Biodiversity and Distribution of *Aspergillus* and Their Toxins in Maize from Western and Eastern Regions of South Africa

Ngum Queenta Nji[1] , Adetunji Modupeade Christianah[2,3,4]* , Ateba Collins Njie[1] ,
Mwanza Mulunda[4]

[1]Department of Microbiology, Faculty of Natural and Agricultural Sciences, North West University, Mafikeng Campus, Mmabatho, South Africa
[2]Department of Biological Sciences, Trinity University, Yaba, Lagos, Nigeria
[3]Food Security and Food Safety Niche Area, Faculty of Agriculture, Science and Technology, North-West University, Mmabatho, South Africa
[4]Department of Animal Health, Faculty of Agriculture, Science and Technology, North West University, Mafikeng Campus, Mmabatho, South Africa
Email: *modupe.adetunji@trinityuniversity.edu.ng

Abstract

Aspergillus species and aflatoxins production are more prevalent during times of high heat and drought. In South Africa, there is frequent occurrence of drought as a result of climate change. The aim of this study was to investigate the biodiversity and distribution of *Aspergillus* species with their corresponding toxins in maize from main maize producing regions of South Africa; [Western Regions (WR) and Eastern Regions (ER)]. One hundred and twenty-three (64 from WR and 59 from ER) maize samples from the two agro-climatic regions in South Africa were analyzed using cultural, molecular and analytical methods. Across agro-climatic regions, *Aspergillus* species contaminated about 62% of the maize samples, while *Aspergillus flavus* was the most prevalent (47.15%) followed by *Aspergillus fumigatus* (4.69%) while the least was *Aspergillus parasiticus* (0.81%). The Western Regions showed a higher distribution of varieties of *Aspergillus* species compared to the Eastern Regions. Aflatoxins contaminated only 27.64% of the maize samples with a mean total aflatoxin concentration of 2.40 μg/kg which is below the South Africa's set standard for total aflatoxin in food (5 μg/kg). About 10.57% of the samples produce aflatoxins above the 5 μg/kg permissible limit for total aflatoxin in foods. The ratio of toxigenic to atoxigenic strains of *Aspergillus flavus* was generally low in all the regions of South Africa. This study could aid policy makers to make informed decisions in developing remediation strategies for *Aspergillus* mycotoxins.

1. Introduction

The filamentous fungus; *Aspergillus flavus* is a cosmopolitan soil-borne saprophytic organism with opportunistic parasitic behaviors to plants, animals and humans. Aflatoxins are metabolites produced mainly by toxigenic strains of *Aspergillus flavus* and *A. parasiticus*, which grow in soil, hay, decaying vegetation and grains [1]. Aflatoxins (AFs) are toxic and carcinogenic to livestock and humans [2] [3] [4]. Contamination by aflatoxin is one of the most serious food and feed safety problems worldwide and causes significant economic losses yearly [5]. Thus, food safety has become a very important issue worldwide and the potential effects of climate change on yields and quality of crops especially for mycotoxins, have received special attention in the past years from a risk analysis perspective [6] [7]. Benkerroum [8] reported past major outbreaks of aflatoxicosis associated with maize-based foods which occurred in West-India (1974), Kenya (1981 and 2004) and Tanzania (2016, 2017). Due to the toxicity and impact of aflatoxins on health, there exist regulatory limits on the quantity of aflatoxins permitted in food and feed in several countries [9] [10].

Climate change plays a major role in the production of AFs in crops by *Aspergillus* spp. [6] [11] [12] [13] [14]. Climate change affects interactions between different mycotoxigenic spp. and toxins produced in foods and feeds [15] [16]. In particular high temperatures and drought stress directly affect maize and the occurrence of *A. flavus* favoring fungal growth, conidiation and spore dispersal, and impairing the growth and development of maize [17]. *Aspergillus flavus* is the dorminant fungal spp. in maize kernels during warm and dry seasons [18] [19] [20] [21] resulting in high levels of aflatoxin contamination of maize in the field [22]. Events related to contamination by aflatoxins are more prevalent during times of high heat and drought which can stress the host plant thereby facilitating infection by *A. flavus* [23].

Mycotoxin toxicity occurs at very low concentrations, therefore sensitive and reliable methods for their detection are required. Different qualitative and quantitative analytical methods having different sensitivity, specificity and accuracy have been developed such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC) liquid chromatography mass spectrometry tandem (LCMS/MS) and many other techniques.

Literature is depleted in information on the distribution and biodiversity of *Aspergillus* species and their toxins in silo stored maize cultivated in different climate regions of the country. Hence, this study aimed at filling the information gap by investigating the biodiversity and distribution of *Aspergillus* species with

their corresponding toxins in maize samples from the different agro-climatic areas of SA. Molecular biomarkers to differentiate the ambiguity between the *Aspergillus* species were employed to identify the different *Aspergillus* species present in SA maize. The presence of the five most important genes in the aflatoxin biosynthesis pathway among *Aspergillus* isolates was also evaluated.

This study will aid policy makers to make informed decisions in developing strategies based on monitoring and characterization of risks, prevention, intervention and remediation strategies for *Aspergillus* mycotoxins, which start from critical points along the production chain such as field, storage, processing and transportation. It will also provide useful information that will enhance global efforts in ensuring production of quality food/feed as well as food security.

