MYCOTOXINS IN *SORGHUM BICOLOR* AND *PENNISETUM GLAUCUM* COLLECTED FROM THE OSHANA REGION OF NORTHERN NAMIBIA

by

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DECLARATION

I, Calvin Rhabelani Bither Kaela, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

10 September 2021

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ABSTRACT

This project formed part of a research collaboration between the Cape Peninsula University of Technology and the University of Namibia (Windhoek, Namibia). The study involved mycological and multiple mycotoxin surveillance of smallholder farms, and processed sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*) products sold at open markets in the Oshana region of northern Namibia. Despite strict regulations worldwide, mycotoxin levels in grains are not regulated in Namibia. Smallholder farming communities in northern Namibia are heavily reliant on sorghum and pearl millet as staple food. The Oshakati smallholder farmers service both the Oshakati and Ondangwa open markets. Sorghum and pearl millet samples were collected from smallholder farmers' households in Oshakati and from randomly selected vending stalls from the Oshakati and Ondangwa open markets. The occurrence of mycotoxigenic fungi on sorghum and pearl millet was determined using conventional mycological methods as well as validated molecular techniques utilizing species-specific primers and quantitative Real-time PCR (qPCR). The concentrations of multiple mycotoxins [aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), ochratoxin A, deoxynivalenol, zearalenone (ZEA), nivalenol and moniliformin] were determined with a validated liquid chromatography with tandem mass spectrometry analytical method. Morphological analyses indicated higher fungal contamination levels in the raw (whole grain) and processed sorghum as compared to pearl millet ($P<0.05$). No *Aspergillus* spp. were detected in the raw sorghum samples. The contamination frequencies of *Fusarium* spp. in raw sorghum and pearl millet were both 80%. *Fusarium* and *Aspergillus* spp. co-occurred in 30% of raw pearl millet samples. In processed samples, contamination with *Fusarium* and *Aspergillus* spp. was detected in 74% and 100% samples, respectively. The infection levels of *Aspergillus* spp. in processed products were higher as compared to *Fusarium* spp. ($P<0.05$). No *Fusarium verticillioides, F. graminearum* and *F. proliferatum* were detected with qPCR in raw sorghum, while *F. verticillioides* (0.08 - 0.1 pg/µl) and *F. subglutinans* (0.03 pg/µl) were detected in 80% of raw pearl millet samples. *Fusarium verticillioides, F. graminearum* and *F. proliferatum* were, however, detected in 95% sorghum malt samples (0.0059 - 16.69 pg/µl). *Fusarium* spp. were detected in 92% pearl millet malt and bran samples (0.07 - 29.05 pg/µl). No mycotoxins were detected in raw sorghum, which corresponds with the fungal qPCR results. No mycotoxins were detected in raw pearl millet, which could be ascribed to the low levels of mycotoxigenic fungi detected with qPCR. The results indicated that contamination with mycotoxins occurred during handling and/or processing. 57% of malt and bran samples contained mycotoxins which are regulated by the European Union (EU). 20 and 54% of processed sorghum and pearl millet samples, respectively, contained AFB_1 (3 - 14 µg/kg). 17% of all bran and malts contained AFB_1 at levels above the regulatory maximum level of 5 µg/kg

set by the European Commission (EC). 9% of bran and malt samples exceeded the fumonisin regulatory level (200 µg/kg) set by the EC for infants and young children. 4% of processed samples contained ZEA at levels far above the recommended EC level of 100 µg/kg. Co-occurrence of FB_1, FB_2, FB_3 and ZEA was detected in both sorghum and pearl millet malts. A strong correlation (R = 0.8 - 0.83) existed between levels of *F. verticillioides* and *F. proliferatum* determined with qPCR and FB_1, FB_2, and FB_3 concentrations in samples. The study provided important information on the degree of fungal and multiple mycotoxin contamination of raw and processed sorghum and pearl millet in the Oshana region of northern Namibia. It confirmed co-contamination of staple grains and therefore possible chronic co-exposure of communities to multiple mycotoxins regulated by the EU. The results will contribute to determining the levels of dietary exposure of these farming communities to multiple mycotoxins. It will make a valuable contribution to the development of technological or practical methods to reduce mycotoxin levels in these staple grains. By monitoring the malting process, the sources of contamination and critical control points could be identified and managed. Mycotoxin awareness campaigns and sustainable education could further contribute to reducing mycotoxin contamination. Ultimately, the project could contribute to food safety and security in northern Namibia.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS AND ACRONYMS

AFB_1	Aflatoxin B_1
CAF	Central Analytical Facility
DON	Deoxynivalenol
ELISA	Enzyme-linked immunosorbent assay
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FGSC	*Fusarium graminearum* species complex
FHB	Fusarium head blight
HPLC	High Performance Liquid Chromatography
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
MON	Moniliformin
NIV	Nivalenol
OTA	Ochratoxin A
PCR	Polymerase Chain Reaction
QPCR	Quantitative Real-Time PCR
SAGL	Southern Africa Grain laboratory
SU	Stellenbosch University
ZEA	Zearalenone

GLOSSARY

Carcinogenic	A cancer causing agent
Characterization	To uniquely identify
Contamination	Non-intended introduction of fungi or toxins
Mycotoxicosis	A disease caused by fungal toxins
Mycotoxigenic fungi	Mycotoxin producing fungi
Mycotoxins	Toxic secondary metabolites produced by certain fungal species
Optimization	The process of fully perfecting the procedure
Primer	A strand of nucleic acids that serves as a starting point for DNA replication
Smallholder farmer	A farmer, owning a small plot of land on which he grows subsistence crops and one or two cash crops, relying mainly on family labour
Mycotoxigenic fungi	Mycotoxin producing fungi

CHAPTER 1

MOTIVATION AND DESIGN OF THE STUDY

1.1 Introduction

Mycotoxins are secondary metabolites of food-borne fungi and are important environmental and carcinogenic agents (Alberts et al., 2017). Contamination of food supplies with mycotoxins is common and impacts dietary staple grains in many regions of the world. Much of Africa's grain stocks are at risk of becoming contaminated with mycotoxins, which further affects food security. Toxicologically significant mycotoxins include aflatoxins, fumonisins, ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZEA) produced by fungal species of the genera *Aspergillus, Fusarium* and *Penicillium*. These toxins cause a variety of biochemical effects (including carcinogenic, mutagenic, teratogenic, estrogenic, neurotoxic, hepatotoxic, nephrotoxic, cytotoxic and immunosuppressive properties) and are implicated in many human diseases (Marasas, 1995; Shephard, 2008). Co-contamination of food and co-exposure of particularly young children to multiple mycotoxins have been extensively reported in low socio-economic communities in African and Latin American countries and is of particular concern (Ezekiel et al., 2014; Torres et al., 2015). Chronic exposure to low levels of mycotoxins further enhances the negative health effects.

Strict regulations of mycotoxins in food exist in developed countries with high levels of food safety control to guard against the harmful effects on human health (Alberts et al., 2017). In low-income countries, mycotoxin laws either are absent or partially implemented, generating situations where mycotoxin exposures are above the thresholds set by health regulators. Populations that are worst affected by mycotoxin contamination of staple crops include smallholder populations where regulation is not in effect, mono-cereal crops are harvested and locally used, and elevated exposure to mycotoxins is the norm (Shephard et al., 2019).

Reviews on mycotoxin contamination of food in Africa concentrated primarily on Central, Eastern and Western Africa (Vismer et al., 2015). However, a large number of dietary mycotoxin cases have been reported in South Africa in the last decade, with fewer reports documented in Botswana, Lesotho, Malawi, Mozambique, Zambia and Zimbabwe (Misihairabgwi et al., 2019). Currently there are only limited reports available on the occurrence of mycotoxins in Namibian staple grains, and no information on exposure levels of communities. Despite strict international regulations, mycotoxins contaminating grain crops are not regulated in Namibia and daily exposure of especially smallholder farming communities remains a concern.

The current study involved mycological and multiple mycotoxin surveillance of raw sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*) produced on smallholder farms and the processed products sold at open markets in the Oshana region of northern Namibia. The study provides important information on the degree of mycotoxigenic fungal and multiple mycotoxin contamination of unprocessed sorghum and pearl millet as well as the transfer of fungi and mycotoxins to processed products sold at local markets. The results will contribute to determining dietary exposure of smallholder farming communities to mycotoxins. The project will potentially have an important impact on the Namibian agricultural industry through the development of sound production and processing practices. Ultimately, the study will make an important contribution to food safety and security in Namibia.

1.2 Statement of research problem

Currently, limited data is available on the occurrence of mycotoxins in Namibian grains and exposure of smallholder farming communities to multiple mycotoxins in their dietary staples. Despite strict international regulations, mycotoxins contaminating staple grains are not regulated in Namibia. Smallholder farmers in northern Namibia utilize undiversified diets due to drought conditions and are heavily reliant on sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*) as a staple food. Relevant geographical areas include the Oshana (sorghum and pearl millet), Okavango (sorghum) and Zambezi [*Zea mays* (maize)] regions of northern Namibia. The Oshana and Okavango regions are semi-arid areas, with frequently occurring droughts. Locally, raw (unprocessed) and processed sorghum and pearl millet products are sold at open markets. The Zambezi region is characterized by maize smallholder farming, as this area experience higher rainfall.

It is imperative for a detailed characterisation and occurrence of mycotoxins amongst the smallholder farmers of sorghum and pearl millet in the Oshana region of northern Namibia. Such a study will provide important information on the degree of contamination by multiple mycotoxins of staple crops produced by smallholder farmers as well as processed products available at local markets.

1.3 Research questions

- What is the occurrence of agriculturally important *Fusarium* and *Aspergillus* spp. in unprocessed sorghum and pearl millet samples obtained from smallholder farmers and processed products from open markets in northern Namibia?
- What are the levels of contamination with multiple mycotoxins of raw sorghum and pearl millet samples obtained from smallholder farmers and processed products from open markets in northern Namibia?

1.4 Objectives of the research

- Determine the occurrence of mycotoxigenic fungi (agriculturally important *Fusarium* and *Aspergillus spp*.) on sorghum and pearl millet using traditional morphological as well as validated molecular techniques involving species-specific primers and quantitative Real-time PCR.
- Determine the concentrations of multiple mycotoxins [aflatoxin B_1 (AFB_1), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), OTA, DON, ZEA, nivalenol (NIV) and moniliformin (MON)] in sorghum and pearl millet samples by validated extraction and liquid chromatography with tandem mass spectrometry (LC-MS/MS) analytical methods.
- Determining the occurrence of fungal species and mycotoxins in raw whole grains and processed grains sold at open markets.

1.5 Ethical clearance

Field study to the Oshana region of northern Namibia: The field study including sampling was performed postharvest during July 2018 by Dr JP Rheeder [Cape Peninsula University of Technology (CPUT)], Prof J Misihairabgwi (University of Namibia, Windhoek, Namibia) and extension officers from the Department of Agriculture, Namibia. Ethical clearance (REC Approval Reference No: CPUT/HW-REC 2018/H5) for the field study to Namibia was obtained from the Department of Health and Wellness Sciences Ethical Clearance Committee (HW-REC) of CPUT. The student, Mr C Kaela, was not involved in the field study and sampling. The student, however, performed all laboratory analyses on the samples at CPUT, including mycological, molecular and analytical chemistry analyses.

Management of the extracted mycotoxins: Solvent extractions of mycotoxins from sorghum and pearl millet were performed in a biohazard fume cabinet. The student wore gloves and protective clothing during experiments. The solvent waste was discarded in 2.5 L glass containers classified according to hazard level, stored in a separate room and removed by a specialist chemical waste company. All solid waste was kept in biohazard containers until removed by the chemical waste company. Glassware was kept separate, and acid washed.

1.6 Delineation of research

- Sorghum and pearl millet samples from southern Namibia were not included in this study.
- Samples were only collected in the Oshana region, where dry climate conditions prevail. Samples from areas with higher rainfall were not included.
- Commercial sorghum and pearl millet samples were not included in the study.
- Only the most important mycotoxins contaminating cereals were included in the study.

1.7 Significance of the research: social and economic impact

It is imperative for a detailed understanding of mycotoxins amongst the smallholder farmers of Namibia. Such a study will provide important information on the degree of contamination by multiple mycotoxins of staple crops produced by smallholder farmers as well as processed products available at local markets in this area. The smallholder farming communities are heavily reliant on sorghum and pearl millet as a staple foodstuff. The results will make an import contribution to determining dietary exposure to multiple mycotoxins of these communities. This project will have important practical applications by identifying steps for intervention to manage the risk of mycotoxin exposure to smallholder farmers and communities, as well as local trade in sorghum and pearl millet food products. Ultimately the project will contribute to food safety and security in northern Namibia.

1.8 Expected outcomes, results and contributions of the research

- A comprehensive understanding of mycotoxins in staple foods amongst smallholder farmers of northern Namibia: A detailed synopsis of mycological and multiple mycotoxins in unprocessed sorghum and pearl millet and processed products at open markets.
- Important information on the co-occurrence of multiple mycotoxins in grains and processed products.
- Important information on the transfer of fungal and multi-mycotoxin contamination from unprocessed cereals to processed products.
- The information will be valuable for future determination of exposure and risk assessment studies.
- The information will be valuable for the development of interventions to create awareness among populations and for the development of practical control strategies.
- The information will be valuable for decision makers concerning policies and regulations on maximum levels of multi-mycotoxins in staple foods.
- The information will make an important contribution to food safety and security in Namibia.
- Publications: Two publications in DHET accredited journals.
- Conferences: Poster presentation at the Joint International Conference of the African Society of Mycotoxicology (ASM) and MYTOX-SOUTH to be held in Stellenbosch during 2022.

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CHAPTER 2

LITERATURE REVIEW

2.1 Sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*): important staple crops in southern Africa

Zea mays (maize) and peanuts are the main staples and sources of revenue in Africa, while sorghum, pearl millet, wheat and cassava are considered important secondary dietary crops (Chiona et al., 2014). Sorghum and pearl millet constitute half of the total cereal crop production on the continent and play an important role in the maintenance of food security (Mukarumbwa et al., 2010; Taylor, 2003). Their high yield rate and adaptation to extreme environmental conditions such as low rainfall, high temperatures and short bursts of heavy rainfall make them suitable for production in most regions in Africa (Mukarumbwa et al., 2010). Sorghum and pearl millet are uniquely drought resistant, i.e. they have a lower water requirement (300-400 mm per annum) during their growth period as compared to maize (500 mm per annum), are more tolerant to high temperatures due to their deep root system, and can be stored for prolonged periods (Amadou et al., 2013; Orr et al., 2016). They are especially ideal crops for cultivation in regions that are semi-arid and tropic, such as sub-Saharan Africa and subtropical East Africa (Mukarumbwa et al., 2010).

As a food commodity, pearl millet is commonly ground into a flour and used to prepare stiff porridge. In West Africa, pearl millet is used as an ingredient of *Koko*, a porridge and drink. On an industrial scale, it is used for the production of biscuits, confectionary, weaning foods, and alcoholic and non-alcoholic beverages (Laminu et al., 2011; Amadou et al., 2013). *Injera,* a sour dough flatbread and national dish of Ethiopia, is produced from sorghum. Sorghum is also processed into instant porridge, e.g. *Morvite* (South Africa) and used for the preparation of non-alcoholic beverages such as *Milo* (Nestlé, South Africa), *Shumba Mahewu* (Delta Beverages, Zimbabwe) and traditional home brewed *Mahewu*. In Namibia, sorghum and pearl millet is ground into flour, which is used to make stiff porridge (Orr et al., 2016). Sorghum and pearl millet are also malted, to activate enzyme activity, and are used in the preparation of traditional fermented beverages such as *Oshikundu* (Misihairabgwi et al., 2018) Sorghum is the key ingredient in the production of traditional cloudy and opaque beer in sub-Saharan Africa. These beers are used for ritual purposes and as a source of income. They are given traditional product names, depending on the place of origin, tribe and brewing method, such as *Umqomboti* (South Africa) and *Doro* (Zimbabwe). In South Africa, Botswana, Zimbabwe, Namibia and Swaziland, there are large-scale commercial production of opaque beer using sorghum malt. Approximately 1.7 billion litres of opaque beer are produced in southern and

eastern Africa per annum (Taylor, 2003). Sorghum is also malted commercially during the production of lager beers. South African Breweries, Delta Beverages (Zimbabwe) and Windhoek Lagers (Namibia) are amongst the main lager producing companies in Africa.

These crops are prone to diseases caused by mycotoxigenic fungi, with resultant contamination with mycotoxins.

2.2 Mycotoxigenic fungal species and mycotoxins in staple grains

2.2.1 *Aspergillus* spp.

Aspergillus is a genus containing more than 250 species, consisting primarily of saprophytic and filamentous fungi. The genus belongs to the phylum *Ascomycota* (Leslie and Summerell, 2006; Yin et al., 2017). Haploid asexual spores germinate and grow into branched filaments or hyphae, forming mycelium. The mycelium of *Aspergillus* spp. participates in sexual reproduction. Mycelial differentiation leads to the formation of asci, sexual spore-forming structures, in which the two nuclei in each cell fuse to form a diploid cell that undergoes meiosis and produces haploid ascospores (Varga et al., 2014). During harsh environmental conditions such as drought and nutrient stress, compact, resistant and dormant sclerotia are produced. When favourable environmental conditions resume, the sclerotia germinate to produce conidia, which are disseminated in the soil and air. *Aspergillus* spp. are food spoilage organisms as well as human and plant pathogens causing a variety of diseases due to the production of mycotoxins. They have, however, a variety of beneficial applications in biotechnology, mainly by acting as host organisms for the molecular expression of genes during antibiotic, organic acid and enzyme production (Keller et al., 2005).

Aflatoxins (AFs) are mycotoxins produced by *Aspergillus* spp. including *A. flavus, A. parasiticus, A. parvisclereteginus* and *A. minisclerotigenes* (Silva et al., 2011). Aflatoxin B_1, B_2, G_1 and G_2 are the four naturally occurring groups of AFs. *A. flavus* produces only the B-type AFs whereas the other species both the B- and G-type AFs. Aflatoxins cause liver cancer, Reye's syndrome, Indian childhood cirrhosis, chronic gastritis and is associated with kwashiorkor. AFs has been classified Group 1 carcinogens by the International Agency for Research on Cancer (Pitt, 2012).

Ochratoxin A (OTA) is mainly produced by *Aspergillus ochraceus, A. carbonarius, A. sclerotiorum* and *Penicillium verrucosum* (Leslie and Summerell, 2006; Shephard et al., 2010). *Penicillium verrucosum* inhabits temperate regions (≤30˚C), whilst *A. ochraceus* is more prevalent in tropical regions of the world. OTA contaminates cereals, coffee, wine, beer and grape juice, and is genotoxic, immunosuppressive, teratogenic and mutagenic and therefore classified a Group 2B mycotoxin (Pitt, 2012).

2.2.2 *Fusarium* spp.

The *Fusarium* genus belongs to the *Ascomycota* phylum, class *Sordariomycetes* and order *Hypocreales*. *Fusarium* spp. are saprophytes and play an important role as decomposers of organic material. It undergoes asexual reproduction through the formation of three types of asexual spores, i.e. microconidia, macroconidia and chlamydospores (Summerell and Leslie, 2011). Chlamydospores, which may be formed singly or in chains, germinate to form fresh mycelia under favourable environmental conditions. *Fusarium* spp. contaminate most cereal crops and are economically very destructive. *Fusarium verticilliodes* and *F. proliferatum* are important plant pathogens occurring worldwide, causing ear, stalk and root rot of maize. These rots reduce both yield and grain quality. Infection of growing plants by *Fusarium* spp. typically occurs through the silks or insect damaged kernels. They grow aggressively in temperate climates, and both soil and seed-borne inoculum can cause infection. Both fungal species produce mycotoxins within the infected kernel.

Fusarium verticilliodes and *F. proliferatum* produce the fumonisin mycotoxins, which are associated with severe mycoses in both humans and animals (Rheeder et al., 2002). Fumonisin B_1 causes leukoencephalomalacia in horses, pulmonary edema in pigs, poor production in poultry, is associated with oesophageal cancer in humans, and immunosuppression and stunting in children (Marasas, 1995), by interfering with sphingolipid metabolism. More than 20 naturally occurring analogues of fumonisins are known, with FB_1 being the most prevalent and toxic.

Fusarium avenaceum, F. cerealis, F. culmorum, F. equiseti, F. graminearum complex*, F. poae, F. proliferatum, F. pseudograminearum, F. sporotrichioides, F. subglutinans* and *F. verticilliodes* produce trichothecene mycotoxins, which cause lifelong health complications in both humans and animals (Nicolaisen et al., 2009; Kazan and Gardner, 2018). Trichothecenes are phytotoxic compounds and act as virulence factors for susceptible plant hosts (Boutigny et al., 2012). Trichothecenes is the largest group of mycotoxins released by *Fusarium* ssp. The most common mycotoxin in the trichothecenes group is deoxynivalenol (DON), which is mainly produced by *F. pseudograminearum* and *F. culmorum* (Nicolaisen et al., 2009). Nivalenol is produced by *F. equiseti* and *F. poae*. DON causes gastrointestinal infection, disturbs intestinal barrier function and immunosuppression after chronic dietary exposure. These *Fusarium* spp. are responsible for Fusarium head Blight (FHB) and crown rot in small grain cereals. Zearalenone (ZEA) is a polyketide mycotoxin and is produced by certain species of the *F. graminearum* complex (Atoui et al., 2011). ZEA contaminates several cereals including maize, barley, oats, rice, sorghum and millet and has been reported in Australia, Europe and North America. ZEA caused changes in the reproductive tract of research and domesticated animals. ZEA has also been found to be associated with multiple oestrogen symptoms, such as reduced

fertility, increased foetal resorption, changes in the endocrine system gland weight and serum hormone levels. This mycotoxin is heat stable and does not denature during processing and cooking (Atoui et al., 2011). Emerging mycotoxins produced by *Fusarium* spp. include enniatins, moniliforomin (MON), beauvericin, and fusaproliferin (Jaji´c et al., 2019).

2.3 Mycotoxins and their effect on human health

Mycotoxins are toxic secondary metabolites produced by certain fungal species, and natural contaminants of grain crops and peanuts (Vismer et al., 2019). Toxicologically significant mycotoxins of concern include aflatoxins, fumonisins, OTA, DON, ZEA, MON and nivalenol (NIV) produced by fungal species of the genera *Aspergillus, Fusarium* and *Penicillium* (Shephard, 2008). Aflatoxins and fumonisins are prevalent in major food and export crops in Southern Africa, with less reports of DON, ZEA, OTA, NIV and MON contamination (Shephard et al., 2010; Ezekiel et al., 2012; Probst et al., 2014; Matumba et al., 2017). These mycotoxins exhibit a variety of biological effects and are implicated in many human diseases (Marasas, 1995; Chilaka et al., 2017).

Mycotoxins can be carcinogenic, mutagenic, teratogenic, estrogenic, neurotoxic, hepatotoxic, nephrotoxic and cytotoxic or may trigger immunosuppression in humans (Marasas, 1995). The type of mycotoxin and the rate at which it is consumed determines the health associated disorder and its severity. Acute toxicity is a result of consumption of high concentrations of mycotoxins in a short period and may result in death (Darwish et al., 2014). Chronic effects may result from consumption of low concentrations of toxins over a long period (Darwish et al., 2014). Acute mycotoxicoses have been documented in Africa, although prolonged exposure to low levels of multiple mycotoxins is a risk factor for human diseases, including cancer and childhood stunting (Chiona et al., 2014; Wild et al., 2015). Preventing chronic exposure, especially in developing countries such as sub-Saharan Africa and parts of Latin America, is of vital importance (Pitt, 2012). Exposure to multiple mycotoxin contamination in staple food commodities enhances the negative health effects in populations (Ezekiel et al., 2014; Torres et al., 2015). Co-contamination of food and co-exposure of particularly young children to multiple mycotoxins have been extensively reported in low socioeconomic areas in African and Latin American countries. Apart from the health implications, mycotoxin contamination of grain crops presents a significant threat to the foreign trade in food and feed.

2.4 Methods to determine the magnitude of fungal contamination in grains

Methods for the detection and identification of mycotoxigenic fungi are a vital part of surveillance studies monitoring the magnitude of contamination of grains during production, storage and processing stages (Faria et al., 2012). Identification methods frequently used are based on (i) morphological traits of fungal species (i) PCR based genotyping based on sequence variation and the presence of key genes (e.g. *EF1*α, *TEF1*-α, IGS; mycotoxin biosynthetic genes such as *FUM1, TRI13* and *TRI17*) (iii) phylogenetic studies (e.g. AFLP, ITS-RFLP and multigene phylogeny) and (iv) Real-time PCR methods using species-specific primers (Valones et al., 2009). However, certain of these techniques are limited by tedious methodology, complex interpretation relying on specialized trained researchers and molecular identification of only targeted fungal species (Marinach-Patrice et al., 2009). Morphological analysis of fungi is considered a traditional mycological technique, allowing the detection and quantification of all fungal species present in a sample. An advantage of this technique is that novel fungal species could also be detected as species-specific parameters are not used. This technique requires, however, training by an experienced researcher on culturing and microscopy techniques (Marinach-Patrice et al., 2009; Faria et al., 2012). Genotyping based on sequence variation and the presence of key genes (e.g. *EF1*α, *TEF1*-α, *IGS* and *ITS*) is also a useful method for detection of all fungal species present in samples. Real-time PCR methods using species-specific primers provides a high level of accuracy in detecting and quantifying fungal species. The technique is useful when only specific fungal species have to be detected (Valones et al., 2009). The technique is, however, limited to the availability of species-specific primers for a specific fungal species. Furthermore, novel species cannot be detected with this method and specialized training in Real-time PCR techniques is required.

2.5 Techniques for quantification of multiple mycotoxins in grains

Various techniques are applied for the detection and quantification of mycotoxins in grains, including ELISA, HPLC and LC-MS/MS (Agriopoulou et al., 2020). A wide range of laboratory kits are available, using ELISA principles. These kits generally provide rapid detection of mycotoxins in grains. The sensitivity and accuracy of ELISA kits vary and should therefore be cautiously selected. Validation of methods is not required (Zhang et al., 2018). The kits are especially applicable to field testing and is used in laboratories lacking sophisticated technologies such as HPLC and LC-MS/MS. HPLC provides an effective method for detection and quantification of mycotoxins. The method requires validation and includes extraction methods prior to HPLC quantification (Agriopoulou et al*.,* 2020). Sophisticated technology is required. However, most universities and research institutes house HPLC instruments. The method is tedious, but accurate results are produced (Agriopoulou et al*.,* 2020). The method is, however, not as sensitive as LC-MS/MS and multiple mycotoxins cannot be quantified in one run (Pascale et al., 2019). LC-MS/MS analyses is currently being applied

worldwide for quantification of multiple mycotoxins in grains (Pascale et al., 2019). This kind of analyses require sophisticated technology and experience, which is only available at the main mycotoxin testing laboratories in South Africa, i.e. the South African Grain Laboratory (SAGL, Pretoria) and the Mass Spectrometry Unit of the Central Analytical Facility (CAF) of Stellenbosch University (SU). This technique is very sensitive, can detect low concentrations of multiple mycotoxins in one run, and provides a high level of precision and accuracy (Pascale et al., 2019). The technique requires, however, extensive validation of the extraction and analytical techniques to ensure accuracy and precision of results (Bessaire et al., 2019).

