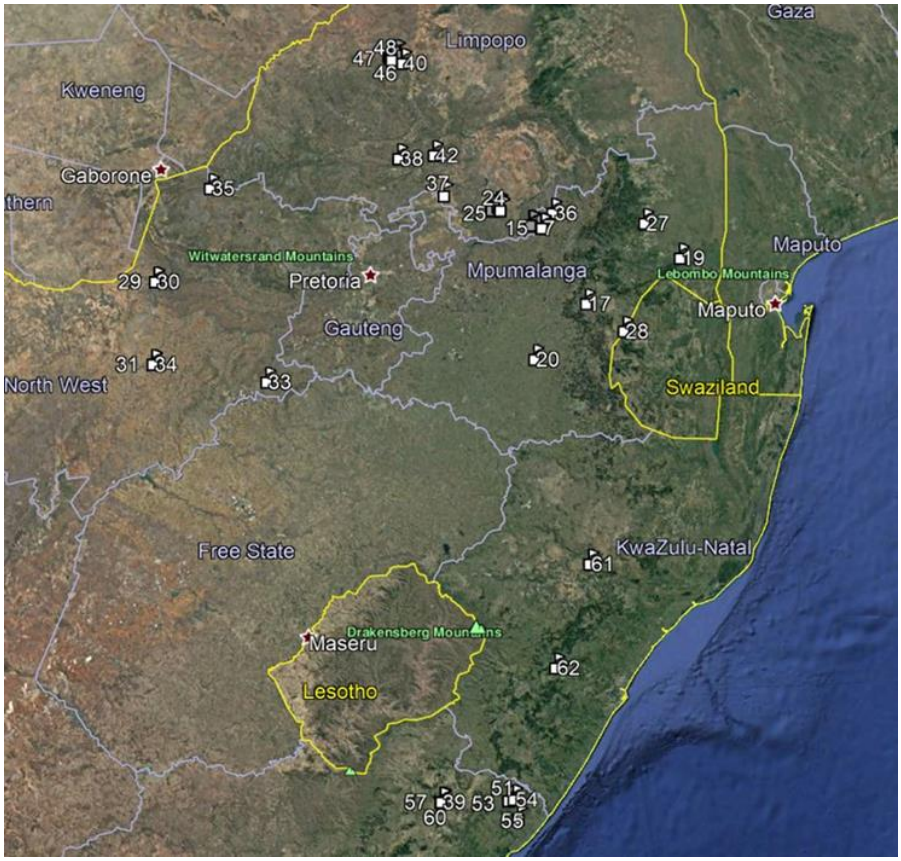


Figure 3. 1: Map of the eastern half of South Africa showing some of the locations where sampling was done. Number indicates sample number (Google Earth Pro).

3.2.2 Isolation of *Fusarium* from maize kernels

Fusarium spp. were isolated from shelled, mixed symptomatic and symptomless maize kernels. The kernels were surface-sterilized by dipping once in 70 % (v/v) ethanol, followed by soaking in 1.60 % (v/v) Sodium hypochlorite (NaOCl) for three minutes, rinsed three times in sterile distilled water and air-dried for 20 minutes on filter paper on the bench of a laminar flow cabinet. One hundred maize kernels from symptomatic and symptomless were plated out for each sample with four kernels per plate on *Fusarium* selective Rose Bengal-Glycerine-Urea medium (RbGU, Merck, South Africa) containing 250 mg chloramphenicol (Medirex Pharmacy, South Africa) to suppress bacterial growth (Van Wyk *et al.*, 1986) in 90 mm Petri dishes.

After five days of incubation at 25 °C, colonies resembling *Fusarium* were selected and purified onto clean ½ strength potato dextrose agar (w/v) (½ PDA, Merck, South Africa) plates. The representative isolates were single-spored by preparing a spore suspension in sterile water and plating out 1 mL into a water agar (WA, Merck) plate,

distributed evenly on the plate and excess liquid poured off. The plates were incubated for 16-24 hours at 25 °C. A single spore was picked up with a sharp needle and plated onto ½ PDA Petri dishes using a stereo microscope (Nikon, Japan) with a light source.

The pure cultures were grown at 25 °C for seven days on ½ PDA (w/v). The pure cultures were preserved by freezing at - 80 °C at the ARC-Grain Crops, Potchefstroom, South Africa. A spore suspension in 15 % (v/v) glycerol was prepared and 1.8 mL of the glycerol spore solution was transferred to a 2 mL cryovial tube (Lasec, South Africa). The cryovial was vortexed before freezing to make sure the spores are not in the bottom of the tube before storage (Leslie and Summerell, 2006).

3.2.3 Morphological identification

All *Fusarium* isolates were cultivated on Carnation Leaf Agar (CLA) and PDA (Leslie and Summerell, 2006) at 25 °C for seven days to observe morphological characters. Morphology characters were described based on shape and size of macroconidia and shape of microconidia on CLA, and the colour of the culture on PDA (Fisher *et al*., 1982). The microscopic characteristics were observed at 40x magnification using a Axio Lab A1 Zeiss microscope (Carl Zeiss, Germany).

3.2.4 Molecular identification

3.2.4.1 DNA extraction

Single spore isolates of *Fusarium* were cultured on ½ PDA at 25 °C for seven days in the dark. Mycelia (ca. 100 µg) were collected from each culture by scraping off the hyphae and spores with a sterile scalpel and depositing it in a sterile Eppendorf tube (Biopiente scientific). Next, DNA was extracted using the NucleoSpin® Microbial DNA Kit (Macherey–Nagel, Germany) according to manufacturer's instructions: 100 µg of the fungal culture was added to 2 mL tube with glass beads; 100 µL Elution buffer BE being added and cells were re-suspended. Lysis Buffer MG 40 µL and liquid proteinase K (Macherey–Nagel, Germany) of 10 µL were added and the NucleoSpin was agitated on a disruptor genie (Scientific Industries, USA) for 20 minutes.

The mixture was centrifuged (Merck Millipore, South Africa) for 30 s at 11 000 g to clean the lid. The DNA binding conditions were adjusted by adding 600 µL of lysis buffer MG; mixed by vortexing and centrifuged for 30 s at 11 000 g. The supernatant (≈500-600 µL) was transferred onto the NucleoSpin® Microbial DNA column. The DNA

column was centrifuged at 30 s and was placed into a fresh collection tube. The first wash of silica membrane was done by adding 500 µL wash buffer BW and centrifuged at 30 s at 11 000 g.

The flow-through was discarded and the column was put back into the collection tube. The second wash buffer B5 of 500 µL was added to the DNA column and centrifuged at 30 s at 11 000 g. The flow-through was discarded and the DNA column was dried by centrifuging for 30 s at 11 000 g to remove residual buffer. The DNA column was placed in a nuclease-free tube and DNA was eluted by adding 100 µL Elution Buffer BE onto the column and incubated at room temperature for 1 minute.

After incubation, the NucleoSpin® Microbial DNA Column was centrifuged for 30 s at 11 000 g, the extracted DNA was stored at - 20 °C until further use. Genomic DNA agarose electrophoresis on 1 % (w/v) gel was done to confirm bands. A molecular marker of 100 bp PLUS™ DNA ladder (Thermo Scientific) was used to determine the concentration of DNA and quality of DNA. The DNA was loaded with 2 µL loading dye from ferments and 1x Tris-borate- EDTA (TBE) (w/v) buffer (Sigma–Aldrich, USA) was used to run the 1 % gel electrophoresis. The gel was stained with 1 µL of gel red nucleic acid (Biotium) and bands were observed under UV light using Gel doc imager documentation system (Bio Rad Laboratories, USA).

3.2.4.2 Polymerase chain reaction

Fungal DNA for *Fusarium* spp. was amplified using primers for the translation elongation factor 1-α (*TEF-1α*) gene region using EF-1 (5′-ATG GGT AAG GAR GAC AAG AC-3′) and EF-2 (5′- GGA RGT ACC GTS ATC ATG TT-3′) supplied by Whitehead Scientific, South Africa (O'Donnell *et al.*, 1998). The PCR mixtures consisted of 1 µL genomic DNA, 12.5 µL OneTaq 2X Master Mix (Inqaba Biotech, South Africa), 0.5 µL of each primer, and 10.5 µL DNA free water (Inqaba Biotech, South Africa). Amplification conditions for the *TEF-1α* gene region included initial denaturation of 5 min at 94 °C; followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C; and a final elongation step of 7 min at 72 °C. The PCRs were performed using a MyCyclerTM Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, Singapore) in a total reaction volume of 25 µL.

3.2.4.3 Sanger DNA sequencing

PCR amplicons were purified using a QIAquick PCR Purification Kit followed by Sanger DNA sequencing that was done at Inqaba Biotech in a single direction using the PCR primers and the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). The sequences were analyzed with an ABI Prism 3730XL Sequencer (Applied Biosystems). The raw sequence was cleaned (base calling) and edges trimmed using ChromasLite version 2.6.6. DNA sequences were submitted to the basic local alignment search tool (nBLAST) on GenBank at the National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the *Fusarium* MLST database hosted by MycoBank (https://fusarium.mycobank.org/) to confirm the identity of isolates.

3.2.5 Phylogenetic analyses

Reference sequences for *Fusarium* spp. were selected based on nBLAST results (Table 3.2). Multiple sequence alignments of the dataset was generated in MAFFT v. 7.427 (Katoh and Standley, 2013) using the auto strategy, with alignment edges manually trimmed in Bioedit v. 7.2.5 (Hall, 1999). The aligned dataset was analysed using Maximum Likelihood (ML) in IQtree v. 2.1.1 (Nguyen *et al.*, 2015). For each dataset the most suitable model was calculated using Modelfinder (Kalyaanamoorthy *et al.*, 2017) and ultrafast bootstrapping approximation done using UFBoot2 using 1 000 replicates (Hoang *et al.*, 2018), both integrated into IQtree. The tree was visualised in Figtree v. 1.4.4 (https://github.com/rambaut/ figtree/releases). The phylogeny tree for *Fusarium* spp. was rooted using *Fusarium equiseti* NRRL 26419 as an outgroup.

Table 3. 2: *Fusarium* reference strains used in phylogenetic analyses

Species	**Isolate number**	***TEF-1α* GenBank accession number**	**Country**	**Host**	**Reference**
Fusarium verticillioides	NRRL 25111	JF740737	USA	Culture	(O'Donnell *et al.*, 2012)
Fusarium verticillioides	NRRL 22172	AF160262	Germany	Maize	(Scauflaire *et al*., 2011)
Fusarium verticillioides	MRC 2228	MH582332	Egypt	Maize	(O'Donnell *et al.*, 2018)
Fusarium verticillioides	MRC 2326	MH582319	USA	Sweet potatoes	(O'Donnell *et al.*, 2018)
Fusarium verticillioides	MRC 2629	MH582328	USA	Maize	(O'Donnell *et al.*, 2018)
Fusarium temperatum	NRRL 25622	AF160301	South Africa	Maize	(Scauflaire *et al.*, 2011)
Fusarium temperatum	CBS 135540	KF956084	Netherlands	Culture	(Al-Hatmi *et al.*, 2016)
Fusarium temperatum	MRC 134	MH582312.1	Zambia	Maize	(O'Donnell *et al.*, 2018)
Fusarium oxysporum	CBS 144134T	MH485044	Germany	*Solanum tuberosum*	(Lombard *et al.*, 2019)
Fusarium oxysporum	CBS 463.91	KU711712	Netherlands	Culture	(Al-Hatmi *et al.*, 2016)
Fusarium oxysporum	CBS 130301	MH485017	USA	Human	(Lombard *et al.*, 2019)
Fusarium subglutinans	MRC 2627	MH582310	USA	Maize	(O'Donnell et al., 2018)
Fusarium subglutinans	NRRL 22016	AF160289	USA	Maize	(Scauflaire *et al.*, 2011)
Fusarium subglutinans	CBS 479.94	MN534036.	South Africa	Maize	(Yilmaz *et al.*, 2021)
Fusarium equiseti	NRRL 26419T	GQ505599	USA	Culture	(O'Donnell et al., 2009)

3.2.6 Fumonisins quantification using ELISA

The use of rapid detection method such as ELISA play a critical role in quantifying total FUM (Kononenko *et al.*, 1999, Ono *et al.*, 2001). Maize kernels were milled using a cyclotec milling machine (Foss, Rhine Ruhr, South Africa) whereby 5 g aliquots from the milled sub-samples was mixed thoroughly before extraction. Fumonisins were extracted in 70 % (v/v) methanol (Microsep, South Africa): water (70:30, v/v) solution. Five gram of the sample as mixed with 25 mL of 70 % methanol/water solvent and the mixture was shaken vigorously for 3 minutes using a laboratory shaker (Analytical Diagnostic Products, USA). The extract (5 mL) was filtered through Whatman # 1 filter (Sigma-Aldrich, USA).

Samples were diluted by adding 100 µL of the extract to a pre-filled with 7.9 mL of deionized water sample dilution bottles and mixed by swirling. The sample was then ready for analysis using the Veratox enzyme-linked immunosorbent assay (ELISA) quantitative fumonisin 5/10 test kit (Neogen Corp, Lansing, MI, USA). Five standards (0 µg/g, 0.5 µg/g. 1 µg/g, 3 µg/g, 6 µg/g) were included in each experiment. All reagents were mixed well by swirling. Next, 100 µL of the conjugate blue-labelled (Analytical Diagnostic Products, USA) was added to the red marked mixing well. This was followed by adding 100 µL of the standards and samples to each red-marked mixing well. A 12-channel pipette was used to mix the liquid in the wells by pipetting up and down three times. The mixture of 100 µL was transferred to the antibody-coated wells and red marked mixing wells were discarded.

A timer was set for five minutes, while the well was mixed for the first minute at room temperature incubation by sliding the microwell holder back and forth. After the five minutes' incubation period, the contents were shaken out and wells were washed five times with deionized water. The wells were dried by tapping on a paper towel. The substrate (100 µL) from the green-labelled bottle (Analytical Diagnostic Products, USA) was added to the wells and incubated for 10 minutes while mixing by sliding. The red stop solution (Analytical Diagnostic Products, USA) of 100 µL was added to the wells to stop the reaction. The plate was mixed thoroughly and the bottom of the plate was wiped dry. The plate was read on a universal microplate reader (Analytical Diagnostic Products, USA) using a 650 nm filter. The results were calculated using Neogen veratox for windows software.

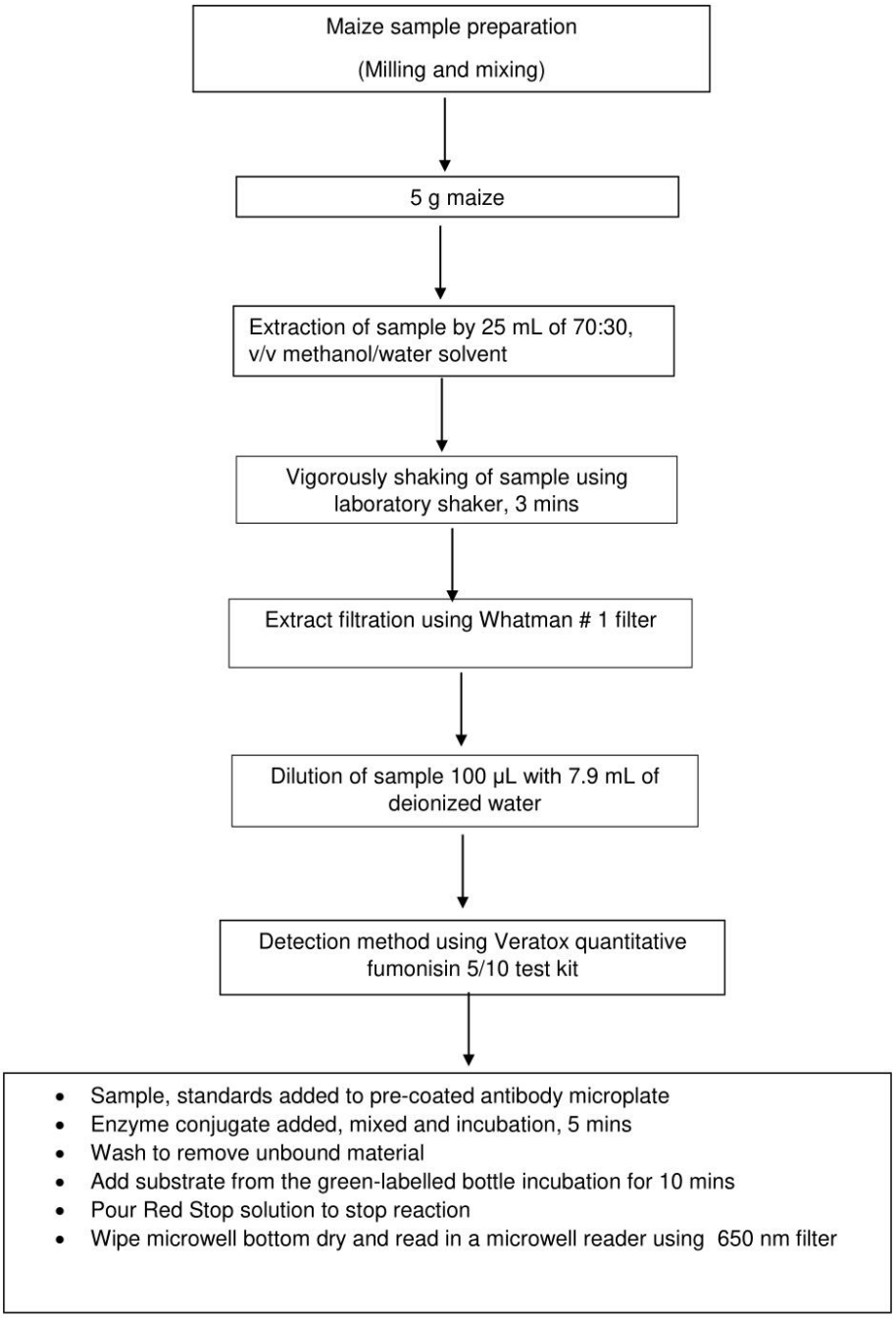


Figure 3. 2: Flow diagram of steps on how fumonisins were extracted using ELISA method.