2. Materials and Methods

2.1. Sampling Site and Collection of Samples

2.1.1. Sampling Site
Figure 1 The dotted lines divide the maize production areas in the country into Western (North West and Western part of Free State) and Eastern (Guateng and Eastern part of Free State) regions [24]. Maize samples were collected from main
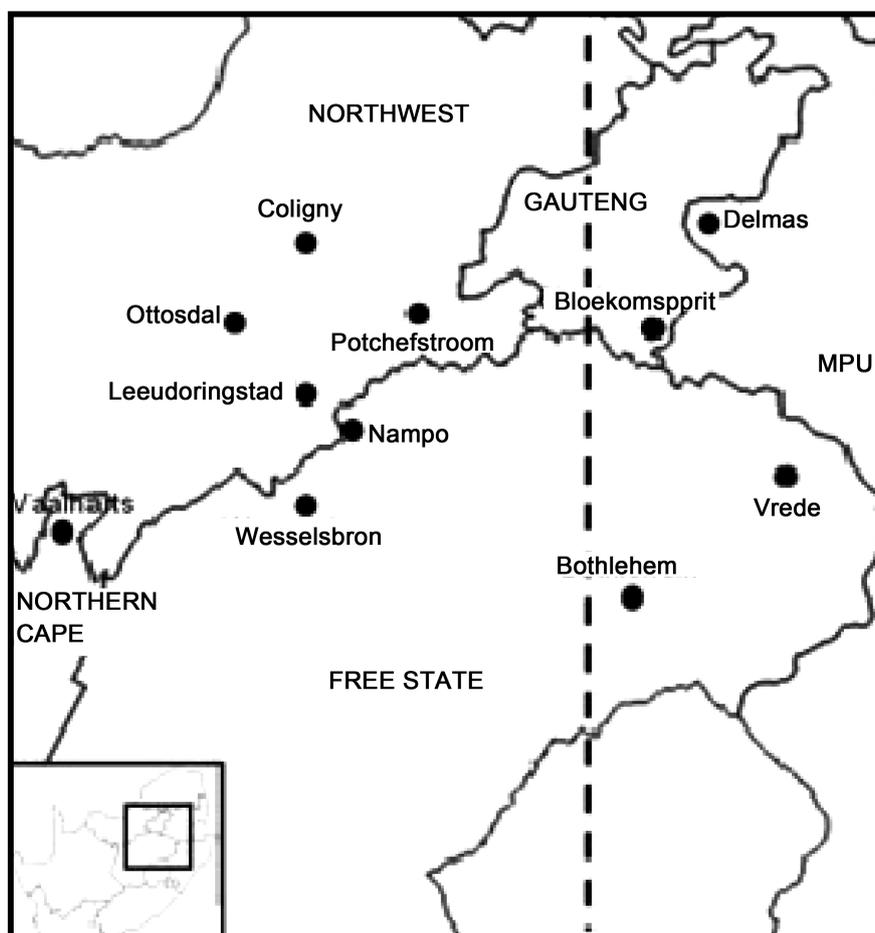

Figure 1. Areas in south Africa where maize samples were collected.

maize producing areas of the different agro-climatic regions of South Africa; Free State (45%), North West (17%) and Guateng (5%) [25]. The Western regions situated in the drier and warmer areas of the country and the Eastern regions with higher rainfall and cooler areas [24]. Samples from these regions were collected from silos managed by SENWESS.

2.1.2. Samples

One hundred and twenty three (123) maize samples were randomly collected from selected silo sites from different agricultural regions of South Africa (Gauteng, North West and Free State). A structured sampling model was used for sampling where 10 kg of maize was taken from different points of the silos while the maize was routinely moved. The collected samples were mixed and 5 kg subsequently collected. The samples were collected separately, finely milled using a warring blender (IKA, Model M20, Germany) and put into sterile zip lock polythene bags, labeled and stored at 4°C prior analysis to arrest any formation of mycotoxins before analysis. Fifty nine samples (59) were from the Eastern region (ER) while sixty four (64) were from the Western region (WR).

2.2. Determination of Moisture Content

The moisture content of samples was determined using the AOAC method [26]. Five grams (5 g) of milled maize samples were dried in an oven (Labcon, Model FSOM4 Maraisburg) at 105°C for 1 hour. Triplicates of each sample were weighed and the initial moisture content (%) calculated as shown in the formula below:

$$\text{Moisture content}(\%) = \frac{(\text{Initial weight} - \text{Final weight})}{\text{Initial weight}} \times 100$$

2.3. Mycobiota Enumeration in Maize Samples

The mycobiota population in the maize samples was enumerated using the dilution plate techniques as described by [27]. Appropriate 10-fold dilutions of milled maize samples in sterile water were spread plated on potato dextrose agar (PDA) and incubated in the dark at 28°C for 72 h. Thereafter, the mycobiota present in each sample was enumerated and reported as colony forming units per gram (cfu/g) of maize.

$$\text{cfu} = \frac{\text{Number of colonies} \times \text{reciprocal of the dilution factor}}{\text{Plating volume}(1\ \text{ml})}$$

The percentage occurrence of each of the isolates was calculated by comparing the ratio of the number of individual organism to the total number of organism present in each sample.