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2.6 Contamination of sorghum and millet with mycotoxigenic fungi and mycotoxins

The most important sorghum stalk rot and grain mould diseases on sorghum is caused by *Fusarium* spp., including *F. andiyazi, F. thapsinum, F. napiforme, F. proliferatum, F. nygamai, F. pseudonygamai,* and *F. verticillioides* (Leslie and Summerell, 2006). In Japan, 52.5% of imported sorghum samples were contaminated with ZEA (60-7260 mg/kg), indicating the presence of the *F. graminearum* spp. complex (FGSC) (Aoyama et al., 2009). A high prevalence of *F. proliferatum*, *F. thapsinum* and *F. verticillioides* and less of *F. sacchari* and *F. beomiforme* was observed in sorghum sampled in Thailand, indicating the possible presence of the fumonisin mycotoxins (Mohamed et al., 2019). Based on Amplified Fragment Length Polymorphisms (AFLP) genetic variation, the *F. thapsinum* populations were the most closely related, the *F. verticillioides* populations were the most distantly related, and the *F. proliferatum* populations were in an intermediate position. This could indicate that *F. thapsinum* is introduced mainly with seed, whilst *F. proliferatum* and *F. verticillioides* may have been contained in seeds or carried over from previous crops. The prevalence of infection of sorghum grains by *Fusarium* spp. in Argentina is high (82.5-99%), with *F. verticillioides*, *F. thapsinum* and *F. andiyazi* the most commonly occurring species, and species within the FGSC occurring in high frequency (Sampietro et al., 2010).

Data on the contamination of sorghum and millet in Africa indicates the co-occurrence of multiple mycotoxins, especially fumonisins and aflatoxins. The simultaneous occurrence of fumonisins and aflatoxins has been documented in sorghum and pearl millet from smallholder farmers under the direction of the International Institute for Tropical Agriculture, Nigeria (Vismer et al., 2015). A surveillance study evaluating the levels of multiple mycotoxins in sorghum from Burkina Faso, Ethiopia, Mali and Sudan resulted in 33% of the 1533 samples contaminated with multiple mycotoxins, including aflatoxins, fumonisins, sterigmatocystin, *Alternaria* toxins, OTA and ZEA (Ssepuuya et al., 2018). Sudan exhibited the lowest prevalence of all mycotoxins analysed. Of the samples analysed, pink sorghum exhibited the highest concentrations of both fumonisins and aflatoxins. Studies on *Fusarium* spp. In sorghum and finger millet in Ethiopia revealed higher contamination in sorghum. A large number of

isolates exhibited a close relationship with the *F. incarnatum–equiseti* spp. complex (Chala et al., 2019). All sorghum samples collected from smallholder farmers' threshing floors and underground storage pits in East Hararghe, Ethiopia were co-contaminated with *Aspergillus* and *Fusarium* spp. Aflatoxin contamination was ≤ 33.10 µg/kg with the highest levels in stored samples, while the fumonisin levels were between 907-2041 µg/kg (Taye et al., 2016).

Little information is available on contamination of sorghum and millet in southern Africa. *F. nygamai* and *F. moniliforme* (syn: *F. verticillioides*) are the main fungi contaminating sorghum and millet in Lesotho and Zimbabwe (Klaasen and Nelson, 1997). In Gaborone, Botswana, 46 traditional sorghum malt, wort, and beer samples were collected from three villages. *F. verticillioide*s contamination was detected in 63% samples and *Aspergillus flavus* in 37%. Aflatoxins were not detected, whilst FB_1 was detected in three malt samples (47-1316 µg/kg), and ZEA in 56%, 48% and 48% of malt (102-2213 µg/kg), wort (26-285 µg/l) and beer (20-201 µg/l) samples, respectively (Nkwe et al., 2005). Several mycotoxin producing *Fusarium* spp. have been isolated from sorghum grain in South Africa (Beukes et al., 2017). *F. andiyazi, F. nygamai, F. thapsinum* and *F. verticillioides are* known fumonisin producers, while species within the FGSC are type B trichothecenes and ZEA producers (Table 2.1). In Namibia, the diversity of fungal metabolites was determined in street vended pearl millet and sorghum ingredients and their transfer rate during fermentation into *Oshikundu*, a traditional Namibian drink (Misihairabgwi et al., 2018). Unacceptable high levels of mycotoxins were detected in the raw grains used for the brewing of *Oshikundu.* AFB_1 was detected in 13% of 40 pearl millet meal samples [mean concentration 0.9±0.7 µg/kg (range 0.1-2.0 µg/kg)] and in 50% of 40 sorghum malt samples [mean concentration 4.5±5.5 µg/kg (range 0.2-25.4 µg/kg)]. However, there remain a huge knowledge gap on the information concerning mycotoxin contamination in Namibian staple grains.

Table 2.1 Mycotoxigenic *Fusarium* spp. associated with South African sorghum and millet [adapted from Beukes et al. (2017)]

Species	South African grain host	Mycotoxins associated with fungal species
Fusarium acuminatum	Sorghum	BEA, DON, HT-2, MON, T-2
F. andiyazi	Sorghum	FUM
F. avenaceum	Sorghum	BEA, FusaC, MON
F. chlamydosporum	Sorghum	HT-2, MON, T-2
F. incarnatum-equiseti species complex	Sorghum	BEA, DON, MON, NIV, ZEA
F. merismoides	Sorghum	ENN
F. napiforme	Millet, sorghum	FUM, MON
F. nygamai	Millet, sorghum	BEA, FUM, MON
F. oxysporum	Sorghum	BEA, FA, FUM, MON, ZEA
F. pseudonygamai	Sorghum	FUM, MON
F. semitectum	Sorghum	BEA, DON, MON, NIV, ZEA
F. solani species complex	Sorghum	DON, FUM, T-2, ZEA
F. subglutinans	Sorghum	BEA, FA, FUM, MON
F. verticillioides (syn: *F. moniliforme*)	Sorghum	BEA, FusaC, FUM, MON
F. graminearum species complex:		
F. acaciae-mearnsii	Sorghum	3-ADON, NIV
F. cortaderiae	Sorghum	3-ADON, NIV
F. meridionale	Sorghum	NIV
F. thapsinum	Sorghum	FA, FUM, MON

BEA, beauvericin; DON, deoxynivalenol; HT-2, HT-2 toxin; MON, moniliformin; T-2, T-2 toxin; FUM, fumonisins; FusaC, fusarin C; NIV, nivalenol; Fx, fusarenon-X; ZEA, zearalenone; AcDON, acetyldeoxynivalenol; ENN, enniatins; FA, fusaric acid; 15-ADON, 15-acetyldeoxynivalenol; 3-ADON, 3-acetyldeoxynivalenol

2.7 Effect of climatic conditions on mycotoxin production

Environmental conditions relating to temperature, water activity and atmospheric CO_2 levels are the most important factors determining the degree of contamination of grain crops with mycotoxigenic fungi and mycotoxins (Paterson and Lima, 2010; Van der Fels-Klerx et al., 2012). Vaughan et al. (2014; 2016) investigated the impact of elevated CO_2 on the interactions between maize and *F. verticillioides* infection. They found that maize simultaneously exposed to elevated CO_2 levels of approx. 800 ppm (approx. 2x the current CO_2) and drought increased maize susceptibility to *F. verticillioides* proliferation and resulted in increased levels of

fumonisin contamination. A three-way interacting study, evaluating the effects of temperature, imposed drought stress and increased CO_2 levels exhibited a profound, statistically significant stimulatory effect on mycotoxin production (approx. 70x the control in *in vitro* studies), especially under drought stress at 37ºC and 650 and 1000 ppm CO_2 exposure. Water stress is an important factor for fumonisin accumulation, particularly in the later phases of maize colonization when water availability decreases. A significant increase in the prevalence of mycotoxigenic fungi and mycotoxins on maize grain was caused by stressful agrometeorological conditions, high temperatures and drought over the period from flowering to waxy maturity of maize (Milićević et al., 2019).

Studies on the relative expression of mycotoxin biosynthetic genes corroborated these findings (Medina et al., 2015). The fumonisin biosynthetic gene, *FUM1*, is significantly affected by changes in environmental stress, is induced under water stress conditions in *F. verticillioides*, and could result in an increased risk of fumonisin contamination of maize. Extreme weather events (temperature, CO_2 and rainfall) lead to plant stress and higher mycotoxin prevalence (Milićević et al., 2019). The risk of mycotoxin contamination increases when crops are grown under conditions of long-term water deficit (Oldenburg et al., 2012).

Several of the agriculturally active areas in Southern Africa have a subtropical or tropical climate, characterized by hot and humid weather, coupled with intermittent rainfall and regular dry spells, which provide the perfect environment for toxigenic fungi to proliferate (Chiona et al., 2014; Mboya and Kolanisi, 2014). Drought spells make it necessary to store grains for long periods. However, this creates environments that are conducive for insect infestation, fungal proliferation and aflatoxin production as most smallholder farmers' storage facilities are characterized by poor pest control and aeration, and a lack of moisture and temperature control measures (Matumba et al., 2015).

Africa is vulnerable to climate instability due to poor adaptive potential (Kruger and Shongwe, 2004). The living standards of many people in Africa, including South Africa, are closely linked to the climate of the region. By 2025, yields from rain-fed farming could be drastically decreased in certain regions, which will further adversely impact food security and intensify malnutrition. In southern Africa, the risk of higher temperatures is higher inland and lower in coastal regions. Given a low to strong rise in greenhouse gas emissions by 2050, the coast temperature is expected to warm by about 1°C and the interior by about 3°C. Floods are most likely to occur, but this does not mean an increase in overall rainfall. Greater evaporation rates are expected to increase the occurrence and severity of drought. There is evidence to suggest that such climate change factors may have a significant effect on crop infection with changing profiles of toxigenic fungi and their associated levels of mycotoxin contamination

(Kruger and Shongwe, 2004; Medina et al., 2015).

2.8 Mycotoxin regulations

Strict guidelines for mycotoxin levels in food exist in high-income countries concurrent with effective food safety control measures, to guard against the harmful effects on human health (Shephard et al., 2019). In low-income nations, either mycotoxin laws are absent or partially implemented, leading to situations where mycotoxin exposures surpass the thresholds set by health regulators. The worst affected population groups include smallholder farming communities where mycotoxin levels are not monitored, as mono-cereal crops are grown and consumed locally. Other factors that intensify the problem include the lack of knowledge of the harmful effects of mycotoxins, as well as conventional applications of grain products not intended for human use during cycles of food insecurity.

Aflatoxins and the fumonisins are the most important mycotoxins contaminating staple grains and are regulated worldwide (Table 2.2). In Africa, only 15 countries have mycotoxin laws, which are primarily linked to aflatoxin exposure in the most common dietary staples [Food and Agriculture Organisation of the United Nations (FAO), 2003]. Despite elevated dietary levels of mycotoxins, there are no policies to regulate them in most countries in Southern Africa. In smallholder farming areas enforcement of legislative policies remains a huge challenge. As a result, high levels of mycotoxins frequently enter the food chain of nations with less stringent or no control measures. The finest quality food products from these countries are exported, with lower quality foods, contaminated with higher levels of mycotoxins, being consumed domestically (Pitt, 2012). In addition, low-income countries face food insecurity due to harsh climatic patterns, economic uncertainty, and a lack of technological capacity (Wagacha and Muthomi, 2008). Apart from economic losses, these factors raise the risk of mycotoxin exposure on a regular basis with the resulting adverse health consequences.

Table 2.2 Regulatory maximum levels for aflatoxins and fumonisins in cereal grains (Ferrigo et al., 2016)

Country	Food commodity	Mycotoxin	Regulatory maximum limit (ppb)	References
Chile	• Feedstuffs	• AFB_1 • AFB_2, AFG_1 and AFG_2	• 5-20 • 20-50	Stoloff et al., 1991

Country	Food commodity	Mycotoxin	Regulatory maximum limit (ppb)	References
China	• Edible oils, rice • Peanuts products, maize • Wheat, barley, oats	• AFB_1 • AFB_1 • AFB_1	• 10 • 20 • 5	Stoloff et al., 1991
Egypt	• Peanuts, oil seeds, cereals and cereal products • Maize	• AFB_1 • AFB_1, AFB_2, AFG_1 and AFG_2	• 5 • 20	Stoloff et al., 1991
European Union	• Cereals, nut and nut products • Milk and Milk products • Ground nuts, copra, maize and maize products	• AFB_1 • AFM_1 • AFB_1	• 2-4 • 0.05 • 20-50	Pittet et al., 2001; Mazumder et al., 2001
Kenya	• Peanuts, peanut products and vegetable oil	• AFB_1, AFB_2, AFG_1 and AFG_2	• 20	Pittet et al., 2001; Mazumder et al., 2001

Country	Food commodity	Mycotoxin	Regulatory maximum limit (ppb)	References
Malawi	• Export quality peanuts	• AFB_1	• 5	Pittet et al., 2001; Stoloff et al., 1991
Nigeria	• Infant food • All other foods • Fluid milk	• AFB_1 • AFB_1 • AFM_1	• 0 • 20 • 1	Stoloff et al., 1991; Mazumder et al., 2001
South Africa	• All foods • Milk and milk products • Maize and maize products	• AFB_1 • AFB_1, AFB_2, AFG_1 and AFG_2 • Total FB_1 and FB_2	• 5 • 10 • 2000-4000	Mazumder et al., 2001; DoH, 2016
United States of America	• All foods • Feed for horses • Feed for livestock and poultry	• AFB_1, AFB_2, AFG_1 and AFG_2 • FB_1 • FB_2	• 20 • 10 000	Mazumder et al., 2001
Zimbabwe	• Foods • Ground nuts, maize, sorghum, peanut butter,	• AFB_1 • AFB_1, AFG_1 and AFB_2	• 5 • 5, 4, 20	Mazumder et al., 2001

Country	Food commodity	Mycotoxin	Regulatory maximum limit (ppb)	References
	cereals, flour and bread			

AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1, AFG_2, aflatoxin G_3; FB_1, fumonisin B_1; FB_2, fumonisin B_2

2.9 Mycotoxin contamination of staple crops in southern Africa

While mycotoxin contamination is a serious concern worldwide, in Southern Africa, little research has been performed on dietary mycotoxins as compared to other regions of the world. This is primarily due to lack of specialized laboratory facilities, insufficient testing resources, capability and skills, and weak surveillance systems (Misihairabgwi et al., 2019). The available reports on mycotoxin contamination in staple crops in Africa mainly focusses on Central, Eastern and Western Africa (Sserumaga et al., 2020; Seetha et al., 2007; Ayalew et al., 2006). In southern Africa, several studies on the prevalence of mycotoxins in smallholder farming populations in South Africa have been recorded, with less reports documented in Namibia, Botswana, Lesotho, Malawi, Mozambique, Zambia, and Zimbabwe. Mycotoxins have been identified in maize, peanuts, barley and barley products, wheat, apple juice and in milk in South Africa over the past decade (Shephard et al., 2010; Rheeder et al., 2016). However, limited studies exist on the presence of mycotoxins in major dietary staples such as maize, peanuts, pearl millet, sorghum and their products in other southern African countries (Warth et al., 2012; Chiona et al., 2014; Matumba et al., 2015).

2.10 Postharvest systems of smallholder farmers in northern Namibia

Postharvest systems include all operations from harvest, drying, threshing, cleaning, storing, grading, marketing and processing of grains (FAO, 2003). Sorghum and pearl millet heads are left to dry on the field before harvesting during May and June. The stems are cut beneath the heads and the heads collected in harvesting baskets for further drying and threshing. Threshing is performed close to the field, by hand, on hardened ground (Figure 2.1). Threshed heads are sun-dried on a threshing floor or on a raised wooden platform until the moisture content is less than 10%. After threshing, the grain is winnowed and stored. The physical damage to heads during threshing and subsequent sun-drying practices could enhance fungal contamination.

Most households in the Oshana region of northern Namibia store their pearl millet in traditional storage baskets (Mallet and du Plessis, 2001). After a field study to the Oshana region of

Namibia, Dr JP Rheeder [Cape Peninsula University of Technology (CPUT)] reported that most smallholder farmers store their grain in traditional storage baskets (Figure 2.2). The traditional granary are large spherical woven baskets made of Mopani branches that are woven together using Mopani bark. The internal surface is plastered using mud from ant and termite hills. The basket has a circular opening on top, which is closed by a lid and sealed by mud once loaded (Mallet and du Plessis, 2001). The storage baskets vary in size and capacity. Some storage baskets used in the North Central region of Namibia are made of Makalawi palm leaves. Due to scarcity of trees in the Oshana region, some farmers have resorted to the use of plastic storage containers, which are commercially available (Figure 2.3). Traditional methods of storage, which include the use of wood ash to guard from insect infection, have been reported in Namibia. These methods could also be sources of fungal contamination.

Figure 2.1 A traditional threshing floor used by smallholder farmers in the Oshana region of Namibia (Photo credit: Dr JP Rheeder)

Figure 2.2 A traditional storage basket used by smallholder farmers in the Oshana region of Namibia (Photo credit: Dr JP Rheeder)

Figure 2.3 Commercial plastic storage containers used by smallholder farmers in the Oshana region of Namibia (Photo credit: Dr JP Rheeder)

2.11 Control strategies for reducing mycotoxins in grains

Surveillance studies indicating exposure of populations to high levels of mycotoxins in their staple grains could alert the Departments of Agriculture and Health of countries to implement suitable control strategies by involving community leaders and policy makers. Several strategies exist for the reduction of mycotoxigenic fungal growth and the production of mycotoxins in food sources. These techniques vary from regulated farming activities, breeding for crops resistant to fungal diseases and insects to diverse methods of physical, chemical and biological management, genetic modification approaches and community-based approaches (Alberts et al., 2017).

Fusarium head blight of wheat is reduced with fungicides, including prochloraz, propiconazole, epoxyconazole, azoxytrobin and cyproconazole (Haidukowski et al., 2005). *Aspergillus* spp. producing aflatoxins can be controlled effectively by oltipraz and chlorophyllin (He and Zhou, 2010). Allyl benzyl and phenyl have been used for the reduction of fumonisins in food products. Calcium hydroxide and hydrogen peroxide are effective for reducing AFB_2 and AFG_2. The use of chemical control methods, however, have several challenges. Fungicides pose a danger to human and animal health and the environment, as certain chemical compounds are not easily degradable and could contaminate soil and water (Larkin and Fravel, 1998). Resistance in fungal strains could develop resulting in a demand for higher concentrations of chemicals, which in turn could lead to an increase in toxic residues in food crops. The use of chemical methods to reduce mycotoxins is expensive, can affect the sensory quality of food, and produce toxic derivatives (Alberts et al., 2017). As a result, there is an increasing interest in alternative control methods.

Several technological methodologies have been implemented to manage pre- and postharvest fungal growth and mycotoxin production, i.e. methods involving clay minerals, plant extracts, antioxidants, biocontrol microorganisms and enzymes (He and Zhou, 2010). Good agricultural management, both pre-harvest and postharvest, and the implementation of hazard analysis and critical control point (HACCP) practices help decrease fungal growth and mycotoxin contamination, but do not prevent them (Alberts et al., 2016). Optimization of agricultural management activities may not always be feasible due to the high costs, the geographical location and the design of the production systems. In Africa, there are limited resources and a scarcity of sophisticated technologies. Population groups that are the worst affected include smallholder farming communities where mycotoxin levels are not monitored, as mono-cereal crops are cultivated and consumed locally. Community-based, culturally acceptable, economically feasible and practical methods, which are sustainable, should also be explored for reducing mycotoxin contamination in staple crops (Misihairabgwi et al., 2018).

2.11.1 Technological control methods

2.11.1.1 Biocontrol microorganisms

The relationship between the host plant, the mycotoxigenic pathogen and the antagonistic biocontrol microorganism is the basis of this strategy. It is based on mechanisms such as competition for nutrients and space, parasitism of the pathogen and antagonism (Alberts et al., 2017). The introduction of atoxigenic *A. flavus* and *A. parasiticus* strains has reduced aflatoxin contamination in peanuts by 74.3-99.9% in the United States (Damann, 2014). *Trichoderma* spp. exhibited efficient biological control against pathogenic fungi through mechanisms such as competition for space, nutrients, and production of secondary metabolites that inhibit or are fungicidal (Calistru et al., 1997; Yates et al., 1999). It also assists in stimulating plant defence mechanisms.

Bacillus subtilis occupies the same area as *F. verticillioides* within the maize plant. It inhibits the growth of the *F. verticillioides* by competitive exclusion (Bacon et al., 2001). Being easy to cultivate, manipulate genetically and generally regarded as safe by the United States Food and Drug Administration, *B. subtilis* has effectively reduced fumonisin contamination in maize. Lactic acid bacteria, such as *Pediococcus pentosaceus,* have also been proved to exhibit anti-fungal activity against *F. verticillioides* and *F. proliferatum in vitro* (Alberts et al., 2017).

2.11.1.2 Plant extracts

Certain phenolic plant compounds are strong antioxidants, which inhibit the activity of key fungal enzymes. The anti-microbial properties of these compounds can be used as an antifungal agent in the pre-harvest, postharvest and storage regulation of fungal growth and related development of mycotoxins in agricultural crops (Alberts et al., 2017). Butylated hydroxyanisole (BHA) and propylparaben (PP) are antioxidants that are produced by organisms such as the algae *Botryococcus braunii* and *Cylindrospermopsis raciborskii* (BHA) and plants such as carrots and olives (PP) (Etcheverry et al., 2002; Reynoso et al., 2002). These antioxidants have demonstrated great potential for control of *F. verticillioides* and *F. proliferatum* growth and fumonisin production at different incubation temperatures and water activities *in vitro*. Other phenolic plant compounds such chlorophorin, iroko, maakianin, vanillic acid, and caffeic acid have been shown to inhibit the growth of *F. verticillioides* and the production of FB_1 (Beekrum et al., 2003).

2.11.1.3 Resistance breeding and genetic modification

Extensive genomic resources are essential for investigations into the biochemical and regulatory pathways of mycotoxin biosynthesis, pathogenesis of fungal-plant interactions and the development of targeted and innovative approaches for breeding and engineering crops for resistance (Brown et al., 2006; Cleveland et al., 2003; Desjardins and Proctor, 2007). Whole

genome sequences and expression sequence tags (ESTs) are important tools for understanding disease caused by fungi, fungal lifecycles and secondary metabolism. Available genomic resources include genetic maps, genome sequences, an EST library and an integrated gene index. Next-generation RNA sequencing was used to study transcriptional changes associated with *F. verticillioides* inoculation in resistant and susceptible maize genotypes by including an extensive range of maize inbred lines (Lanubile et al., 2014). The technique generated useful data on genetic markers involved in recognition, signalling and controlling host resistance mechanisms. It also provided quantification of expression, thus enabling interpretation of defence responses. The data provides an important genomic resource for the development of disease resistant maize genotypes.

Comprehensive knowledge on the biochemical and molecular mechanisms involved in natural resistance of crops is imperative for the further development of resistance to *Fusarium* infection and insect infestation in crops (Cleveland et al., 2003). With this approach, regions on chromosomes associated with natural resistance to insect invasion, fungal contamination or mycotoxin production are identified, resistant traits mapped, and resistant lines crossed with commercially acceptable lines.

Expression profiles for maize genes during infection with *F. verticillioides* indicated up-regulation of genes encoding a range of proteins related to cell rescue, defence and virulence in both resistant and susceptible maize lines, including pathogenesis related (PR) proteins [e.g. chitinase (reducing chitin in fungal membrane); permatin (fungal hyphae leak and rupture)]; proteins involved in detoxification response (e.g. cytochrome P450 monoxygenase, peroxidases and glutathione-S-transferases); heat-shock proteins (regulating folding of resistance proteins); and proteinase inhibitors (Lanubile et al., 2010). Resistance in maize lines could be due to constitutive defence mechanisms that resist fungal infection. In resistant maize lines defence-related genes, encoding constitutively expressed PR, detoxification enzymes and β-glucosidases, were transcribed at high levels before infection, and provided defence against the fungus. In susceptible maize lines, defence genes are induced as a response to pathogen infection, though not sufficiently enough to prevent progress of the disease.

Mapping of chromosomal regions encoding *Fusarium* ear mould resistance as quantitative trait loci (QTL) and the employment of marker-assisted QTL in selection for *Fusarium* ear mould resistance are valuable tools being developed for maize hybrid development (Duvick, 2001). Ear mould resistance can be mapped as QTL using large segregating plant populations. Molecular markers linked to these QTL could be valuable during inbred development.

2.11.1.4 Adsorbents

The negative effects of mycotoxins in foods can be reduced by the incorporation of clay adsorbents during food processing. Fumonisins and aflatoxins can be tightly and selectively bound by the phyllosilicate clay montmorillonite, rendering them bio inactive and nontoxic in the gastrointestinal tract of experimental animals (Alberts et al., 2017).

2.11.2 Community-based methods

2.11.2.1 Diet diversification and practical methods

Diet diversification, replacing mycotoxin prone foods with other dietary options, could be beneficial (Misihairabgwi et al., 2018). It could, however, be problematic to convert to a diverse diet in most parts of southern Africa due to harsh environmental conditions, prolonged droughts and economic constraints (Alberts et al., 2019). Hand sorting, shelling, winnowing, dehulling and milling are common postharvest activities practiced, reducing mycotoxin levels in staple food (Van der Westhuizen et al., 2011; Misihairabgwi et al., 2019). Hand sorting of cereal grains removes visibly infected and damaged kernels and resulted in a notable reduction of fumonisin levels (Shephard et al., 2010; Vismer et al., 2019). Hand-sorting followed by a 10 min water wash method resulted in 84% reduction of FB_1, 62% reduction in the probable daily intake (PDI) and 52% reduction in urinary excretion of FB_1. Effective reduction of AFB_1 (88%) in maize was achieved by using a sequence of dehulling, soaking for 72 h and sun drying for 4.5 h (Misihairabgwi et al., 2019).

2.11.2.2 Creating awareness

A lack of knowledge on the dangers of mycotoxins in raw and processed sorghum and pearl millet raises the risk of mycotoxin exposure (Matumba et al., 2015; Misihairabgwi et al., 2019). A survey performed in Zambia indicated that only 7% of the interviewed participants had knowledge of the existence of mycotoxins (Misihairabgwi et al., 2019). Smallholder farmers should be trained in order to raise their awareness. Radio and television programmes, newspapers, magazines and social media platforms could be used as means to disseminate information on mycotoxin exposure and prevention methods. Primary and secondary school curricula should be reviewed to include information on mycotoxins to help create awareness at an early age. Public seminars and workshops will assist in creating public awareness. The World Health Organisation (WHO) made several recommendations for mycotoxin mitigation and management. These included an adaptive strategy, educational programs, improved laboratory and surveillance capacities and the development of early warning systems (Chilaka et al., 2017). Other methods, such as introducing realistic and inexpensive household-level mycotoxin reduction strategies to efficiently minimize toxicity, are becoming increasingly important (Alberts et al., 2017; Shephard et al., 2019).