3.3 RESULTS

3.3.1 Morphological characterization

From the 107 symptomatic and symptomless maize kernel samples, 49 *Fusarium* isolates were retrieved and characterized based on morphological characters with 41 isolates collected from Limpopo (19 samples), Mpumalanga (13 samples), North West (7) and Eastern Cape (2 samples) identified as *F. verticillioides,* six as *F. temperatum* isolates collected from Limpopo (3 samples), Mpumalanga (2 samples) and one sample from North West, two samples from Limpopo as *F. subglutinans*, and *F. oxysporum*. The identification was based on typical *Fusarium* structures observed using the *Fusarium* Laboratory Manual (Leslie and Summerell, 2008). Morphological characters for *F. verticillioides* included macroconidia that were 3-septate on CLA (Figure 3.3 A), microconidia formed in long chains and in clusters that arised from monophialides (Figure. 3.3 B-C) and the colony on PDA was defined by white mycelia developing violet pigment as the culture age (Figure 3.3 D-E). Pigmentation in the agar varies, ranging from no pigmentation or greyish orange to dark violet in others.

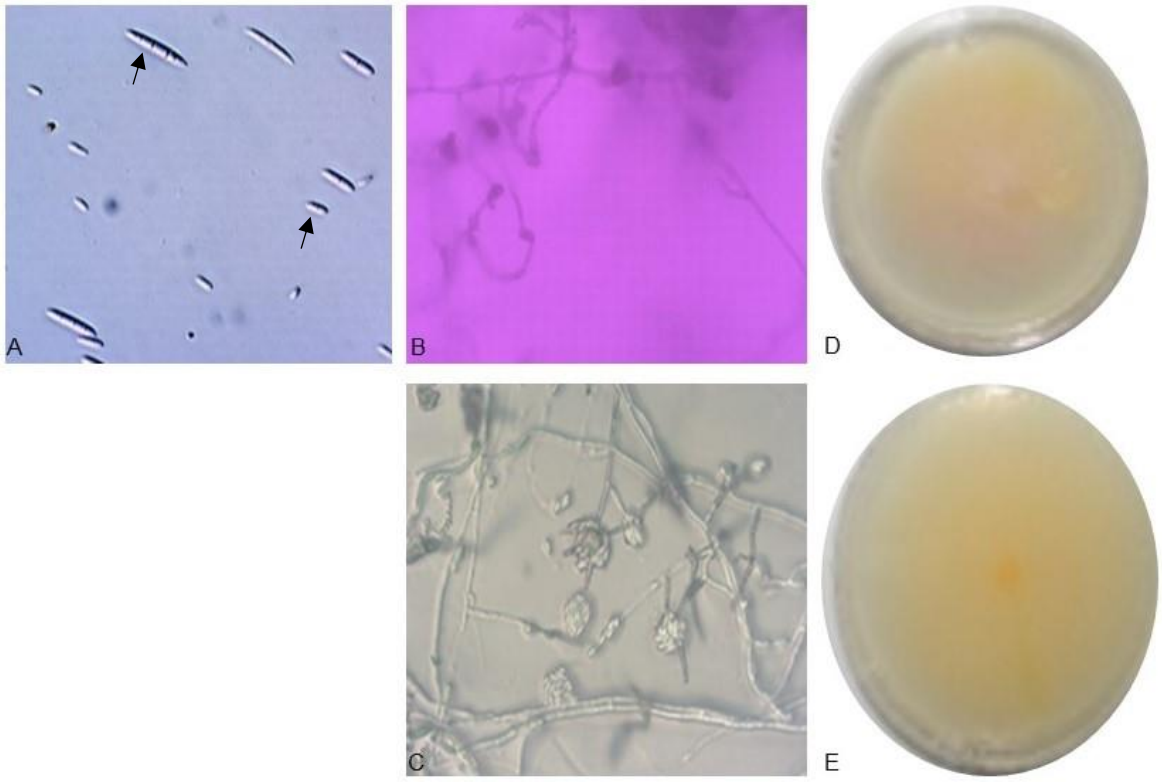


Figure 3. 3: *Fusarium verticillioides*: A= macroconidia, B-C= microconidia in chains and clusters under light microscope at 40X magnification; D-E = greyish orange to dark violet colony on PDA.

In *Fusarium subglutinans* the macroconidia were produced rarely, usually 3-septate (Figure 3.4 A), microconidia borne in false heads on CLA on mostly polyphialide, and sometimes monophialide conidiophores (Figure 3.4 B) and the colony on PDA start with white mycelial growth that forms a violet pigmentation on the agar (Figure 3.4 C-D).

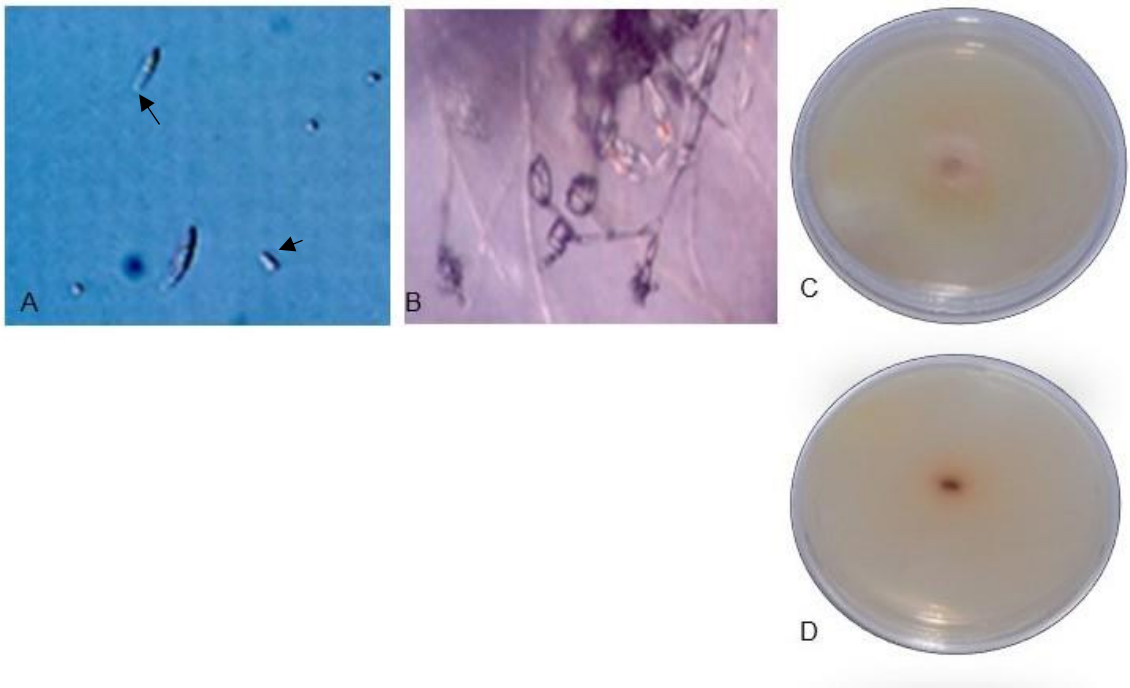


Figure 3. 4: *Fusarium subglutinans:* A= macroconidia and microconidia B= false heads on CLA under light microscope at 40X magnification; C-D= orange colony forming violet pigmentation colony on PDA.

The macroconidia of *F. temperatum* were rare, curved and 3-septate (Figure 3.5 A). The microconidia produced formed false heads on monophialides on CLA media (Figure 3.5 B). The colony on PDA was characterized by white mycelium that produced a yellowish pigment (Figure 3.5 C-D).

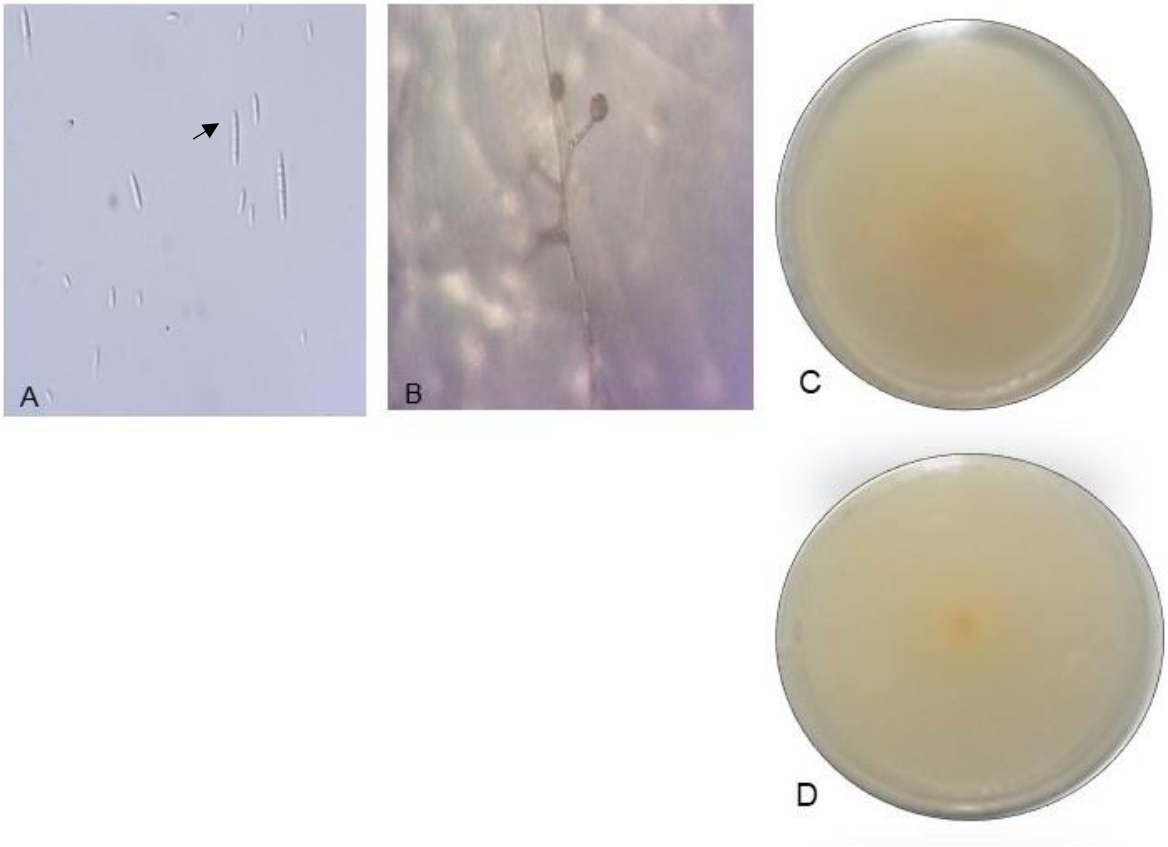


Figure 3. 5: *Fusarium temperatum:* A= macroconidia, B= false heads on CLA under light microscope at 40X magnification, C-D= yellowish colony on PDA.

Fusarium oxysporum macroconidia were curved and pointed on CLA (Figure 3.6 A). The microconidia were produced in false heads on short monophialides (Figure 3.6 B) and the colony on PDA were white cottony mycelium with purple under surface (Figure 3.6 C-D).

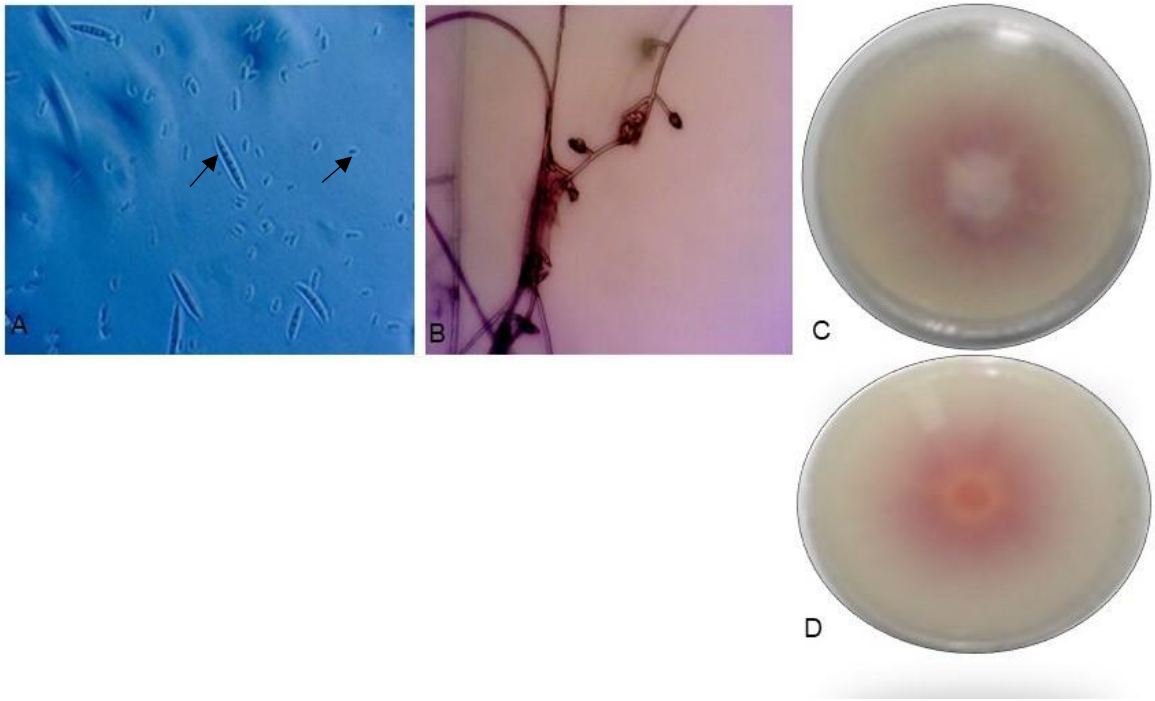


Figure 3. 6: *Fusarium oxysporum:* A= macroconidia and microconidia; B= microconidia in false heads under light microscope at 40X magnification, C-D= purple colony on PDA.

3.3.2 Molecular characterization

3.3.2.1 DNA extraction and PCR assay

The DNA bands were visualized using gel electrophoresis to confirm DNA integrity and concentration (Figure 3.7). The PCR assay of the *TEF-1α* was successfully performed and amplicon product size of approximately 650 base pairs (bp) indicated in Figure 3.8.

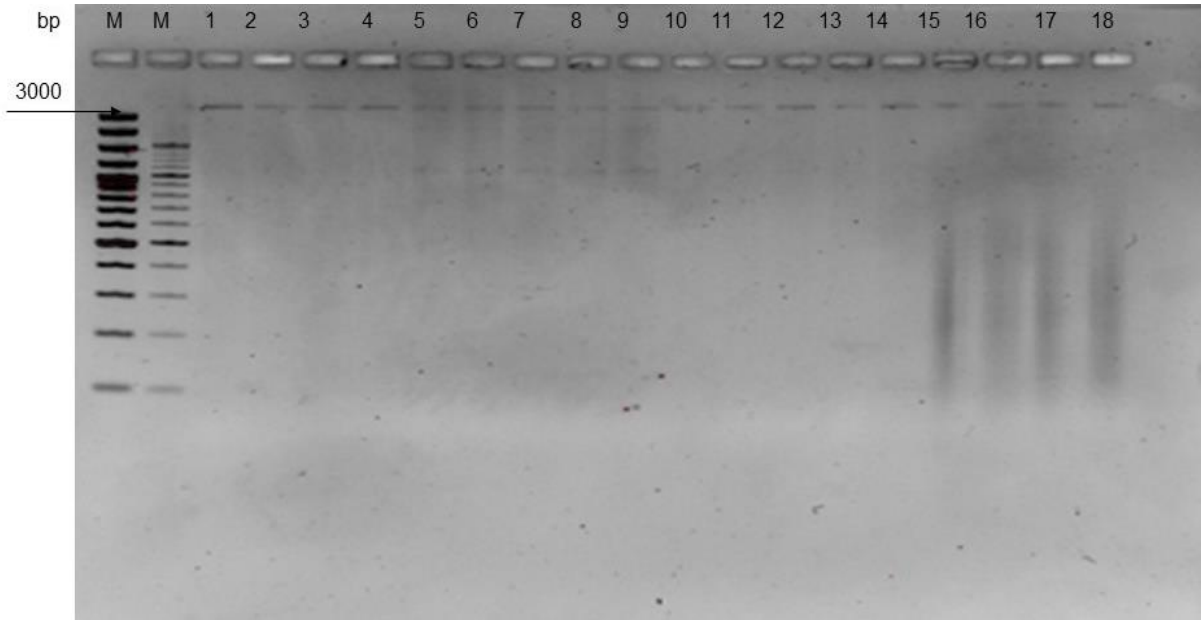


Figure 3. 7: *Fusarium* genomic DNA 1.5 % agarose gel. M: molecular markers (O' Gene Ruler 100 bp PLUS^{TM} DNA ladder, Thermo Scientific), lane 1-18 = isolates 1.2; 2.4; 5.1; 6; 10.1; 10.3; 12.2; 14; 15.1; 16.2; 24.2; 29.2; 30.1; 40; 30.3; 82; 85 and 87.