Percentage of appearance (PA) was calculated according to the following formula:

$$\text{PA} = \frac{\text{Number of samples with the same genera or spp.}}{\text{Total number of samples of genera of spp.}}$$

2.4. Isolation and Purification

The previously cultured plates were returned to the incubator for additional 2 - 4 days for isolation of surface sample mycoflora and purification [28] [29]. Purified isolates from various plates were grouped on the basis of their colony morphology (colour and pigmentation of colony) and characteristics of spores (sclerotial production) [30] [31] [32] among others. Actual identification was carried out using the Polymerase Chain Reaction (PCR) method with different primers. A representative from all the various groups was scrapped using a wooden spatula and put in special extraction tubes (lysis tube) prior extraction of DNA.

2.5. Molecular Identification of *Aspergillus* Isolates

Extraction of the genomic DNA of fungal isolates was done using the Zymo Research kit (Zymo-Research fungal/Bacterial Soil Microbe DNA, D6005, USA), following the manufacture's protocols. A reaction volume of 25 μl was used containing 7 μl nuclease-free water, 12 μl PCR Master Mix (Biolabs), 1.5 μl each of oligonucleotide forward and reverse primers (3 μm) and 3 μL template DNA mixed in the PCR tubes [33]. Amplification of the Internal Transcribed Spacer Region (ITS1–5.8S-ITS2- ITS; approximately 550 - 600 bp) of fungal isolates from the maize was done through the Polymerase Chain Reaction (PCR), using the universal ITS1 primer (5'-CTTGGTCATTTA GAGGAAGAAGTAA-3') and ITS 4 (5'-TCC TCC GCT TAT TGA TATGC-3) with intial denaturation at 95˚C for 2 min, 35 cycles of 95˚C for 30 s, 54˚C for 30 s, and 72˚C for 1 min, and an additional 72˚C for 10 min [34]. Species level differentiation of closely related *Aspergillus* species was done by amplification and sequencing of the β-tubulin (benA) and calmodulin (CaM) genes. Camodulin primer (CMD5-5'-CCGAGTACAAGGARGCCTTC-3' and CMD6-5'CCGATR GAGGTCATR ACGTGG-3') with approximately 550 bp amplicon size at 94˚C for 1 min, 35 cycles of 94˚C for 1 min, 55˚C for 30 s, and 72˚C for 1 min, and a final extension of 72˚C for 10 min as the PCR optimization condition [35]. β-tubulin primer *ben A* (ben2f-5-GGTAACCAAATCGGTGCTGCTTTC-3' and bt2b 5'-ACCCTCAGTGTAGTGACCCTTGGC-3') with 500 bp amplicon size and PCR optimization condition with initial denaturation of 94˚C for 3 min, 32 cycles of 94˚C for 45 s, 55˚C for 45 s, and 72˚C for 1 min, and a final extension at 72˚C for 5 min [36]. These primers were commercially synthesised by Inqaba biotechnical Industrial (Pty) Ltd (Pretoria, South Africa). The PCR amplicons were analyzed by electrophoresis on 1 % (w/v) agarose gel using $1 \times$ TAE (Tris acrylamide-EDTA) buffer. The gels were run for about 160 min at 120 V on a 20 cm gel to ensure separation of bands, and then detected by ethidium bromide fluorescence on a UV trans illuminator to confirm the expected size of the amplicons and visualised using Chemi Doc Image Analyzer [37].

Sequencing was conducted on an ABI Prism 3500 XL DNA Sequencer (Applied Biosystems, Foster City, California) at the Inqaba Biotechnical Industrial,

Pretoria, South Africa. Finch TV software version 1.4.0 was used to analyse chromatograms, resulting from sequencing reaction for good quality sequence assurance. The resulting chromatographs were blasted in the NCBI database (http://www.ncbi.nlm.nih.gov) with the Basic Local Alignment Search Tool (BLASTn) for homology in order to identify probable organisms in question [38]. The sequences were later deposited in the GenBank for allocation of accession number.

2.6. Aflatoxigenicity Test of *Aspergillus* Isolates

2.6.1. Cultural Method for Detection of Aflatoxigenic Fungi in Maize

Yeast Extract Sucrose (YES) Agar (yeast extract; 20 g, sucrose; 150 g, agar; 20 g and $MgSO_4$; 0.5 g) was used as described by Criseo, Bagnara [39]. Mycelia plugs of *Aspergillus* isolates were inoculated on petri dishes containing YES medium in duplicates and the isolates incubated unilluminated at 28°C for 3 - 14 days. The YES agar plates checked on the 7th and 14th day for aflatoxin producing ability by the ammonium hydroxide vapour-induced colour change test as described by Jefremova, Ostrý [40]. 2 ml of concentrated ammonium hydroxide solution (W/V, 25%,) was placed on the inside of the lid of the inverted petri dish containing the isolate on YES agar and left for 5 - 10 minutes. Colour change at the reverse side of the agar plate was then observed for those that tested positive. Plates that tested negative were re-examined on the 14th day for colour change.