2.12 Conclusions

During this study the occurrence of mycotoxigenic fungi (agriculturally important *Fusarium* and *Aspergillus spp*.) was determined on sorghum and pearl millet in northern Namibia using traditional morphological as well as validated molecular techniques involving species-specific primers and qPCR. The concentrations of multiple mycotoxins (AFB_1, FB_1, FB_2, FB_3, OTA, DON, ZEA, NIV and MON) in samples were determined by validated extraction and LC-MS/MS analytical methods. Determinations were performed in raw whole grains as well as processed grains sold at open markets.

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CHAPTER 3

THE INCIDENCE OF *FUSARIUM* AND *ASPERGILLUS* SPP. IN *SORGHUM BICOLOR* AND *PENNISETUM GLAUCUM* SAMPLES FROM NOTHERN NAMIBIA

3.1 Introduction

Aspergillus, Fusarium and *Penicillium* spp. are known to produce mycotoxins in a wide variety of food items (Alberts et al., 2019). Mycotoxins are secondary fungal metabolites toxic to humans and animals (Leslie et al., 2005). *Fusarium* spp. can contribute to significant production losses in both *Zea mays* (maize) and sorghum (*Sorghum bicolo*r) and other cereal crops by causing stalk rots, ear rots, and grain moulds. In the United States (US) alone, mycotoxins cause an annual economic losses of about US$ 1.4 billion and are estimated to contaminate one fifth of the food crops in the rest of the world (Alberts et al., 2019). In 1985, maize contamination with aflatoxin was estimated at US$ 75–100 million, and recently between US$ 52.1 million and US$ 1.68 billion annually in the US (Alberts et al., 2019).

Mycotoxins have a range of negative biological effects in humans and animals and are involved in many human illnesses (Alberts et al., 2017). In the Centane and Butterworth districts of the former Transkei region of the Eastern Cape Province, a positive correlation was observed between the consumption of maize and the incidence of oesophageal cancer (Rheeder et al., 1991). Maize is the staple food in these regions and *F. verticillioides* (previously known as *F. moniliforme*), *F. subglutinans* and *F. graminearum* amongst the main fungal contaminants (Rheeder et al., 1991; Shephard et al., 2019). A high incidence of *Fusarium* contamination was also linked to high rates of oesophageal cancer in China (Rheeder et al., 1991). Fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) producing strains *of F. verticilliodes* have been isolated, purified and characterized from maize that originated from one of these high oesophageal cancer regions.

The most important sorghum stalk rot and grain mould diseases are caused by *Fusarium* spp., including *F. andiyazi, F. proliferatum, F. napiforme, F. thapsinum, F. nygamai, F. pseudonygamai,* and *F. verticillioides* (Leslie and Summerell, 2006). A high prevalence of *F. thapsinum*, *F. proliferatum*, and *F. verticillioides* and less of *F. sacchari* and *F. beomiforme* was observed in sorghum sampled in Thailand, indicating the possible presence of the fumonisin mycotoxins (Mohamed et al., 2019). The prevalence of infection of sorghum grains by *Fusarium* spp. in Argentina is high (82.5-99%), with *F. verticillioides*, *F. thapsinum* and *F. andiyazi* the most evident, and species within the *Fusarium graminearum* spp. complex (FGSC) in high frequency (Sampietro et al., 2010).

Data on the contamination of sorghum and millet in Africa indicates contamination with *Fusarium* and *Aspergillus* spp. and the co-occurrence of multiple mycotoxins, especially aflatoxins and fumonisins. The co-occurrence of fumonisins and aflatoxins has been reported in sorghum and pearl millet from smallholder farmers under the direction of the International Institute for Tropical Agriculture, Nigeria (Vismer et al., 2019). Occurrence of mycotoxins in samples implies the presence of fungal contamination. A surveillance study evaluating the levels of multiple mycotoxins in sorghum from Burkina Faso, Ethiopia, Mali and Sudan resulted in 33% of 1533 samples contaminated with multiple mycotoxins, including aflatoxins (AFs), fumonisins, sterigmatocystin, *Alternaria* spp. toxins, ochratoxin A and zearalenone (ZEA) (Ssepuuya et al., 2018). Sudan reported the lowest incidence and average concentration of all examined mycotoxins with the highest concentrations of fumonisins and AFs detected in pink sorghum. Studies of *Fusarium* spp. isolates from sorghum and finger millet in Ethiopia revealed higher contamination in sorghum. A large number of isolates exhibited a close relationship with the *F. incarnatum-equiseti* spp. complex (Chala et al., 2014). All sorghum samples collected from smallholder farmers' threshing floors and underground storage pits in East Hararghe, Ethiopia were contaminated with both *Fusarium* and *Aspergillus* spp. AF contamination was ≤33.10 µg/kg with the highest levels in stored samples, while the fumonisin levels were between 907-2041 µg/kg (Taye et al., 2016).

There is little data available on sorghum and millet contamination in southern Africa. *Fusarium nygamai* and *F. verticillioides* are the main fungi contaminating sorghum and millet in Lesotho and Zimbabwe (Klaasen and Nelson, 1997). Forty-six traditional sorghum malt, unfermented beer and fermented beer samples were obtained from three villages in Gaborone, Botswana, and *F. verticillioide*s and *A. flavus* contamination detected in 63% and 37% of samples, respectively. AFs were not detected, whilst FB_1 was detected in three malt samples (47-1316 µg/kg), and ZEA in 56%, 48% and 48% of malt (102-2213 µg/kg), unfermented beer (26-285 µg/l) and fermented beer (20-201 µg/l) samples, respectively (Nkwe et al., 2005). In South Africa, the following *Fusarium* spp. were found to be associated with sorghum: *Fusarium acuminatum, F. andiyazi, F. avenaceum, F. chlamydosporum, F. merismoides, F. napiforme, F. nygamai, F. oxysporum, F. pseudonygamai, F. semitectum, F. solani spp. complex, F. subglutinans, F. verticillioides*, as well as species from the FGSC, including *F. acaciae-mearnsii, F. cortaderiae, F. meridionale* and *F. thapsinum*, while *F. napiforme and F. nygamai* were associated with millet (Beukes et al., 2017). These species are associated with the production of several mycotoxins. The diversity of fungal metabolites in street vended pearl millet and sorghum ingredients and their transfer rate during fermentation into *Oshikundu*, a traditional Namibian drink, was determined (Misihairabgwi et al., 2018). Unacceptable high levels of mycotoxins were detected in the raw grains used for the brewing of *Oshikundu.* AFB_1

was detected in 13% of 40 pearl millet meal samples [mean concentration 0.9±0.7 µg/kg (range 0.1-2.0 µg/kg)] and in 50% of 40 sorghum malt samples [mean concentration 4.5±5.5 µg/kg (range 0.2-25.4 µg/kg)]. However, there remain a huge knowledge gap concerning contamination of Namibian sorghum and pearl millet with mycotoxigenic fungi and mycotoxins.

Methods for the identification of *Fusarium* and *Aspergillus* spp. and the detection of unknown isolates are a vital part of surveillance studies monitoring the magnitude of contamination of grains with mycotoxigenic fungi during the production, storage and processing stages (Sampietro et al., 2010; Shephard et al., 2019). Methods for fungal characterization have historically depended on the morphological features of fungal species and the host's diagnostic symptoms, as well as the presence of fungi in the affected tissues (Gherbawy and Voigt, 2010). The traditional methods of characterization include physical macroscopic description of colonies on appropriate culture media, measuring and observing parameters such as colony growth, texture and pigment (Marasas et al., 2001; Dhoro, 2010). The macroscopic characteristics vary depending on incubation time, temperature and culture media used. Conventionally, toxigenic fungal species are identified using morphology and cross-fertility techniques (Leslie et al., 2001; Sampietro et al., 2010). Microconidial chains as well as the presence of polyphialides assist in distinguishing between *Fusarium verticillioides* and *F. proliferatum* (Visentin et al., 2009).

Leslie and Summerell (2006) developed an identification key for *Fusarium* spp. based on important morphological characteristics. Their laboratory manual presents an overview of *Fusarium* biology and the techniques involved in isolating, identifying and characterizing individual organisms and the populations in which they occur. Genetic, morphological and molecular methods were integrated into this volume dedicated to the identification of *Fusarium* spp. The manual contains descriptions of new and old species, the toxins produced by these fungi and the diseases which they cause. It assists in bridging the gap between phylogenetic and morphological taxonomy (Leslie and Summerell, 2006).

A combination of morphological growth patterns and mating type tests distinguishes *Fusarium subglutinans* from other *Fusarium* spp. (Faria et al., 2012; Marasas et al., 2001). Clinical detection includes fertility tests with mating type lineages. These methods, in terms of the materials used, are relatively simple and cost-effective, but can be laborious, and it may take weeks or months to obtain accurate results. These approaches are often highly dependent on the skills of the analyst (Faria et al., 2012). Fungi can be classified according to their ability to synthesize secondary metabolites such as antibiotics, mycotoxins, polyketides and alkaloids. Though all secondary metabolites are chemically diverse, they are produced via a few common biosynthetic pathways, often linked to morphological development (Keller et al., 2005). A study

by Adetitun et al. (2015) suggested that fungi can be classified based on their sensitivity to antifungal agents. The study showed that *Aspergillus niger*, A. *flavus, A. fumigatus, Mucor racemosus, Rhizopus stolonifer* and *F. oxysporium* were all susceptible to the antifungal compounds griseofulvin and ketoconazole. Biochemical characteristics such as isozyme profiles have also been used to distinguish between two groups of *F*. *graminearum*. There was a high degree of similarity in isozymes patterns among the isolates of the same group, regardless of geographical origin, which suggested that isolates within a given group are descendants of the same ancestral population. Large variation in isozyme patterns between specific groups of isolates suggested that they were of distinctly different ancestry (Láday et al., 2000).

Due to similarities between closely related species (e.g. *A. parasiticus* and *A. nomius*), morphological identification is not uncomplicated (Rodrigues et al., 2007; Marasas et al., 2001). *Aspergillus* spp. strains are effectively identified and differentiated using selective culture media, which rely on unique biochemical characteristics (Rodrigues et al., 2007). *Aspergillus* selective agar (AFPA) is used to determine the presence of toxigenic *A. flavus* in foods. *Aspergillus flavus* produces an intense yellow orange colour at the colony base. The coloration is due to the reaction of ferric ions from ferric citrate with aspergillic acid that forms a yellow-coloured complex (Sigma, 2004). Coconut cream agar (CCA) is also useful in identifying aflatoxin producing strains. When cultivated on this medium, aflatoxigenic fungal strains fluoresce typically blue under UV-light (Rodríguez-Lázaro et al., 2013). When cultivated on Czapek dox agar, *A. flavus* and *A. parasiticus* strains display a yellow/green colour and distinctly dark green colonies, respectively (Rodríguez-Lázaro et al., 2013).

The aim of this study was to determine the occurrence of the most important mycotoxigenic fungal species in raw sorghum and pearl millet (*Pennisetum glaucum*) samples collected from smallholder farmers and processed samples obtained from open markets in the Oshana region of northern Namibia. Morphological and biochemical characteristics were used to determine the incidence of *Fusarium* and *Aspergillus* spp.

3.2 Materials and Methods

3.2.1 Collection of sorghum and pearl millet samples

Sorghum and pearl millet samples (± 2 kg) were collected postharvest during July 2018 from ten randomly selected smallholder farmer households in Oshakati (Table 3.1) and from 13 and 10 randomly selected vending stalls from the Oshakati and Ondangwa open markets in the Oshana region of northern Namibia (Table 3.2). Twenty unprocessed whole grain samples (ten sorghums and ten pearl millets) were collected from the households and 23 processed samples from the open markets, namely ten malted sorghum, 11 malted pearl millet and two pearl

millet bran from the open markets. The Oshakati smallholder communal farmers service both the Oshakati and Ondangwa open markets. Figure 3.1 depicts geographical maps of (A) the different Provinces of Namibia, (B) the Oshana region indicating the locations of Oshakati and Ondangwa, and (C) the sampling sites (N1-N10) near Oshakati, as determined with GPS (Table 3.1). Standardised sampling protocols adapted from "The *Fusarium* Laboratory Manual" (Leslie and Summerell, 2006) were followed. Labelling of the samples was done according to procedure described by Safrinet (1999). The first three letters in the sample name (NAM) denoted the locality. The numeric value in the sample name represented the number of the sampling site. The last letter denotes the substratum ("M" for pearl millet and "S" for sorghum). The samples were packed in sterilized absorbent sample bags, which were individually placed in polythene bags and stacked into large plastic storage containers. Samples were sent by courier to the Cape Peninsula University of Technology (CPUT), Bellville campus. Upon reception, the samples were stored at 4˚C until analysed. Analyses commenced a week after the arrival of samples.

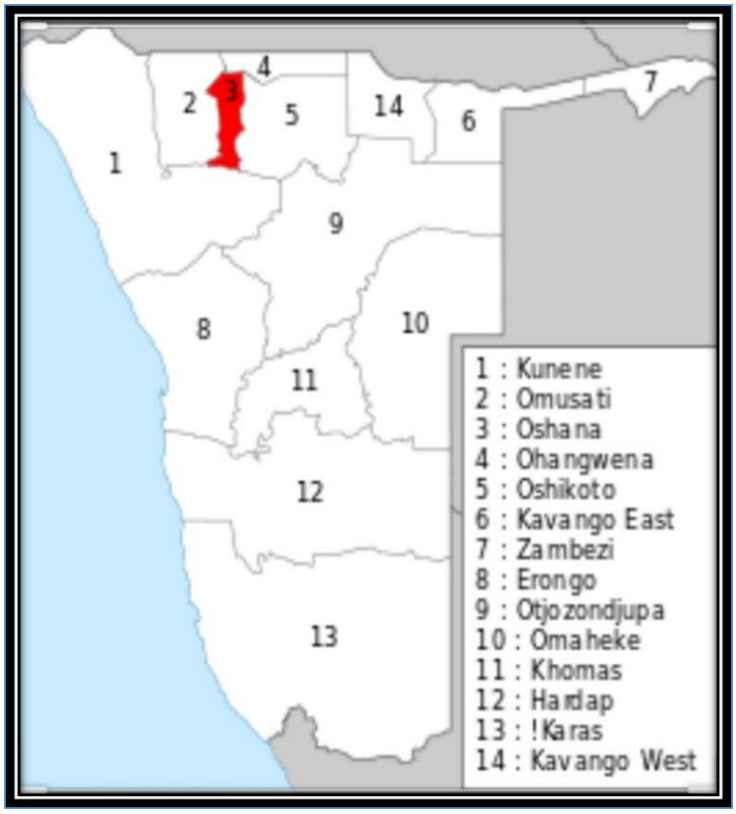
Samples were collected as part of an international collaboration between CPUT and the University of Namibia (UNAM, Windhoek, Namibia). The field study team consisted of Dr JP Rheeder (CPUT), Dr J Misihairabgwi (UNAM) and agricultural extension officers from the Department of Agriculture, Namibia. Ethical clearance for the field study was obtained (Registration Number NHREC: REC- 230408-014) from the Health and Wellness Research Ethics Committee (HW-REC) of CPUT.

3.2.2 Isolation and enumeration of mycotoxigenic fungal species from raw whole grain samples

3.2.2.1 Isolation of *Fusarium* spp.

A subsample of whole grain sorghum and pearl millet kernels (100 g) was surface-sterilised by soaking for 1 min in 3.5% sodium hypochlorite (NaOCl) and rinsed twice with sterile distilled water (Leslie and Summerell, 2006). One hundred kernels from each sample were plated out; five kernels per Petri dish, on modified Malt extract agar (MEA) laced with 150 mg/L novobiocin and incubated at 25˚C for 10-14 days (Figure 3.2). *Fusarium* spp. colonies were identified by their morphological structures (Figure 3.3), sub-cultured onto fresh MEA and single spores prepared according to the methods described by Leslie and Summerell (2006).

A


1 : Kunene
2 : Omusati
3 : Oshana
4 : Ohangwena
5 : Oshikoto
6 : Kavango East
7 : Zambezi
8 : Erongo
9 : Otjozondjupa
10 : Omaheke
11 : Khomas
12 : Hardap
13 : !Karas
14 : Kavango West

B


C46
C41
B1
Oshakati
Okahao
Ondangwa
Onayena
Constituency
Amarika
Etosha
National Pa
Okaukuejo
Ombika

C

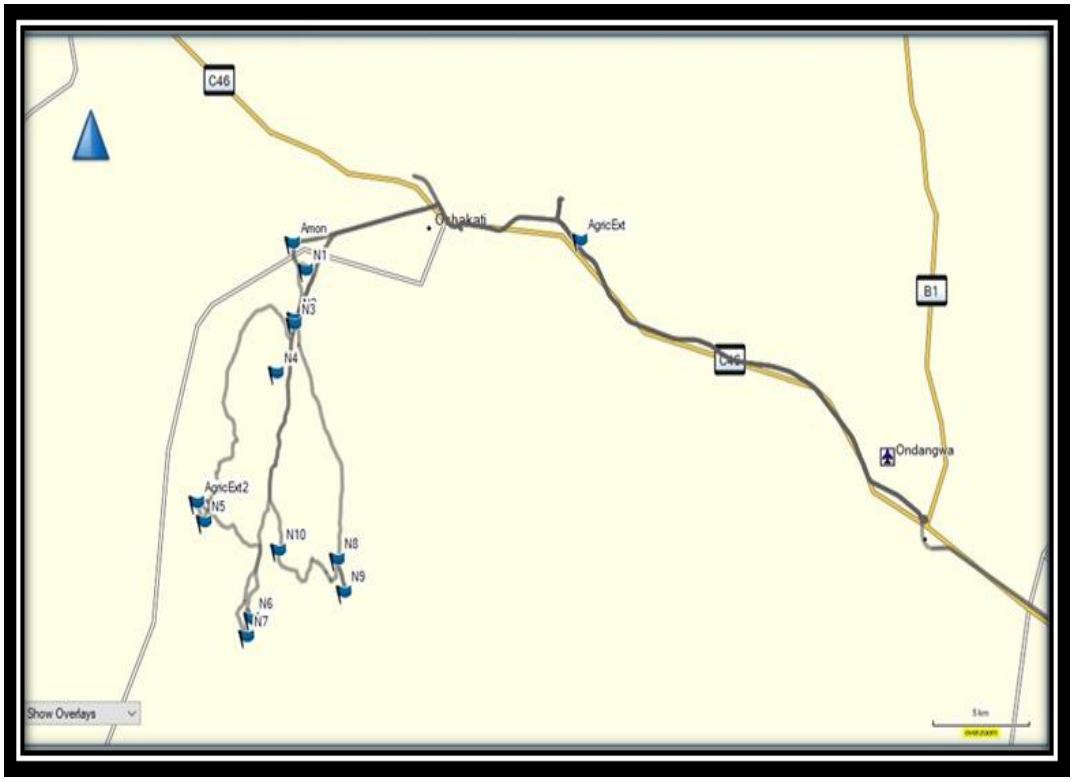


Figure 3.1 Geographical maps of Namibia indicating: A, the Oshana region in northern Namibia (red); B, the locations of Oshakati and Ondangwa, and C, the sampling sites (N1-N10) near Oshakati (Wikimedia, 2016; Google Maps, 2020; Photo credit: Dr JP Rheeder)

Table 3.1. Sampling sites and description of the raw whole grain samples collected in Oshakati

Sampling site / Agricultural extension office	GPS co-ordinates	Sample no.	Sample description	Sample type
N1	S 17°48.2623' / E 015°37.2028'	NAM-1S	Sorghum	Raw grain
		NAM-1M	Pearl millet	Raw grain
N2	S 17°49.4188' / E 015°36.8340'	NAM-2S	Sorghum	Raw grain
		NAM-2M	Pearl millet	Raw grain
N3	S 17°49.5494' / E 015°36.8073'	NAM-3S	Sorghum	Raw grain
		NAM-3M	Pearl millet	Raw grain
N4	Not recorded	NAM-4S	Sorghum	Raw grain
		NAM-4M	Pearl millet	Raw grain
N5	S 17°54.2552' / E 015°33.7664'	NAM-5S	Sorghum	Raw grain
		NAM-5M	Pearl millet	Raw grain
N6	S 17°56.5680' / E 015°35.3790'	NAM-6S	Sorghum	Raw grain
		NAM-6M	Pearl millet	Raw grain
N7	S 17°56.9902' / E 015°35.2063'	NAM-7S	Sorghum	Raw grain
		NAM-7M	Pearl millet	Raw grain

Sampling site / Agricultural extension office	GPS co-ordinates	Sample no.	Sample description	Sample type
N8	S 17°55.1538' / E 015°38.2789'	NAM-8S	Sorghum	Raw grain
		NAM-8M	Pearl millet	Raw grain
N9	S 17°55.9310' / E 015°38.5087'	NAM-9S	Sorghum	Raw grain
		NAM-9M	Pearl millet	Raw grain
N10	S 17°54.9360' / E 015°36.2855'	NAM-10S	Sorghum	Raw grain
		NAM-10M	Pearl millet	Raw grain
Agricultural extension office 1	S 17°47.5476' / E 015°46.5086'	-	-	-
Agricultural extension office 2	S 17°53.7969' / E 015°33.5177'	-	-	-

Table 3.2 Processed samples collected at open markets in Oshakati and Ondangwa

Sample no.	Sample description	Sample type	Sampling location
NAM-11	Sorghum	Malt	OSH M
NAM-12	Sorghum	Malt	OSH M
NAM-13	Sorghum	Malt	OSH M
NAM-14	Sorghum	Malt	OSH M
NAM-15	Sorghum	Malt	OSH M
NAM-16	Sorghum	Malt	OSH M
NAM-17	Pearl millet	Bran	OSH M
NAM-18	Pearl millet	Malt	OSH M
NAM-19	Pearl millet	Malt	OSH M
NAM-20	Pearl millet	Malt	OSH M
NAM-21	Pearl millet	Malt	OSH M
NAM-22	Pearl millet	Malt	OSH M
NAM-23	Pearl millet	Malt	OSH M
NAM-24	Sorghum	Malt	ONDW M
NAM-25	Sorghum	Malt	ONDW M
NAM-26	Sorghum	Malt	ONDW M
NAM-27	Sorghum	Malt	ONDW M
NAM-28	Pearl millet	Bran	ONDW M
NAM-29	Pearl millet	Malt	ONDW M
NAM-30	Pearl millet	Malt	ONDW M

Sample no.	Sample description	Sample type	Sampling location
NAM-31	Pearl millet	Malt	ONDW M
NAM-32	Pearl millet	Malt	ONDW M
NAM-33	Pearl millet	Malt	ONDW M

OSH M, Oshakati market; ONDW M, Ondangwa market

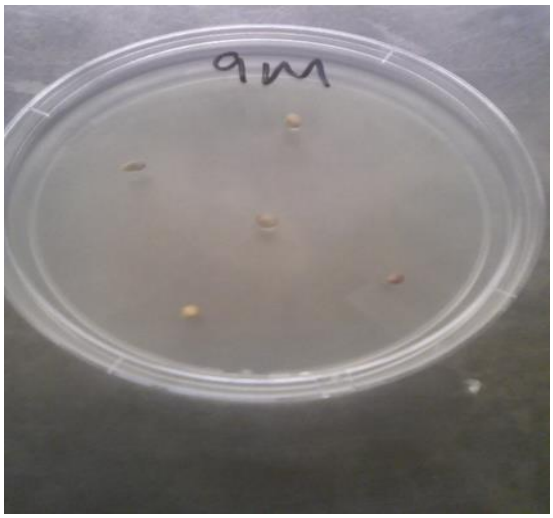


Figure 3.2 Malt extract agar (MEA) supplemented with novobiocin (150 mg/L) and inoculated with raw whole grain kernels

Figure 3.3 Raw whole grain kernels after 5 days of incubation at 25˚C on Malt extract agar (MEA) supplemented with novobiocin (150 mg/L)

3.2.2.2 Preparation of pure cultures by preparing *Fusarium* spp. single spores

After 14 days of incubation, a sterile inoculation loop was used to scrap off the edges of the suspected *Fusarium* sp. colony and the cells transferred to a test tube containing 10 ml sterilized distilled water (Leslie and Summerell, 2006). One millilitre of the suspension was transferred to a 1.5% water agar plate. The plate was dried at room temperature and incubated at 25°C overnight. A dissection microscope was used to examine germinated spores (Figure 3.4) and a scalpel blade used to transfer it onto a fresh Potato dextrose agar (PDA) plate (Figure 3.5), where after it was incubated at 25°C and 30°C. A compound microscope and a dissecting microscope were both used in the morphological identification and single spore isolation. All isolated fungi were recorded against their sample of origin, their frequency of isolation and pigment colour at 25°C. The information assisted in determining the exact region with the highest frequency of a certain fungal species.

Figure 3.4 A germinated *Fusarium* spp. single spore on a 1.5% water agar plate

Figure 3.5 Transfer of a germinated *Fusarium* sp. single spore onto fresh Potato dextrose agar (PDA) with the aid of a scalpel blade

3.2.2.3 Isolation of *Aspergillus* spp.

A subsample of kernels was surface sterilized as described above. The kernels were plated onto AFPA medium (Sigma, 2004) and incubated at 30˚C for 5 days. The presence of the unique yellow-orange pigment visible at the bottom of the agar plates was used to determine the presence of *A. flavus* and/or *A. parasiticus* spp. in samples. Single-spore colonies of *Aspergillus* spp. were obtained as described above for *Fusarium* spp.

3.2.3 Isolation and enumeration of mycotoxigenic fungal species from processed samples

Processed samples (malted sorghum and pearl millet, and bran) (1 g) were tested for contamination with *Fusarium* and *Aspergillus* spp. by using the dilution plate method (10^{-1} to 10^{-10}), as described by Leslie and Summerell (2006) and Sigma (2004). For detection of *Fusarium* spp., dilutions were plated out on Van Wyk's *Fusarium* selective medium (Andrews and Pitt, 1986) and for *A. flavus* and *A. parasiticus* on AFPA medium (Sigma, 2004). The number of colony forming units (cfu/g sample) for both the *Fusarium* and *Aspergillus* spp. were recorded.

3.3 Statistical analysis

The NCSS software (2019) was used for statistical analysis. Data were subjected to natural log (ln) transformation of all variables and analysed within a generalised linear model ANOVA. Multiple comparisons were analysed using the Tukey-Kramer's multiple comparison procedure. Generally, $P < 0.05$ was used as statistical significance.