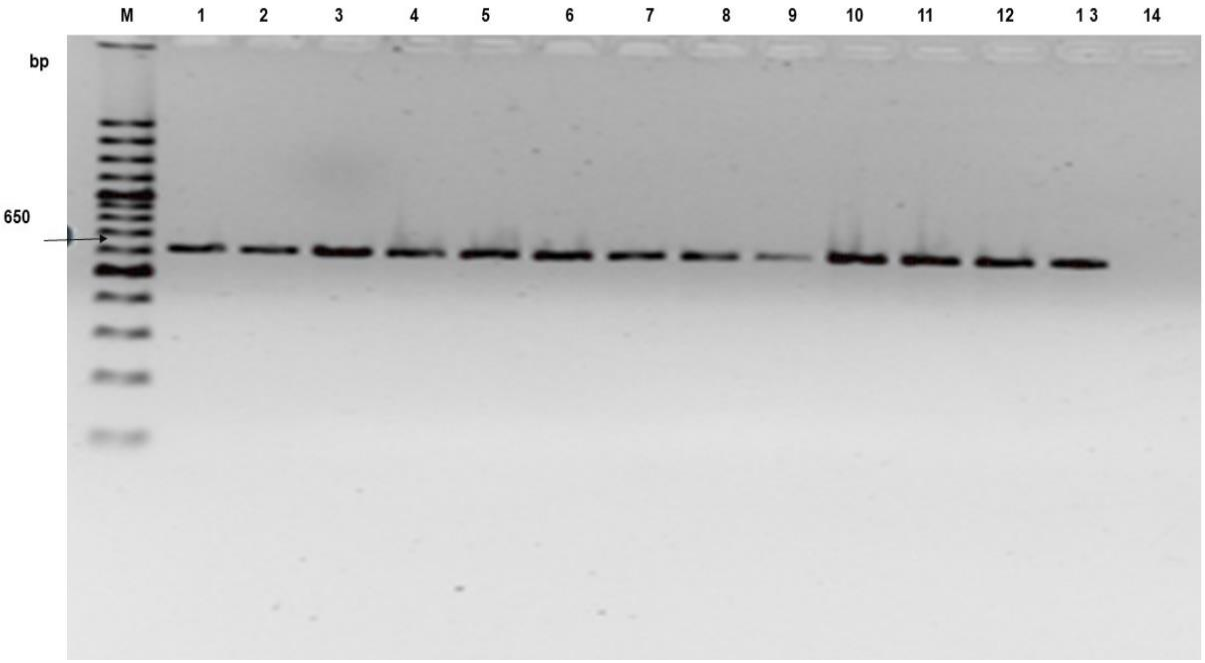


Figure 3. 8: PCR amplicons of *TEF-1α* with approximately 650 base size (bp) *Fusarium* fungal isolates 1.5 % agarose gel. M: marker (O' GeneRuler 100 bp DNA ladder, Thermo Scientific), lane 1-14 = isolates 1.2; 2;4; 6; 10.1; 10.3; 12.2; 14; 15.1; 16.2; 82; 25; 91 and negative control (ntc).

3.3.2.2 Phylogenetic analysis

The obtained sequences from the 49 isolates were subjected to nBLAST analysis on the GenBank and the *Fusarium* MLST databases and the highest similarity match for each isolate was indicated in Table 3.3. The results from both databases were consistent for all the isolates, and no discrepency occurred in the species names. The phylogenetic analyses based on Parsimony and Maximum Likelihood resulted in high bootstrap support for all the species clades (Figure 3.9). Both the nBLAST results and the bootstrap support of the clades of the phylogenetic tree supports the morphological identification. The four identified species grouped into two species complexes which are represented by the *Fusarium fujikuroi* species complex (*F. verticillioides*, *F. subglutinans,* and *F. temperatum*) and *Fusarium oxysporum* species complex (*F. oxysporum*). Strong bootstrap support (value = 98) associated 41 isolates with *F. verticillioides.* The *F. verticillioides* grouped into five lineages while *F. temperatum* grouped into two lineages with a strong bootstrap value of 100 and *F. subglutinans* and *F. oxysporum* identified with strong bootstrap (values=100) respectively, (Figure 3.9).

Table 3. 3: *Fusarium* MSLT and Gene Bank database results based on *TEF-1α* region of *Fusarium* isolates retrieved from maize kernel collected from smallholder farmers in South Africa

Isolate number	Species	Accession #	Species	Accession #
	MSLT database		Gene Bank database	
1.1	*F. verticillioides*	MH582327	*F. verticillioides*	MN689177
1.2	*F. verticillioides*	MH582330	*F. verticillioides*	MN223455
2	*F. verticillioides*	MH582332	*F. verticillioides*	KC964129
4	*F. verticillioides*	MH582315	*F. verticillioides*	MN223455
5.1	*F. verticillioides*	MH582327	*F. verticillioides*	MN689177
5.2	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
6	*F. subglutinans*	MH582310	*F. subglutinans*	JX456583
7	*F. verticillioides*	MH582324	*F. verticillioides*	KF993992
8.1	*F. verticillioides*	MH582328	*F. verticillioides*	MN223454
8.2	*F. verticillioides*	MH582328	*F. verticillioides*	MN689177
9.1	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
9.2	*F. verticillioides*	MH582324	*F. verticillioides*	KF993992
10.1	*F. verticillioides*	MH582332	*F. verticillioides*	MN223455
10.2	*F. verticillioides*	MH582328	*F. verticillioides*	MN223454
11.1	*F. verticillioides*	MH582327	*F. verticillioides*	KC964129
11.2	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
12.2	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
13	*F. verticillioides*	MH582327	*F. verticillioides*	MN689177
14	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
15.1	*F. verticillioides*	MH582324	*F. verticillioides*	KF993992
15.3	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
16.1	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
16.2	*F. verticillioides*	MH582332	*F. verticillioides*	KC964129
17.1	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
18	*F. temperatum*	MH582313	*F. temperatum*	MT237887
20.1	*F. temperatum*	MH582313	*F. temperatum*	KC964112
22.1	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
24.1	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
24.2	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
25.1	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
25.2	*F. temperatum*	MH582312	*F. temperatum*	MH013344
25.3	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
28.1	*F. verticillioides*	MH582332	*F. verticillioides*	MN223455
29.1	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
29.2	*F. verticillioides*	MH582332	*F. verticillioides*	KC964129
34	*F. temperatum*	MH582312	*F. temperatum*	MH013344
37	*F. temperatum*	MH582312	*F. temperatum*	MH013344
38	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129

Table 3.3 (continued): *Fusarium* MSLT and GenBank database results based on *TEF-1α* region of isolates retrieved from maize kernels

Isolate number	Species	Accession #	Species	Accession #
	MSLT database		Gene Bank database	
47	*F. temperatum*	MH582312	*F. temperatum*	MH013344
48	*F. oxysporum*	FJ985292	*F. oxysporum*	MN507110
50	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
56	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
74	*F. verticillioides*	MH582324	*F. verticillioides*	KC964129
82	*F. verticillioides*	MH582327	*F. verticillioides*	MN223455
84	*F. verticillioides*	MH582330	*F. verticillioides*	KF499581
85	*F. verticillioides*	MH582327	*F. verticillioides*	MW720602
87	*F. verticillioides*	MH582330	*F. verticillioides*	MN223455
88	*F. verticillioides*	MH582332	*F. verticillioides*	KC964129
91	*F. verticillioides*	MH582330	*F. verticillioides*	KF499581

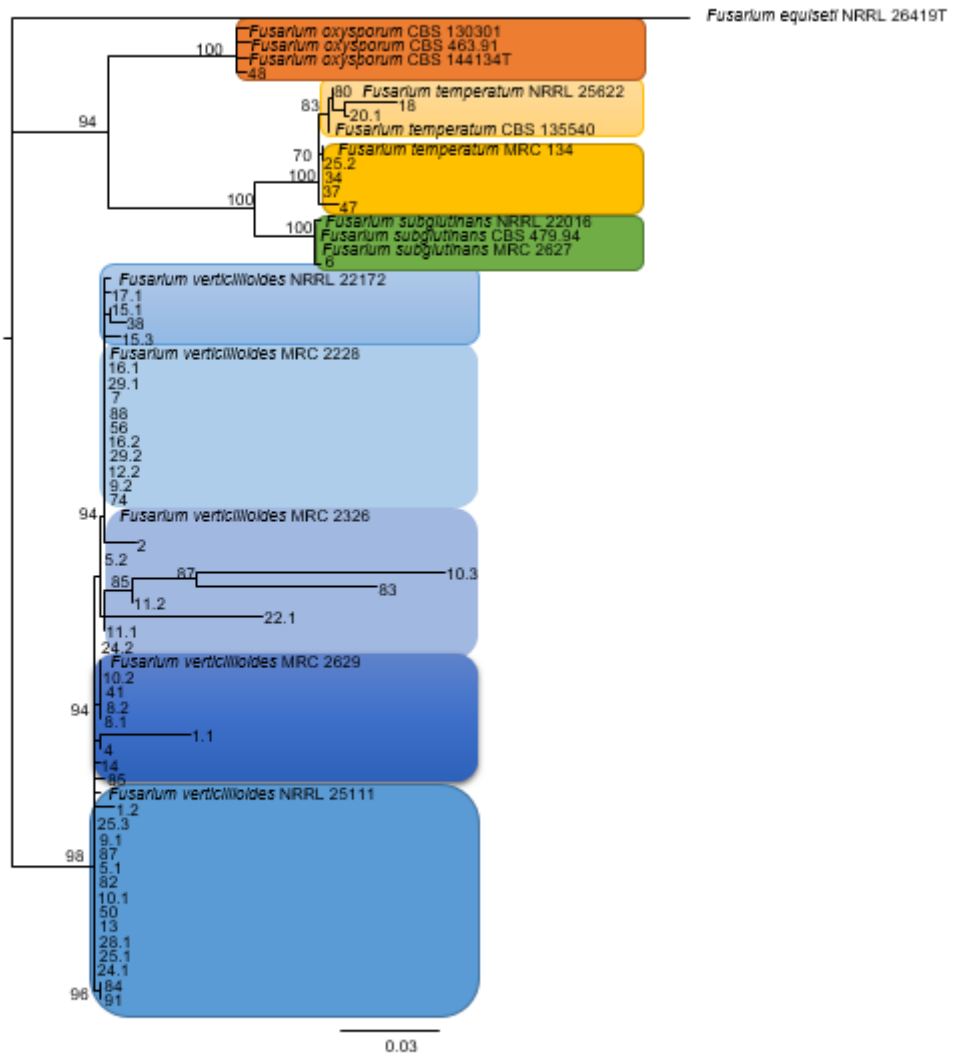


Figure 3. 9: Phylogenetic tree based on Parsimony and Maximum Likelihood inferred from partial *TEF-1α* sequences from *Fusarium* spp. isolated from maize kernels. The tree is rooted with *Fusarium equiseti* NRR 26419 as an outgroup.

3.3.3 Fumonisin analysis results

The ELISA analysis of the maize kernel samples collected in South Africa indicated that 91 % of the samples collected from KwaZulu-Natal were contaminated with fumonisins, with 55 % of the samples exceeding the maximum allowable limit of 2 000 µg/kg of total fumonisins (Table 3.4). Limpopo had the second highest incidence of

fumonisin contamination, with 39 % positive samples and 35 % of the total samples that had fumonisin levels exceeding 2 000 µg/kg. Samples from the Eastern Cape had the third highest positivity rate at 22 %, followed by Mpumalanga at 13 % and the North West at 7 %. All samples that were positive for fumonisin contamination exceeded the maximum allowable limit in Mpumalanga and North West Province (Table 3.4).

Table 3. 4: Incidence of fumonisin contamination in maize kernels collected from smallholder farmers in South Africa

Province	Samples analysed	Positive samples (%)	Sample % >2 000 µg/kg
Eastern Cape	9	22	11
KZN	11	91	55
Limpopo	23	39	35
Mpumalanga	40	13	13
North West	14	7	7

3.4 DISCUSSION

The morphological analysis in this study showed that *F. verticillioides* was the most prevalent *Fusarium* species isolated from maize kernels collected from subsistence farmers in Limpopo, Mpumalanga, North West and Eastern Cape, followed by *F. temperatum*. This is in agreement with findings by Rheeder *et al*. (1993) and Ncube *et al*. (2011). However, *F. temperatum* was previously classified as *F. subglutinans* and in 2011, *F. subglutinans* was divided into *F. temperatum* and *F. subglutinans sensu stricto* (Scauflaire *et al*., 2011). The mycotoxin profile of *F. temperatum* is not yet fully clarified in maize. Nevertheless, an assessment of mycotoxin production by 60 strains of *F. temperatum* cultivated on rice (*Oryza sativa* L.) showed that all strains produced beauvericin, moniliformin, fusaric acid, and fusaproliferin (Pfordt *et al*., 2020). In addition, a higher prevalence and aggressiveness of *F. temperatum* compared to *F. subglutinans* was found in German maize cultivation areas (Pfordt *et al*., 2020). All strains of *F. temperatum* consistently produced beauvericin but moniliformin and fumonisins were produced only by a single isolate of *F. temperatum* as reported by Scauflaire *et al*. (2012). The same results were reported for *F. temperatum* strains from Argentina (Fumero *et al*., 2015). *Fusarium temperatum* infection also causes maize seedling blight and root rot (Varela *et al*., 2013).

Since this study has shown that *F. temperatum* is the second most common *Fusarium* spp. in maize produced by subsistence farmers in Limpopo, Mpumalanga and North West, it is thus important that further studies are conducted in South Africa to better understand the mycotoxin production by *F. temperatum* with particular focus on beauvericin which has insecticidal, antimicrobial, antiviral and cytotoxic activities (Zhang *et al*., 2016). Beauvericin has a potential for use as a bio-pesticide in crop production. *Fusarium proliferatum*, a common fumonisin-producing *Fusarium* sp., was not found in maize kernels from this study. This is possibly due to the dominance by *F. verticillioides*, as indicated by Gaige *et al*. (2020) that prior colonization of maize tissues by *F. verticillioides* reduced subsequent colonization by *F. proliferatum*.

The morphology of *F. temperatum* were similar to *F. subglutinans* and these two species could not be distinguished based just on morphology. Nevertheless, isolation of *Fusarium* spp. using plating out techniques on selective media followed by morphological identification and single spore purification is still important to generate reference cultures for use in databases for further identification by molecular methods and future research.

KwaZulu-Natal, Limpopo and the Mpumalanga area close to the Limpopo Province had more samples that were positive for FUM contamination compared to the North West Province. This could be due to agricultural practices such as planting farm saved seed that is prevalent in the Eastern Cape, KwaZulu-Natal, Limpopo and the Mpumalanga (Ncube *et al*., 2008, Phokane *et al*., 2019). *Fusarium verticillioides* is an endophyte of maize that can infect maize ears systemically from infected seed (Oren *et al*., 2003). The Eastern Cape, KwaZulu-Natal and Limpopo Provinces are well known hot spots for *F. verticillioides* infection (Aveling *et al*., 2020) and concomitant FUM contamination in maize (Marasas *et al*., 2001, Ncube *et al*., 2011, Phokane, 2018) possibly due to hot and dry conditions that occur during the maize growing season. These conditions are known to promote FUM production in maize (Janse van Rensburg, 2012).

The Department of Health in South African has implemented new regulations for fumonisins B_1 and B_2 limits in maize in 2016 (DoH, 2016). The maximum levels for fumonisins B_1 and B_2 in raw maize grain, intended for further processing was set at 4 000 µg/kg, while maize products for direct human consumption is set at 2 000 µg/kg.

Despite these regulations, subsistence farmers are still exposed to high levels of fumonisins since they produce grain for their own consumption. Their grain is not regulated or tested for FUM contamination. This indicates that monitoring and enforcing this regulation in an informal environment is impractical due to food insecurity. Therefore, studies by Alberts *et al*. (2019) highlighted control measures to reduce mycotoxin contamination in rural areas of South Africa should focus on agricultural practices such as grain sorting to discard mouldy kernels, planting *Fusarium* and FUM adapted maize varieties, control of insects, particularly the stem borer, *Busseola fusca*, hermetic storage procedures, and farmer education through extension services.

CHAPTER 4: Fumonisin-producing *Aspergillus* species isolated from maize kernels in South Africa

ABSTRACT

Aspergillus niger has recently been reported to produce fumonisin B_2 (FB_2). In this study, fumonisin-producing *Aspergillus* species isolated from maize kernels collected from smallholder farmers were investigated. Maize kernels were collected from five provinces in South Africa, namely Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga, and North West Provinces. A total of 107 maize kernel samples were collected from which *Aspergillus* spp. were isolated and identified. Selected colonies were purified and morphologically grouped into genus and species groups. Sanger DNA sequencing using the internal transcribed spacer (ITS) and secondary marker Calmodulin (*CaM*) gene regions and phylogenetic analysis using parsimony were performed to confirm morphological grouping of isolates. The ability of *Aspergillus* isolates from maize to produce fumonisin B_2 was assessed by analyzing the secondary metabolite profile of the isolates using QTOF-HRMS analysis. Based on morphological and molecular identification six *Aspergillus* sections were identified belonging to section *Nigri* and section *Flavi*. The results of the metabolite profiling showed that five *A. niger* isolates produced fumonisin B_2 in Yeast Extract Sucrose Agar. This is the first report of FB_2 production by *A. niger* isolated from maize in South Africa. The production of FB_2 by *A. niger* has implications in mitigating the risks of fumonisin contamination in maize kernels since *Fusarium* spp. and *Aspergillus* spp. differ in their ecological roles, their mode of infection and responses to management practices. Further studies to determine the extent of fumonisin production by *A. niger* under field conditions in South Africa need to be performed in various environments.

4.1 INTRODUCTION

Maize (*Zea mays* L.) is an important staple food in South Africa and the average intake per person per day may exceed 300 g (Shephard *et al.*, 2007). However, maize kernels are often contaminated with fumonisins (FUM). Fumonisin contamination of maize kernels is a major problem in all farming systems in South Africa. Numerous studies have been conducted focusing on *Fusarium verticillioides* as the major FUM producer in maize (Fandohan *et al.*, 2015). However, *Aspergillus* species have been reported to cause *Aspergillus* ear rot in maize and may induce the development of mycotoxins to cause food safety concerns (Ogara *et al.*, 2017). *Aspergillus* species reported to produce FUM (FB_2 and FB_4) are *A. niger* and *A. welwitschiae* (Ráduly *et al.*, 2020). Fumonisin production by *A. niger* isolated from maize kernels has not been reported in South Africa. Logrieco *et al.* (2014) indicated that the FB_2/FB_1 ratio was significantly higher in maize samples with *A. niger* infection. Fumonisin B_2 was detected in isolates of *A. niger* in Europe (Frisvad *et al.*, 2007) Fumonisin has serious adverse health effects on livestock and humans (Desjardins, 2006). Desjardins (2006) further mentioned that FUM are responsible for equine leukoencephalomalacia, a serious disease in horses (*Equus caballus* L.) and porcine pulmonary edema, a serious disease in swine (*Sus scrofa domesticus* L.). Fumonisin is linked to human oesophageal cancer and potential neural tube defects (Marasas *et al.*, 2004). Fumonisin B_2 is more cytotoxic than FB_1, because FB_2 inhibits sphingolipid biosynthesis via inhibition of sphinganine N-acyltransferase (Gutleb *et al*., 2002).