2.6.2. Thin Layer Chromatographic Method for Qualitative Detection of Aflatoxins Producing Isolates among *Aspergillus* Species

Extraction of aflatoxin from the isolates was done in accordance with Midorikawa, Pinheiro [41] and Yin, Yan [42]. The 7-day old *Aspergillus* isolates on YES agar were divided into two equal halves using sterile surgical blades (one half for TLC analysis while the other half was kept for other analyses). The first half of the agar containing the isolates was scooped into 50 ml centrifuge tubes and 15 - 20 ml of 70% methanol-water (70:30) added to it and kept on a shaker for 30 minutes. Thereafter, it was centrifuged at 5000 rpm for 5 minutes and the extracts decanted into clean tubes. The extracts were then evaporated to dryness by air blowing in the dark evolution chamber. The residues were then reconstituted with 500 μl of 100% methanol. A 20 μl volume of each of the reconstituted extracts were spotted on a 20 × 20 glass backed 250 μm thick silica gel coated TLC plates (Merck KGaA, Darmstadt Germany) and developed in the TLC tank containing the mobile phase; chloroform-ethyl acetate-propane-2-ol (90:5:5, v/v/v). The presence of aflatoxins was determined by viewing the plates under the UV light at 365 nm for the presence of a bright blue or blue green fluorescence at the same migration level with the total aflatoxin standard (Sigma Aldrich) on the silica plate.

2.6.3. Detection of Aflatoxin Producing Gene in *Aspergillus* Isolates

The DNA of suspected *Aspergilla* were examined for the presence of five important aflatoxin-producing genes (*aflR*, *aflJ*, *aflM*, *aflQ*, *aflD* and *omt-A*) present in

the aflatoxin biosynthesis pathway by PCR method, using previously reported primer sets [43] [44]. Optimization of polymerase chain reaction conditions was done separately for the different target genes. A reaction volume of 25 µl was used containing 7 µl nuclease-free water, 12 µl PCR Master Mix, 1.5 µl each of oligonucleotide forward and reverse primers (3 µm) and 3 µL template DNA mixed in the PCR tubes [33]. The PCR amplified products were resolved on 1% agarose gel and stained with ethidium bromide. After electrophoresis, gel was removed from the gel-casting platform and exposed to UV transillumination.

The following primers were used for the detection of the aflatoxin producing gene: Nor primer (Nor 1-5-ACC GCT ACG CCG GCA CTC TCG GCA C-3 and Nor 2-5-GTT GGC CGC CAG CTT CGA CAC TCC G-3 with 400 bp amplicon with the Nor (*AflD*) gene); Ver primer (Ver 1-5-GCC GCA GGC CGC GGA GAA AGT GGT-3 and Ver 2-5-GGG GAT ATA CTC CCG CGA CAC AGC C-3 with 537 bp amplicon with Ver (*AflM*) gene); Omt primer (Omt 1-5-GTG GAC GGA CCT AGT CCG ACA TCA C-3 and Omt 2-5-GTC GGC GCC ACG CAC TGG GTT GGG G-3 with a 797 amplicon size having the gene *OmtA*); *AflR* primer (*AflR* 1-5-TAT CTC CCC CCG GGC ATC TCC CGG-3 and *AflR* 2-5-CCG TCA GAC AGC CAC TGG ACA CGG-3 with 1032 bp amplicon size having the *AflR* gene); and the *AflJ* primer (*AflJ* F-5-TGA ATC CGT ACC CTT TGA GG-3 and *AflJ* R-5-GGA ATG GGA TGG AGA TGA GA-3 with 737 bp amplicon size having the gene *AflJ* gene). The PCR optimization conditions for the primers are presented in Table 1.

2.7. Liquid Chromatographic Analyses for Fungal Isolates

2.7.1. Liquid Chromatography Mass Spectrometry Mass Tandem (LCMS/MS) Parameters

An LC-MS/MS method was designed and validated for the analysis of 123 analytes. A Waters Acquity UPLC system coupled to a Xevo TQS mass spectrometer

Table 1. Polymerase chain reaction conditions for aflatoxin primers.

Primer code (gene)	Pre-denaturation	Denaturation	Annealing	Elongation	
Nor 1&2 (Nor(aflD)	94˚C—10 min	94˚C—1 min	65˚C—1 min	72˚C—2 min (33 cycles)	72˚C—5 min (1 cycle
Ver 1&2 (Ver(aflM)	95˚C—4 min	95˚C—1 min	58˚C—1 min	72˚C—30 sec (30 cycles)	72˚C—10 min (1 cycle
Omt 1&2 (omt)	94˚C—5min	94˚C—1 min	72˚C—2 min	72˚C—2 min (33 cycles)	72˚C—10 min (1 cycle
AflR 1&2 (aflR)	95˚C—4 min	95˚C—1 min	60˚C—1 min	72˚C—30 sec (30 cycles)	72˚C—10 min (1 cycle
AflJ F&R (aflJ)	95˚C—10 min	95˚C—50 sec	58˚C—50 sec	72˚C—2 min (30 cycles)	72˚C—10 min (1 cycle