3.4 Results

3.4.1 Isolation and enumeration of mycotoxigenic fungal species from raw whole grain samples

3.4.1.1 Isolation and incidence of *Fusarium* and *Aspergillus* spp.

Table 3.3 summarises the incidence of *Fusarium* spp., *A. flavus* and *A. parasiticus* on sorghum and pearl millet raw whole grain samples. 132 *Fusarium* spp. and 27 *Aspergillus* spp. single spore isolates were obtained. In both sorghum and pearl millet, a contamination frequency of 80% was observed for *Fusarium* isolates, while only 15% of the samples yielded *Aspergillus spp*. The formula for calculating the percentage (%) contamination frequency was adopted from Sreenivasa et al. (2010):

Contamination frequency (%) = number of samples infected by fungi/total number of samples analysed x 100.

3.4.2 Isolation and enumeration of mycotoxigenic fungal species from processed samples

Following the dilution plate method, 74 and 100% of the processed samples tested positive for the presence of *Fusarium* and *Aspergillus* spp. contamination, respectively (Table 3.4). Contamination was quantified as cfu/g and ranged from 2×10^1 - 7×10^7.

3.5 Discussion

Sorghum and pearl millet are important staple crops in northern Namibia. These grains are uniquely drought resistant, i.e. they have a lower water requirement during their growth period as compared to maize, are more tolerant to high temperatures due to their deeper root system, and can be stored for prolonged periods (Mukarumbwa and Mushunje, 2010). They are the main staple grains cultivated in the Oshana region of northern Namibia, which is dominated by smallholder farming. Sorghum and pearl millet are used daily for the preparation of stiff porridge (Orr et al., 2016). The grains are also malted and used for the preparation of traditional fermented beverages such as *Oshikundu* (Misihairabgwi et al., 2018). Raw and processed products are home consumed and sold at local markets. Despite strict international regulations on the occurrence of mycotoxins in staple grains, mycotoxins are not regulated in Namibia (Alberts et al., 2019). Microbial surveillance and human exposure studies are currently performed worldwide to determine and reduce the level of exposure of populations to mycotoxigenic fungi that produce carcinogenic mycotoxins in staple grains. Knowledge on the occurrence of mycotoxigenic fungi and mycotoxins in Namibian grains is limited. The current study evaluated the contamination of raw and processed sorghum and pearl millet grains for the main mycotoxigenic fungal species, i.e. *Fusarium* and *Aspergillus* spp.

A total of 132 *Fusarium* spp. isolates and 27 *Aspergillus* spp. isolates were obtained from culturing whole grain sorghum and pearl millet samples. All these samples were collected from households in Oshakati. These results could be attributed to a variety of factors, which include differences in kernel strength, postharvest handling or fungal resistance on a molecular level. In general, raw whole grain samples NAM-1, NAM-2, NAM-3 and NAM-8 exhibited the highest level of fungal contamination. The contamination frequency of *Fusarium* spp. in sorghum was 80%, with NAM-1S (42%), NAM-2S (27%) and NAM-3S (39%) exhibiting the highest percentage kernel infection. No *Aspergillus* spp. were detected in the sorghum samples This is in line with findings of Rensburg et al. (2011). The contamination frequency of *Fusarium* spp. in pearl millet was 80%, with NAM-1M (9%), NAM-2M (4%) and NAM-3M (5%) exhibiting the highest percentage kernel infection. The contamination frequency of *Aspergillus* spp. in pearl millet samples was 33% with NAM-2M (10%), NAM-3M (10%) and NAM-8M (10%) exhibiting the highest percentage kernel infection. *Fusarium* and *Aspergillus* spp. co-occurred in 30% of raw pearl millet samples collected at households in Oshakati.

Contamination with *Fusarium* and *Aspergillus spp*. was detected in 74% and 100%, respectively, of processed samples obtained from open markets in Oshakati and Ondangwa. NAM-12S ($6x10^5$ cfu/g), NAM-19M ($17x10^5$ cfu/g), NAM-24S ($3x10^5$ cfu/g) and NAM-27S (range 6-$17x10^5$ cfu/g) contained the highest levels of *Fusarium* sp. contamination, with the highest incidence in pearl millet. NAM-12S and NAM-19M were obtained from the Oshakati market, whereas NAM-24S and NAM-27S was obtained from the Ondangwa market. The infection levels of *Aspergillus* spp. in processed products were higher as compared to *Fusarium* spp. ($P<0.05$). The highest contamination levels with Aspergillus spp. were observed with NAM-11S ($7x10^7$ cfu/g), NAM-18M ($1.1x10^7$ cfu/g), NAM-19M ($1.2x10^7$ cfu/g) and NAM-25S ($2x10^6$ cfu/g). NAM-11S, NAM-18M and NAM-19M were obtained from the Oshakati market, whereas NAM-25S was obtained from the Ondangwa market. *Fusarium* and *Aspergillus* spp. co-occurred in 74% of processed samples sold at open markets.

The co-occurrence of *Fusarium* and *Aspergillus* spp. in processed sorghum and pearl millet is a serious concern, as these fungal species produce the carcinogenic fumonisin and aflatoxin mycotoxins. The fumonisins are classified Group 2B carcinogens by the International Agency for Research on Cancer (IARC) and is associated with neural tube defects, stunting in children and oesophageal cancer (Alberts et al., 2019). Aflatoxins are classified Group 1 carcinogens by IARC and poses a serious threat to human and animal health by causing hepatotoxicity, teratogenicity, immunotoxicity as well as liver cancer (Wild et al., 2015).

The high levels of fungal contamination in raw and processed samples could be attributed to pre- and postharvest agricultural and processing practices (Alberts et al., 2017). Most smallholder farmers keep a portion of their yield to use as seed for the next farming season. The continued inbreeding with no introduction of new varieties reduces hybrid vigour and plant resistance to pathogenic fungi. The resultant grain could be of poor quality and more susceptible to mycotoxigenic fungal infection. After the growing season, crops are left to dry on the field. The drying process reduces water activity and induces stress on the crops, making them susceptible to fungal infection. The kernel pericarp or hull acts as a protective layer against pathogens. Most of the processing either involves removal of the hull or destruction by threshing and grinding. This exposes the powdery endosperm to fungal infection. Most of the storage units in northern Namibia are made of mud, dung, straw and bark which harbour microorganisms including mycotoxigenic fungi. The moisture and temperature conditions during malting also provide an ideal environment for fungi to proliferate, and could lead to an exponential increase in mycotoxin concentrations (Tangni and Larondelle, 2014). Contamination may be further enhanced by microflora originating from home-based malting plants (Tangni and Larondelle, 2014). By critically monitoring the grain production process from

planting, through to harvesting, processing and marketing, the sources of contamination and critical control points could be identified and managed. Mycotoxin awareness campaigns and education on the subject will greatly contribute to reducing mycotoxin contamination during processing of sorghum and pearl millet. This could involve peer-to-peer training to improve awareness and knowledge, the introduction of community-based mycotoxin reduction methods such as washing of grains and the dissemination of community-specific goods agricultural and storage practices (Alberts et al., 2017).

Most members of the *Fusarium* genus are morphologically similar or are cryptic species (Mavhunga, 2013). This makes it increasingly challenging and inaccurate to rely only on morphological features for identification (Leslie and Summerell, 2006; Mavhunga, 2013). The traditional methods for identification and characterization of mycotoxigenic fungi are currently complemented with molecular based approaches such as polymerase chain reaction (PCR) (McClenny, 2005). PCR-based genotyping based on sequence variability and the presence of certain genes such as elongation factor 1-alpha (*EF1α*), translation elongation factor 1-alpha (*TEF1-α*), intergenetic spacer region (*IGS*), and mycotoxin biosynthetic genes such as *FUM1*, *TRI13* and *TRI17* has become useful and more reliable fungal identification methods (Dhoro, 2012; Sampietro et al., 2010). In comparison to fungal culturing approaches, PCR does not require the existence of viable fungal species for implementation and can be carried out with very small amounts of biological material (Dhoro, 2010). A number of studies have indicated molecular characterization based on the ITS region as the potential barcode for most fungal species due to the high degree of interspecific variability, preserved primer sites and multiple copy nature in the genome (Fajarningsih, 2016). However, tedious methodology, optimization, complex interpretation based on specialized trained researchers and molecular identification of only targeted fungal species limit these techniques. Molecular methods such as PCR and next-generation sequencing can be used to identify the isolates further to species level.

Table 3.3 The incidence of *Fusarium* spp. and total *Aspergillus flavus and A. parasiticus*, expressed as percentage (%) kernel infection, in sorghum and pearl millet raw whole grain samples

Sample	Description	Sample type	Location	*Fusarium* spp. (%)	*A. flavus* and *A. parasiticus* (%)
NAM-1S	Sorghum	Grain	OSH household	42	0
NAM-1M	Pearl millet	Grain	OSH household	9	0
NAM-2S	Sorghum	Grain	OSH household	27	0
NAM-2M	Pearl millet	Grain	OSH household	4	10
NAM-3S	Sorghum	Grain	OSH household	39	0
NAM-3M	Pearl millet	Grain	OSH household	5	10
NAM-4S	Sorghum	Grain	OSH household	2	0
NAM-4M	Pearl millet	Grain	OSH household	2	0
NAM-5S	Sorghum	Grain	OSH household	1	0
NAM 5M	Pearl millet	Grain	OSH household	1	0
NAM-6S	Sorghum	Grain	OSH household	0	0

Sample	Description	Sample type	Location	*Fusarium* spp. (%)	*A. flavus* and *A. parasiticus* (%)
NAM-6M	Pearl millet	Grain	OSH household	0	0
NAM-7S	Sorghum	Grain	OSH household	0	0
NAM-7M	Pearl millet	Grain	OSH household	2	0
NAM-8S	Sorghum	Grain	OSH household	2	0
NAM-8M	Pearl millet	Grain	OSH household	1	10
NAM-9S	Sorghum	Grain	OSH household	14	0
NAM-9M	Pearl millet	Grain	OSH household	0	0
NAM-10S	Sorghum	Grain	OSH household	5	0
NAM-10M	Pearl millet	Grain	OSH household	2	0

OSH, Oshakati

Table 3.4 Contamination with *Fusarium* spp. and total *Aspergillus flavus* and *A. parasiticus,* expressed *as* colony-forming units (cfu) per gram (g) in malt and bran samples of sorghum and pearl millet

Sample no.	Description	Sample type	Location	*Fusarium* spp. (cfu/g)	*A. flavus* and *A. parasiticus* (cfu/g)
NAM-11	Sorghum	Malt	OSH market	0	7×10^{7}
NAM-12	Sorghum	Malt	OSH market	6×10^{5}	9×10^{6}
NAM-13	Sorghum	Malt	OSH market	2×10^{4}	6×10^{4}
NAM-14	Sorghum	Malt	OSH market	7×10^{3}	2×10^{4}
NAM-15	Sorghum	Malt	OSH market	4×10^{2}	1.1×10^{2}
NAM-16	Sorghum	Malt	OSH market	0	1×10^{6}
NAM-17	Pearl millet	Bran	OSH market	5×10^{3}	7×10^{3}
NAM-18	Pearl millet	Malt	OSH market	2.3×10^{3}	1.1×10^{7}
NAM-19	Pearl millet	Malt	OSH market	1.7×10^{6}	1.2×10^{7}
NAM-20	Pearl millet	Malt	OSH market	0	8×10^{6}
NAM-21	Pearl millet	Malt	OSH market	0	7×10^{6}
NAM-22	Pearl millet	Malt	OSH market	1.2×10^{4}	5×10^{6}
NAM-23	Pearl millet	Malt	OSH market	7×10^{2}	3×10^{5}
NAM-24	Sorghum	Malt	ONDW market	3×10^{5}	3×10^{3}
NAM-25	Sorghum	Malt	ONDW market	1×10^{4}	2×10^{7}
NAM-26	Sorghum	Malt	ONDW market	2×10^{4}	2×10^{3}
NAM-27	Sorghum	Malt	ONDW market	6×10^{5}	9×10^{1}
NAM-28	Pearl millet	Bran	ONDW market	2×10^{4}	2×10^{3}
NAM-29	Pearl millet	Malt	ONDW market	5×10^{4}	$1x\ 10^{2}$
NAM-30	Pearl millet	Meal	ONDW market	2×10^{1}	4×10^{1}
NAM-31	Pearl millet	Meal	ONDW market	0	2×10^{1}
NAM-32	Pearl millet	Malt	ONDW market	0	4×10^{5}

Sample no.	Description	Sample type	Location	*Fusarium* spp. (cfu/g)	*A. flavus* and *A. parasiticus* (cfu/g)
NAM-33	Pearl millet	Malt	ONDW market	1×10^1	5×10^2

OSH, Oshakati; ONDW, Ondangwa

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CHAPTER 4

MOLECULAR CHARACTERIZATION OF FUNGAL SPECIES IN *SORGHUM BICOLOR* AND *PENNISETUM GLAUCUM* SAMPLES FROM NORTHERN NAMIBIA

4.1 Introduction

In the past, identification of fungal species in grains mainly involved simple and cost effective methods based on morphology, phenotypic characteristics, fertility, growth rate and mating tests (Faria et al., 2012). However, pathogen detection using traditional methods can often be misleading, erroneous and time-consuming and also depends on the ability of the fungus to be cultured and on the expertise of the researcher (Marinach-Patrice et al., 2009; Faria et al., 2012). The characterization of macroscopic and microscopic features of filamentous fungi are somewhat subjective, frequently resulting in inconclusive or erroneous descriptions (Marinach-Patrice et al., 2009). Owing to hybridisation, cryptic speciation, and convergent evolution, morphological characters can often be deceptive (Raja, et al., 2017). Furthermore, dual nomenclature, which resulted from the different morphotypes of the sexual and asexual stages in some fungal life cycles, was a common practice in mycology until recently, and created some confusion among scientists. According to the new International Code of Nomenclature for algae, fungi, and plants, this practice is no longer appropriate (Raja et al., 2017). Those complexities led to the development of more effective ways of fungal characterization (Marinach-Patrice et al., 2009).

A number of research groups have adopted the use of the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis for rapid and sensitive identification of fungal species (Marinach-Patrice et al., 2009). This method analyses the protein content of an unknown isolate as a species-specific spectrum, usually between 2 and 20 kDa, which is then defined by comparison with reference spectra in a database (Becker et al., 2014). Based on the similarity between spectra, the microorganism is described as belonging to the same genus, species or sub-species (Cassagne et al., 2016). In the detection of pathogenic species in food and the environment, the use of the MALDI-TOF technique has proven useful. Recently, this technique was applied for the identification of grain spoilage fungi and 44 of 48 fungal strains was accurately identified (Drissner et al., 2017; Marinach-Patrice et al., 2009). The major advantage of this technique is that sample preparation and analysis is achievable in an hour as compared to at least a week for morphological methods and at least two days

for molecular methods. However, the disadvantage of this method is the lack of standardized method protocols and limited reference spectra databases. A clear understanding of the concepts that underlie this technology is also essential (Triest et al., 2015; Marinach-Patrice et al., 2009; Marzano et al., 2020). Due to the limitations, this method should complement and not replace other methods such as molecular based characterization methods (Drissner et al., 2017).

Molecular techniques utilising polymerase chain reaction (PCR) are extremely valuable as they allow for fast and specific detection of fungal species (Visentin et al., 2009). Conventional PCR imitates *in vitro* the natural DNA replication ability on a large scale (Valones et al., 2009). This method requires the identification of the DNA target, which is used to develop primers that will hybridize specifically to the target sequence (Valones et al., 2009). The use of conventional PCR has its own limitations as the requirement of agarose or polyacrylamide gel for electrophoresis makes the process laborious and more prone to errors and contamination i.e. the introduction of unwanted foreign DNA from sources such as skin and dander (Biji et al., 2012). It is also challenging to differentiate band sizes of slightly different molecular sizes, therefore lacking quantification capacity. Experimentally, extra care should be exercised, as the use of reagents such as ethidium bromide is known to be carcinogenic (Valones et al., 2009).

Quantitative Real-time PCR (qPCR) improves on the limitations of conventional PCR. It relies on dyes or fluorescent probes such as SYBR Green 1 that allow for the continuous monitoring of the amplified product. SYBR Green 1 binds non-specifically to the duplexes of DNA generated during amplification (Valones et al., 2009). Application of a TaqMan probe, which is labelled with two fluorescent moieties, is another way to generate fluorescence of a targeted region of the internal sequence that needs to be amplified (Valones et al., 2009). TaqMan probes degrade during amplification and release a reporter that emits light. A light signal detector analyses the emission of light and creates a graph from the absorption readings obtained after each PCR cycle (Valones et al., 2009). The signal generated is a reflection of the product formed (Valones et al., 2009). Real time-PCR requires the use of a thermos cycler equipped with an optical fluorescence capture system as well as a computer with software (available from several manufacturers) capable of capturing data and conducting a final reaction analysis (Valones et al., 2009). Data obtained from qPCR is used to establish the baseline of the reaction, generate standard curves, calculate threshold values (Ct), percentage (%) efficiency, slopes, correlation coefficients (R^2) and for absolute quantification (ThermoFisher, 2015).

Microbial diagnosis and management of food spoilage organisms are two main areas of Food Microbiology (Klancnik et al., 2012). Microbiological quality management systems are constantly being used in food chain processes to minimize the risk of contamination for consumers (Rodríguez-Lázaro et al., 2013). For the identification of pathogens and other microorganisms in food, traditional, time-consuming and labour-intensive culture methods are no longer adequate for the level of quality control and diagnostic laboratories' need to produce rapid results (Klancnik et al., 2012). As a result, the availability of accurate, fast and validated test systems to diagnose the presence, absence, and the degree of contamination of pathogens are becoming increasingly important in the food industry as well as in agriculture (Rodríguez-Lázaro et al., 2013). The introduction of qPCR has improved on the detection limit, selectivity, specificity, sensitivity and speed of analyses with a turnaround time of less than 24 hours (Rodríguez-Lázaro et al., 2013). In food samples, qPCR is able to detect DNA or RNA of contaminants such as spoilage microorganisms and pathogens, DNA of toxigenic fungi and bacteria, as well as DNA associated with trace components (e.g. allergens such as nut proteins) or undesirable components for food authenticity (e.g. cow's milk in goat's milk cheese) (Klancnik et al., 2012).

Quantitative Real-time PCR has been successfully applied for the identification of mycotoxigenic fungal species and associated mycotoxin production. Several *Fusarium* spp. genes are involved in the biosynthesis of the trichothecene mycotoxins, most of which are found in the *Tri* gene cluster (Astrid Bauer et al., 2015). Designing the *Tri-5* gene-specific primer pairs made it possible to distinguish between *Fusarium* spp. that produce trichothecenes and non-producing species using PCR-based assays (Tox5 PCR) (Sreenivasa et al., 2008). Research was conducted on 172 samples of randomly harvested winter wheat from farmers' fields in various areas of Bavaria, South Germany. The goal was to use qPCR methods to detect the presence of *Tri-5* gene producing fungi and to determine the correlation between the presence of the *Tri-5* gene and deoxynivalenol (DON) concentration. It also aimed to evaluate the extent of *F. graminearum* and *F. culmorum* infection. The *Tri-5* gene was present in 86% of all contaminated samples and was associated with a detectable amount of DON. *F. graminearum* is the most prevalent species associated with FHB and considered the predominant trichothecene producer (Astrid Bauer et al., 2015).

A qPCR assay was developed for the identification and quantification of *F. graminearum* based on primers targeting the *PKS13* gene which is involved in zearalenone (ZEA) biosynthesis (Atoui et al., 2011). The investigation was carried out on 32 maize samples. A strong correlation (R^2=0.760) was observed between *F. graminearum* DNA concentration and ZEA content in the maize samples. In another study, qPCR assays based on the elongation

factor 1α (*EF1α*) gene and mycotoxin analyses were conducted on 11 *Fusarium* spp. isolated from 24 wheat and 24 maize samples collected from Danish wheat and maize fields, respectively. The qPCR assays were highly sensitive and specific, and the results corresponded well with the mycotoxin data (Nicolaisen et al., 2009). *Fusarium* spp. isolates (64) from 44 sorghum samples collected in Karnataka, India after the 2004–2005 growing seasons were analysed using molecular methods (Sreenivasa et al., 2008). Multiplex PCR characterization was performed on *Fusarium* spp. using two separate sets of primers and targeting two distinct regions, i.e. the internal transcribed spacer (*ITS*) region and the *FUM1,* a fumonisin biosynthesis gene*,* cluster. All *Fusarium* spp. tested positive with the *ITS* primer set. From these isolates, 53 out of 64 were positive for the *FUM1* primer set and further analysis revealed that the 53 isolates produced fumonisins. These findings demonstrated that the molecular based technique could be used to classify a group of *Fusarium* spp. based on its ability to produce fumonisins (Sreenivasa et al., 2008). A qPCR method, utilizing species-specific primers adopted from Nicolaisen et al (2009), was successfully used to quantify the occurrence of *F. subglutinans*, *F. verticillioides, F. graminearum* and *F. proliferatum* in maize samples collected at 14 localities in South Africa during the 2008 and 2009 growing seasons (Boutigny et al., 2012).

Limited data is available on the occurrence of mycotoxigenic fungi in Namibian staple grains. Smallholder farmers in northern Namibia produce raw sorghum and pearl millet as well as processed products, which are used for home consumption and are sold at open markets in the area (Misihairabgwi et al., 2019). There is an urgent need for microbial surveillance studies among these farming communities to provide information on the degree of contamination by mycotoxigenic fungi of raw sorghum and millet as well as contamination transferred during processing. The aim of this study was to detect and quantify mycotoxigenic *Fusarium* and *Aspergillus* spp. in raw and processed *Sorghum bicolor* (sorghum) and *Pennisetum glaucum* (pearl millet) from northern Namibia. Raw whole grain (unprocessed) and processed sorghum and pearl millet samples were collected from Oshakati and from open markets in Oshakati and Ondangwa, in the Oshana region of northern Namibia. Four fungal species were identified and quantified in samples by employing qPCR and species-specific primers, i.e. *F. verticillioides, F. proliferatum, F. subglutinans* and *F. graminearum.*

4.2 Materials and Methods

4.2.1 Sorghum and pearl millet samples

Raw whole grain and processed sorghum and pearl millet samples were collected postharvest during July 2018 from the households of ten smallholder farmers in Oshakati and from two open markets in Oshakati and Ondangwa located in the Oshana region of northern Namibia.

Details of the sampling sites and sample descriptions are included in Chapter 3 (Section 3.2.2, Figure 3.1, and Tables 3.1 and 3.2).

4.2.2 Extraction of DNA from sorghum and pearl millet

Raw and processed sorghum and pearl millet samples (20 g) were ground to a powder using a C and N Laboratory mill, size 8' (Christy and Norris Ltd. Engineers, Chelmsford, England). Prior to DNA extraction, 5 g of the sample powder was further homogenized into a fine powder in liquid nitrogen using a mortar and pestle. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Cat. no. 69104) according to the procedure supplied by the manufacturer. The procedure was modified by the addition of freshly prepared 10 ml cetyltrimethylammonium bromide (CTAB)/polyvinyl-pyrrolidone (PVP) lysis buffer, 40 µl Proteinase K (10 mg/ml) and incubation for 2 hours at 65˚C in a shaker at 200 rpm. A Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific, Inqaba Biotechnical Industries) was used to determine the DNA concentration of each sample. The quality of the genomic DNA (gDNA) was visualized on a 0.8% agarose gel laced with ethidium bromide (5 µl) and run in TAE 1X buffer (Tris-acetate and EDTA) electrophoresis at 70 V for 45 minutes. A molecular weight marker, λ Hind III (ThermoFisher Scientific Cat. no. SM0101) (3 µl), containing a loading dye (2 µl), was included. Genomic DNA samples were diluted to a final concentration of 30 ng/µl and stored at -20 °C until analysed with qPCR.

4.2.3 Extraction of DNA from pure fungal cultures

Four *Fusarium* spp. reference cultures were used in this study, i.e. *F. subglutinans* MRC 8553, *F. verticillioides* MRC 826, *F. graminearum* MRC 6010 and *F. proliferatum* MRC 8550 [Cape Peninsula University of Technology (CPUT) AMHBI culture collection]. *Aspergillus* reference strains were obtained from the American Type Culture Collection (ATCC, Virginia, USA) [*A. parasiticus* [CBS100926 AP1, CBS103.57 AP2 and CBS571.65 (AP3)] and *A. flavus* [CBS100927 AF1, CBS100.45 AF2 and CBS114062 AF3] and from the CPUT AMHBI culture collection [*A. parasiticus* (0200), *A. flavus* (0645 and 3954)].

Fungi were cultured in 100 ml Potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks at 26 °C on a rotary shaker for 14 days. The mycelium was harvested by filtration using a sterilized muslin cloth, and ground to a powder in liquid nitrogen using a mortar and pestle. 50 mg of each sample was weighed into 2 ml micro centrifuge tubes and used for extraction of gDNA. A DNeasy Plant Mini Kit (Qiagen Cat. no. 69104) was used to extract the gDNA according to a procedure adapted from Boutigny et al. (2011). A Nanodrop 2000 Spectrophotometer (Inqaba Biotechnical Industries) was used to determine the DNA concentration of each sample. The absorbance ratios A260/A280 and A260/A230 were used to determine the purity

of the gDNA. The quality of the gDNA was visualized on a 1.5% agarose gel containing ethidium bromide (5 µl) in TAE 1X buffer (Tris-acetate and EDTA) electrophoresis at 70 V for 45 minutes using a λ Hind III molecular weight marker (Thermo Fisher Scientific Cat. no. SM0101). The pure fungal DNA was diluted to a final concentration of 2.5 ng/µl and stored at -20 °C until analysed with qPCR.

4.2.4 Conventional PCR

Primer specificity for the detection of *Fusarium* spp. was confirmed with conventional PCR by the laboratory during previous projects (data not shown). Conventional PCR was used to determine the primer specificity for detecting *A. flavus* and *A. parasiticus* (Sardiñas et al., 2010). Primer sets FLAVIQ1 (5′ GTCGTCCCCTCTCCGG 3′) and FLAQ2 (5′CTGGAAAAAGATTGATTTGCG 3′) were tested against type strains of *A. flavus* whilst primer sets FLAVIQ1 (5′ GTCGTCCCCTCTCCGG 3′) and PARQ2 (5′ GAAAAAATGGTTGTTTTGCG 3′) were tested against *A. parasiticus* type strains. Two different reactions were performed using different polymerase enzymes and reaction mixtures (Table 4.1). The PCR reaction for *A. parasiticus* was carried out using the following conditions: initial denaturation step for 5 min at 95 °C, followed by 25 cycles of 30 s at 95 °C (denaturation), 30 s at 69.3 °C (annealing) and 30 s at 72 °C (extension) and a final elongation step of 5 min at 72 °C. The amplification reaction for *A. flavus* was carried out using the following conditions: initial denaturation of 4 min and 30 s at 95 °C, 30 cycles of 30 s at 95 °C (denaturation), 20 s at 60 °C (annealing), 35 s at 72 °C (extension) and final elongation step for 3 min at 72 °C. Both procedures were adopted from Sardinas et al. (2010) and the reactions performed using a T100 Thermal Cycler PCR (Bio-Rad, California, USA). PCR products were detected in 2% agarose ethidium bromide gel in 1X TAE buffer (Tris-acetate and EDTA) for an electrophoresis at 70 V for 45 min. 150 ng of template DNA was used for the DreamTaq master mix reaction and was calculated as follows:

150 ng/concentration of DNA (ng/µl) = Volume (µl) of DNA template added to the reaction mixture.