Aspergillus niger is considered a primary species in section *Nigri* on maize. The black *Aspergillus* section *Nigri* represents closely genetically related species (*A. niger* and *A. welwitschiae*) that are reported to be associated with maize kernels and they have a potential to produce FB_2. It can colonize maize in the pre-harvest period, during harvest or post-harvest, even at storage and FUM production can occur at any of these stages (García-Díaz *et al.*, 2020, Susca *et al.*, 2014). Some of the species of the black *Aspergillus* form symptomless infection on several crops, such as grape vine (*Vitis vinifera* L.), Damask rose (*Rosa* × *damacaena* Mill.) and Japanese yew (*Taxus cuspidata* Siebold & Zucc.) (Palencia *et al*., 2014, Perrone *et al*., 2008, Wani *et al*., 2010). Detailed research on their characterization of FB_2 on maize kernels has not been done worldwide (Logrieco *et al.*, 2014).

Logrieco *et al.* (2014) indicated that some strains of *A. niger* can produce FB_2 and to a lesser extent FB_4. *Aspergillus niger* is used in industrial production and is granted the GRAS (Generally Regarded as Safe) status by the Food and Drug Administration of the United States (Susca *et al.*, 2014). However, because some strains of *A. niger* are reported to produce mycotoxins such as ochratoxin and fumonisins (Palencia *et al.*, 2010), it is important to assess correct identification and FUM production by strains of *Aspergillus* (Susca *et al.*, 2014). *Aspergillus* characterization on maize is required to understand the contribution of *A. niger* on FUM production. This study characterized *Aspergillus* species in maize ears and the potential production of FB_2 by *Aspergillus* section *Nigri* from maize kernels collected from smallholder farms in South Africa.

4.2 MATERIALS AND METHODS

4.2.1 Origin of samples

Maize kernels were collected randomly from smallholder farmers in the Eastern Cape, KwaZulu-Natal, Limpopo, Mpumalanga, and North West Provinces in South Africa during post-harvest season. A total number of 107 samples ranging in weight from 700g to 2kg were obtained from the five provinces (Figure. 4.1; Table 4.1). The *Aspergillus* maize samples were analyzed concurrently with *Fusarium* isolations within a month upon arrival. They were shelled and packed into brown bags labelled with the location and contact information of the farmers. The shelled maize samples were stored at 4 °C until fungal isolation was done.

Table 4. 1: Number of maize kernel samples collected from smallholder farmers per area and province

Province	Area	Samples #
Eastern Cape	Alfred Nzo District Municipality	11
Eastern Cape	Amathole District Municipality	2
Kwazulu-Natal	Umzinyathi District Municipality	11
Kwazulu-Natal	Cedara Research Stations	1
Kwazulu-Natal	Makhathini Research Stations	1
Limpopo	Sekhukhune District Municipality	22
Limpopo	Waterberg District Municipality	11
Mpumalanga	Gert Sibande District Municipality	13
Mpumalanga	Nkangala District Municipality	4
Mpumalanga	Ehlanzeni District Municipality	16
North West	Ngaka Modiri Molema District Municipality	14
North West	Dr. Kenneth Kaunda District Municipality	1

Figure 4. 1: Map of eastern half of South Africa showing the sample locations. Number refer to the sample number (Google Earth Pro).

4.2.2 Isolation and morphology identification.

4.2.2.1 Isolation of *Aspergillus*

A hundred kernels from each symptomatic and symptomless maize sample were surface disinfected by dipping once in 70 % (v/v) ethanol, followed soaking in 1.60 % (v/v) sodium hypochlorite (NaOCl) for three minutes rinsed three times in sterile distilled water and air-dried for 20 minutes on filter paper on the bench of a laminar flow cabinet, where after four maize kernels was placed on each of 25 Petri (90 mm) dishes containing Dichloran Rose-Bengal medium amended with 250 mg Chloramphenicol to suppress bacterial contamination (King Jr *et al.*, 1979). Plated kernels were incubated at 25 ˚C for seven days. Fungal colonies representing *Aspergillus*, *Mucorales* and *Stenocarpella macrospora* were purified onto ½ PDA plates. The representative isolates were single-spored by adding 1 mL of sterile water onto the fungal culture and dislodging the spores. The spores were plated on water agar and incubated for 16-24 hours at 25 ˚C. A single germinating spore was picked and transferred on ½ PDA plates. The pure cultures were incubated for seven days and fungal isolates were preserved and used for DNA extraction.

4.2.2.2 Fungal cultures preservation

The pure cultures were preserved by freezing at - 80 °C (Nuaire, Inc, UK) at the ARC-Grain Crops, Potchefstroom, South Africa. A spore suspension in 15 % (v/v) glycerol was prepared and 1.8 mL of the glycerol spore solution was transferred to a 2 mL cryovial tube (Lasec, South Africa). The cryovial was vortexed before freezing to ensure that the spores are not in the bottom of the tube before storage (Leslie and Summerell, 2006).

4.2.2.3 Morphological Identification

Aspergillus isolates were plated on Czapek Yeast Autolysate Agar (CYA) (Thermo Fisher Scientific, MA, USA) and Malt Extract Agar (MEA) (Merck, Darmstadt, Germany) using the three-point inoculation pattern (Frisvad *et al.*, 2019, Samson *et al*., 2014). The plates were incubated for seven days at 25 °C in the dark and additional CYA plates were incubated at 5 °C, 30 °C and 37 °C (Samson *et al*., 2014). The colony characters used for identification were the growth rate, colony texture, degree of sporulation, mycelia colour, and colony reverse colour. The shape of the conidial heads, presence or absence of metulae between vesicle and phialides (uniseriate or

biseriate), colour and shape of stipes were observed from MEA plates (Samson *et al*., 2014) using an Axio Lab A1 Zeiss microscope (Carl Zeiss, Germany). The colony diameters were measured using a digital vernier caliper (Tork craft, SA) measuring the size of the colony to the edge of growth. *Mucorales* and *Stenocarpella macrospora* were also among other potential maize pathogens isolated and identified.

4.2.3 Molecular identification

4.2.3.1 DNA extraction

DNA extraction for *Aspergillus* isolates technique is the same as the method used in chapter 3. For detailed steps chapter three can be used as a reference.

4.2.3.2 Polymerase chain reaction

Aspergillus DNA was amplified using primers for internal transcribed spacer (ITS) region (ITS 1 and ITS4) (Whitehead Scientific, South Africa) and secondary marker Calmodulin (*CaM*) (CMD5 and CMD6, Whitehead Scientific) (Samson *et al.*, 2014). The PCR mixtures consisted of 1 µL (10 ng) genomic DNA, 12.5 µL OneTaq 2X Master Mix (Inqaba Biotech, South Africa), 0.5 µL (0.4 µM) of each primer, and 10.5 µL DNA free water (Inqaba Biotech, South Africa). Amplification conditions for *CaM* gene were initial denaturation of 5 min at 94˚C; followed by 35 cycles of 30 s at 94 °C, 30 s at 52 °C and 60 s at 72 °C ; and a final elongation step of 7 min at 72 °C. Conditions for PCR amplification of the ITS gene region consisted of an initial denaturation of 5 min at 94 °C ; followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 60 s at 72 °C ; and a final elongation step of 7 min at 72 °C. The PCRs were performed using a MyCyclerTM Thermal Cycler (Bio-Rad Laboratories) in a total reaction volume of 25 µL. The primers and annealing temperature used for this study are indicated in Table 4.2.

Table 4. 2: Primers and annealing temperatures used

Gene region	Primer	Annealing temp ºC	Sequence	References
ITS	ITS 1	55 ºC	TCC GTA GGT GAA CCT GCG G	(Samson *et al*., 2014)
	ITS 4		TCC TCC GCT TAT TGA TAT GC	
CaM	CMD5	52 ºC	CCG AGT ACA AGG ARG CCT TC	(Samson *et al*., 2014)
	CMD6		CCG ATR GAG GTC ATR ACG TGG	

4.2.3.3. Sanger DNA sequencing

PCR amplicons were purified using a QIAquick PCR Purification Kit followed by Sanger DNA sequencing that was done at Inqaba Biotech in a single direction using the PCR primers and the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK). The sequences were analyzed with an ABI Prism 3730XL Sequencer (Applied Biosystems). The raw sequences were cleaned (base calling) and edges trimmed using Chromas version 2.6.6. DNA sequences were submitted to the basic local alignment search tool (nBLAST) on GenBank at National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and MycoBank (http://www.mycobank.org/) to confirm identity of isolates.

4.2.4 Phylogenetic analyses

Reference sequences for *Aspergillus* spp. (10 references for ITS; 23 references for *CaM*) and 4 references for *Mucorales* and *Stenocarpella* species recovered (using ITS region) were selected based on nBLAST results indicated in Tables 4.3, 4.4 and 4.5 respectively. Multiple sequence alignments were generated with MUSCLE in the Molecular Evolutionary Genetics Analysis version 7.0 (MEGA7.0) (Kumar *et al.*, 2016). Maximum parsimony analysis of the sequences was executed using MEGA7.0 program. The phylognetic tree was constructed and evaluated by 1000 bootstrap replication. The phylogenetic trees for *Aspergillus* spp. were rooted with *Aspergillus paradoxus* Fennell & Raper and the phylogenetic tree for *Mucorales* was rooted with *Stenocarpella macrospora* CBS 117560. Bootstrap analyses were performed to determine branching point confidence intervals (1 000 replicates) for the most parsimonious trees generated for the data sets. A phylogeny ITS, and *CaM* trees were constructed with MEGA 7 software.

Table 4. 3: NCBI reference sequences for *Aspergillus* spp. based on the ITS gene region

Aspergillus Species	**Strain number**	**GeneBank Accession #**	**Source**	**Origin**	**Reference**
Aspergillus niger	ATCC1688	NR111348	Culture	Brazil	(Baron *et al*., 2018)
Aspergillus niger	ND58	MG659652	Mixed ration feed	Zimbabwe	(Nleya *et al*., 2020)
Aspergillus brasiliensis	CBS 101740	NR_111414	Soil	Brazil	(Baron *et al*., 2018)
Aspergillus brasiliensis		KM491891	Surface water	Portugal	(Oliveira *et al*., 2015)
Aspergillus tubingensis	NRRL 4875T	NR_131293	Culture	USA	(Peterson, 2008)
Aspergillus tubingensis	ND8	MG659602	Dairy feed	Zimbabwe	(Nleya *et al*., 2020)
Aspergillus flavus	NRRL 1957	AF027863	Soil	Japan	(Ito *et al*., 1998)
Aspergillus flavus	TUHT100	LN482496	feed	Saudi Arabia	(Gherbawy *et al*., 2016)
Aspergillus flavus	ATCC 16883	NR_111041	Soil	USA	(Schoch *et al*., 2014)
Aspergillus paradoxum	NRRL 2162	NR_138266	TYPE material	USA	(Peterson, 2000)

Table 4. 4: NCBI reference sequences for *Aspergillus* spp. based on the *CaM* gene region

***Aspergillus* Species**	**Strain number**	**GeneBank Accession #**	**Source**	**Origin**	**Reference**
Aspergillus niger	ITEM 4501	AJ964872	Grapes	Italy	(Perrone *et al.*, 2006)
Aspergillus niger	DTO 422-A3	MT261281	Cereals	Nigeria	(Ezekiel *et al.*, 2020)
Aspergillus niger	DUCC6001	MN619777	Furniture	Korea	(Choi *et al.*, 2019)
Aspergillus niger	13L06I1	KT150506	Air	South Korea	(Lee and Yamamoto, 2015)
Aspergillus welwitschiae	AW2017	MH021601	Rice	China	(Ying *et al.*, 2019)
Aspergillus welwitschiae	IHEM 17902	MH644965	Culture	Belgium	(D'hooge *et al.*, 2019)
Aspergillus tubingensis	CBS 134.48T	AJ964876	Grapes	Italy	(Perrone *et al.*, 2006)
Aspergillus tubingensis	27L05I1	KT150507	Unknwon	South Korea	(Lee and Yamamoto, 2015)
Aspergillus. brasiliensis	NRRL 26652	EF661161	Culture	USA	(Peterson, 2008)
Aspergillus brasiliensis	IHEM 3766	MH644891	Culture	Belgium	(D'hooge *et al.*, 2019)
Aspergillus brasiliensis	IHEM 3766	MH644891	Culture	Belgium	(D'hooge *et al.*, 2019)
Aspergillus parasiticus	CMV002B8	MK451480	Soil	South Africa	(Visagie and Houbraken, 2020)
Aspergillus parasiticus,	CBS 100926T	AY017584	Culture	USA	(Peterson, 2008)
Aspergillus flavus	CMXY8113	MG991523	Clinical	china	(Xu *et al.*, 2020)
Aspergillus flavus	CBS 569.61T	FN594583	Culture	South Africa	(Visagie and Houbraken, 2020)
Aspergillus flavus	BF11	MK304457	Feeds	Algeria	(Bouti *et al.*, 2020)
Aspergillus paradoxum	NRRL 2162	EF669692	TYPE material	USA	(Kathuria *et al.*, 2015)

Table 4. 5: NCBI references strains included for *Mucor*, *Rhizopus* and *Stenocarpella* species retrieved based on the ITS region

Species	**Strain number**	**GeneBank Accesion #**	**Source**	**Origin**	**Reference**
Mucor circinelloides	CBS 195.68	NR_126116	Culture	France	(Schwarz *et al*., 2006)
Mucor fragilis	IA1I1F3	KX421452	Lepidoptera	Brazil	(Poitevin *et al*., 2018)
Rhizopus oryzae	CBS 126971	KJ744361	Sugar beet	Germany	(Liebe *et al*., 2016)
Stenocarpella macrospora	CBS 117560 T	NR_157418	Maize	South Africa	(Lamprecht *et al*., 2011)

4.2.5 Extraction of secondary metabolite produced by *Aspergillus* isolates

From the pure cultures of *Aspergillus niger* cultivated on both Czapek Yeast Autolysate agar and Yeast Extract Sucrose agar grown at 25 °C for seven days, three 6-mm agar plugs were cut randomly from the plate using a cork drill. The agar plugs were placed in a 1.5 mL vial and 500 µL of a solvent mixture of methanol-dichloromethane-ethyl acetate (1:2:3) containing 1 % (v/v) formic acid. The plugs were placed in an ultrasonic bath for 60 minutes. The extract was then transferred to a clean vial and evaporated with an evaporator system (Genevac, England). The residues were re-dissolved ultrasonically for 10 minutes in 400 µL methanol solution containing 0.6 % (v/v) formic acid, 0.02 % (v/v) hydrochloric acid, and 2.5 % (v/v) water. The aliquots were filtered into an HPLC vial using 0.45 µm minisart RC4 filters (Smedsgaard, 1997).

4.2.6 Ultra High Performance Liquid Chromatography fumonisin analysis apparatus and conditions

Prior to mass spectrometric sample analysis, chromatographic separation was realized using an Elute HPG 1300 UHPLC-system (Bruker Daltonics, Bremen, Germany). A 30 µL sample extract was injected using a PAL HTC-xt (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separation was performed on a Nucleodur C18 Gravity-SB column (75 x 2 mm, 1.8 µm) (Macherey-Nagel) and run at 40 °C in Elute column oven systems (Bruker Daltonics, Bremen, Germany). Gradient elution conditions using MeCN+0.1 % (v/v) formic acid (FA) (A) and H_2O+0,1 % (v/v) FA (B), were applied as follows: 0.0 min 5 % A, 2.0 min 5 % A, 3.9 min 25 % A, 6.5 min 70 % A, 7.5 min 70 % A, 9.0 min 95 % A, 12.0 min 95 % A, 12.2 min 5 % A, 15.0 min 5 % A. Additionally, a flow gradient of 350 µL/min respective 450 µL/min (3.9-12.2 min) was included. The first two minutes of each run were discarded as waste. The software Compass HyStar (versions 4.1) (Bruker Daltonics, Bremen, Germany) was used for the operation of the UHPLC system.

4.2.7 Quadruple Time Of flight –High Resolution Mass Spectrometry Apparatus and Conditions

An Impact II QTOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was utilized for Quadruple Time Of flight –High Resolution Mass Spectrometry (QTOF-HRMS) experiments. Ionization in the mass spectrometers was performed using an Apollo II ESI source (Bruker Daltonics, Bremen, Germany) with the following parameters: Ionization was performed in positive and negative mode at 4.5 kV and 3.0 kV, respectively. The optimum ionization mode for each mycotoxin in the given sample material was determined individually. Dry gas temperature was set to 220 °C at a flow rate of 10.0 L/min. Nebulizer gas pressure was 2 bar. A mass range of 50 1000 *m/z* was covered and full scan and MS^2 data were recorded at a spectra rate of 4 Hz. Data-independent acquisition in broadband collision-induced dissociation (bbCID) mode was chosen for MS/MS experiments. Fragmentation took place in a collision-induced dissociation cell using nitrogen. Spectral acquisition was performed at alternating collision energies of 24 eV and 36 eV. Sodium formate cluster ions were used for instrument mass calibration and for re-calibration of individual raw data files. The software Compass OtofControl (software versions 4.1, Bruker Daltonics, Bremen, Germany) was used for the operation of the mass spectrometer and for data acquisition. Data processing was executed with the software TASQ (version 2.2, Bruker Daltonics, Bremen, Germany) and DataAnalysis (version 4.1, Bruker Daltonics, Bremen, Germany). Evaluation criteria included retention time and the detection of the principal ion and at least one confirmatory fragment ion with a maximum mass deviation of 5 µg/g.