(Waters, Milford, MA, USA) was used to analyse the samples, equipped with Mass LynxR (version 4.1) and Quan Lynx R (version 4.1) software (Waters, Manchester, UK) for data acquisition and processing. A ZORBAX Eclipse XDB C18-column (1.8 μm, 100 × 2.1 mm) was applied (Agilent Technologies, Diegem, Belgium). The mobile phase consisted of water/methanol (55/45, v/v), adjusted with 9mg potassium bromide and 1ml 65% nitric acid at a flow rate of 0.4 mL/min. The gradient elution programme started at 100% mobile phase. Then, the mobile phase increased with a linear increase to 99% in the 20th minute. Duration of each LC run was 30 minutes including re-equilibration. The capillary voltage was 3 kV and nitrogen applied as spray gas. Source and desolvation temperatures were set at 120˚C and 400˚C respectively. The argon collision gas pressure was 9×10^{-6} bars, cone gas flow was 35 L/h and desolvation as flow was 800 L/h. For increased sensitivity and selectivity, the instrument was operated in the selected reaction monitoring (SRM) mode and two SRM transitions monitored for each analyte. Matrix-matched calibration plots were constructed for the determination of AFB_1, AFB_2, AFG_1 and AFG_2. The limit of detection (LOD) was calculated as three times the standard error of the intercept divided by the slope of the standard curve; the limit of quantification (LOQ) was computed in a similar way except for the standard error, which was by a factor of six. The calculated LOD and LOQ were verified by the signal-to-noise ratio (s/n), which was more than 3 and 10 respectively in accordance with the IUPAC guidelines [45].

2.7.2. High Performance Liquid Chromatography

This is a quantitative determination method for the extraction of aflatoxins. Extraction of aflatoxins from the samples was done using the Easi-Extract Aflatoxin (R-Biopharm Rhone LTD) immuno-affinity columns kits in conjunction with HPLC or LC-MS/MS as described in the product's extraction kit. The total aflatoxin content of the samples was determined and quantified from maize samples with a high performance liquid chromatography column (HPLC) using the Shimadzu corporation model (Kyoto, Japan). LC-20AB liquid chromatography equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, SIL-20A auto sampler, RF-10AxL fluorescence detector, RID-10A refractive index detector and SPD-M20A photodiode array detector linked to LC solutions version 1.22 software release. The chromatographic separation of analytes and standards was performed by passing through a reverse phase Symmetry column C18 (Waters). The oven temperature was maintained at 30˚C. Peak areas and retention times of mycotoxins were used to determine the amount of specific mycotoxins per sample based on those of standard mycotoxins using a calibration curve. To determine AFs, the fluorescence detector RF 10AXL was coupled with a Coring cell (CoBrA cell) (DR Weber Consulting, Germany) as electrochemical cell for the derivatisation of AFs. The mobile phase consisted of water/methanol (55/45, v/v), adjusted with 9 mg potassium bromide and 1 ml 65% nitric acid. The HPLC method used was validated by determining its linear-

ity, accuracy and sensitivity. Linearity was determined by constructing calibration curves from standards of AFB$_1$, AFB$_2$, AFG$_2$, AFG$_2$, total aflatoxin (AF$_{tot}$) and from extracts of blank samples of previously analyzed maize samples that did not contain any of the aflatoxins. Linear range was examined at 3 different concentrations (0.025 μg/ml, 0.25 μg/ml and 2,5 μg/ml). The matrix-matched calibration curves were built by spiking blank samples with selected aflatoxin standards after the extraction process. Calibration curves were constructed by plotting peak areas against concentration and linear functions applied to the calibration curves. Matrix effect (ME) was calculated for each analyte by comparing the slope of the standard calibration curve with the matrix-matched calibration curve for the same levels of concentration. Sensitivity of the methodology or system used was evaluated by limit of detection (LOD) and limit of quantification (LOQ), estimated for a signal-to-noise ratio (S/N) ×3 and ×10 respectively from chromatograms of samples spiked at the lowest level validated. Accuracy was evaluated through recovery studies and determined by calculating the ratio of the peak areas for each aflatoxin by analyzing the samples spiked before and after extraction at three additional levels of 25, 50, and 100 μg/kg for all aflatoxins analyzed (AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$ AF$_{tot}$). Quantification of the toxins was performed by measuring peak areas, retention time and comparing them with the relevant standard calibration curves.

3. Results

3.1. Moisture Content and Mycobiota Count in Maize Sample

The moisture content of the maize samples ranged from 7.38% - 8.84% across both regions, with ranges of 7.38% - 8.81% and 7.64% - 8.84% for ER and WR respectively. The mean moisture content for maize in both regions (8.30% and 8.26%) is not significantly different (p > 0.05) from one another (Table 2).

Mycobiota count in the maize ranged from 0 - 48 cfu/g and 0 - 23 cfu/g for WR and ER respectively, with mean count in WR (9.75 cfu/g) higher than those obtained in the ER (5.93 cfu/g). Sample 045/M/2015 from ER had the highest *Aspergillus* spp. load (9 cfu/g), with fungal load ranging from 0 - 7.33 cfu/g and 0 - 9 cfu/g for WR and ER respectively (Table 2).

Table 2. Mean moisture content, fungal and *Aspergillus* load in maize samples.