Table 4.1 Reaction mixtures for conventional PCR to determine the primer specificity for detection of *Aspergillus flavus* and *Aspergillus parasiticus* (Sardiñas et al., 2010)

2X Phusion master mix reaction mixture		**DreamTaq master mix reaction mixture**	
Reagent	**Volume (µl)**	**Reagent**	**Volume (µl)**
2X Phusion master mix ($MgCl_2$, dNTP, buffer, Taq DNA polymerase)	10	**10X DreamTaq buffer**	5.0
Forward primer (10 µM)	1.0	**Forward primer (10 µM)**	10.0
Reverse primer (10 µM)	1.0	**Reverse primer (10 µM)**	10.0
Template DNA (30 ng/µl)	2.0	**DNA template** ($\frac{150\ ng}{[DNA] ng\mu l^{-1}}$)	It varied
DMSO	0.6	**dNTP mix**	5.0
Nuclease free H_2O	5.4	**Nuclease free H_2O**	Fill up to 50
		DreamTaq polymerase	0.25
Total volume	**20.0**	**Total volume**	**50.0**

4.2.5 Quantitative Real-time PCR (qPCR)

4.2.5.1 Optimization and preparation of standard curves

Quantitative Real-time PCR was performed with a Bio-Rad CFX96 Real-time PCR detection system (Bio-Rad, California, USA). Optimization reactions were carried out separately on four reference strains of *Fusarium* spp. (*F. verticillioides* MRC 826*, F. subglutinans* MRC 8553*, F. graminearum* MRC 6010 and *F. proliferatum* MRC 8550) and two *Aspergillus* spp. reference strains (*A. parasiticus* AP1 and *A. flavus* AF3) using non-contaminated (control) sorghum and pearl millet matrixes. Control sorghum and pearl millet were obtained from the Mycotoxin analysis laboratory, of the Southern Africa Grain Laboratory (SAGL, Pretoria) and from store bought birdseed and sorghum meal. The *Fusarium* species-specific primers used in this experiment were adapted from Nicolaisen et al. (2009) (Table 4.2). The primer design was based on the alignments of the *EF1α* gene. The *Aspergillus* species-specific primers were designed based on the sequence alignments of the ITS2 region of several strains from different origins retrieved from nucleotide databases, as described by Sardiñas et al. (2010) (Table 4.2).

Six-fold serial dilutions of DNA (2.5-2.44×10^3 ng/µl) from all six *Fusarium* and *Aspergillus* spp. (Table 4.3) were prepared against control sorghum and pearl millet DNA matrixes (30 ng/µl), respectively, and used to prepare standard curves. Different annealing temperatures were used in trial and error experiments to establish the optimum conditions for qPCR (Nicolaisen

et al., 2009; Sardiñas et al., 2010) and to determine acceptable ranges of the assay parameters (Bustin, 2009).

4.2.5.2 Quantification of *Fusarium* and *Aspergillus* spp. in sorghum and pearl millet samples using qPCR

Quantitative Real-time PCR was carried out using SYBR green polymerase, *Aspergillus* spp. and *Fusarium* spp. primers (Table 4.2) in a total volume of 25 µl, standard curve samples and master mix prepared as described in Table 4.3. All reactions were carried out in triplicate in 96-well plates. Each 96-well plate included standard curve, two negative control and two non-template control samples. The initial concentration of target species DNA was expressed as ng/µl of the total DNA collected per sample. The different species were amplified using the respective experimental protocols summarized in Table 4.4.

4.3 Results

4.3.1 The quality and concentration of gDNA extracted from sorghum, pearl millet and fungal cultures

The concentrations of gDNA were determined with a Nanodrop 2000 Spectrophotometer and the quality of gDNA determined visually following gel electrophoresis, on a Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad, California, USA) (Figures 4.1 and 4.2). Overall, the quality and concentrations of the respective gDNA samples were high. Certain lanes, however, contained degraded DNA, observed as smears. The degraded samples were repeated and analysed until acceptable gDNA was obtained. The highest concentration of DNA was obtained from NAM-21 (335 ng/µl; pearl millet malt), and the lowest concentration from birdseed (30.4 ng/µl; control pearl millet).

4.3.2 Conventional PCR to determine the primer specificity for detection and quantification of *A. flavus* and *A. parasiticus*

Conventional PCR was performed to verify primer specificity in order to distinguish between the various *A. flavus* and *A. parasiticus* strains by using sorghum and pearl millet matrixes, respectively. Figures 4.3 and 4.4 present the amplification obtained from using the DreamTaq polymerase (Thermo Scientific Cat. no. EP0701) and 2X Phusion polymerase (Phusion High-Fidelity PCR master mix) (Thermo Scientific MAN0012771) PCR reactions, respectively. Table 4.4 and 4.5 summarize the results obtained in the reactions. In both enzyme reactions, no amplification was observed, except for the reaction with AF2 and AF3 using the 2X Phusion polymerase.

Table 4.2 The primer sets used for qPCR detection and quantification of *Fusarium* and *Aspergillus* spp.

Target species	Primer Name	Primer sequence (5′–3′)	Source
F. graminearum	FgramB379 fwd FgramB411 rev	CCATTCCCTGGGCGCT CCTATTGACAGGTGGTTAGTGACTGG	Nicolaisen et al., 2009
F. proliferatum	Fpro220 fwd Fpro270 rev	CTTCGATCGCGCGTCCT CACGTTTCGAATCGCAAGTG	Nicolaisen et al., 2009
F. subglutinans	Fsub565 fwd Fsub622A rev	GTCATTGGTATGTTGTCGCTCAT GTGATATGTTAGTACGAATAAAGGGAGAAC	Nicolaisen et al., 2009
F. verticillioides	Fver356 fwd Fver412 rev	CGTTTCTGCCCTCTCCCA TGCTTGACACGTGACGATGA	Nicolaisen et al., 2009
A. flavus	FLAVIQ1 fwd FLAQ2 rev	GTCGTCCCCTCTCCGG CTGGAAAAAGATTGATTTGCG	Sardinas et al., 2010
A. parasiticus	FLAVIQ 1 fwd PAR Q2 rev	GTCGTCCCCTCTCCGG GAAAAAATGG TTGTTTTGCG	Sardinas et al., 2010

Table 4.3 Preparation of the PCR master mix to obtain a six-fold dilution of target DNA

Reagent	Volume in 25 µl	Total volume in master mix
Molecular grade H_2O	9.25 µl	222 µl
SYBR Green 1	12.5 µl	300 µl
Forward Primer (10 mM)	0.625 µl	16.25 µl
Reverse Primer (10 mM)	0.625 µl	16.25 µl

Table 4.4 Experimental protocols for *Fusarium* and *Aspergillus* spp. qPCR

Target species	qPCR cycle						
	1	2	3	4	5	6	7
Fusarium graminearum	95°C; 10 min	95°C; 15 s	72°C; 15 s	95°C; 15 s	72°C; 0.05 s	-	95°C; 0.5 s
F. verticillioides	95°C; 10 min	95°C; 15 s	66°C; 15 s	72°C; 15 s	95°C; 10 s	72°C; 0.05 s	95°C; 0.5 s
F. proliferatum	95°C; 10 min	95°C; 15 s	66°C; 15 s	72°C; 15 s	95°C; 10 s	72°C; 0.05 s	95°C; 0.5 s
F. subglutinans	95°C; 10 min	95°C; 15 s	64°C; 15 s	72°C; 15 s	95°C; 10 s	72°C; 0.05 s	95°C; 0.5 s
Aspergillus flavus	95°C; 10 min	95°C; 15 s	60°C; 60 s	-	95°C; 10 s	65°C; 0.05 s	95°C; 0.5 s
A. parasiticus	95°C; 10 min	95°C; 15 s	60°C; 60 s	-	95°C; 10 s	65°C; 0.05 s	95°C; 0.5 s

Protocols were adapted and modified from the procedure described by Boutigny et al. (2011). For each reaction, 40 cycles were included. qPCR cycle steps 1-7, temperatures and incubation time frames to achieve initial denaturation, denaturation, annealing, extension and final elongation of DNA fragments

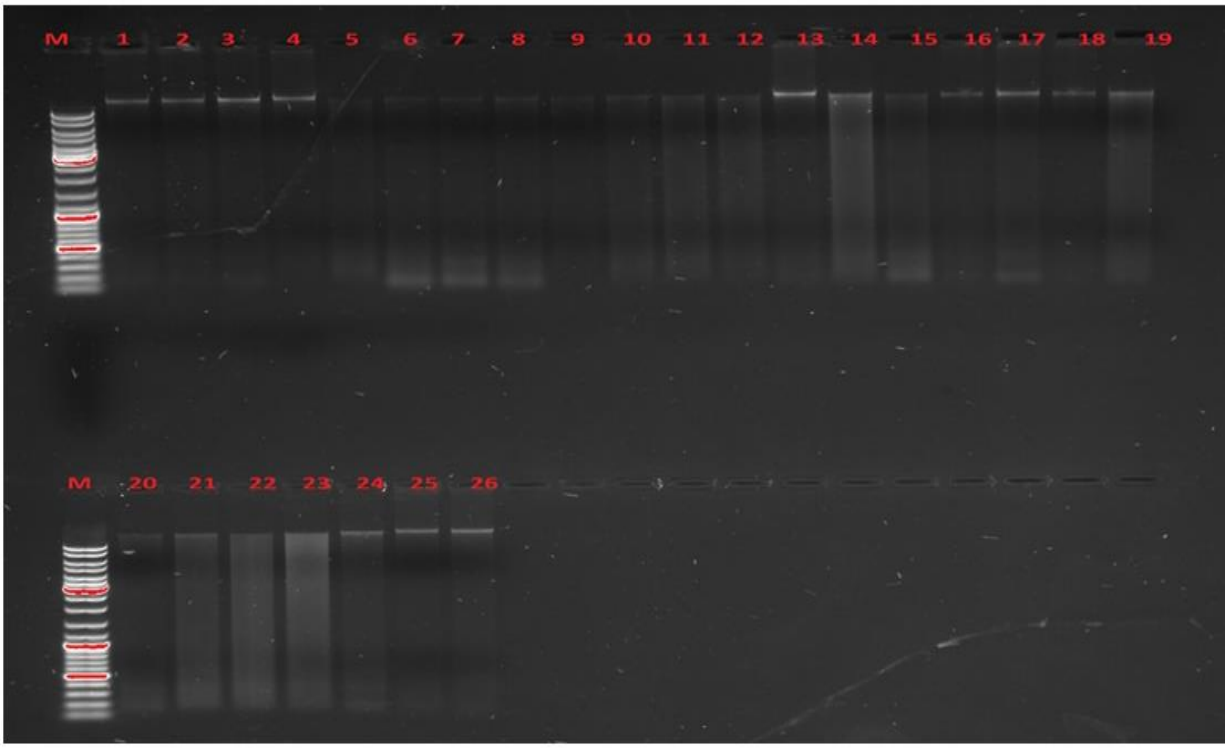

Figure 4.1 Agarose gel electrophoresis (0.8%) of gDNA extracted from processed and unprocessed sorghum and pearl millet samples. Lane M, molecular weight marker (λ Hind lll); Lane 1, NAM-10 S; Lane 2, NAM-9 M; Lane 3, NAM-9 S; Lane 4, NAM-8 M; Lane 5, NAM-8S; Lane 6, NAM-7 M; Lane 7, NAM-7 S; Lane 8, NAM-6 M; Lane 9, NAM-6S; Lane 10, NAM-5M; Lane 11, NAM-5S; Lane 12, NAM-4M; Lane 13, NAM-4S; Lane 14, NAM-3M; Lane 15, NAM-3S; Lane 16, NAM-2M; Lane 17, NAM-2S; Lane 18, NAM-1M; Lane 19, NAM-1S; Lane 20, NAM-16; Lane 21, NAM-15; Lane 22, NAM-14; Lane 23, NAM-13; Lane 24, NAM-12, Lane 25, NAM-11; Lane 26, NAM-10M

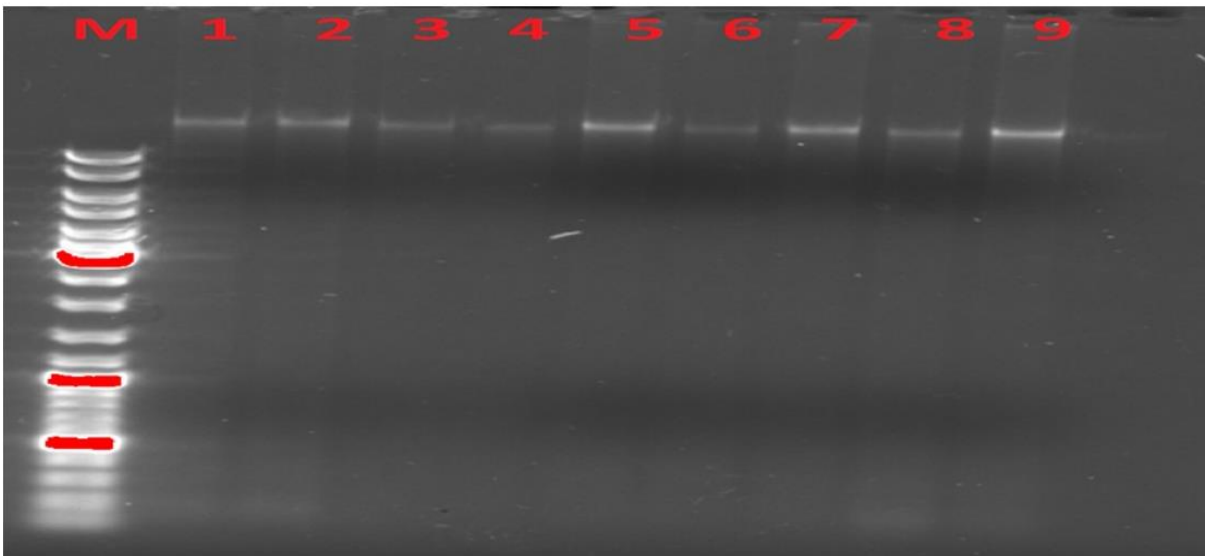

Figure 4.2 Agarose gel electrophoresis (1.5%) of the gDNA extracted from reference *Aspergillus* spp. strains. Lane M, molecular weight marker (λ Hind III); Lane 1*, A. flavus* 3954; Lane 2, *A. flavus* 0645; Lane 3*, A. parasiticus* 0200; Lane 4*, A. flavus* AF3; Lane 5*, A. flavus* AF2; Lane 6, *A. flavus* AF1; Lane 7, *A. parasiticus* AP3; Lane 8, *A. parasiticus* AP2; Lane 9, *A. parasiticus* AP1

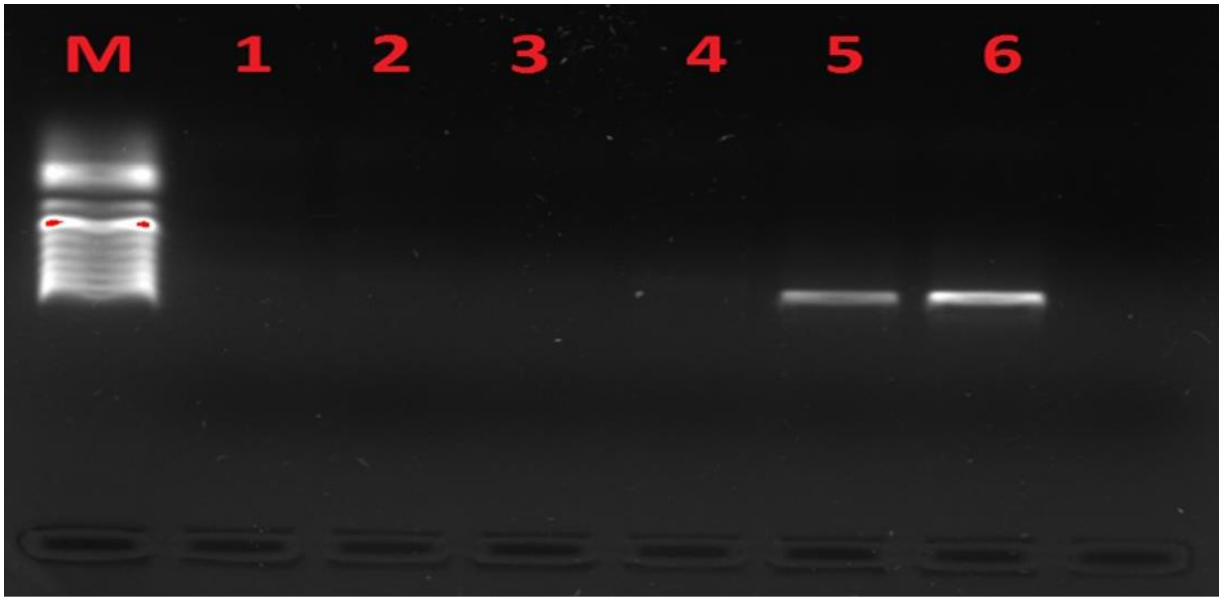

Figure 4.3 PCR amplification of species-specific DNA fragments of reference *Aspergillus* spp. strains using 2X Phusion polymerase. Lane M, molecular weight marker (λ Hind III); Lane 1, *A. parasiticus* AP1; Lane 2, *A. parasiticus* AP2; Lane 3, *A. parasiticus* AP3; Lane 4*, A. flavus* AF1; Lane 5*, A. flavus* AF2; Lane 6, *A. flavus* AF3

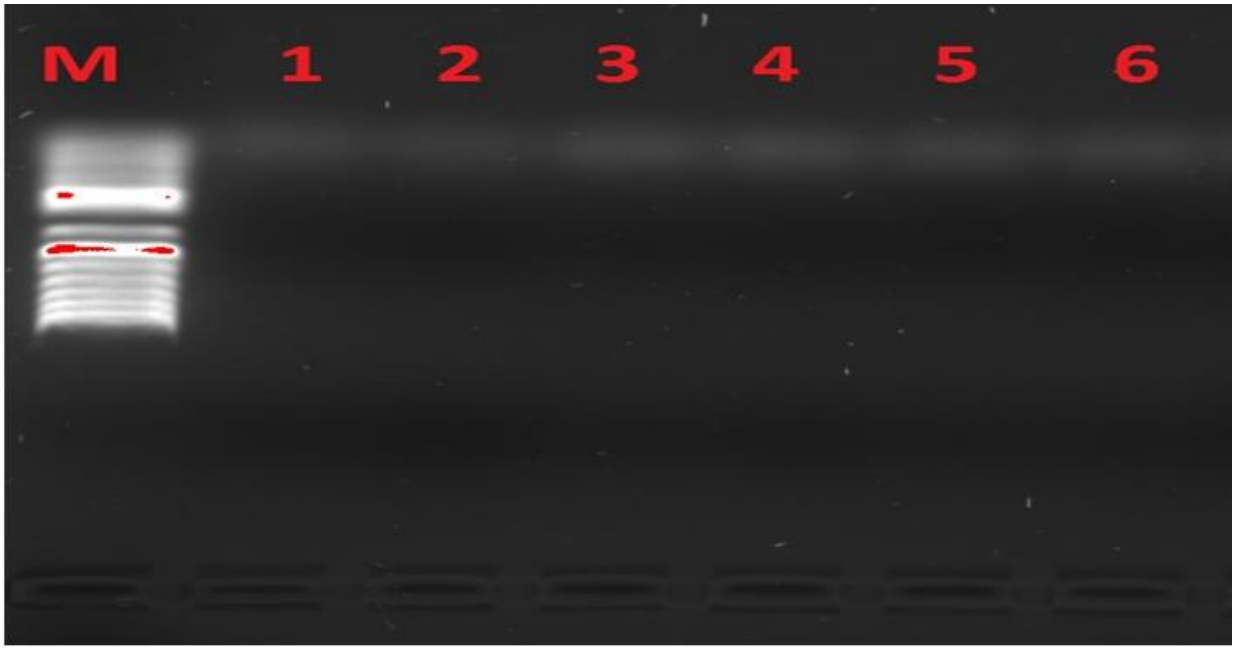

Figure 4.4 PCR amplification of species-specific DNA fragments of reference *Aspergillus* spp. strains using DreamTaq polymerase. Lane M, molecular weight marker (λ Hind III); Lane 1, *A. parasiticus* AP1; Lane 2, *A. parasiticus* AP2; Lane 3, *A. parasiticus* AP3; Lane 4, *A. flavus* AF1; Lane 5, *A. flavus* AF2; Lane 6, *A. flavus* AF3

Table 4.5 Conventional PCR reactions determining the specificity of *A. flavus and A. parasiticus* spp. primers using DreamTaq and 2X Phusion polymerases

Fungal species	Strain	DreamTaq polymerase reaction (+/-)	2X Phusion polymerase reaction (+/-)
Aspergillus parasiticus	AP1	-	-
A. parasiticus	AP2	-	-
A. parasiticus	AP3	-	-
A. flavus	AF1	-	-
A. flavus	AF2	-	+
A. flavus	AF3	-	+
A. parasiticus	0200	-	-
A. flavus	0645	-	-
A. flavus	3954	-	-

-, no amplification observed; +, amplification observed

4.3.3 Optimization of the qPCR conditions

The optimum conditions for qPCR were determined by running trial and error experiments and modifying the conditions in the protocols by changing the annealing temperatures (Nicolaisen et al., 2009; Sardiñas et al., 2010). Standard curves, including a range of gDNA concentrations, were analysed to confirm acceptable ranges of the parameters: slopes (-3.1 to -3.6), correlation coefficients (R^2>0.99) and PCR efficiency (90-110%) (Boutigny et al., 2012; Bustin et al., 2009; Nicolaisen et al., 2009).

4.3.4 Optimization of *Aspergillus* species-specific qPCR identification

The standard curves obtained for *A. flavus* AF1 and *A. parasiticus* AP2 against pearl millet and sorghum matrixes are presented in Figures 4.5 and 4.6, respectively. None of the *Aspergillus* spp. reaction values were within range, confirming no primer specificity (Table 4.6).

Table 4.6 Specificity of SYBR green polymerase for species-specific detection of *Aspergillus* spp.

Sample	Annealing Temp (˚C)	Efficiency (%)	Correlation Coefficient (R^2)	Slope
AF1	60	164	0.888	- 2.365
AP2	60	92128 x 10^7	0.008	0.039

AF1, *Aspergillus flavus*; AP2, *Aspergillus parasiticus*

4.3.5 Optimization of *Fusarium* species-specific qPCR identification

Quantitative Real-time PCR was optimized using both sorghum and pearl millet as matrix. The results are summarized in Table 4.7. Figures 4.7 and 4.8 present the standard curve and melt curve, respectively, of *F. subglutinans* against a sorghum matrix. Although all the standard curve parameters were within range, the control sorghum produced an interfering peak like the *F. subglutinans* reference culture. *F. subglutinans* could therefore not by quantified in sorghum samples. Figure 4.9 presents the standard curve of *F. graminearum* against a millet matrix. Co-eluting peaks were observed (between Log SQ -4.2 and -5.4), but were out of the range of interest (between Log SQ -2.8 and 0.6), and did not affect the interpretation of results.

4.3.6 Quantitative Real-time PCR

Quantitative Real-time PCR was performed in a 96-well plate to identify and quantify the four *Fusarium* spp. in all the sorghum and pearl millet samples. The analyses were performed in triplicate and included standard curve samples, two no DNA template samples and two negative controls. The mean DNA concentrations of *F. verticillioides, F. graminearum* and *F. proliferatum* detected in sorghum are summarized in Table 4.8. The limit of detection for this study was 0.00244 pg/µl. The most prevalent contaminant was *F. verticillioides*, which was detected in 45% of the samples, followed by *F. proliferatum* (15%) and *F. graminearum* (10%). Sample NAM-27 was contaminated with all three *Fusarium* spp., whilst 15% of the samples exhibited co-contamination of two *Fusarium* spp., with *F. verticillioides* present as one of the contaminants in each case. All the contaminated samples were processed and sold at markets. 44% of the contaminated sorghum samples originated from the Ondangwa market and 56% from the Oshakati market.

Twenty-two samples of pearl millet were tested for *F. verticillioides*, *F. graminearum*, *F. proliferatum* and *F. subglutinans* contamination (Table 4.9). The detection limit for this activity was 0.00244 pg/µl. *F. verticillioides* (64%) was predominant, followed by *F. subglutinans* (22.7%), *F. graminearum* (13.6%) and *F. proliferatum* (0.05%). Sample NAM-17 was infected with all four *Fusarium* spp., while NAM-23 contained three *Fusarium* spp. Of the pearl millet samples, 18%-exhibited co-contamination with two *Fusarium* spp. *F. verticillioides* was present in all the contaminated samples. All the contaminated samples were processed grains and market bought with 36% and 64% of the contaminated samples originated from the Ondangwa and Oshakati markets, respectively.

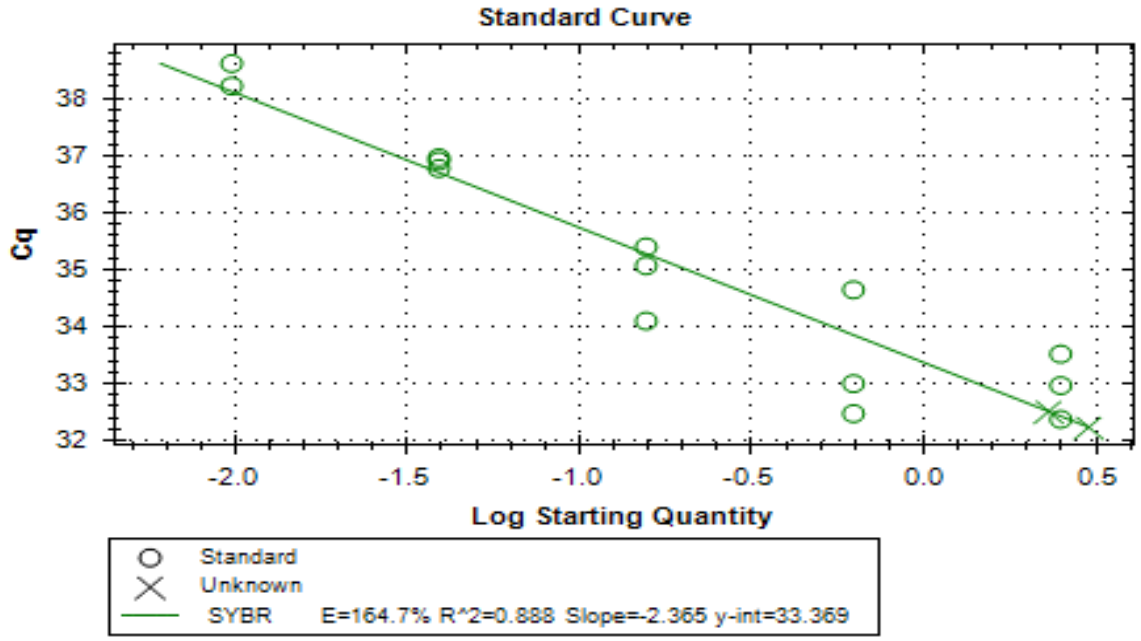

Figure 4.5 The standard curve of *Aspergillus flavus* AF1 against a millet matrix (R^2 = 0.888). Cq, quantification cycle.