4.3 RESULTS

4.3.1 Morphological characterization of *Aspergillus* isolates

Morphology grouping of 48 isolates (Mpumalanga 20 isolates),Limpopo (16 isolates), North West (8 isolates) and Eastern Cape (4 isolates) from the 107 maize kernels samples based on colony colour, type of spores and colony size were identified into two *Aspergillus* sections; belonging to section *Nigri* from maize samples collected from Limpopo (13 isolates), Mpumalanga (18 isolates), North West (7 isolates), Eastern Cape (three isolates) and one sample from KwaZulu-Natal and section *Flavi* two isolates from Limpopo, Mpumalanga (three isolates) and one from Eastern Cape.

Aspergillus section *Nigri* was predominant and other sections were less frequent from the maize kernels.

4.3.1.1 *Aspergillus* section *Nigri*

Aspergillus section *Nigri* was the most isolated (41 isolates). Four species were identified namely *Aspergillus niger; A. tubingensis; A*. *brasiliensis* and *A*. *welwitschiae* (Figures 4.2-4.7) and their morphological features are described below.

Aspergillus niger: The colony diameters after seven days of incubation at 25^{o}C; MEA 55-60 mm; CYA 60 mm. The colonies on MEA were black velvety with thick mat of floccose mycelia beneath the colonies and at the edges and the underside showed dark cream to yellowish colour (Figure 4.2). Colonies on CYA were black onto thick cream mycelia with white mycelia at the edges and the reverse was cream. The density of the conidial heads was higher on MEA compared to CYA media. The conidia heads were biseriate and globose in shape with phialides covering the entire vesicle.

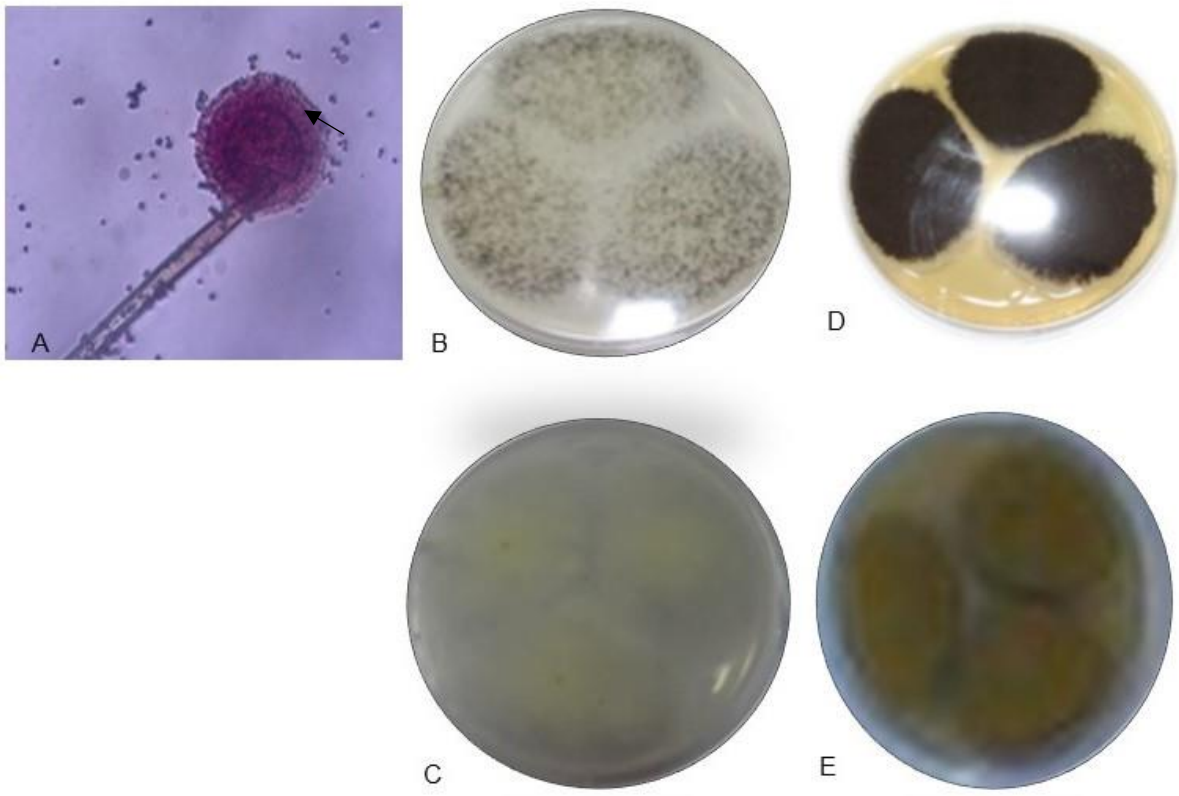


Figure 4. 2: *Aspergillus niger* A= biseriate conidia head and vesicle under light microscope at 40X magnification; B-C = CYA colonies and D-E = MEA colonies.

Aspergillus tubingensis: The colony diameters after seven days of incubation at 25ºC; MEA 40-60 mm; CYA 40-55 mm. The colonies appeared black in colour with white edges on MEA, whereas white colonies with high density conidia at centre were exhibited on CYA. The conidial heads were dark brown to black and slightly floccose (Figure 4.3). The conidiophores was biseriate with a spherical shape.

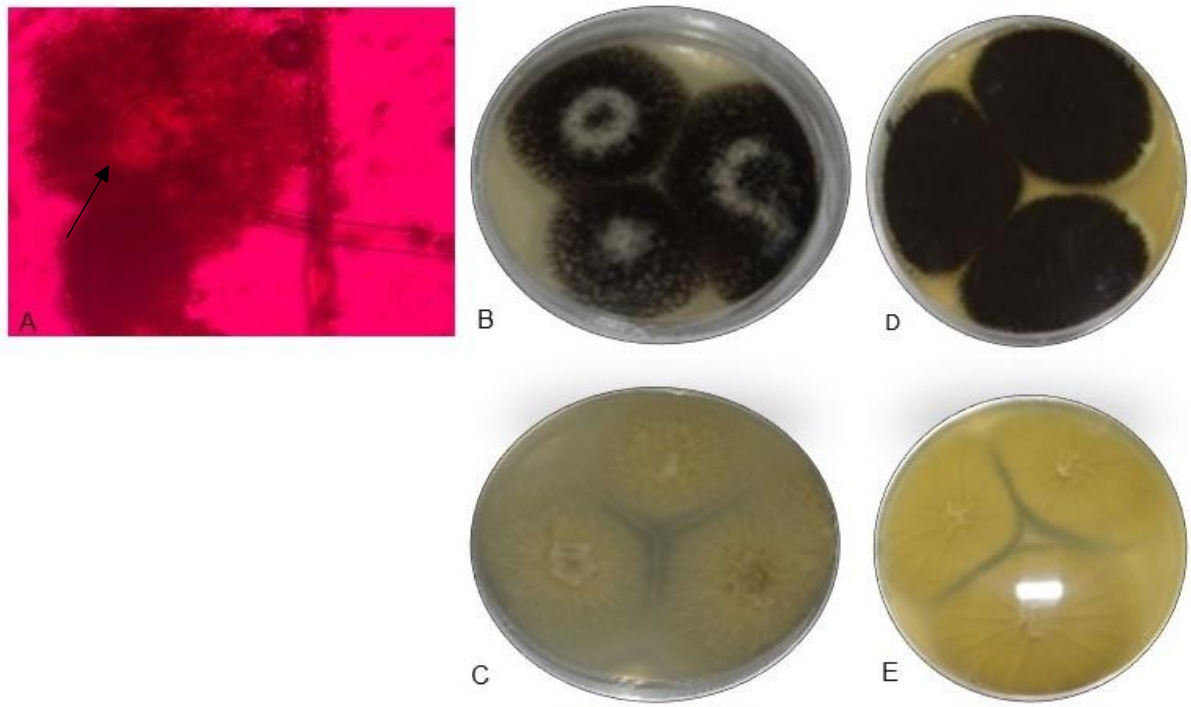


Figure 4. 3: *Aspergillus tubingensis* A= biseriate conidia head and vesicle under light microscope at 40X magnification; B-C = CYA colonies and D-E= MEA colonies.

Aspergillus brasiliensis: The colony diameters after seven days of incubation at 25ºC; MEA 40-60 mm; CYA 44-58 mm. The colonies on CYA media started white then turning dark brown forming sporulation rings with white and rough mycelia. The reverse had rings of grey to cream at the centre (Figure 4.4). On MEA the colonies were black with white mycelia at the edges. The conidial head was biseriate and globose shaped, with metuale covered the entire vesicle (Figure 4.4).

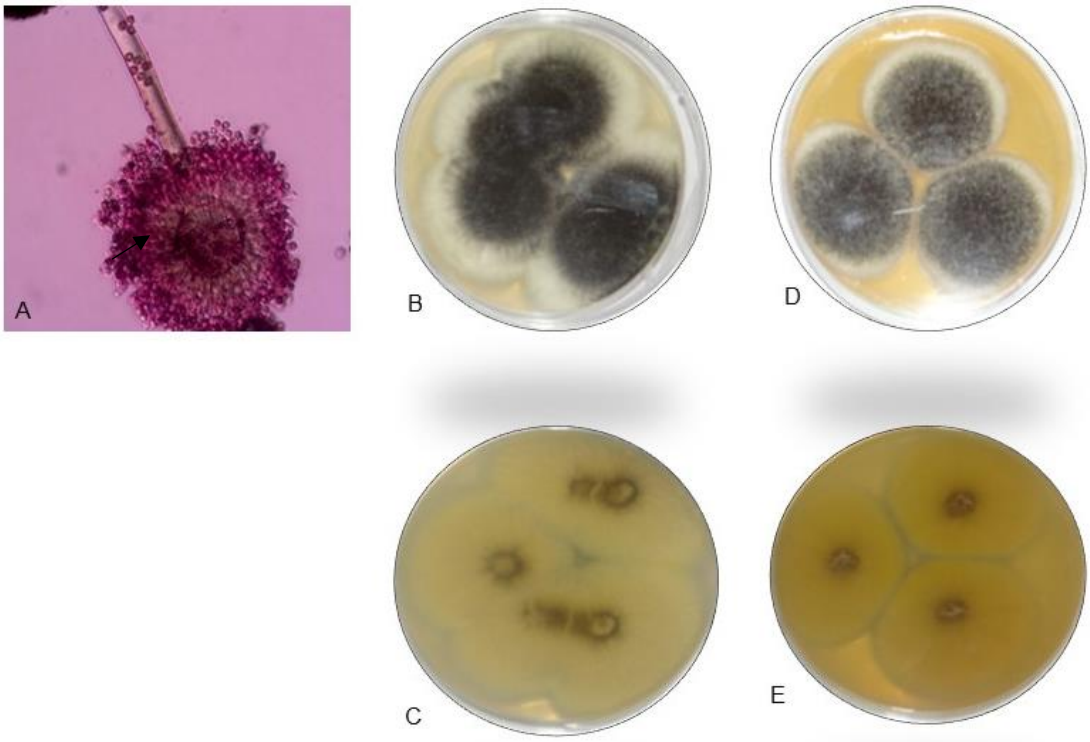


Figure 4. 4: *Aspergillus brasiliensis* A= biseriate conidia head and vesicle under light microscope at 40X magnification; B-C= CYA colonies and D-E = MEA colonies.

Aspergillus welwitschiae: The colony diameters after seven days of incubation at 25ºC; CYA 47-60 mm; MEA 42-60 mm. The colonies grew rapidly in CYA and colonies were brown to black in colour with white mycelium and light yellow underside. On MEA the colonies were dark brown with white edges (Figure 4.5).

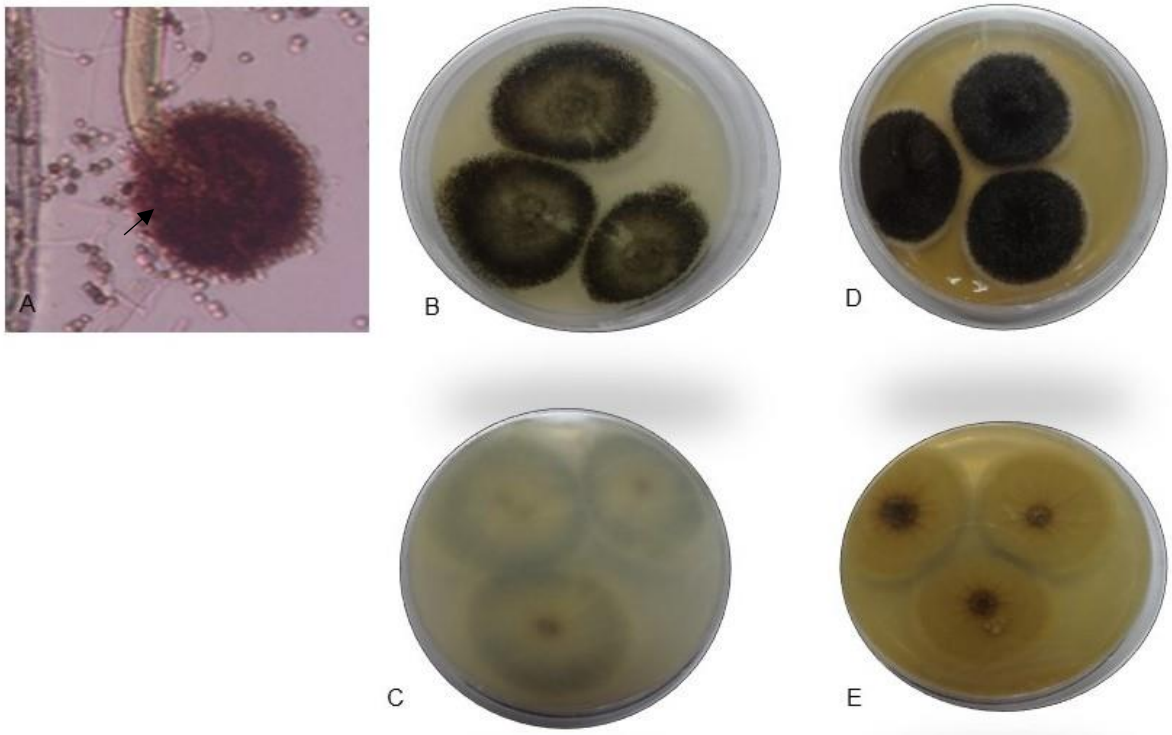


Figure 4. 5: *Aspergillus welwitschiae* A= biseriate conidia head and vesicle under light microscope at 40X magnification; B-C= CYA colonies and D-E= MEA colonies.

4.3.1.2 *Aspergillus* section *Flavi*

Six isolates were identified as *Aspergillus* section *Flavi*, namely five *Aspergillus flavus* and one isolate as *A*. *parasiticus.* The morphological features were based on greenish coloured spores.

Aspergillus flavus: The colony diameters after seven days of incubation at 25ºC; CYA 30 mm; MEA 60 mm; initially the mycelial colour of *A*. *flavus* was white and after seven-day incubation period, the colony on MEA media was olive green with rough conidia and reverse colour was cinnamon brown (Figure 4.6). The sporulation began at the centre and covered the surface of the colony. Colonies on CYA were green with light yellowish colour. Reverse was pale yellow. The species were uniseriate and metuale covered the entire vesicle (Figure 4.6).

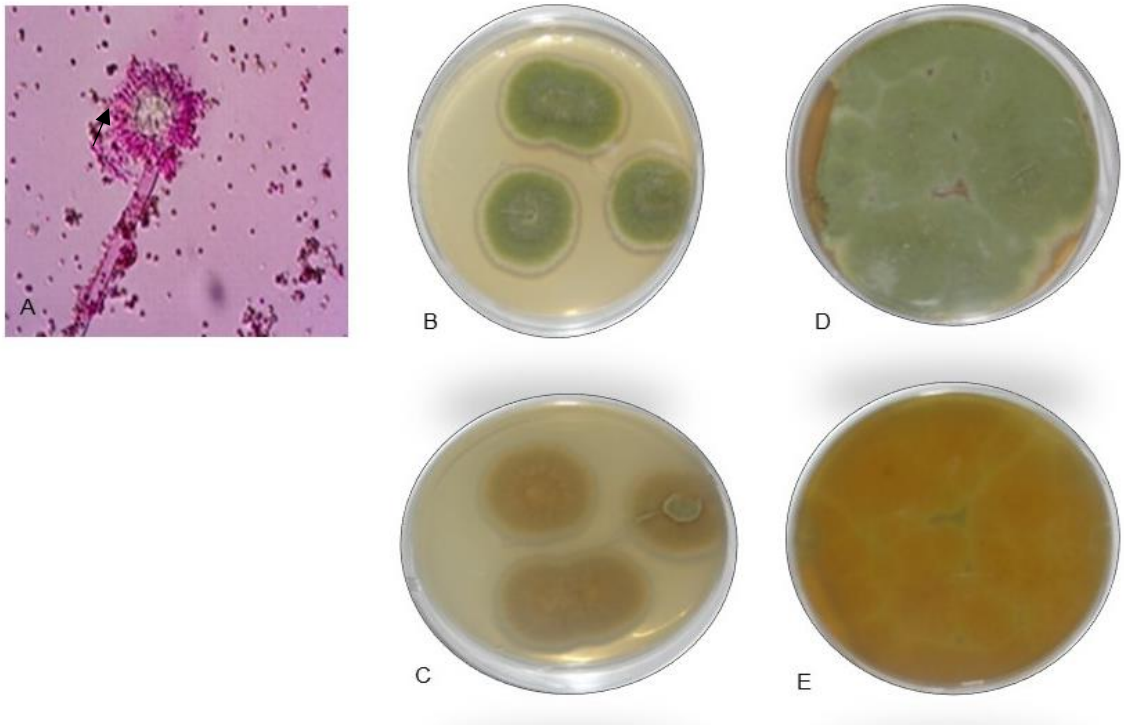


Figure 4. 6*: Aspergillus flavus* A= uniseriate conidia head and vesicle under light microscope at 40X magnification; B-C= CYA colonies and D-E= MEA colonies.