	Regions	Range	Mean ± SD
Moisture (%)	WR (n = 64)	7.64 - 8.84	8.30 ± 0.27
	ER (n = 59)	7.38 - 8.81	8.26 ± 0.26
Fungi (cfu/g)	WR (n = 64)	0 - 48.00	9.75 ± 9.55
	ER (n = 59)	0 - 23.00	5.93 ± 14.76
Aspergillus (cfu/g)	WR (n = 64)	0 - 7.33	0.82 ± 6.05
	ER (n = 59)	0 - 9.00	1.09 ± 6.42
SD = Standard deviation			

3.2. Mycobiota Distribution in Maize from Different Regions

A total of 123 maize samples were analyzed (WR; 64 and ER; 59). Fungi spp. belonging to eight genera; *Fusarium*, *Aspergillus*, *Penicillium*, *Rasamsonia*, *Talaromyces*, *Paecilomyces*, *Byssochlamys* and *Verticillium* were isolated from the maize and four distinct genera *Talaromyces*, *Paecilomyces*, *Byssochlamys*, *Verticillium* found in maize were from the ER (Table 3). *Fusarium* spp. had the highest distribution while *Penicillium* spp. remained the least distributed among other fungal spp. from both regions (Figure 2).

Table 3. Frequency of Mycobiota distribution in maize.

| Fungi isolate	No. of infected samples from each Region		Frequency of isolation (%) from each Regions		Total No. of infected samples	Total frequency of isolation (%)
	Western Region (n = 64)	Eastern Region (n = 59)	Western Region (64)	Eastern Region (59)	(n = 123)	(n = 123)
Aspergillus spp.	47	29	73.44	49.15	76	61.79
Fusarium spp.	52	44	81.25	74.58	96	78.05
Penicillium spp.	12	3	18.75	5.08	15	12.2
Other Fungi	31	10	48.44	16.95	41	33.33
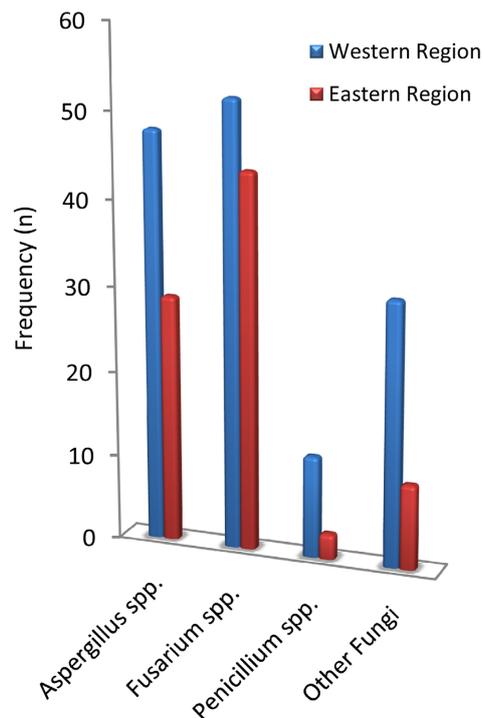
Figure 2. Fungi distribution in maize in different regions.

3.3. Molecular Identification of *Aspergillus* Isolates

Out of the total 123 samples, 76 (29 from ER and 47 from WR) were morphologically identified as *Aspergillus* isolates and these represented 11 Operational Taxonomic Unit (OTU) and four different *Aspergilli* sections; *Aspergillus* flavi section (*A. flavus, A. parasiticus, A. oryzae*); *Aspergillus* nigri section (*A. niger, A. awamori, A. brasiliensis, A. luchuensis, A. welwitschiae*); *Aspergillus* fumigate section (*A. fumigatus*) and *Aspergillus* usti section (*A. insuetus, A. ustus, A. aff. ustus, A. mnutus*) by matching the sequences of the isolates with ITS sequences in the database, as well as those of partial *benA* and *caM* genes of closely related type strains in the GenBank (Table 4). Accession number were then assigned to each isolates after successful submission to the genbank. *Aspergillus* spp. contaminated 61.79% of the maize samples with *Aspergillus flavus* being the most frequent (WR; 73.44%, ER; 49.15%.) in both region.

Table 4. Molecular identification of *Aspergillus* isolates.

Group	Isolates	Description	Fungal ID (ITS)	Fungal ID (CMD)	Fungal ID (BT2)
1	22, G275, 76, 100, 24, 40, 15, 55, 74, 66, 68, 50, 17, 51, 108, 58, 57, 117, 49, 27, 16, 29, 105, 107, 87, 102, 91, 5	Greenish colony without scerotia	*A. flavus* *A. oryzae*	*A. flavus*	*A. flavus*
2	108, 59, 36, 111, 113, 110, 1, 192, 48, 32, 6, 54, 37, 67, 109, 72, 51, 94, 84, 95, 62, 2,	Greenish yellow colony with scerotia	*A. oryzae*	*A. flavus*	*A. flavus*
3	G208, 47, 88, 26, 13, 35, 31, 21, 118, 46, 119, 114, 53, 99, 40, 107, 10, 70, 6, 101, 45, 16, 64, 80, 82, 104	Greenish brown colony with timidly visible scerotia	*A. flavus*	*A. flavus*	*A. flavus*
4	38	Dark green	*A. parasiticus*	*A. parasiticus*	*A. parasiticus*
5	108, 16	Greenish thallus structure	*A. aff. ustus* *A. ustus* *A. insuetus*	*A. insuetus* *A. mnutus*	*A. insuetus*
6	27, 119	Blue grey	*A. fumigatus*	*A. fumigatus*	*A. fumigatus*
7	119, 64, 19, 6, 87, G218, 103, 111, 70	Dark green to grey	*A. fumigatus*	*A. fumigatus*	*A. niger* *A. brasiliensis*
8	111, 12, 42	Dark with yellow tones on the reverse side	*A. niger*	*A. niger*	*A. niger* *A. awamori*
9	108, 1, 70	Black	*N/A*	*A. luchuensis*	N/A
10	115, 45, 80, 84, 2	Black	*A. niger*	*A. welwitschiae*	*A. niger*
11	58, 95	Dark brown to black	*A. brasiliensis* *Aspergillus sp.*	*A. brasiliensis*	*Rasamsonia columbiensis*