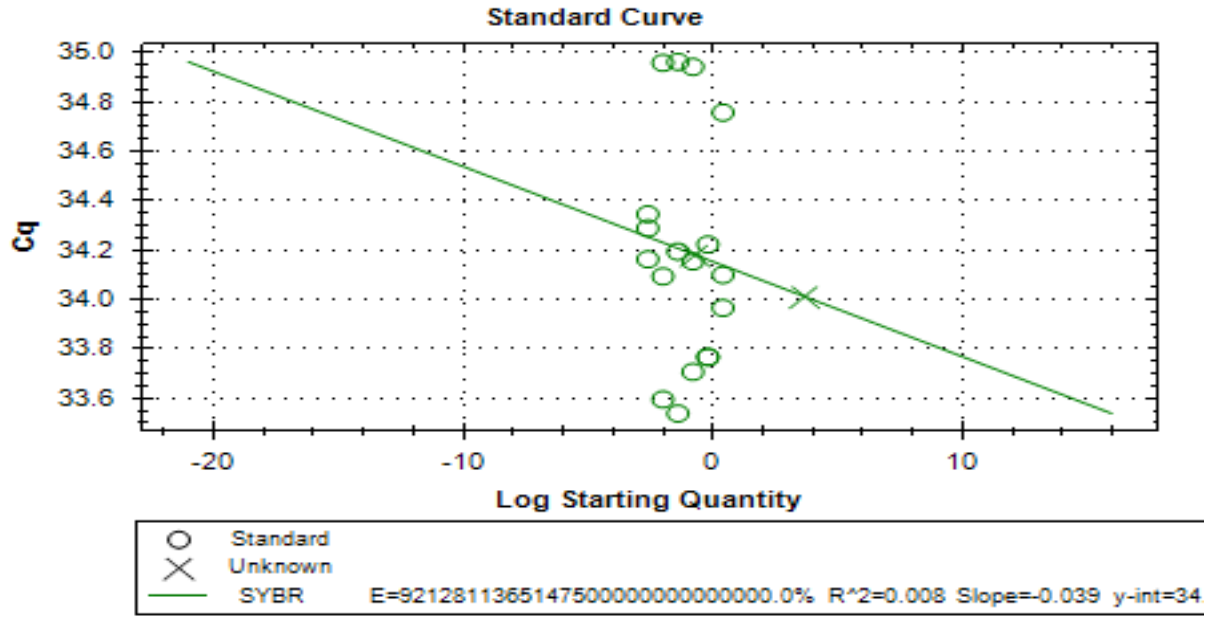

Figure 4.6 The standard curve of *Aspergillus parasiticus* AP2 against a sorghum matrix (R^2 = 0.008). Cq, quantification cycle.

Table 4.7 Optimum conditions for *Fusarium* spp. qPCR

Reference fungal strains	Matrix	Annealing Temperature (˚C)	Efficiency (%)	Correlation coefficient (R^2)	Slope
F. graminearum **MRC 6010**	Pearl millet	64	101.3	0.993	-3.292
F. graminearum **MRC 6010**	Sorghum	62	117.3	0.993	-2.966
F. proliferatum **MRC 8550**	Pearl millet	64	106.2	0.992	-3.182
F. proliferatum **MRC 8550**	Sorghum	66	122.7	0.994	-2.877
F. subglutinans **MRC 8553**	Pearl millet	64	96.0	0.996	-3.422
F. subglutinans **MRC 8553**	Sorghum	64	99.3	0.997	-3.339
F. verticillioides **MRC 826**	Pearl millet	66	101.0	0.991	-3.298
F. verticillioides **MRC 826**	Sorghum	60	106.6	0.992	-3.174

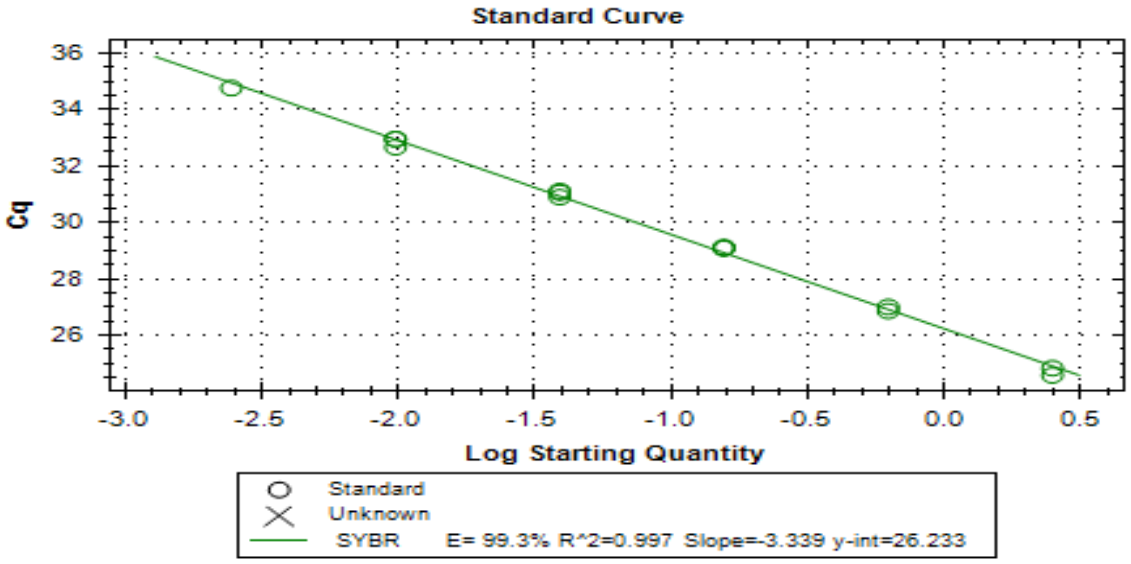

Figure 4.7 The standard curve of *F. subglutinans* MRC 8553 against a sorghum matrix (R^2 = 0.997)

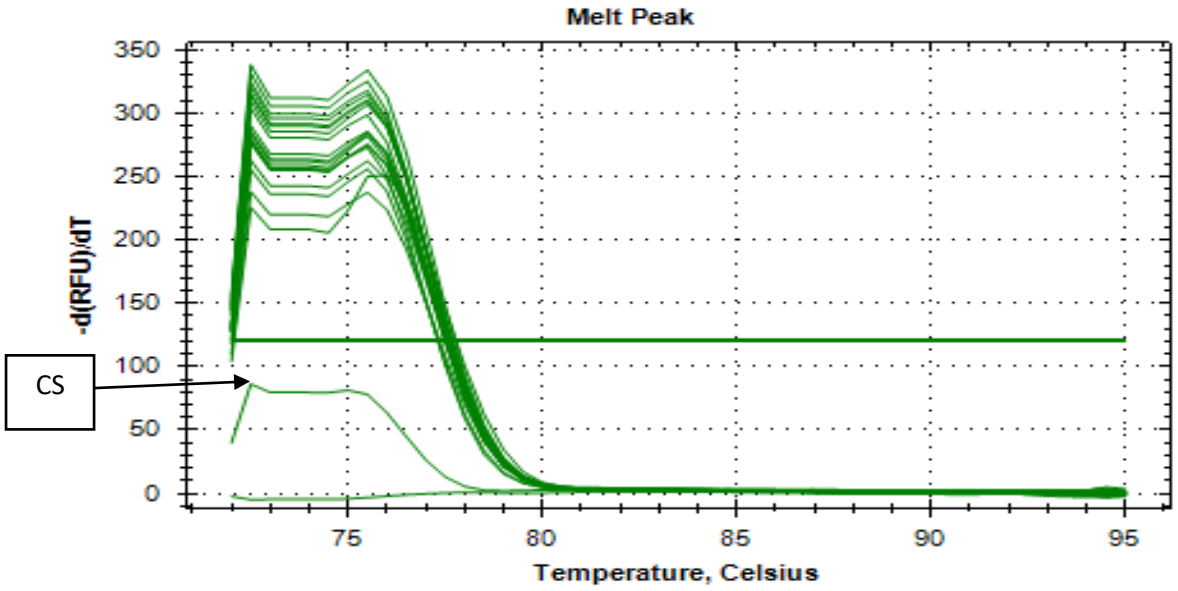

Figure 4.8 The melting curve of *F. subglutinans* MRC 8553 against a sorghum matrix. CS, control sorghum without fungal contamination

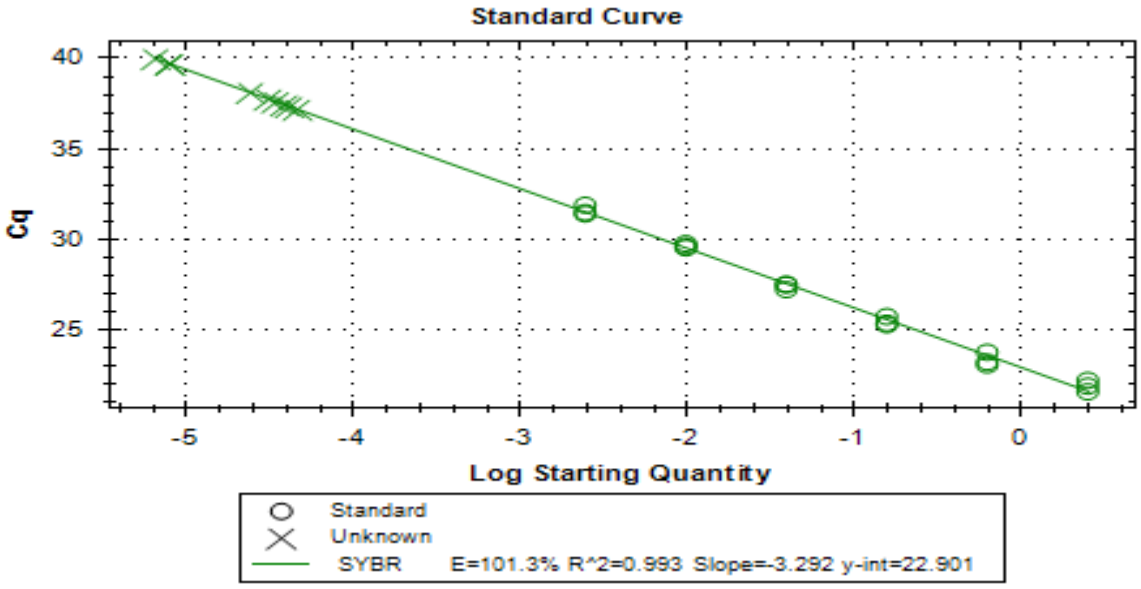

Figure 4.9 The standard curve of *F. graminearum* MRC 6010 against a millet matrix (R^2 = 0.993)

Table 4.8 Concentrations (pg/µl) of *Fusarium* spp. DNA identified in unprocessed and processed sorghum samples from the Oshana region of Namibia

Sample no.	*Fusarium verticillioides*	*Fusarium graminearum*	*Fusarium proliferatum*
NAM-1S	ND	ND	ND
NAM-2S	ND	ND	ND
NAM-3S	ND	ND	ND
NAM-4S	ND	ND	ND
NAM-5S	ND	ND	ND
NAM-6S	ND	ND	ND
NAM-7S	ND	ND	ND
NAM-8S	ND	ND	ND
NAM-9S	ND	ND	ND
NAM-10S	ND	ND	ND
NAM-11	2.76 ± 0.05^{a}	0.11 ± 0.05^{a}	ND
NAM-12	1.24 ± 0.47^{b}	ND	ND
NAM-13	9.6 ± 1.98^{c}	ND	ND
NAM-14	1.52 ± 0.17^{b}	ND	ND
NAM-15	ND	ND	ND
NAM-16	1.49 ± 0.21^{b}	ND	ND
NAM-24	0.00595 ± 2.02^{d}	ND	0.6 ± 0.5^{a}
NAM-25	0.7 ± 018^{e}	ND	ND
NAM-26	16.69 ± 2.5^{f}	ND	0.37 ± 0.3^{a}
NAM-27	4.08 ± 1.47^{g}	0.11 ± 016^{a}	0.59 ± 0.6^{a}

Values represent means of triplicate determinations ± standard deviations. ND, none detected. Statistical differences ($p < 0.05$) in a column are indicated with different letters

Table 4.9 Concentrations (pg/µl) of *Fusarium* spp. DNA identified in unprocessed and processed pearl millet samples from the Oshana region of Namibia

Sample no.	*Fusarium verticillioides*	*Fusarium graminearum*	*Fusarium proliferatum*	*Fusarium subglutinans*
NAM-1M	ND	ND	ND	ND
NAM-2M	0.08 ± 0.00^{a}	ND	ND	ND
NAM-3M	0.10 ± 0.00^{b}	ND	ND	0.03 ± 0.00^{a}
NAM-4M	ND	ND	ND	ND
NAM-6M	ND	ND	ND	ND
NAM-7M	ND	ND	ND	ND

Sample no.	*Fusarium verticillioides*	*Fusarium graminearum*	*Fusarium proliferatum*	*Fusarium subglutinans*
NAM-8M	ND	ND	ND	ND
NAM-9M	ND	ND	ND	ND
NAM-10M	ND	ND	ND	ND
NAM-17	29.05 ± 2.76^{c}	0.04 ± 0.01^{ab}	0.19 ± 0.00a	2.03 ± 0.57^{b}
NAM-18	1.31 ± 0.20^{d}	ND	ND	ND
NAM-19	0.30 ± 0.10^{e}	ND	ND	ND
NAM-20	0.66 ± 0.27^{f}	ND	ND	ND
NAM-21	0.69 ± 0.03^{f}	ND	ND	ND
NAM-22	0.36 ± 0.02^{g}	ND	ND	0.04 ± 0.00^{c}
NAM-23	0.62 ± 0.3^{f}	0.04 ± 0.00^{a}	ND	0.06 ± 0.00^{d}
NAM-28	0.07 ± 0.06^{abh}	ND	ND	ND
NAM-29	ND	ND	ND	ND
NAM-30	0.11 ± 0.00^{h}	ND	ND	ND
NAM-31	0.60 ± 0.56^{abdefh}	0.02 ± 0.01^{b}	ND	ND
NAM-32	0.52 ± 0.18^{efg}	ND	ND	0.06 ± 0.03^{d}
NAM-33	0.35 ± 0.16^{efg}	ND	ND	ND

Values represent means of triplicate determinations ± standard deviations. ND, none detected. Statistical differences ($p < 0.05$) in a column are indicated with different letters

4.4 Discussion

Sorghum and pearl millet are important staple grains in Africa, they account for half of the total cereal production on the continent and play a crucial role in the preservation of food security (Mukarumbwa et al, 2010; Misihairabgwi et al., 2019). In Namibia, sorghum and pearl millet are key ingredients in the preparation of porridge, non-alcoholic traditional drink *Oshikundu* and commercial beers such as Windhoek lagers (Amadou et al., 2013; Misihairabgwi et al., 2018). Mycotoxin toxicity is one of the key issues affecting the production of sorghum and pearl millet due to its threat to human and animal health (Misihairabgwi et al., 2018). *Fusarium verticillioides, F. proliferatum, F. subglutinans and F. graminearum* are known to be the spp. that cause the most pre-harvest mycotoxin contamination, while *Aspergillus flavus* and *A. parasiticus* is the most serious concern during storage of grain (García-Díaz et al., 2020). To date, there is limited knowledge on the occurrence of mycotoxins and mycotoxin producing-fungi along the complete sorghum and pearl millet production chain in northern Namibia. The present study focuses on the occurrence of the main mycotoxin-producing *Aspergillus* and *Fusarium* spp. in the Oshana region of northern Namibia, mainly focussing on the stored

household grain and the processed and unprocessed grain sold on the open markets of Ondangwa and Oshakati.

High quality DNA was extracted from sorghum and pearl millet samples as well as from liquid cultures of the reference *Fusarium* and *Aspergillus* spp. The DNA used in this study was extracted from 2 g of each sample. This was done to reduce the errors caused by non-uniform distribution of *Fusarium* spp. in samples (Brandfass and Karlovsky, 2008). Although a higher amount of plant tissue might be desirable, it could saturate the mini extraction column and have a negative effect on the efficacy of DNA extractions (Brandfass and Karlovsky, 2008). The DNA reading on the Thermo Scientific Nanodrop 2000 Spectrophotometer showed all the DNA samples used in these experiments have high molecular weight fragments with an A260/280 ratio between 1.8 and 2.0. According to Abdel-Latif and Osman (2017) this is the standard assay for high quality gDNA. In order to ensure that both the fungal and plant cell walls were properly broken and a high yield of gDNA was obtained during extraction, a liquid nitrogen homogenize step was included in the protocol for both *Aspergillus* and *Fusarium* spp. pure culture strains as well as for the sorghum and pearl millet samples.

Phenolics are considered as the main contaminants in plant and fungal DNA preparation (Calderón-Cortés et al., 2010). Phenolics, being strong oxidizing agents, decrease the yield and purity of DNA by binding covalently to the isolated DNA, thus inhibiting further enzymatic reactions of DNA, such as PCRs (Sarwat et al., 2006). The CTAB and the addition of PVP, an antioxidant, to the extraction buffer can assist in eliminating phenolics in DNA extracted from plants and fungi. To ensure complete removal of phenols from the fungal gDNA, a phenol chloroform isoamyl (PCI) and a chloroform isoamyl (CI) step was added as modifications to the protocol provided by the manufacturer. According to Abdel-Latif and Osman (2017), high quality gDNA should not be contaminated by polysaccharides and phenols. Contamination by these substances would render the DNA non-amplifiable (Sarwat et al., 2006).

The lack of amplification of *Aspergillus* spp. in both conventional PCR enzyme reactions indicated that there was no primer specificity. The AF2 and AF3 reactions exhibited a false positive reaction. The expected product of the PCR reaction was between 100 to 150 bp. The product of the AF2 and AF3 reaction were less than the expected molecular weight, therefore the false positive reaction could be due to the presence of primer dimers. Dimethyl sulfoxide (DMSO) is a known to enhance PCR amplification by improving specificity and reproducibility (Simonović et al., 2012). The addition of DMSO in both conventional PCR assays performed in this study did not result in any significant changes to the results when compared to the controls without DMSO.

According to the MIQE (minimum information necessary for evaluating qPCR experiments) guidelines the following criteria apply for the optimization and validation of qPCR (Bustin et al., 2009): a slope between -3.1 and-3.6, a correlation coefficient (R^2) >0.99 and a PCR efficiency between 90 and 110%. The negative controls should give no reading and no peaks should be observed on the melting curve (Nicolaisen et al., 2009). For optimization of the *Aspergillus* qPCR reactions the two standard curves created by the pairs FLAVIQ1 / FLAQ2 and FLAVIQ1 / PARQ2 lacked linearity across the range of concentrations used and displayed a correlation coefficient <0.99 in all reactions. The slopes of the standard curves for *A. flavus* and *A. parasiticus* were −2.365 and 0.039, respectively, corresponding to amplification efficiencies of 164% and 92128 x 10^7 %. The non-template control exhibited no amplification. All the values were out of the acceptable ranges, and this served to confirm the lack of primer specificity as suggested by the two approaches used in conventional PCR.

For optimization of the *Fusarium* qPCR reactions, the standard curves generated by applying *F. graminearum*, *F. proliferatum*, *F. verticillioides* and *F. subglutinans* primers showed linearity across the spectrum of six-fold serial dilution concentrations used. They also showed strong correlation coefficients, suggesting very low inter-assay variability in all cases. The slope of the standard curves and the amplification efficiencies attained were within the acceptable range. All the non-template control values showed no reading except for the reaction of *F. subglutinans* against a sorghum matrix. It should be noted that all the primer sets used in this study were originally designed and optimized for reactions against a maize matrix (Nicolaisen et al., 2009). The *F. subglutinans* primer sequence might hybridize to the sorghum DNA, hence the PCR reaction amplified a piece of the sorghum DNA and produced a false positive result. For this reason, the presence of *F. subglutinans* in the sorghum samples from Namibia could not be investigated using the maize-optimized primer pair. To rule out the possibility of fungal contamination in the control sorghum, two different samples of control sorghum (store bought sorghum meal and control sorghum sample from SAGL) were used as matrixes in separate PCR experiments and produced the same result. To confirm the purity of the control sorghum samples, they were plated out onto PDA and analysed for fungal contamination and the results were negative in both respects.

Quantitative Real-time PCR was performed to detect and quantify contamination of sorghum and pearl millet by four different *Fusarium* spp. In this study, the use of species-specific primers allowed the detection and quantification of the *Fusarium* spp. in samples. It also allowed for the detection of the co-occurrence of *Fusarium* contaminants. It should, however, be noted that qPCR can only be applied to grain samples to detect and quantify fungal species (i) that are known, (ii) for which species-specific primers are available, and (iii) for which

species-specific primers have been optimized for the relevant grain matrix. The method is not applicable for detection of novel fungal species or where species-specific primers are not available. It should therefore be used to complement other methods, such as morphological methods, and genotyping based on sequence variation and the presence of key genes (e.g. *EF1*α, *TEF1*-α, *IGS* and *ITS*), in order to ensure that the correct fungal species are targeted. qPCR results obtained for samples contaminated with more than one *Fusarium* spp. may also mean higher subsequent mycotoxin levels.

Mycotoxigenic fungi can infect grains in the field, causing ear rots and head blight resulting in mycotoxin contamination (Munkvold, 2017). It is important to determine when contamination with mycotoxigenic fungi occurs and whether it is linked to the appearance of the related mycotoxins so as to help understand the fungal life cycle and curb the fungal infection and the subsequent mycotoxin contamination (García-Díaz et al., 2020). This can be achieved by applying the Hazard Analysis Critical Control Points (HACCP) approach (Codex Alimentarius Commission, 2009) to identify the critical control points from the time of planting, processing, transportation, handling and marketing. The HACCP principle may be used in conjunction with a Multiplex Real Time PCR protocol designed and optimized using species-specific primers as were used in this exercise and known primers that are linked to the biosynthetic pathways of specific mycotoxins e.g. *FUM* cluster genes that are expressed during fumonisin biosynthesis (Steogonpień et al., 2011). Genes such as the *EF1*α as used in this study are well characterized in many *Fusarium* spp. (Nicolaisen et al., 2009). It is useful to detect and measure the biomass of a broad range of *Fusarium* spp. However, this gene cluster cannot be used to distinguish between toxigenic and non-toxigenic *Fusarium* spp. Mycotoxin cluster genes have been used to design primers from genes that are directly involved in the mycotoxin biosynthesis (Horevaj et al., 2011). The use of such primers can easily be adopted to improve the methods used in this study, as the toxigenic strains present in a sample can be quantified directly.

In vitro experiments in which 17 different strains of *Fusarium* spp. were inoculated on maize, sorghum and pearl millet samples, revealed that the amount of toxin produced by each strain varied with each substrate (Vismer et al., 2019). This could be linked to the difference in the infection rates of each substrate by a given strain of *Fusarium spp.* In this study, the infection rate of *F. proliferatum* was significantly lower than that of *F. graminearum* in pearl millet samples as compared to sorghum samples. In another experiment, Atoui (2011) established a positive and strong correlation between *F. graminearum* gDNA and ZEA content in maize samples. This confirms that the differences in mycotoxin levels recorded in the experiments by Vismer et al. (2019) may have arisen from the differences in the infection rates by the

different fungal strains. More research needs to be done on the fungal, host and environmental interactions as several factors affecting pathogenicity, virulence and mycotoxin production. *F. graminearum* DNA content lower than 500 pg of DNA / mg of maize may ensure food safety according to a study by Atoui (2012). However, this method is not completely reliable because the absence of a certain mycotoxigenic fungus does not guarantee the absence of mycotoxins. A certain mycotoxin can be produced by more than one strain of mycotoxigenic fungi e.g. both *A. flavus* and *A. parasiticus* produce aflatoxin B_1 (Hassan, 2019). Grain fungal infection results in loss of quality in the grain. A follow up study can be done to determine if there is a correlation between the amount of fungal target DNA (qPCR) and the grain quality.

4.5 Conclusion

Sorghum and pearl millet cereals are susceptible to contamination by mycotoxigenic fungi. This work was a preliminary study of the degree of contamination by mycotoxigenic fungi in the staple grains consumed by the smallholder farming communities in the Oshana region of northern Namibia. The study mainly focussed on the fungal contaminants in stored grain and market-ready, processed sorghum and pearl millet meal, bran and malt. *Aspergillus* spp. could not be detected using both conventional PCR and qPCR due to primer non-specificity. The primer sets used on both *A. flavus* and *A. parasiticus* were originally designed and optimized for a maize matrix. Further research will be done to optimize this process. The results also indicated that *F. verticillioides*, *F. graminearum*, *F. proliferatum* and *F. subglutinans* spp. are able to colonize both sorghum and pearl millet samples. *F. subglutinans* in sorghum could not be quantified as the reaction gave a false positive result with the control samples. The *Fusarium* spp. primer sets used in this study were designed for a maize matrix hence there is need for further research. However, there is need for application of good agricultural practices and proper aseptic techniques during the whole grain production line to minimise contamination by mycotoxigenic fungi.

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CHAPTER 5

QUANTIFICATION OF MULTIPLE MYCOTOXINS IN RAW AND PROCESSED *SORGHUM BICOLOR* AND *PENNISETUM GLAUCUM* WITH A VALIDATED LC-MS/MS TECHNIQUE

5.1 Introduction

Sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum*) constitute half of the total cereal crop production in Africa and play a pivotal role in the maintenance of food security (Taylor, 2003; Mukarumbwa and Mushunje, 2010). Their high yields and adaptation to extreme environmental conditions such as low rainfall, high temperatures and short bursts of heavy rainfall make them suitable for production in most regions in Africa (Mukarumbwa and Mushunje, 2010). They are especially ideal crops for cultivation in regions that are semi-arid to tropical, such as sub-Saharan Africa and subtropical East Africa (Diouf, 2016). Sorghum and pearl millet are the main staple crop in the Oshana region of northern Namibia where such climatic conditions prevail. In this area, grains are mainly cultivated by smallholder farmers, used for household consumption and sold at local markets in raw and processed forms. Postharvest, the clusters of grains are thrashed, winnowed and kept in storage baskets for extended periods. Sorghum and pearl millet are ground into flour and used to prepare porridge, confectionary, and alcoholic and non-alcoholic beverages.