Aspergillus parasiticus: The colony diameters after seven days of incubation at 25ºC; CYA 10-15; MEA 42-45 mm. Colonies on CYA were green with mycelia forming sporulation rings with light in colour underside (Figure 4.7). The colonies on MEA were green velvet appearance with white edges and reverse was dull brown. The isolate was uniseriate with a radiate conidia head with a pyriform to globose vesicle (Figure 4.7).

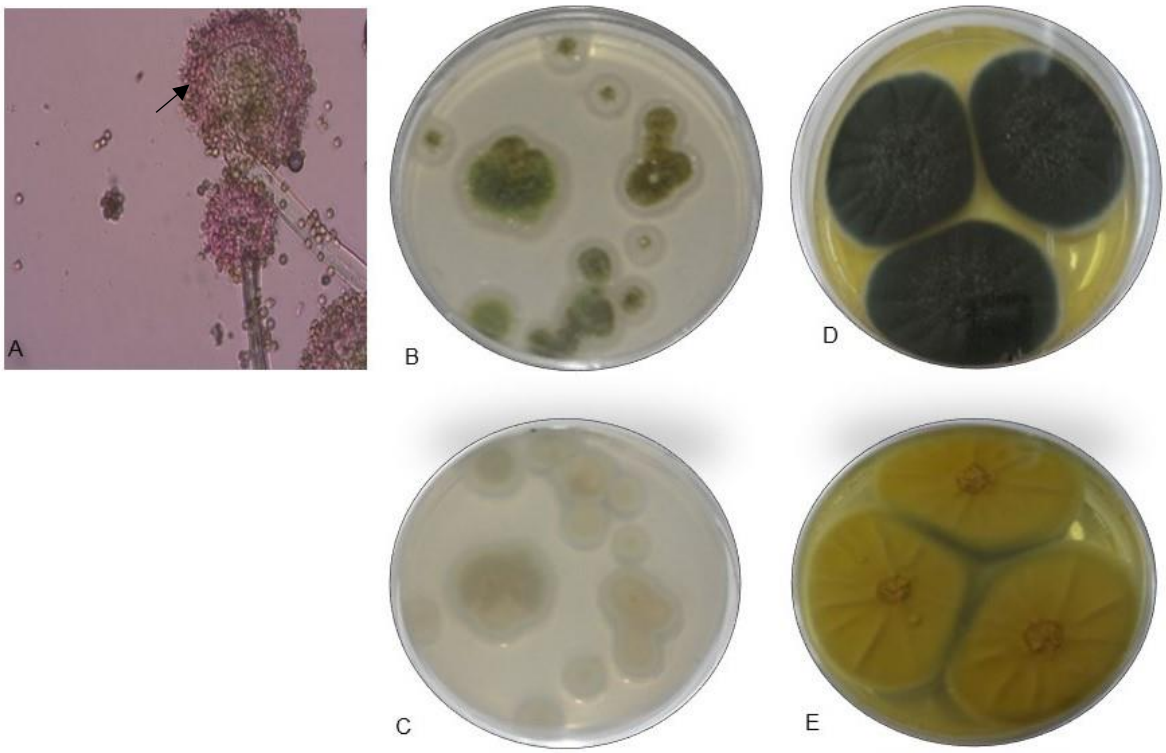


Figure 4. 7: *Aspergillus parasitic*us A= uniseriate conidia head and vesicle; B-C= CYA colonies under light microscope at 40X magnification and D-E= MEA colonies.

4.3.2 Molecular characterization

4.3.2.1 DNA and PCR amplification

The quality and concentration of the extracted target DNA were determined by running a 1.5 % (w/v) gel electrophoresis stained with 1 µL Gel-Red (Figure 4.8) for *Aspergillus* isolates. The PCR amplification of the ITS and *CaM* gene regions for *Aspergillus* spp. resulted in PCR products with band sizes of approximately 600bp (*CaM*), and 600bp ITS respectively, (Figures 4.9-4.10).

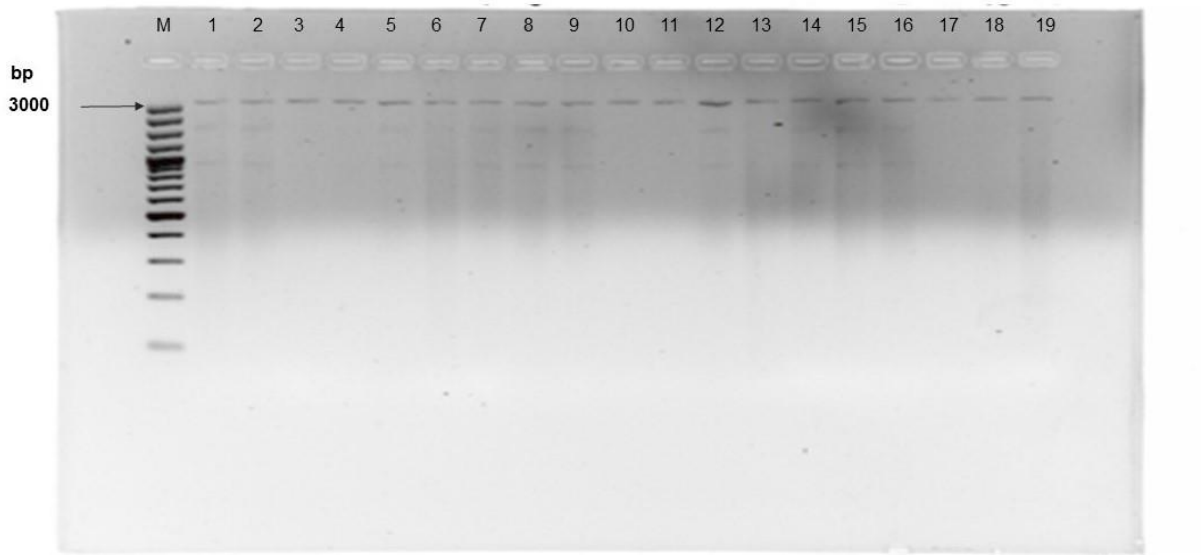


Figure 4. 8: *Aspergillus* genomic DNA 1.5 % agarose gel. M= molecular markers (O' Gene Ruler PLUS^{TM} DNA ladder, Thermo Scientific) lane 1-19= isolates 1; 9; 10; 18; 29; 35; 47; 60; 64; 65; 67; 68; 71; 75; 76; 78; 82; 84; 92.

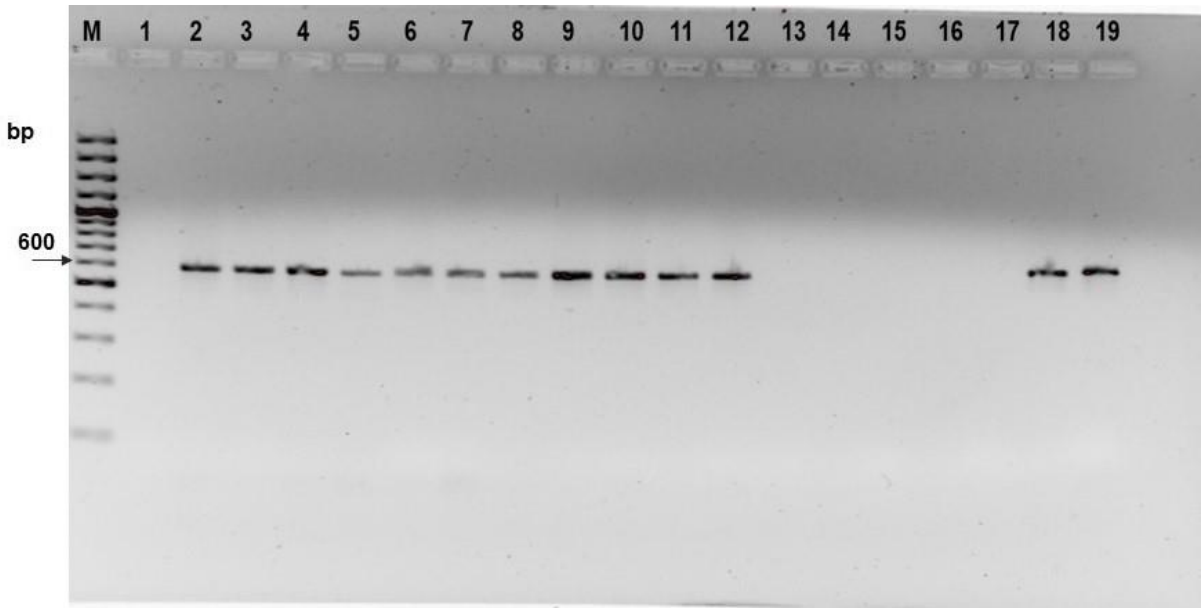


Figure 4. 9: PCR amplicons for *CaM* gene region for *Aspergillus* isolates with approximately 600 bp product size on 1.5 % agarose gel. M= marker (O' GeneRuler 100 bp DNA ladder, Thermo Scientific), lane 1-19= negative control; 1; 10; 18; 78; 65; 47; 29; 77; 76; 84; 4; 35; 9; 67; 8; 71; 92; 63.

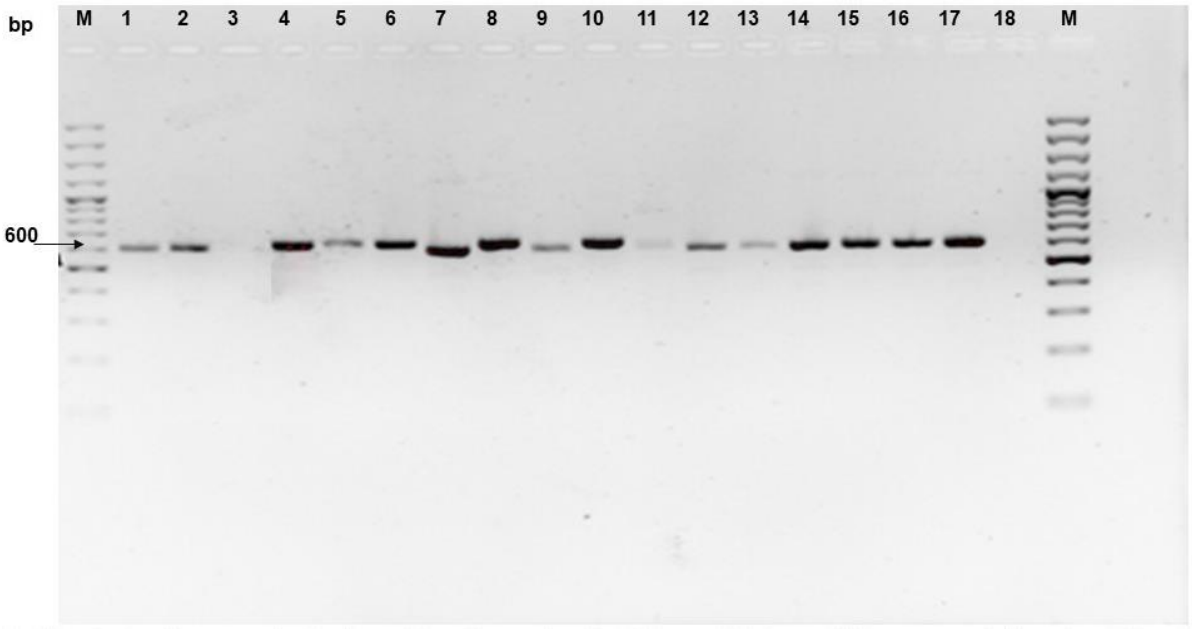


Figure 4. 10: PCR product for ITS gene region for *Aspergillus* with approximately 600 bp product size on 1.5 % agarose gel. M= marker (O' GeneRuler 100 bp DNA ladder, Thermo Scientific), lane 1-18= isolates 2; 3; 28; 30; 31; 41; 52; 55; 70; 56; 92; 89; 76; 77; 29; 47; 64 and negative control.

4.3.2.2 DNA sequencing nBLAST search results

The NCBI and MycoBank nBLAST results for ITS region identified 14 *Aspergillus* spp. isolates belonging to *Aspergillus* section *Nigri* and section *Flavi* (Table 4.6). Section *Nigri* was represented by five isolates of *A*. *niger*; five isolates of *A*. *brasilliensis*; two isolates of *A*. *tubingensis* and section *Flavi* by two isolates of *A*. *flavus*. Other species retrieved by ITS gene region were *Mucor fragillis*, represented by 11 isolates; followed by five isolates of *Mucor circinelloides,* three isolates of *Rhizopus oryzae* and one isolate identified as *Stenocarpella macrospora* (Table 4.6).

The DNA sequencing from secondary marker calmodulin identified 34 *Aspergillus* spp. belonging to four *Aspergillus* sections namely section *Nigri* represented by *A*. *niger* (7 isolates); *A*. *welwitschiae* (5 isolates); *A*. *tubingensis* (10 isolates) and A. *brasiliensis* (8 isolates) (Table 4.7). *Aspergillus* section Flavi was represented by *A*. *flavus* (3 isolates) and *A*. *parasiticus* (1 isolate).

Table 4.6: NCBI database and MycoBank database results of ITS from maize kernels

Isolate number	Species	Accession#	Species	Accession #
	NCBI database		MycoBank database	
4	*Aspergillus niger*	MG659679	*Aspergillus niger*	WM 04.470
8	*Aspergillus niger*	MK841442	*Aspergillus niger*	WM 04.470
12	*Aspergillus niger*	MG659652	*Aspergillus niger*	WM 04.470
38	*Aspergillus niger*	MK841442	*Aspergillus niger*	WM 04.470
52	*Aspergillus niger*	MT965558	*Aspergillus niger*	CBS 263.65
3	*Aspergillus brasiliensis*	MK450632	*Aspergillus brasiliensis*	CBS 101740
24	*Aspergillus brasiliensis*	MK450632	*Aspergillus brasiliensis*	CBS 246.65
86	*Aspergillus brasilienis*	MW534829	*Aspergillus brasiliensis*	ATCC MYA-4553
87	*Aspergillus brasiliensis*	MK450632	*Aspergillus brasiliensis*	ATCC MYA-4553
93	*Aspergillus brasiliensis*	MK450632	*Aspergillus brasiliensis*	ATCC MYA-4553
17	*Aspergillus tubingensis*	MT495451	*Aspergillus tubingensis*	KF435032
33	*Aspergillus tubingensis*	MZ314731	*Aspergillus tubingensis*	UOA/HCPF 8806
48	*Aspergillus flavus*	MT447498	*Aspergillus flavus*	UOA/HCPF 8374A
59	*Aspergillus flavus*	MK841461	*Aspergillus flavus*	WM 12.3
20	*Mucor fragillis*	MK910073	*Mucor fragilis*	KX421448
23	*Mucor fragilis*	MN069560	*Mucor fragilis*	KX421446
25	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421446
27	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421452
32	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421446
34	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421452
36	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421452
40	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421446
51	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421446
62	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421446
31	*Mucor circinelloides*	KF381073	*Mucor circinelloides*	KF381073
41	*Mucor circinelloides*	KP132468	*Mucor circinelloides*	KJ744378
45	*Mucor fragilis*	MK910073	*Mucor fragilis*	KX421452
55	*Mucor circinelloides*	MK501847	*Mucor circinelloides*	CNRMA9.1173
56	*Mucor circinelloides*	KP132468	*Mucor circinelloides*	CNRMA9.1173
61	*Mucor circinelloides*	KP132468	*Mucor circinelloides*	CNRMA16.563
54	*Rhizopus oryzae*	MT448910	*Rhizopus oryzae*	CNRMA14.515
57	*Rhizopus oryzae*	MK910073	*Rhizopus oryzae*	CNRMA9.360
92	*Rhizopus oryzae*	MT448910	*Rhizopus oryzae*	CNRMA14.515
39	*Stenocarpella macrospora*	KY012337	*Stenocarpella macrospora*	CBS 128560

Table 4.7: NCBI database and MycoBank database results of *CaM* from maize kernels

Isolate number	Species	Accession #	Species	Accession #
	NCBI database		MycoBank database	
1	*Aspergillus niger*	MT261281	*Aspergillus niger*	ITEM 11448
7	*Aspergillus niger*	MH614646	*Aspergillus niger*	CNRMA14.514
36	*Aspergillus niger*	KT150506	*Aspergillus niger*	CNRMA16.712
37	*Aspergillus niger*	KT150506	*Aspergillus niger*	CNRMA14.514
51	*Aspergillus niger*	MN619777	*Aspergillus niger*	CNRMA16.712
53	*Aspergillus niger*	KT150506	*Aspergillus niger*	CNRMA16.712
65	*Aspergillus niger*	MH614646	*Aspergillus niger*	CNRMA14.514
5	*Aspergillus welwitschiae*	MH644965	*Aspergillus welwitschiae*	ITEM 4730
10	*Aspergillus welwitschiae*	MH021601	*Aspergillus welwitschiae*	CNRMA15.743
18	*Aspergillus welwitschiae*	MH644965	*Aspergillus welwitschiae*	ITEM 4730
21	*Aspergillus welwitschiae*	MH021601	*Aspergillus welwitschiae*	CNRMA15.743
23	*Aspergillus welwitschiae*	MH614649	*Aspergillus welwitschiae*	CNRMA20.256
3	*Aspergillus brasiliensis*	MH644891	*Aspergillus brasiliensis*	ITEM 4539
24	*Aspergillus brasiliensis*	AM295176	*Aspergillus brasiliensis*	ITEM 6139
42	*Aspergillus brasiliensis*	MH644891	*Aspergillus brasiliensis*	ITEM 4539
62	*Aspergillus brasiliensis*	MH644891	*Aspergillus brasiliensis*	ITEM 4539
72	*Aspergillus brasiliensis*	MH644891	*Aspergillus brasiliensis*	ITEM 4539
74	*Aspergillus brasiliensis*	MH644891	*Aspergillus brasiliensis,*	ITEM 4539
78	*Aspergillus brasiliensis*	MH644891	*Aspergillus brasiliensis*	ITEM 4539
80	*Aspergillus brasiliensis*	MH644891	*Aspergillus brasiliensis*	ITEM 4539
17	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA16.450
29	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA19.572
34	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA19.572
44	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA19.572
66	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA16.450
69	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA16.450
73	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA19.572
76	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA19.572
77	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	ITEM 13328
84	*Aspergillus tubingensis*	KX768540	*Aspergillus tubingensis*	CNRMA19.572
47	*Aspergillus parasiticus*	MK451480	*Aspergillus parasiticus,*	CNRMA20.247
59	*Aspergillus flavus*	MK304457	*Aspergillus flavus*	CNRMA19.418
82	*Aspergillus flavus*	MG991523	*Aspergillus flavus*	CNRMA19.418
88	*Aspergillus flavus*	MN416021	*Aspergillus flavus*	CNRMA20.195

4.3.3 Phylogenetic analysis

The isolates identified morphologically were confirmed with ITS and *CaM* gene region sequences. Morphologically similar isolates were separated by molecular identification. Phylogenetic anaysis was based on maximum parsimony where isolates of the same species were grouped in the same clade (Figures 4.11- 4.12).