3.4. Frequency of Isolation of *Aspergillus* Isolates

Thirteen different *Aspergillus* spp. were isolated from the 11 OTUs group out of which only six were found in maize from the ER while all the spp. were found in maize from the WR in varying loads, spp. such as *A. parasiticus*, *A. insuetus*, *A. awamori*, *A. mnutus*, *A. ustus* as well as *A. aff. ustus* were not found in Eastern regions (Table 5). Amongst the *Aspergillus* spp. isolated, *A. flavus* was the most common spp with the highest frequency of isolation from both regions (47.15%) as compared with other spp. *Aspergillus parasiticus* had the least frequency of isolation (0.81%) and was found only in the WR.

3.5. Incidence of Aflatoxin Producing Genes in *Aspergillus* Species

The result of screening for aflatoxin producing genes in the *Aspergillus* section flavi revealed that none of the isolates from both regions possessed the AflR gene (Figure 3(a)). The hierarchy of distribution of the other genes screened for in isolates from WR and ER is; *aflM* (33%) > *aflJ* (19%) > *aflD* (8%) > *Omt-A* (3%) and *aflM* (17%) > *aflJ* (7%) > *aflD* (7%) > *Omt-A* (2%). The aflatoxin producing ability of the isolates was also confirmed with the cultural and TLC methods. Only 14% and 5% of the isolates in WR and ER respectively tested positive using the cultural NRDCA method and 6.25% and 1.7% with TLC method (Figure 3(b)). A 9.09% of the isolates from both region tested positive for aflatoxigenicity using the polyphasic approach (molecular, cultural and TLC), 20.45% were positive for two methods while 70.45% were positive for at least one of the methods.

Table 5. Percent frequency of isolation of *Aspergillus* isolates across different regions.

| *Aspergillus* isolate	Total number sample (n = 123)	Frequency of isolation (%)	Eastern Region		Western Region	
			Sample (n = 59)	Frequency of isolation (%)	Sample (n = 64)	Frequency of isolation (%)
A. flavus (*A. oryzae*)	58	47.15	21	35.59	37	57.81
A. fumigatus	8	6.5	5	8.47	3	4.69
A. niger	3	2.44	0	0	3	4.69
A. parasiticus	1	0.81	0	0	1	1.56
A. brasiliensis	2	1.63	1	1.69	1	1.56
A. luchuensis	2	1.63	2	3.39	1	1.56
A. aff. ustus	2	1.63	0	0	2	3.13
A. awamori	3	2.44	0	0	3	2.42
A. insuetus	2	1.63	0	0	2	1.63
A. mnutus	2	1.63	0	0	2	1.63
A. ustus	2	1.63	0	0	2	1.63
A. welwitschiae	5	4.07	4	3.25	1	0.81

(a)