Grains are vulnerable to contamination by mycotoxigenic fungi and their associated mycotoxins, which are implicated in many human and animal diseases, including cancer, immunosuppression, neural tube defects and stunting in children (Alberts et al., 2019). The most important mycotoxins contaminating dietary staple grains include aflatoxin B_1 (AFB_1), fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZEA), nivalenol (NIV) and moniliformin (MON). Exposure to multiple mycotoxin contamination in staple grains enhances the negative health effects in populations, while the prevention of chronic exposure is of critical importance (Ezekiel et al., 2014; Torres et al., 2015; Pitt, 2012). In high-income countries, the levels of mycotoxins in staple grains are effectively regulated, while in low-income countries, including many African countries, regulations do not exist or are poorly implemented (Shephard et al., 2019; Alberts et al., 2019)

The most important sorghum stalk rot and grain mould diseases resulting in mycotoxin contamination is caused by *Fusarium* spp., including *F. andiyazi, F. proliferatum, F. napiforme,*

F. nygamai, F. thapsinum, F. pseudonygamai, and *F. verticillioides* (Leslie and Summerell, 2006). In Japan, 52.5% of imported sorghum samples were contaminated with zearalenone (60-7260 mg/kg), mainly produced by *F. semitectum* (Aoyama et al., 2009). A high prevalence of *F. thapsinum*, *F. proliferatum*, and *F. verticillioides*, and less of *F. sacchari* and *F. beomiforme* was observed in sorghum sampled in Thailand, indicating the possible presence of the fumonisin B (FB) mycotoxins (Mohamed et al., 2019). Data on the contamination of sorghum and pearl millet in Africa indicates the co-occurrence of multiple mycotoxins, especially aflatoxins (AF), produced by *Aspergillus* spp., and FB. The co-occurrence of AF and FB has been reported in sorghum and pearl millet from smallholder farmers under the direction of the International Institute for Tropical Agriculture in Nigeria (Vismer et al., 2015). A surveillance study evaluating the levels of multiple mycotoxins in sorghum from Burkina Faso, Ethiopia, Mali and Sudan resulted in 33% of 1533 samples contaminated with multiple mycotoxins, including AF, FB, sterigmatocystin, *Alternaria* toxins, OTA and ZEA (Ezekiel et al., 2018). Sudan exhibited the lowest incidence and average concentration of all mycotoxins analysed with the highest concentrations of AF and FB recorded in pink sorghum. Phylogenetic studies of *Fusarium* spp. isolates in Ethiopia revealed higher contamination in sorghum than in finger millet. A large number of isolates exhibited a close relationship with the *F. incarnatum–equiseti* spp. complex (Chala et al., 2019). All sorghum samples collected from smallholder farmers' threshing floors and underground storage pits in East Hararghe, Ethiopia, were contaminated with both *Aspergillus* and *Fusarium* spp. AF contamination was ≤ 33.10 µg/kg with the highest levels in stored samples, while the FB levels were between 907 and 2041 µg/kg (Taye et al., 2016).

Limited information is available on contamination of sorghum and pearl millet in southern Africa. *F. nygamai* and *F. moniliforme* (= *F. verticillioides*) are the main fungi contaminating sorghum and pearl millet in Lesotho and Zimbabwe (Klaasen and Nelson, 1997). In Gaborone, Botswana, 46 traditional sorghum malt, wort, and beer samples were collected from three villages and *F. verticillioide*s and *A. flavus* contamination detected in 63% and 37% samples, respectively. AF was not detected, whilst FB_1 was detected in three malt samples (47-1316 µg/kg), and ZEA in 56%, 48% and 48% of malt (102-2213 µg/kg), unfermented beer (26-285 µg/L) and fermented beer (20-201 µg/L) samples, respectively (Nkwe et al., 2005). A diversity of fungal metabolites was determined in street vended pearl millet and sorghum ingredients and their transfer rate during fermentation into *Oshikundu*, a traditional Namibian drink (Misihairabgwi et al., 2018). High levels of mycotoxins were detected in the raw grains used for the brewing of *Oshikundu.* AFB_1 was detected in 13% of 40 pearl millet meal samples [mean concentration 0.9±0.7 µg/kg (range 0.1-2.0 µg/kg)] and in 50% of 40 sorghum malt samples [mean concentration 4.5±5.5 µg/kg (range 0.2-25.4 µg/kg)]. Contamination of raw

and processed sorghum and pearl millet with mycotoxins in northern Namibia is a concern. Compared to other regions worldwide, relatively few studies have been carried out in this field, mainly due to the lack of sophisticated laboratory equipment, insufficient funds, expertise and limited surveillance systems (Misihairabgwi, 2018).

Various techniques are applied for the detection and quantification of mycotoxins in grains, including enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC) and liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Agriopoulou et al., 2020). A wide range of laboratory kits are available which rely on the ELISA principles (Zhang et al., 2018). These kits generally provide rapid detection of mycotoxins in grains, while validation of the method is not required. The sensitivity and accuracy of ELISA kits vary and should therefore be cautiously selected (Zhang et al., 2018). The kits are especially applicable to field testing and are used in laboratories lacking sophisticated technologies such as HPLC and LC-MS/MS. HPLC provides an effective method for detection and quantification of mycotoxins (Agriopoulou and*.,* 2020). The method requires validation and includes extraction methods prior to HPLC quantification and sophisticated technology is required. However, most universities and research institutes house HPLC instruments. The method is tedious, and requires a skilled analyst but produces accurate results (Agriopoulou et al*.,* 2020). The method is, however, not as sensitive as LC-MS/MS and multiple mycotoxins cannot be quantified in one run (Pascale et al., 2019). LC-MS/MS analyses is currently being applied worldwide for quantification of multiple mycotoxins in grains (Pascale et al., 2019). This kind of analyses require sophisticated technology and experience, which is only available in specialized laboratories. The South African Grain Laboratory (SAGL, Pretoria) and the Mass Spectrometry Unit of the Central Analytical Facility (CAF) of Stellenbosch University (SU) house state of the art LC-MS/MS instruments and are considered the main mycotoxin testing laboratories in South Africa. This technique is considered very sensitive, can detect low concentrations of multiple mycotoxins in one run, and provides a high level of precision and accuracy (Woo et al., 2019). The technique requires, however, extensive validation of the extraction and analytical techniques to ensure accuracy and precision of results (Bessaire et al., 2019).

The aim of the study was to determine the concentrations of AFB_1, FB, ZEA, OTA, DON, NIV and MON in raw (unprocessed) and processed sorghum and pearl millet collected from smallholder farmers and open markets in the Oshana region of northern Namibia by using a validated LC-MS/MS analytical technique. The study provides important information on the degree of contamination of staple grains produced by the smallholder farmers servicing local markets in this region.

5.2 Materials and Methods

5.2.1 Chemicals

Whatman filter paper, acetonitrile, formic acid and methanol were obtained from Merck (Darmstadt, Germany). Water for all experiments was successively purified by reverse osmosis followed by Milli-Q water purification (Millipore, Massachusetts, USA).

5.2.2 Sorghum and pearl millet samples

Raw and processed sorghum and pearl millet samples were collected postharvest during July 2018 from the households of ten smallholder farmers in Oshakati and from two open markets in Oshakati and Ondangwa, located in the Oshana region of northern Namibia (Chapter 3). These markets are serviced by smallholder farmers in the area. The sampling locations (N1-N10) are indicated on the geographical map (Chapter 3; Figure 3.1C). Samples were collected as part of an international collaboration between Cape Peninsula University of Technology (CPUT) and the University of Namibia (UNAM, Windhoek, Namibia). The field study team consisted of Dr JP Rheeder (CPUT), Dr J Misihairabgwi (UNAM) and agricultural extension officers from the Department of Agriculture, Namibia. Ethical clearance for the field study was obtained (Registration Number NHREC: REC- 230408-014) from the Health and Wellness Research Ethics Committee (HW-REC) of CPUT. Blank sorghum and pearl millet (100 g each) containing no mycotoxins, were obtained from the Southern Africa Grain Laboratory (SAGL, Pretoria) for spiking experiments and for the preparation of matrix matched calibration curves. Samples were ground to a fine meal in a laboratory mill (Falling Number ABStockholm, Sweden) and placed in airtight containers at a temperature of -20 °C before processing.

5.2.3 Analytical standards

Analytical standards of FB_1, FB_2 and FB_3 (purity ≥97%) were obtained from the Mycotoxicology Research Group of the Institute of Biomedical and Microbial Biotechnology, CPUT. Analytical standards of AFB_1 (Cat no A6636), OTA (Cat no 32937), ZEA (Cat no Z2125), DON (Cat no D0156), MON (Cat no M5269) and NIV (Cat no 34141) were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany). Individual stock solutions (0.1 mg/ml) of AFB_1, OTA and ZEA were prepared in acetonitrile, MON in methanol, while FB_1, FB_2 and FB_3, DON and NIV were prepared in acetonitrile-H_2O (1:1). Working solutions in acetonitrile-H_2O (1:1) containing (i) AFB_1, ZEA and OTA (200 ng/ml) individual concentrations, and (ii) FB_1, FB_2, FB_3, DON, NIV and MON (5 µg/ml) individual concentrations were prepared using aliquots of the stock solutions.

5.2.4 Multi-mycotoxin analyses

Multiple mycotoxin concentrations (AFB_1, FB_1, FB_2, FB_3, OTA, DON, ZEA, NIV and MON) were determined in blank sorghum and pearl millet, method validation samples, and in the raw and processed sorghum and pearl millet samples (three replicates).

5.2.4.1 Extraction method

Multiple mycotoxins were extracted from the raw and processed grain samples following the method described by Alberts et al. (2019). Extraction solvent [methanol: acetonitrile: water; (25:25:50); 100 ml] was added to ground samples (10 g) and the mixture placed on a shaker for 30 min at 80 rpm. The extracts were centrifuged at 4000 x g for 10 minutes at 4˚C in a refrigerated Sorvall RC-3B centrifuge (DuPont, Norwalk, Connecticut, USA). The supernatant was diluted (1:1) with methanol: water (25:75), filtered (Whatman No. 4 filter paper) and analysed with LC-MS/MS. FAPAS (Cat no T22110QC; The Food and Environmental Research Agency, York, England; contains DON, ZEA and NIV) and Biopure (Cat no QCM3C2; Industrial Analytical, Kyalami, South Africa; contains FB_1, FB_2 and FB_3) certified quality control samples, containing mycotoxins in the expected concentration ranges, were included in each run.

5.2.4.2 LC-MS/MS analyses

LC-MS/MS analyses of samples was undertaken in conjunction with the Mass Spectrometry Unit of the Central Analytical Facility of Stellenbosch University. The mycotoxins were separated on a reversed-phase BEH C_{18} column (2.1x100 mm; particle size 1.7 µm; Waters, Milford, MA, USA) and analysed with positive electrospray ionisation (ESI) (Capillary voltage 3.5 kV; Cone voltages: FB, 50 V; DON, 35 V; ZEA, 20 V) in the multiple reaction monitoring (MRM) mode in a Waters Acquity Ultra Performance Liquid Chromatograph (UPLC) coupled to a Waters Xevo TQ tandem quadrupole mass spectrometer. Eluent A was water and eluent B was acetonitrile, both containing 0.1% formic acid. The elution gradient consisted of an initial mobile phase composition (2% B) held constant for 0.5 min, followed by a linear gradient to 40% B within 7 min and to 70% B over 3 min, followed by a 1-minute wash step at 100% B and finally a 3-minute column re-equilibration to 2% B for a total run time of 15 minutes. The flow rate of the mobile phase was 0.35 ml/min. For each compound, one precursor and two product ions were monitored, one product ion for quantification and one for confirmation (Table 5.1). A calibration curve consisting of matrix-matched standards for each mycotoxin was used to compensate for matrix effects in the analysis. Matrix-matched standard solutions were prepared utilising an extract prepared from blank sorghum and pearl millet.

5.2.5 Validation of the extraction and chromatographic methods

Validation of the extraction and chromatographic methods was performed in collaboration with SAGL and CAF, SU according to guidelines of the United States Department of Health and Human Services and Food and Drug Administration (FDA, 2017). Methods were validated in terms of selectivity, percentage (%) recovery, accuracy, calibration curve regression parameters and limit of quantitation (LOQ).

5.2.5.1 Selectivity. The ability of an analytical method to distinguish and measure the analyte in the presence of other components in a sample is called selectivity. Samples included: (i) blank sorghum and pearl millet samples, and (ii) blank sorghum and pearl millet spiked with the respective mycotoxins. LC-MS/MS chromatograms and data were examined for the presence of co-eluting or interfering compounds.

5.2.5.2 Percentage (%) recovery. Recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the analyte in a solvent. Control sorghum and pearl millet samples were spiked with the respective mycotoxins at expected levels (Tables 5.2 and 5.3), and thereafter subjected to the extraction method and LC-MS/MS analyses. The % recovery was determined by comparing the analytical results for the extracted samples with pure standards that represented 100% recovery.

5.2.5.3 Limit of Quantification (LOQ). In a sample, the LOQ is the lowest quantity of an analyte that can be quantitatively measured with sufficient precision and accuracy. Serial dilutions of the lowest calibration curve concentrations were prepared and analysed. The peak areas were plotted as a function of the analyte concentrations (µg/kg). Analyte response at LOQ was taken as five times the response compared to the blank response.

5.2.5.4 Accuracy. Accuracy was determined by the repeated examination of samples with known amounts of the respective mycotoxins to determine the precision. The relative standard deviation for repeatability (RSDr) was determined relative to the theoretical values of each mycotoxin.

5.2.5.5 Calibration curve. The association between instrument reaction and known concentrations of the analytes is shown by a calibration curve. For compiling matrix-matched calibration curves, four to nine working standard serial dilutions were prepared with blank extract as solvent, as described above. Following LC-MS/MS analyses, the peak areas (LC-

MS response factors) were plotted as a function of the analyte concentrations (µg/kg). The correlation coefficients and regression equations were determined for each calibration curve.

5.2.5.6 Quality control samples. In order to verify the precision and accuracy of the analytical method, certified quality control samples [FAPAS (Cat no T22110QC) and Biopure (Cat no QCM3C2)] containing the mycotoxins at the expected concentrations ranges were included.

5.2.6 Correlation between *Fusarium* spp. occurrence and mycotoxin levels

Correlations were determined between the concentrations of the respective multiple mycotoxins and levels of *F. verticillioides*, *F. graminearum*, *F. proliferatum* and *F. subglutinans* in samples as determined with a validated quantitative Real-time PCR method (qPCR) (Chapter 4).

5.2.7 Statistical analyses

The NCSS Version 11 (NCSS, 2019) software was used for statistical analysis. Data was analysed within a generalised linear model ANOVA. $P<0.05$ was used as statistical significance. Correlation coefficients (R) were determined using R software, version 4.02 (Harris, 2018). The fungal genomic DNA values used in this exercise were obtained by using qPCR (Chapter 4).

5.3 Results and discussion

Smallholder farmers in northern Namibia utilize undiversified diets due to drought conditions and are heavily reliant on sorghum and pearl millet as a staple foodstuff (Misihairabgwi et al., 2018). The Oshakati smallholder farming communities produce sorghum and pearl millet for home consumption and service both the Oshakati and Ondangwa open markets in the Oshana region of northern Namibia. Despite strict international regulations, mycotoxins contaminating grains are not regulated in Namibia. This could establish situations where exposures to mycotoxin surpass the thresholds set by regulatory bodies for health. Communities that are the most affected include smallholder farmers where mycotoxin levels are not monitored, as mono-cereal crops are cultivated and consumed locally.

In this study, contamination of raw and processed sorghum and pearl millet with multiple mycotoxins was evaluated in northern Namibia. Planting of grain crops takes place during November and harvesting during June and July of the next year. Sorghum and pearl millet heads are left to dry on the field before harvest. Postharvest, clusters of grains are thrashed, winnowed and kept in storage baskets for extended periods. Wood ash is added as an

antimicrobial agent to whole grains during storage (Oguntade and Adekunle, 2010). The raw grains are ground into flour and used to prepare porridge, confectionary, and alcoholic and non-alcoholic beverages. Production of malted sorghum and pearl millet involves cleaning, soaking in water (42-46% moisture content) and germination for 4-5 days, which produces solubilized nutrients and results in an increase in temperature (Agbor Asuk et al., 2020). Raw and processed sorghum and pearl millet samples were collected postharvest respectively from smallholder farmers in the Oshakati region, and from open markets in Oshakati and Ondangwa. The levels of multiple mycotoxins in samples were determined using a validated LC-MS/MS analytical technique.

The LC-MS/MS analytical specifications for each mycotoxin are summarised in Table 5.1. The results of the analytical validation experiments concerning sorghum and pearl millet are summarized in Tables 5.2 and 5.3, respectively. LC-MS/MS chromatograms of the respective analytical standards were separately compared with chromatograms and data of the blank sorghum and millet matrixes as well as selected samples. The results indicated that the blank sorghum and pearl millet contained no mycotoxins. Selectivity of the method was confirmed by the absence of co-eluting peaks (Figure 5.1). The percentage recoveries for the individual mycotoxins (68-95%) remained constant between runs. Results indicated that the extraction of FB_1 and FB_2 was more effective ($P<0.05$) from pearl millet than from sorghum. The LOQ levels for AFB_1 (2 µg/kg), OTA (20 µg/kg), DON (100 µg/kg), ZEA (10 µg/kg), NIV (10 µg/kg) and MON (10 µg/kg) were the same for sorghum and pearl millet, while the LOQ levels for FB_1, FB_2 and FB_3 (5-20 µg/kg) were higher in sorghum than in pearl millet (2-10 µg/kg). These differences could be attributed to the polarity of the extraction solvent in relation to the solubility of the mycotoxins in the grains. Replicate analysis of samples containing known amounts of the respective mycotoxins, resulted in means within 15% from the theoretical values, confirming the accuracy of the method. For increased accuracy, matrix-matched calibrations curves were prepared in order to correct the ionization influence of the sorghum and pearl millet matrixes (Alberts et al., 2019). Coefficients of determination (R^2), which indicates the degree of linearity of the respective mycotoxins' calibration curves, were >0.993 (Figure 5.2). Mycotoxin concentrations in the FAPAS and Biopure certified quality control samples were within the ranges specified by the supplier for each mycotoxin during each LC-MS/MS run.

Table 5.1 LC-MS/MS conditions for quantification of multiple mycotoxins by positive ESI at capillary voltage 3.5 kV

Analyte	Cone Voltage (V)	Precursor Ion	Quantifier Ion (Collision Energy) (V)	Qualifier Ion (Collision Energy) (V)
Aflatoxin B_1	50	313	285 (23)	241 (37)
Fumonisin B_1	50	722.3	334.3 (40)	352.3 (38)
Fumonisin B_2 and B_3	50	706.3	318.3 (40)	336.3 (40)
Ochratoxin A	22	404.2	239 (24)	221 (40)
Deoxynivalenol	35	397.1	203.2 (15)	231.2 (12)
Zearalenone	20	319.1	185.0 (23)	187.0 (19)
Nivalenol	15	313.2	175 (25)	295 (8)
Moniliformin	30	97	41 (18)	N/A

N/A, not applicable

Table 5.2 Validation parameters of the analytical method for quantification of multiple mycotoxins in sorghum

Analyte	Spike level (µg/kg)	Recovery (%)	LOQ (µg/kg)	RSDr (%)	Coefficient of determination (R^2)
Aflatoxin B_1	10	95	2	1	0.9949
Fumonisin B_1	1000	86	5	2	0.9940
Fumonisin B_2	500	68	20	4	0.9950
Fumonisin B_3	500	76	20	1	0.9958
Ochratoxin A	10	82	20	1	0.9949
Deoxynivalenol	500	86	100	4	0.9975
Zearalenone	200	91	10	3	0.9938
Nivalenol	500	87	10	2	0.9999
Moniliformin	500	85	10	3	0.9959

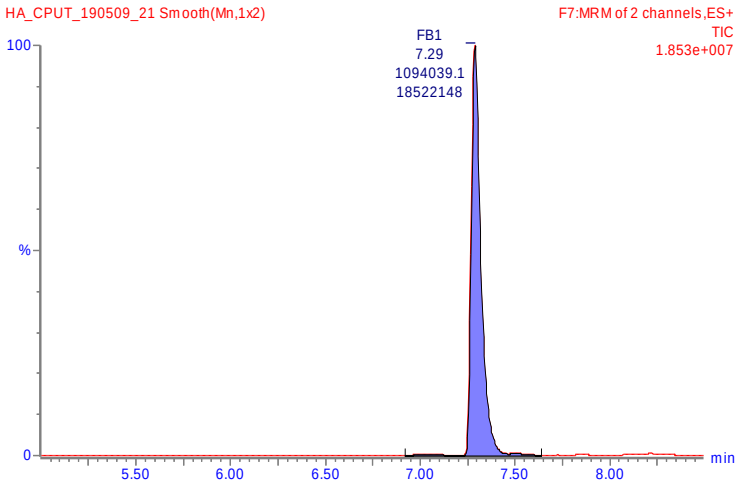
LOQ, Limit of quantification; RSDr, Relative standard deviation for repeatability; Spike level, supplementation of control grain with a mycotoxin to a specific concentration level

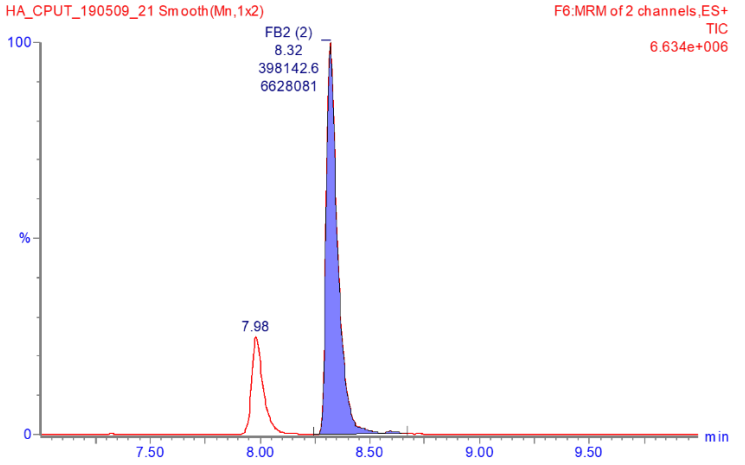
Table 5.3 Validation parameters of the analytical method for quantification of multiple mycotoxins in pearl millet

Analyte	Spike level (µg/kg)	Recovery (%)	LOQ (µg/kg)	RSDr (%)	Coefficient of determination (R^2)
Aflatoxin B_1	10	95	2	2	0.9940
Fumonisin B_1	1000	90	2	1	0.9975
Fumonisin B_2	500	70	10	3	0.9951
Fumonisin B_3	500	80	10	2	0.9963
Ochratoxin A	10	83	10	2	0.9989
Deoxynivalenol	500	85	100	4	0.9980
Zearalenone	200	90	10	2	0.9990
Nivalenol	500	86	10	1	0.9947
Moniliformin	500	85	10	1	0.9943

LOQ, Limit of quantification; RSDr, Relative standard deviation for repeatability; Spike level, supplementation of control grains with a mycotoxin to a specific concentration level

A

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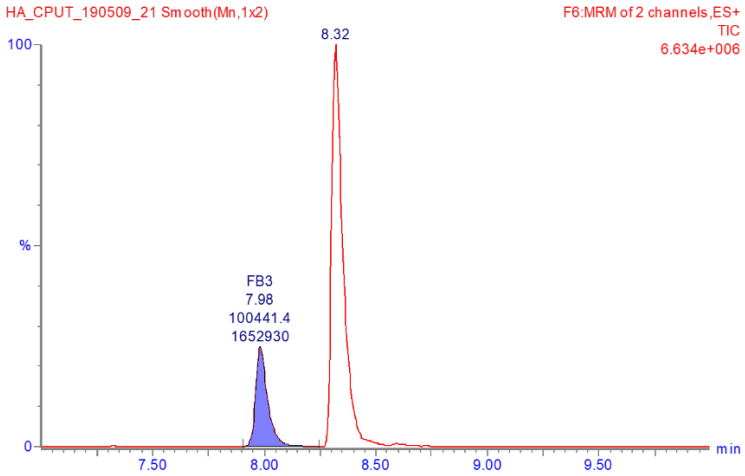
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100
%
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min
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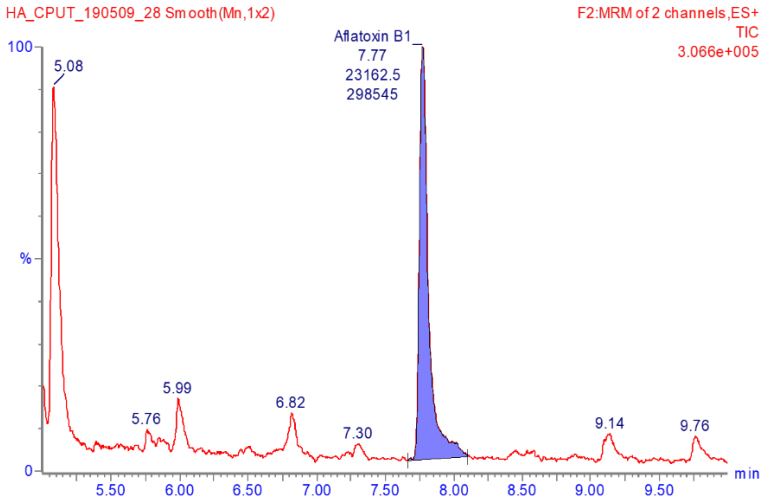
8.00
8.50
9.00
9.50

B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298545
5.08
5.99
5.76
6.82
7.30
9.14
9.76
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9.00
9.50

C

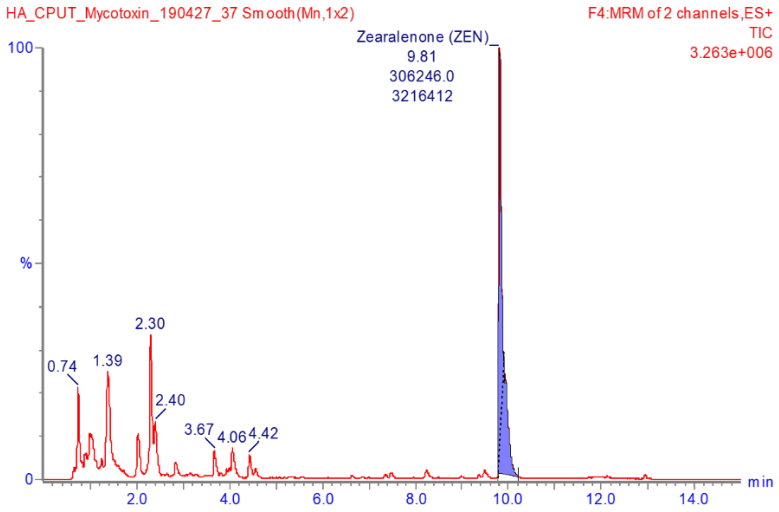


Figure 5.1 LC-MS/MS chromatograms presenting in blue: A, fumonsin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) detected in pearl millet bran sample NAM-17; B, aflatoxin B_1 (AFB_1) detected in pearl millet malt sample NAM-20, and C, zearalenone (ZEA) detected in sorghum malt sample NAM-24. Fumonisins B_1 and B_2 were also detected in NAM-24 (chromatograms not shown)

No mycotoxins were detected in any of the raw sorghum and pearl millet samples collected from 10 households of smallholder farmers in Oshakati (Table 5.4). This could mainly be attributed to good agricultural practices. Wood ash added to grains could have effectively limited fungal contamination during storage, by increasing the pH and reducing water activity (a_w) (Tangni and Larondelle, 2014). The hot climatic conditions would further assist in reducing a_w levels. The grain is stored in airtight plastic containers and woven baskets, which are plastered with clay, thereby reducing aeration and microbial activity. The non-detectible levels of mycotoxins in the raw grain samples do not necessarily imply that mycotoxigenic fungi are absent. Fungal species may be present in low cell numbers or in a dormant state. The combined antimicrobial properties of wood ash and the low a_w during storage keeps *Fusarium* spp. spores dormant. *Fusarium* spp. are considered field fungi and require an a_w of 0.98 to 0.995 for growth (Tangni and Larondelle, 2014).