The phylogenetic tree obtained with *CaM* had five lineages grouped into 7 major clusters (Figure 4.11). *Aspergillus* section *Nigri* cluster 1 included a sub-cluster 1 with 2 strains which grouped together with *A*. *niger* (MT261281) reference strain, sub cluster 2 within cluster 1 included three strains which grouped together with *A*. *welwitshiae* (MH644965) and one strain showed homology to *Aspergillus welwitshiae* (MH021601) within cluster 1.

Cluster 2 was represented by six strains identified as *A*. *niger* (AJ964872; KT150507; MN619777). Cluster 3 was represented by 10 strains that identified as *A*. *tubingensis* (KT150507; AJ964876). Cluster 4 was represented by eight strains that grouped with reference strains *A*. *brasiliensis* (MH644891; AM295176; EF661161).

The last cluster 5 had two sub clusters. Sub cluster 1 was represented by one strain identified as *A*. *parasiticus* grouped with MK451480 and AY017584. The last sub cluster 2 was represented by three strains identified as *A*. *flavus* (MK304457; FN594583 and MG991523) (Figure 4.11).

Phylogenetic tree based on ITS three lineages grouped into four major clusters (Figure. 4.12). Cluster 1 was represented by five strains grouped together with *A. niger* NR 111348. Cluster 2 was represented by 2 strains identified as *A*. *tubingensis* (MG659602 and NR 131293). Cluster 3 grouped 4 strains with reference strains (NR 111414 and KM491891) which identified as *A*. *brasiliensis*. The last cluster 4 was represented by two strains identified as *A*. *flavus* (AF027863; LN482496 and NR 111041) (Figure 4.12).

Mucor fragillis was among the other species identified with ITS gene region (Figure. 4.13). Eleven strains grouped with *M. fragillis* (KX4421452). Cluster 2 identified as *M. circinelloides* represented by five strains which showed homology to NR126116. Cluster 3 was represented by three strains identified as *Rhizopus oryzae* which

grouped together with KJ744361. The last cluster 4 was represented by one strain and it grouped together with reference *Stenocarpella macrospora* (NR157418), which was used as an outgroup to root the phylogenetic tree.

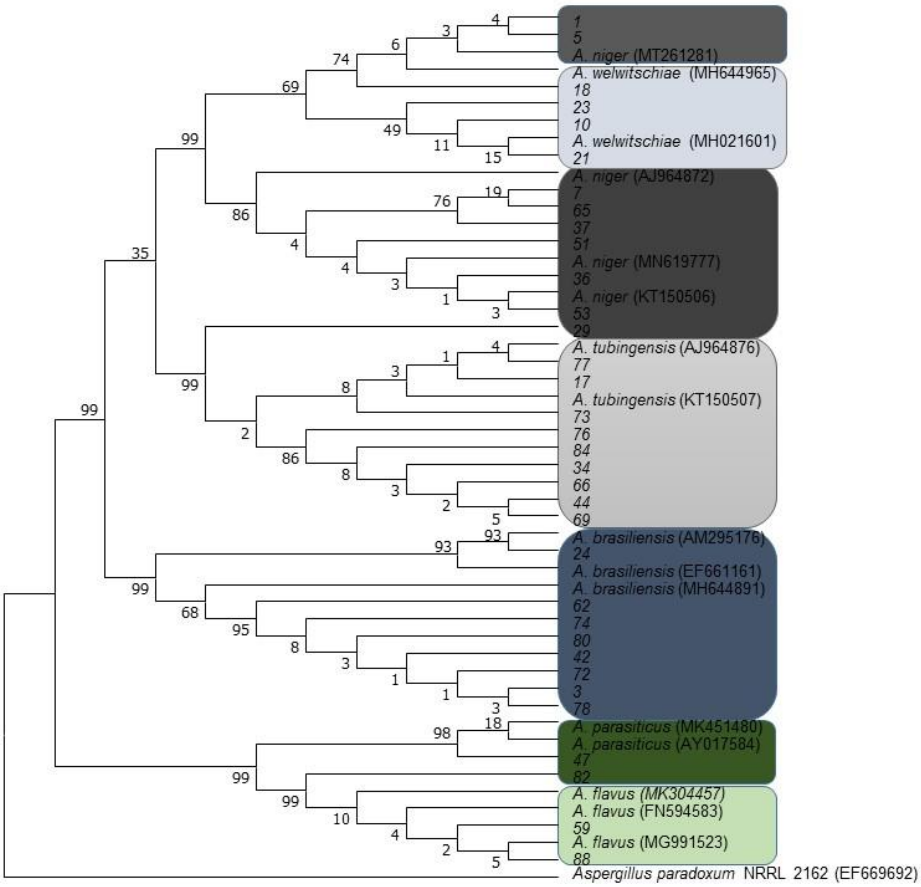


Figure 4. 11: Phylogenetic tree based on Maximum Parsimony from *CaM* sequences from *Aspergillus* spp. from maize kernels. The tree was rooted with *Aspergillus paradoxum* NRRL 2162.

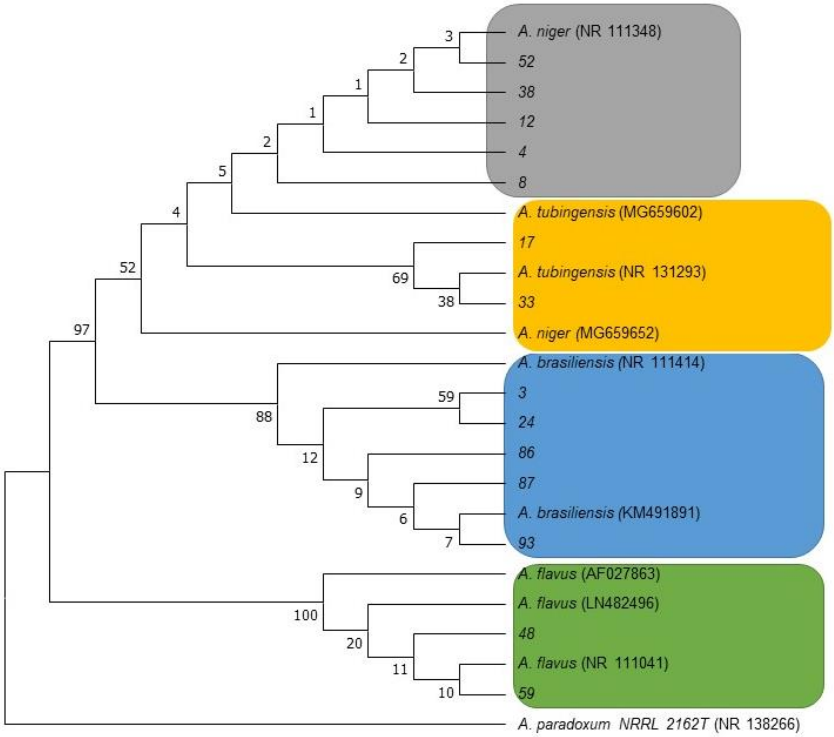


Figure 4. 12: Phylogenetic tree based on Maximum Parsimony from ITS sequences from *Aspergillus* spp. from maize kernels. The tree was rooted with *Aspergillus paradoxum* NRRL 2162.

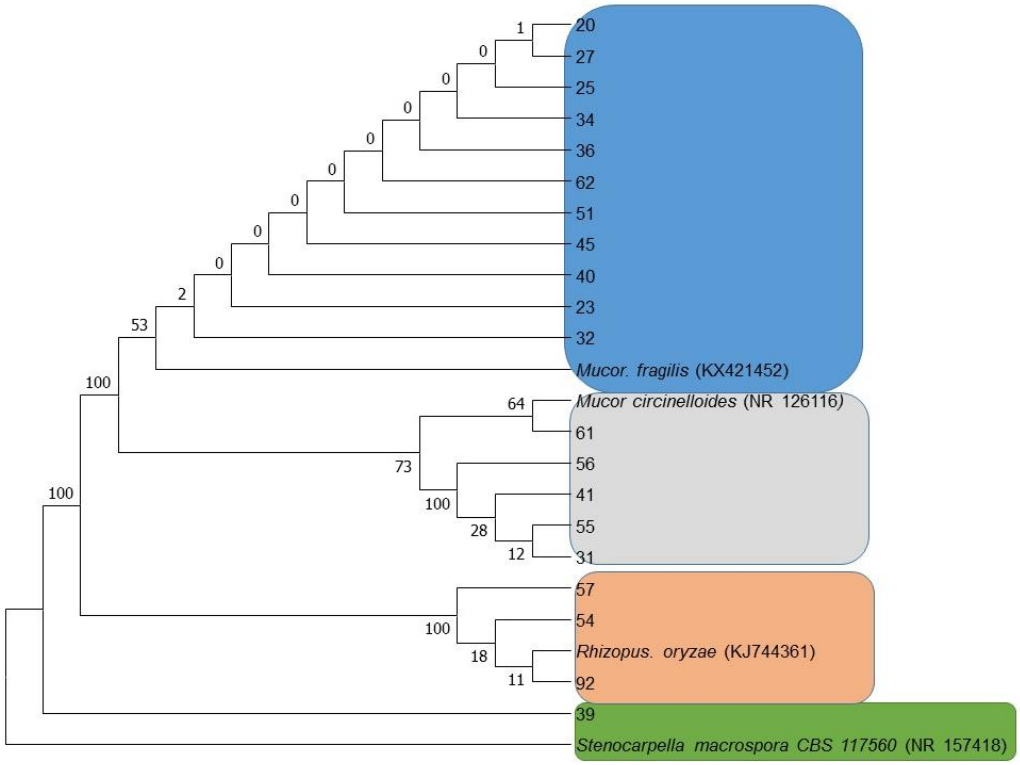
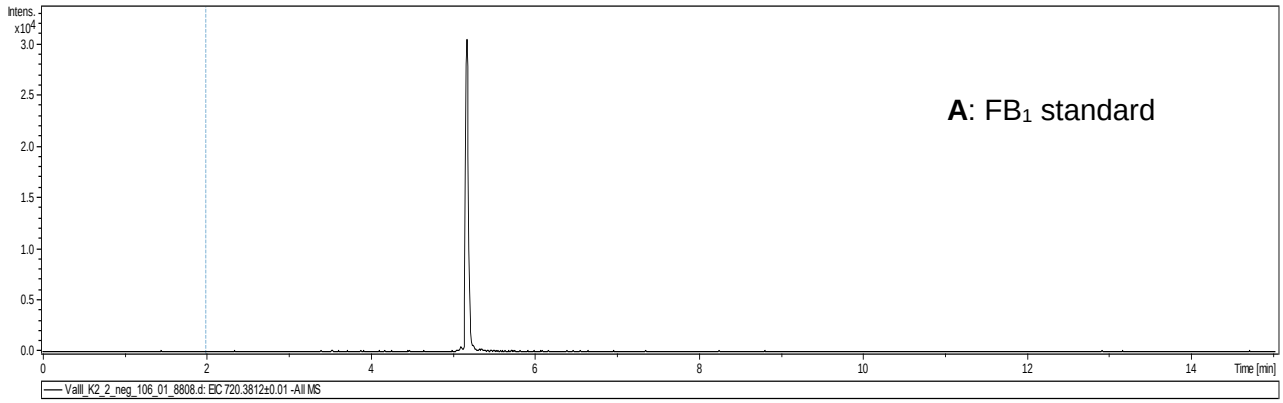


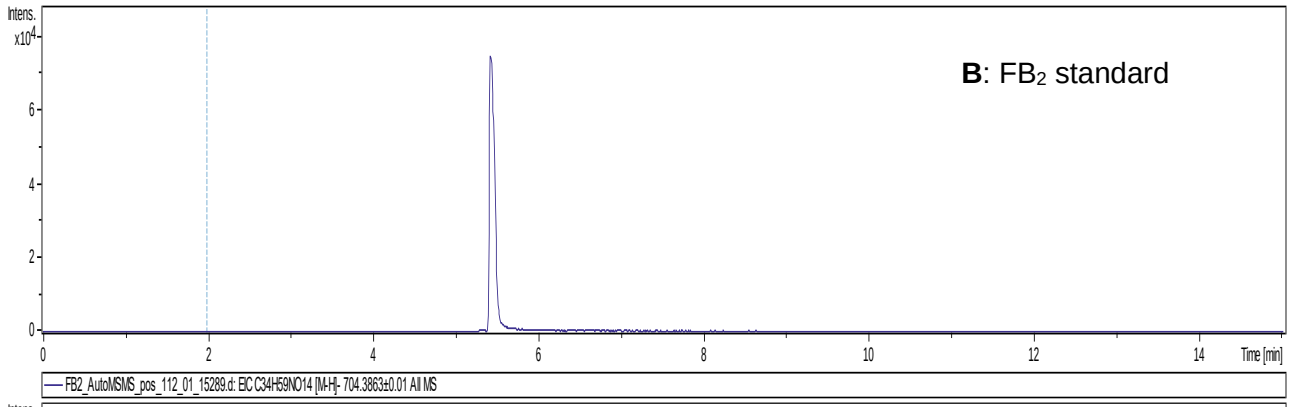
Figure 4. 13: Phylogenetic tree based on Maximum Parsimony from ITS sequences from *Mucor* and *Rhizopus* spp. from maize kernels. The tree was rooted with *Stenocarpella macrospora* CBS 117560.

4.3.4 Fumonisin production by *Aspergillus niger* isolates

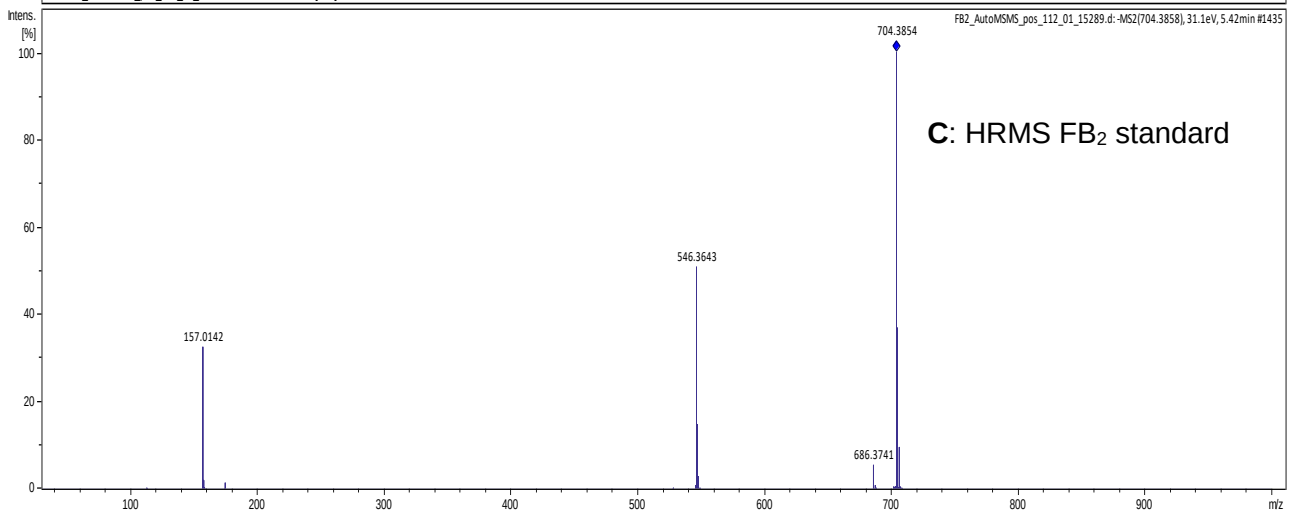
The screening test for FB_2 from 20 *Aspergillus niger* isolates indicated that five *A. niger* isolates cultured on YES produced FB_2. The detection of FB_2 from *Aspergillus* isolates was confirmed by comparison of its retentiontime and high resolution mass spectrum with those of the FB_2 standard (retention time = of 5.42 min) (Figure 4.14 (C). Fumonisin B_2 production on yeast extract sucrose agar (YES) by five *A. niger* isolates from Mpumalanga (two isolates), Limpopo (one isolate), Eastern Cape (two isolates) Province as measured by QTOF-HRMS (Figure 4.14).



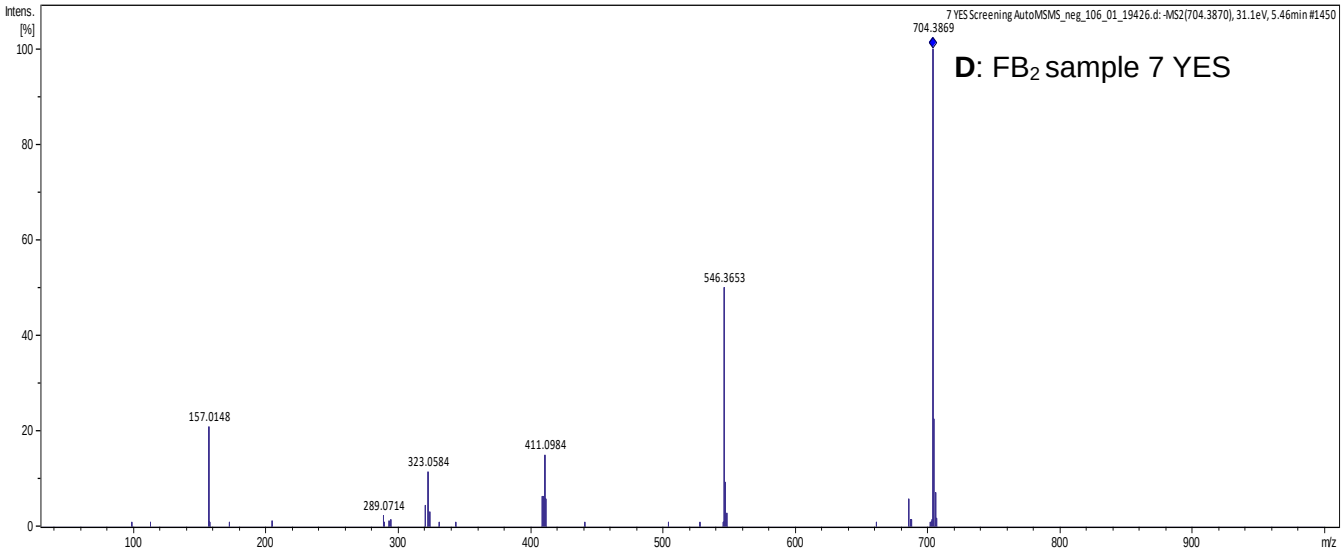
A: FB_1 standard



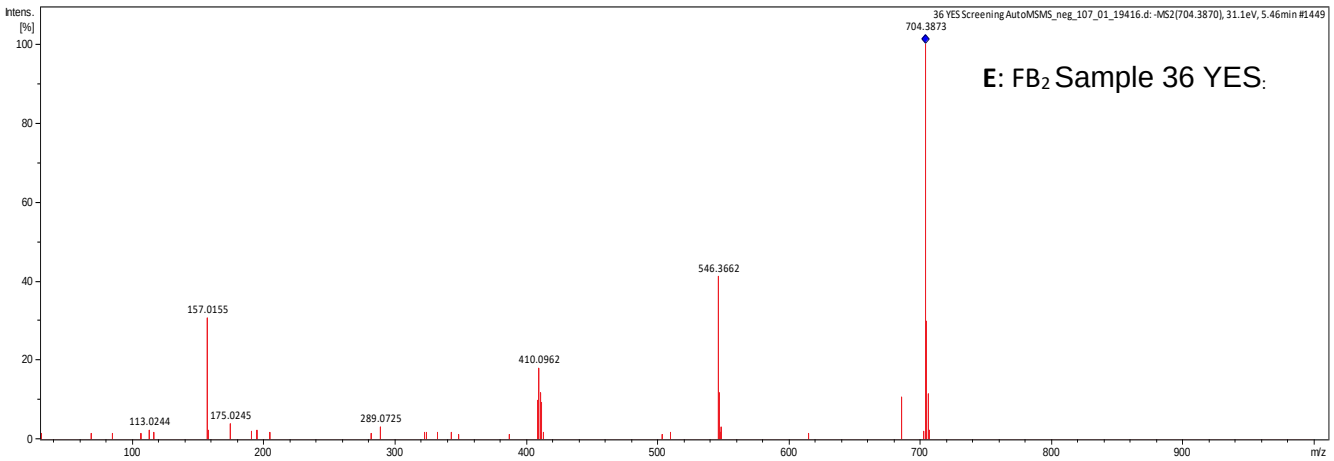
B: FB_2 standard



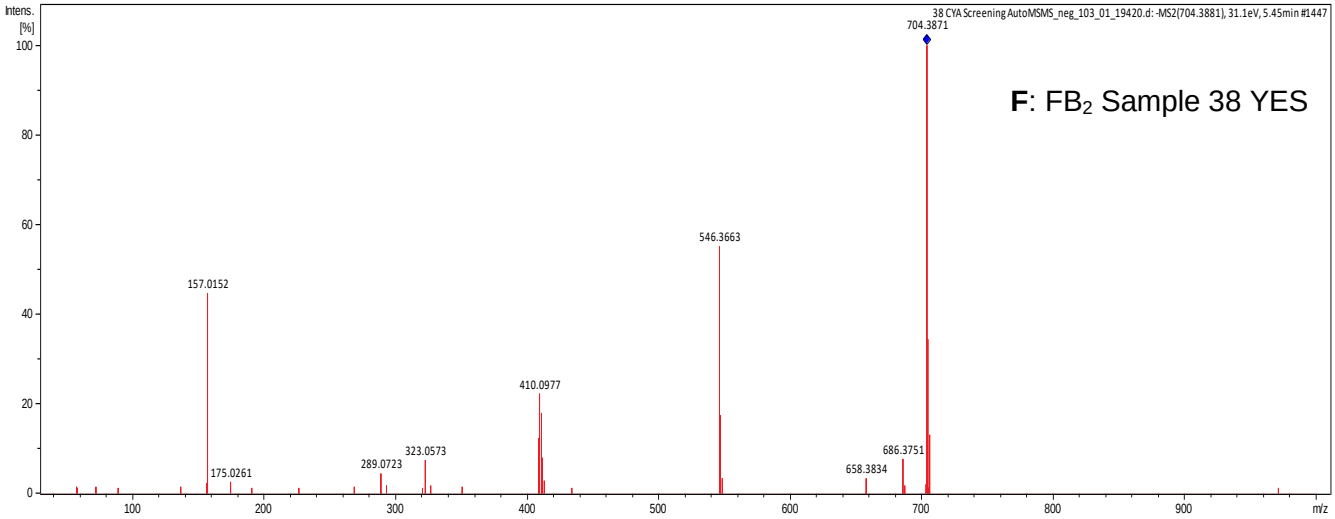
C: HRMS FB_2 standard



D: FB_2 sample 7 YES



E: FB_2 Sample 36 YES:



F: FB_2 Sample 38 YES

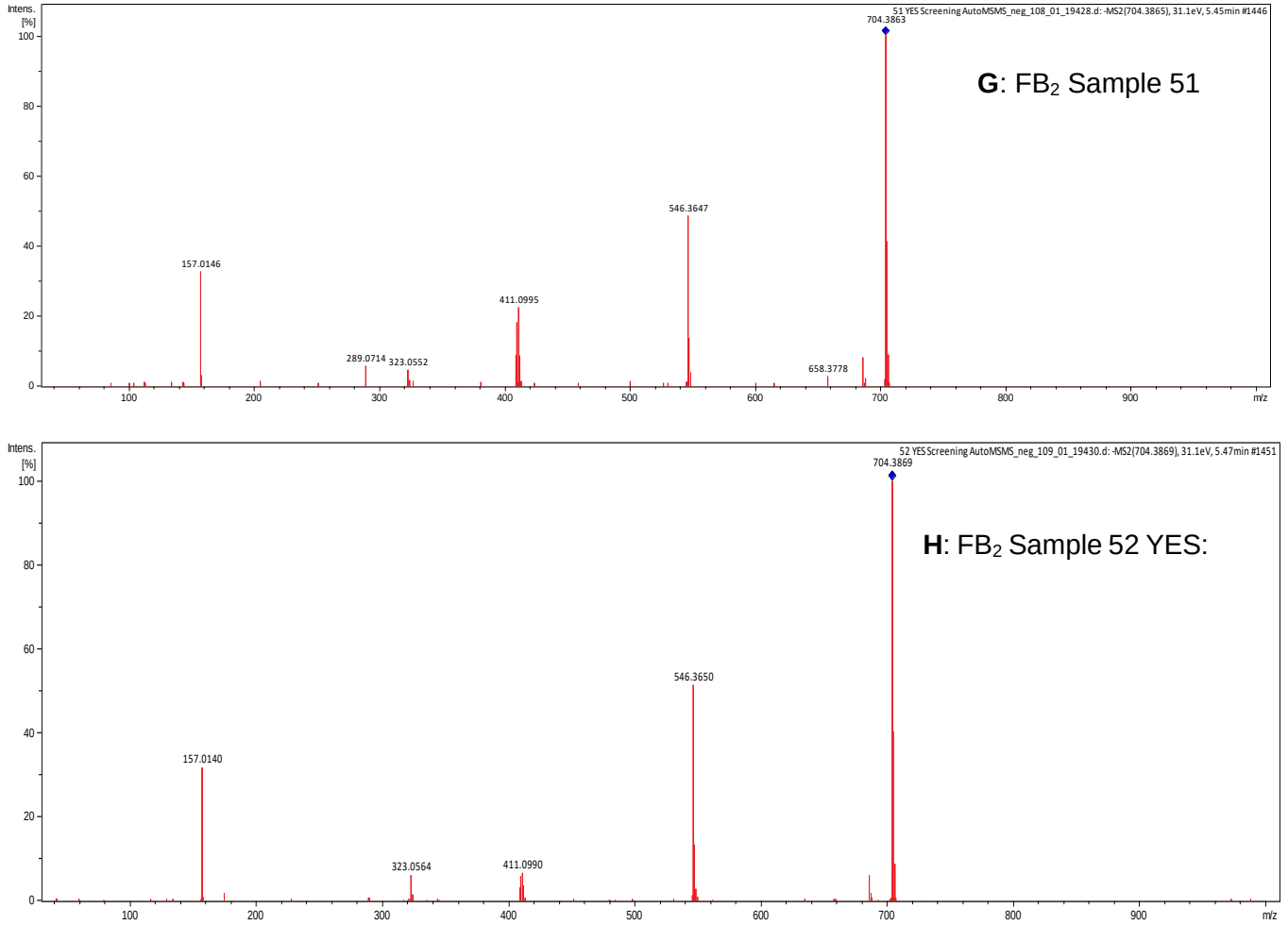


Figure 4. 14: Fumonisin B1 Standard (A), Fumonisin B_2 Standard (B). FB_2 production by *A. niger* sample 7, 36, 38, 51 and 52 cultivated on YES clearly show distinct peaks from fumonisin B2 (C, D, E, F, G & H).

4.4 DISCUSSION

The identification of *Aspergillus* species based on morphological and molecular techniques indicated that *Aspergillus* section *Nigri* was predominant with strains represented by *A*. *niger*; A. *tubingesis*; *A*. *brasilliensis* and *A*. *welwitshiae. Aspergillus* section Flavi had minor occurrences represented by *A*. *flavus* and *A*. *parasiticus*. The high frequency of *Aspergillus* section *Nigri* on maize indicate the increased risk of mycotoxin contamination in maize produced by smallholder farmers. This is particularly important with FUM which are linked to oesophageal cancer in humans and animal diseases induced by consuming contaminated maize food products. The results of molecular analysis confirmed the morphological identification. The phylogenetic analysis divided strains into different clades for *CaM* and ITS gene

regions, closely related *Aspergillus* isolates that could not easily separated by morphological identification were identified by DNA sequencing.

Five of the 20 *A. niger* isolates tested produced FB_2. This is the first study to demonstrate FUM-producing *A. niger* isolates from maize produced in South Africa. Fumonisin production by *A. niger* can have severe implications beyond the maize value chain. For instance, the ability of *A. niger* to produce FB_2 has many consequences because *A. niger* is used in biotechnological applications, which increases the potential for FUM contamination of foods and feeds in which *A. niger* has been reported to grow. In addition, *A. niger* has long been used for the production of single-cell protein for a range animal feed (Christias *et al*., 1975, Singh *et al*., 1991, Oboh *et al*., 2002). *Aspergillus niger* is also extensively used to produce citric acid and extracellular enzymes as well as transformation host in the bio-technological industries (Schuster *et al*., 2002, Karaffa and Kubicek, 2003, Blumenthal, 2004, Goldberg *et al*., 2006).

This study further showed that the five FUM-producing *A. niger* isolates only produced FB_2 and no FB_1 was produced. This is in agreement with studies by Frisvad *et al*. (2007). The production of FB_2 by *A. niger* has implications in mitigating the risks of FUM contamination in maize kernels. *Fusarium* spp. and *Aspergillus* spp. differ in their mode of infection and responses to management practices (Logrieco *et al*., 2014). For example, *F. verticillioides* is a field pathogen that produces FUM before harvest whereas *A. niger* is both a field and storage pathogen that infects maize in the field as well as during storage. Therefore, it is important to not only target pre-harvest control measures but also use post-harvest control measures to control the growth of *A. niger* during storage. Storage practices such as hermetic storage that controls insects as well as fungal growth and concomitant mycotoxin contamination are critical in the management of post-harvest losses (Adler and Ncube, 2020).

Maize is a staple food in South Africa, and the average intake per person per day may exceed 300 g (Shephard *et al*., 2007). Therefore, this high maize intake highlights the relevance of FUM mycotoxin research, given that the recommended daily intake of food contaminated with mycotoxins was calculated based on the average intake of maize in developed countries where statistics are easily available. However, human consumption of maize is far less than in developing countries. The prevalence of FUM-

producing *A. niger* in maize kernels from this study mainly originating from emerging farmers with limited agricultural inputs indicates that mitigation measures for mycotoxins in food and feed is required to reduce exposure in such high risk populations. Further studies to determine FUM production by *A. niger in planta* need to be conducted in various environments in South Africa. Occurance of fumonisin B_2 in maize has serious health implication to humans and animals because FB_2 is cytotoxic and further quantitative data on FB_2 produced by *A. niger* is critical to understand the extent of *A. niger* FB_2 contamination.

CHAPTER 5: General conclusion

The morphological characterization of *Fusarium* and *Aspergillus* species is an important part in the identification of fungi where morphology characters are described and isolates are grouped according to their morphology characters and further identification using molecular techniques. This study has confirmed the presence of the primary maize ear rot pathogens, *Fusarium* and *Aspergillus* species, in maize produced by smallholder farmers in South Africa. Limpopo had high infection of *Fusarium* spp. and *Aspergillus* infection was more prevelant in Mpumalanga. Infection of maize with these fungal pathogens result in reduced quality of maize kernels and the potential risk of mycotoxins produced by *Fusarium* and *Aspergillus* species in maize collected in South Africa.

The phylogenetic analysis of *Fusarium* using *TEF-1α* indicated that *Fusarium verticillioides* is the main Fusarium ear rot pathogen, followed by *Fusarium temperatum* in maize collected in South Africa. The molecular identification of *Aspergillus* using ITS and Calmodulin separated isolates into different *Aspergillus* sections and clades. The *Aspergillus* section *Nigri* was the most prevalent species, indicating the risk of mycotoxins from samples collected in smallholder farmers. The results of the metabolite profiling showed that five *Aspergillus niger* isolates produced fumonisin B_2 (FB_2) *in vitro*. This is the first report of FB_2 production by *A. niger* isolated from maize in South Africa. The production of FB_2 by *A. niger* has implications on mitigating the risks of fumonisin contamination in maize kernels since *Fusarium* spp. and *Aspergillus* spp. differ in their mode of infection and response to management practices. For instance, *A. niger* is a storage fungus while *F. verticillioides* is a field fungus, indicating that *A. niger* can also increase fumonisin contamination of grain during storage.

Fumonisin analysis of the maize kernel samples indicated that 91 % of the samples collected from KwaZulu-Natal Province were contaminated with fumonisins, with 55 % of the samples exceeding the maximum allowable limit of 2 000 µg/kg set by the Department of Health. Limpopo Province had the second highest incidence of fumonisin contamination, with 39 % positive samples and 35 % of the total samples that had fumonisin levels exceeding 2 000 µg/kg. Samples from the Eastern Cape had the third highest positivity rate at 22%, followed by Mpumalanga at 13 % and the North

West at 7 %. All samples that were positive for fumonisin contamination exceeded the maximum allowable limit in Mpumalanga and North West Provinces. These results indicate that smallholder farmers are chronically exposed to high levels of fumonisins. Therefore, there is a need for more awareness and mycotoxin control measures to reduce the risk mycotoxin exposure in these communities. Farmers can mitigate their exposure to fumonisins by sorting maize kernels, washing of maize kernels and hermetic storage conditions. Possible future work emerging from this study should include profiling of *Fusarium temperatum* mycotoxins in maize since it was the second common *Fusarium* species in maize collected in South Africa. In addition, fumonisin production by *A*. *niger in planta* need to be determined in field trials under different climatic conditions.

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Appendix

Growth mediums and buffer compositions

Fusarium selective media

Glycerol 10 g, Urea 1 g, L-alanine , 0.5 g, PCNB (Pentachloronitrobenzene) 1 g, Rose Bengal 0.5 g, and Agar 15 g.

Aspergillus selective media

Peptone 3 g, Soy Peptone 2 g, Dextrose 12.85 g, di-Potassium Hydrogen Phosphate 0.65 g, Magnesium Sulphate 0.5 g, Rose Bengal 0.05 g, Chloramphenicol 0.1 g and Agar 13 g.

Potato Dextrose Agar

Dextrose 20 g, Agar 15 g and Potato Extract 4 g.

Carnation Leaf Agar

Agar 20 g, leaf pieces.

Czapek Yeast Extract Agar

Sodium nitrate 2 g, Potassium chloride 0.5 g, Magnesium glycerophosphate 0.5 g, Ferrous sulphate 0.01 g, Potassium sulphate 0.35 g, Sucrose 30 g and Agar 12 g.

Malt Extract Agar

Malt extract 30 g, Soy Peptone 5 g and Agar 15 g.

Water Agar

Agar 15 g.

TBE Buffer

Tris base 108 g, boric acid 55 g, 40 ml 0.5 M EDTA solution (pH 8.0).