Processed samples collected at open markets in Oshakati and Ondangwa contained mycotoxins (AF and FB) regulated by the European Union (EU) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (ZEA) (Table 5.5). *Aspergillus flavus* and *A. parasiticus* are known to contaminate grains during storage with subsequent production of

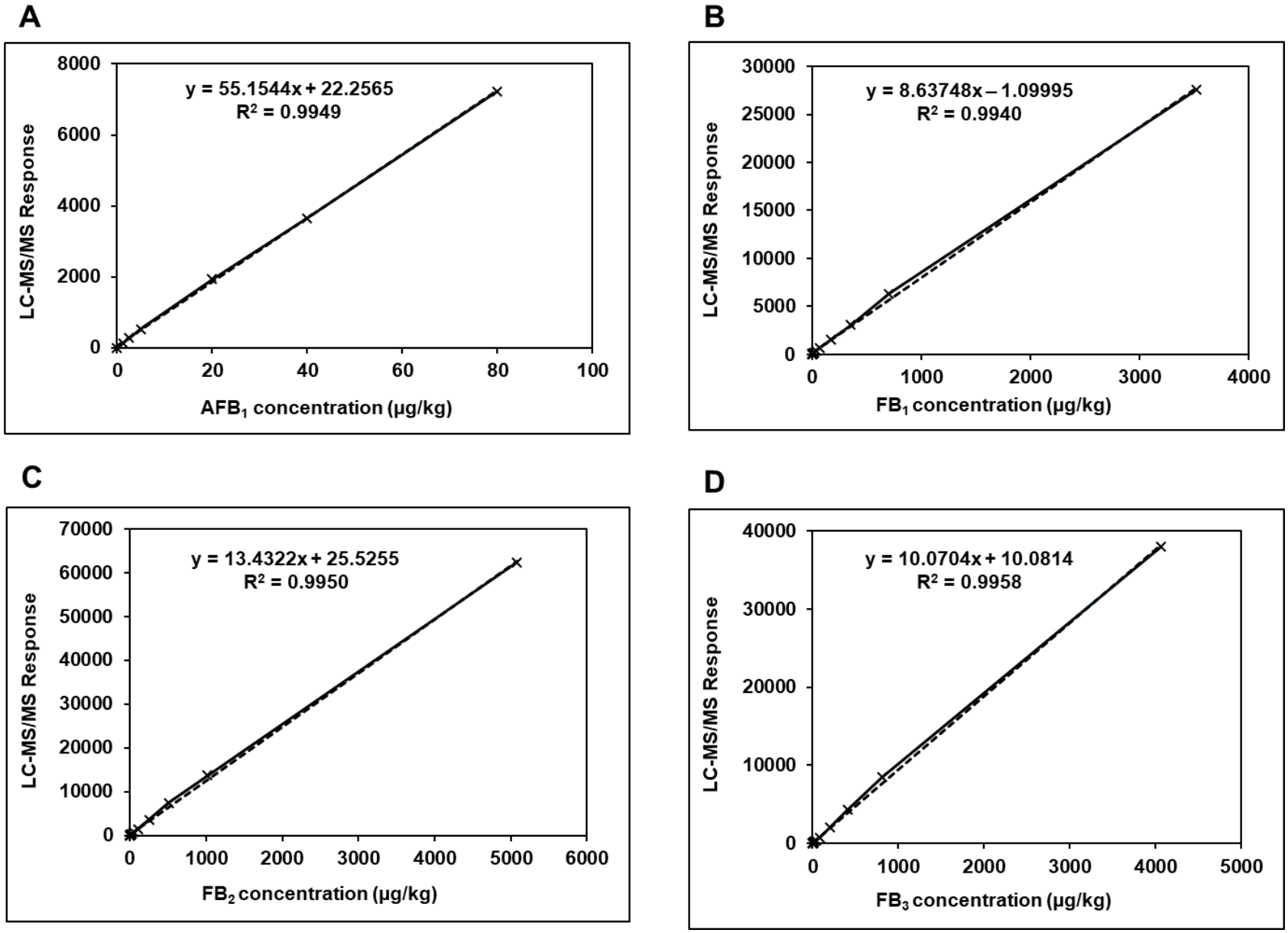
carcinogenic aflatoxins (Tangni and Larondelle, 2014). In the current study, 20 and 54% of processed sorghum and pearl millet samples, respectively, contained AFB_1 (3-14 µg/kg). 17% of all bran and malts contained AFB_1 at levels above the regulatory maximum level of 5 µg/kg set by the European Commission (Bessaire et al., 2019), with one sample containing 14 µg/kg. Aflatoxins is classified a Group 1 carcinogen by the Agency for Research on Cancer (IARC) and poses a serious threat to human and animal health by causing hepatotoxicity, teratogenicity, immunotoxicity as well as liver cancer (Pitt, 2012).

FB_1 (33%), FB_2 (10%) and FB_3 (5%) were detected in sorghum and pearl millet bran and malt samples at concentrations below EU regulatory levels of 1000 µg/kg (Table 5.5). 9% of bran and malt samples, however, exceeded the fumonisin regulatory level (200 µg/kg) set by the EC for infants and young children. FB_1 is classified a Group 2B carcinogen by IARC and is associated with neural tube defects, stunting in children and oesophageal cancer (Alberts et al., 2019) 4% of processed samples contained ZEA at levels far above recommended EC levels (100 µg/kg) (Ferrigo et al., 2016). JECFA has established a provisional maximum tolerable daily intake (PMTDI) for ZEA of 0.5 µg/kg of body weight (Burger et al., 2014). ZEA is an estrogenic mycotoxin affecting male and female reproductive systems. Contamination by ZEA is mostly caused by *F. graminearum, F. equiseti, F. culmorum, F. cerealis* and *F. semitectum* and ZEA contamination often co-occurs with DON (Ferrigo and., 2016). However, there were non-detectible levels of DON contamination in the processed samples. Co-occurrence of FB_1, FB_2, FB_3 and ZEA was detected in both sorghum and pearl millet malts. Co-exposure to numerous mycotoxins of young children in particular, is of significant concern (Alberts et al., 2017). Continued exposure to low concentrations of mycotoxins is also a risk factor for human diseases as it is linked to the development of tumours, neural tube defects as well as childhood stunting.

The correlation matrix indicated a strong correlation (R = 0.8-0.83) between levels of *F. verticillioides* and *F. proliferatum* determined with a validated qPCR method (Chapter 4) and FB_1, FB_2, and FB_3 concentrations (Figure 5.3). There was no correlation between the respective *Fusarium* spp. and DON, ZEA, NIV and MON levels. These results confirm the efficacy of these methods for evaluating specific *Fusarium* spp. and multiple mycotoxin contamination in grain samples. No correlation was observed between levels of *Fusarium* spp. detected with morphological methods and mycotoxin levels (data not shown).

The moisture and temperature conditions during malting provide an ideal environment for fungi to proliferate, and could lead to an exponential increase in mycotoxin concentrations (Agbor Asuk et al., 2020). Contamination may be further enhanced by microflora originating from

home-based malting plants (Noots et al., 1999). The lack of sanitary ware, non-sterilization of equipment and lack of knowledge could result in increased mycotoxin levels. By critically monitoring the grain production process from planting, through to harvesting, processing and marketing, the sources of contamination and critical control points could be identified and managed. Community specific mycotoxin awareness and sustainable education will greatly contribute to reducing mycotoxin contamination during processing of sorghum and pearl millet. This could involve peer-to-peer training to improve awareness and knowledge, the introduction of community-based mycotoxin reduction methods such as washing of grains and the dissemination of community-specific good agricultural and storage practices (Alberts et al., 2017). Understanding the impact of mycotoxins on human health is critical to further improve the management processes through the development of informed policy strategies and eventually the implementation of regulations. This could ultimately contribute to food safety and security in northern Namibia where communities are exposed to multiple mycotoxins in their staple diet.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AFB1 concentration (µg/kg)
B
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R² = 0.9940
LC-MS/MS Response
FB1 concentration (µg/kg)
C
y = 13.4322x + 25.5255
R² = 0.9950
LC-MS/MS Response
FB2 concentration (µg/kg)
D
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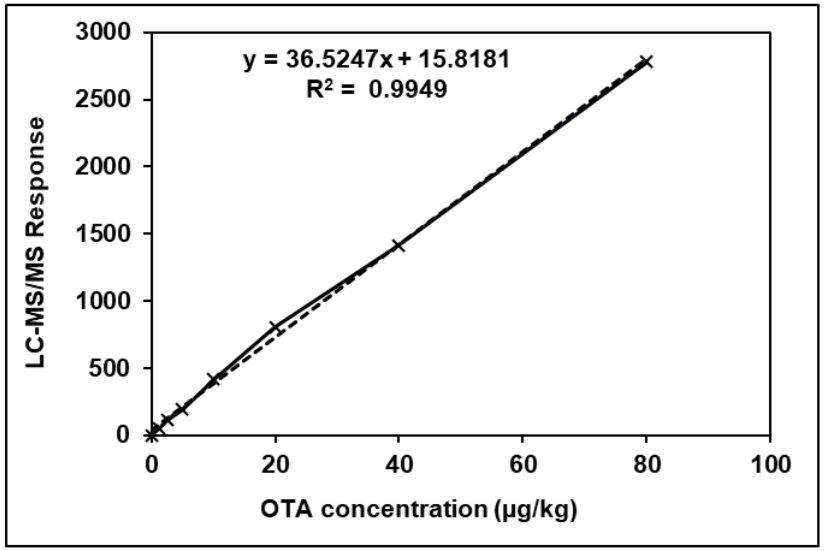
LC-MS/MS Response
FB3 concentration (µg/kg)

E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R² = 0.9949
LC-MS/MS Response
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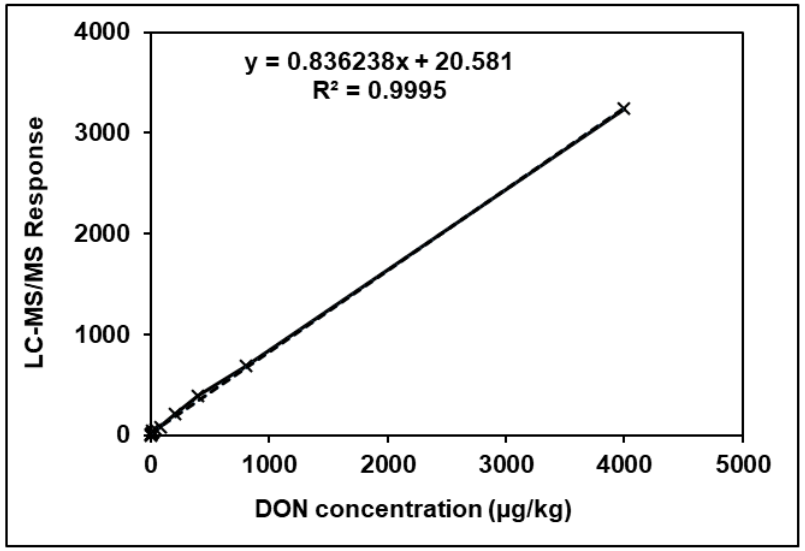
1500
2000
2500
3000
0
20
40
60
80
100

F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R² = 0.9995
LC-MS/MS Response
DON concentration (µg/kg)
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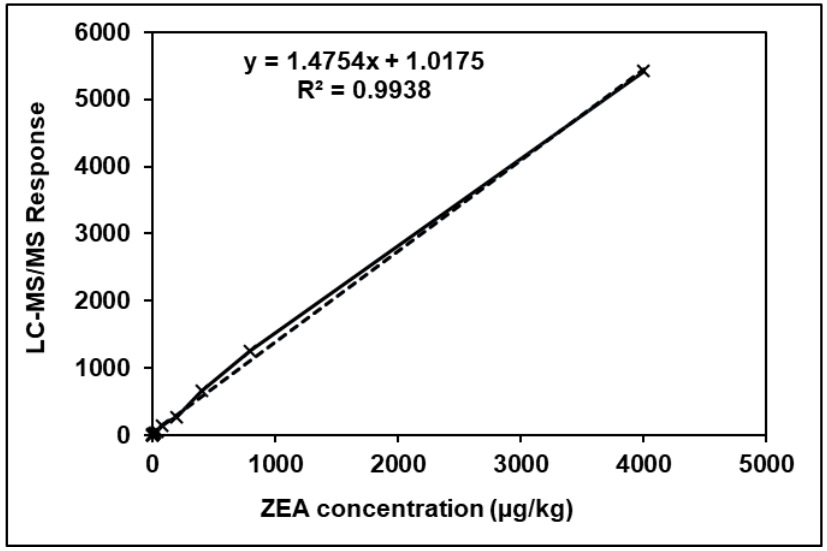
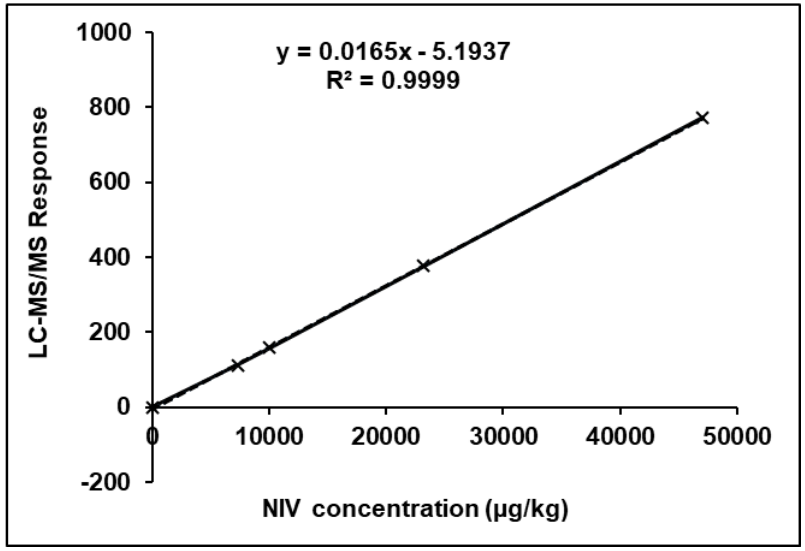
1000
2000
3000
4000
0
1000
2000
3000
4000
5000

G


y = 1.4754x + 1.0175
R² = 0.9938
LC-MS/MS Response
ZEA concentration (µg/kg)
0
1000
2000
3000
4000
5000
6000
0
1000
2000
3000
4000
5000

H


y = 0.0165x - 5.1937
R² = 0.9999
LC-MS/MS Response
NIV concentration (µg/kg)
-200
0
200
400
600
800
1000
0
10000
20000
30000
40000
50000

I

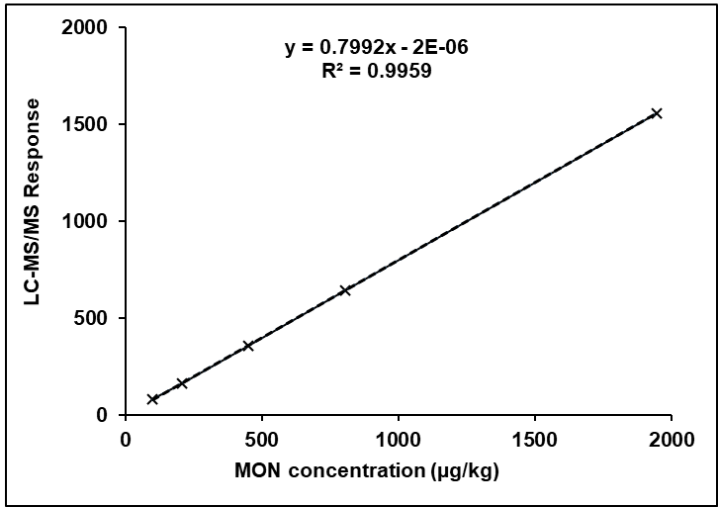


Figure 5.2 Matrix-match calibration curves, indicating regression equations and coefficients of determination (R^2). A, aflatoxin B_1 (AFB_1); B, fumonisin B_1 (FB_1); C, fumonisin B_2; D, fumonisin B_3; E, ochratoxin A (OTA); F, deoxynivalenol (DON); G, zearalenone (ZEA); H, nivalenol (NIV); I, moniliformin (MON). Solid lines represent actual values. Dashed lines indicate trend lines

Table 5.4 Concentrations (µg/kg) of multiple mycotoxins present in raw sorghum and pearl millet samples collected from households of smallholder farmers in Oshakati

Sample no.	Sample description	Sample type	Sampling location	AFB_1	FB_1	FB_2	FB_3	OTA	DON	ZEA	NIV	MON
NAM-1S	Sorghum	Raw grain	N1	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-1M	Pearl millet	Raw grain	N1	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-2S	Sorghum	Raw grain	N2	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-2M	Pearl millet	Raw grain	N2	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-3S	Sorghum	Raw grain	N3	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-3M	Pearl millet	Raw grain	N3	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-4S	Sorghum	Raw grain	N4	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-4M	Pearl millet	Raw grain	N4	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-5S	Sorghum	Raw grain	N5	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-5M	Pearl millet	Raw grain	N5	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-6S	Sorghum	Raw grain	N6	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-6M	Pearl millet	Raw grain	N6	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-7S	Sorghum	Raw grain	N7	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-7M	Pearl millet	Raw grain	N7	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-8S	Sorghum	Raw grain	N8	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-8M	Pearl millet	Raw grain	N8	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-9S	Sorghum	Raw grain	N9	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-9M	Pearl millet	Raw grain	N9	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-10S	Sorghum	Raw grain	N10	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-10M	Pearl millet	Raw grain	N10	ND	ND	ND	ND	ND	ND	ND	ND	ND

Sampling locations (N1-N10) are indicated on the geographical map of Namibia (Chapter 3, Figure 3.1). AFB_1, aflatoxin B_1; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; OTA, ochratoxin A; DON, deoxynivalenol; ZEA, zearalenone; NIV, nivalenol; MON, moniliformin; ND, none detected

Table 5.5 Concentrations (µg/kg) of multiple mycotoxins present in processed sorghum and pearl millet samples collected from open markets in Oshakati and Ondangwa

Sample no.	Sample description	Sample type	Sampling location	AFB_1	FB_1	FB_2	FB_3	OTA	DON	ZEA	NIV	MON
NAM-11	Sorghum	Malt	OSH M	ND	18 ± 0.00	ND	ND	ND	ND	ND	ND	ND
NAM-12	Sorghum	Malt	OSH M	11 ± 0.88	ND	ND	ND	ND	ND	ND	ND	11
NAM-13	Sorghum	Malt	OSH M	ND	69 ± 0.25	<LOQ	ND	ND	ND	ND	ND	ND
NAM-14	Sorghum	Malt	OSH M	ND	15 ± 0.18	ND	ND	ND	ND	ND	ND	ND
NAM-15	Sorghum	Malt	OSH M	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-16	Sorghum	Malt	OSH M	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-17	Pearl millet	Bran	OSH M	<LOQ	562 ± 22.65	117 ± 4.97	38 ± 1.31	ND	ND	ND	ND	ND
NAM-18	Pearl millet	Malt	OSH M	6 ± 0.69	ND	ND	ND	ND	ND	ND	ND	ND
NAM-19	Pearl millet	Malt	OSH M	5 ± 0.75	ND	ND	ND	ND	ND	ND	ND	ND
NAM-20	Pearl millet	Malt	OSH M	14 ± 1	ND	ND	ND	ND	ND	ND	ND	ND
NAM-21	Pearl millet	Malt	OSH M	4 ± 0.35	ND	ND	ND	ND	ND	ND	ND	ND
NAM-22	Pearl millet	Malt	OSH M	4 ± 0.35	ND	ND	ND	ND	<LOQ	ND	ND	ND
NAM-23	Pearl millet	Malt	OSH M	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND
NAM-24	Sorghum	Malt	ONDW M	ND	245 ± 15.56	42 ± 3.77	<LOQ	ND	ND	3184 ± 412.35	ND	ND
NAM-25	Sorghum	Malt	ONDW M	3 ± 0.15	ND	ND	ND	ND	ND	ND	ND	ND
NAM-26	Sorghum	Malt	ONDW M	ND	63 ± 0.19	<LOQ	<LOQ	ND	ND	ND	ND	ND
NAM-27	Sorghum	Malt	ONDW M	ND	73 ± 15.64	<LOQ	<LOQ	ND	ND	19 ± 7.26	ND	ND
NAM-28	Pearl millet	Bran	ONDW M	3 ± 0.42	ND	ND	ND	ND	ND	ND	ND	ND
NAM-29	Pearl millet	Malt	ONDW M	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND
NAM-30	Pearl millet	Malt	ONDW M	ND	ND	ND	ND	ND	ND	ND	ND	ND
NAM-31	Pearl millet	Malt	ONDW M	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND
NAM-32	Pearl millet	Malt	ONDW M	4 ± 0.27	ND	ND	ND	ND	<LOQ	ND	ND	ND
NAM-33	Pearl millet	Malt	ONDW M	ND	ND	ND	ND	ND	ND	ND	ND	ND

Values represent means ± standard deviations of three replicates. AFB_1, aflatoxin B_1; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FB_3, fumonisin B_3; OTA, ochratoxin A; DON, deoxynivalenol; ZEA, zearalenone; NIV, nivalenol; MON, moniliformin; OSH M, Oshakati market; ONDW M, Ondangwa market; LOQ, limit of quantification; ND, none detected

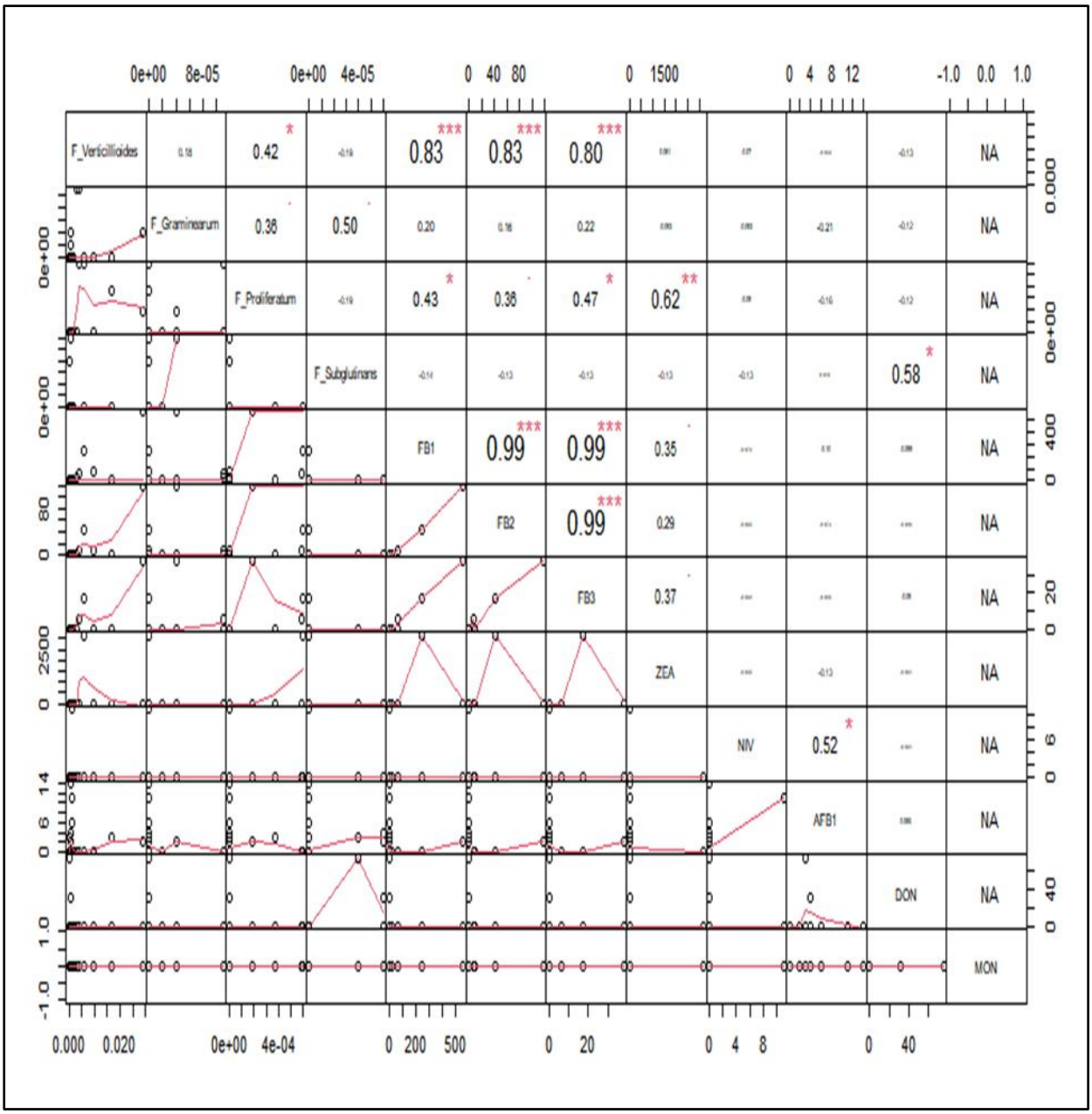


Figure 5.3 Correlation matrix indicating correlation coefficients (R) obtained between *F. verticillioides, F. graminearum, F. proliferatum, F. subglutinans,* Fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), zearalenone (ZEA), nivalenol (NIV), aflatoxin B_1 (AFB_1), deoxynivalenol (DON) and moniliformin (MON) levels

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