(b)
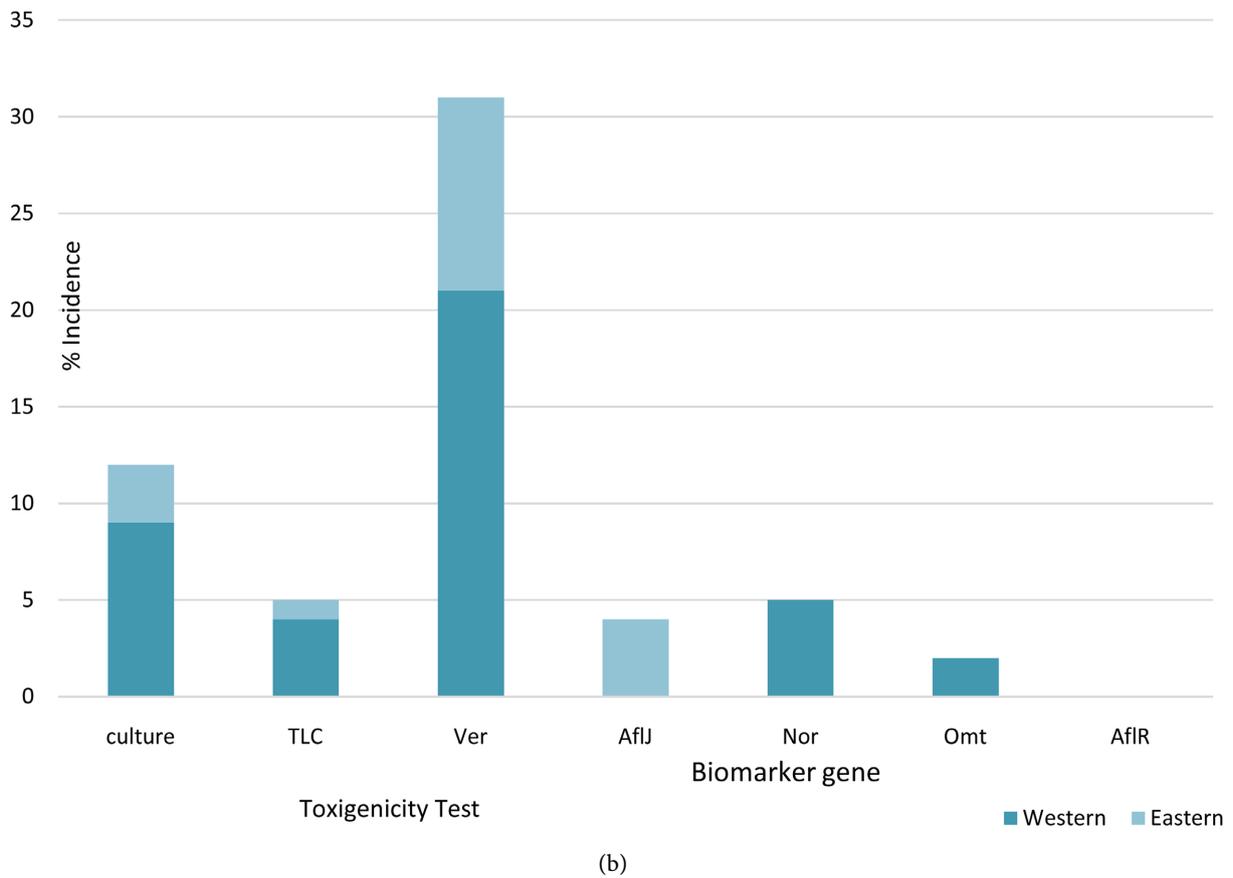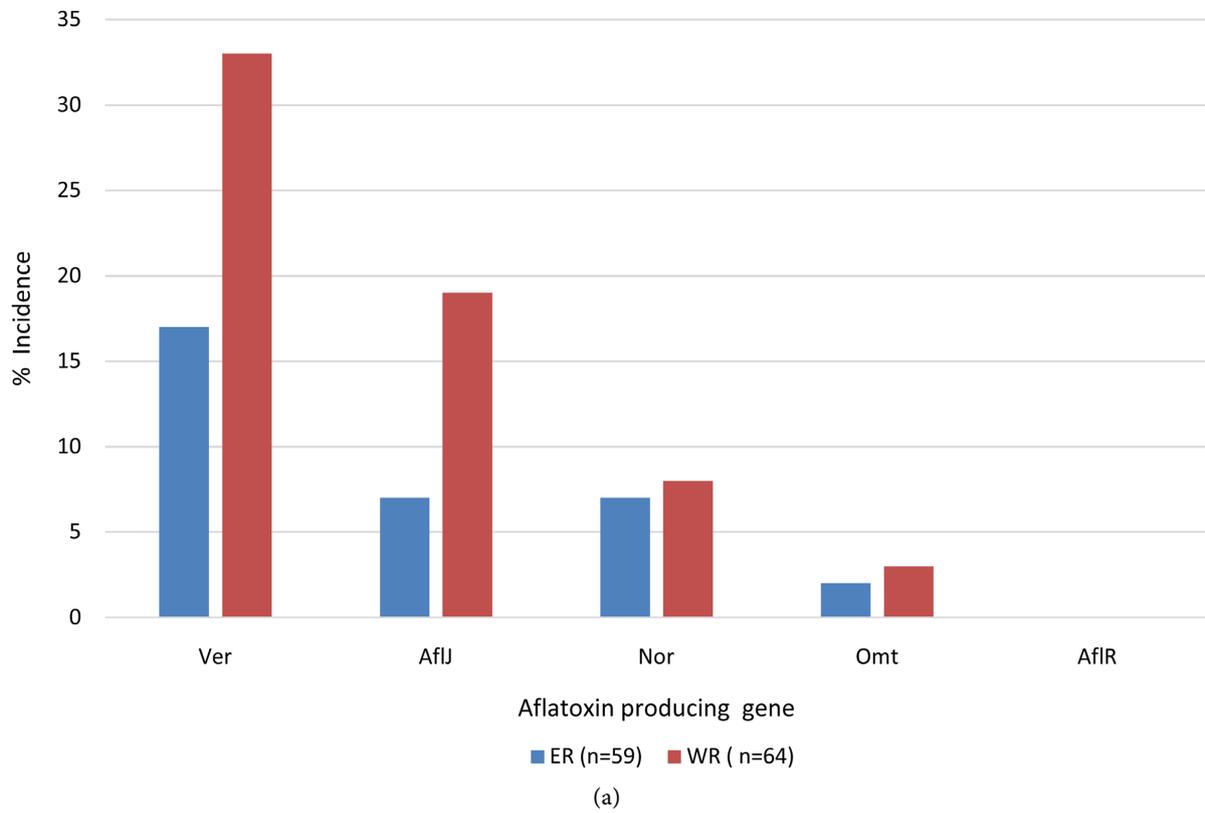
Figure 3. (a) Distribution of Aflatoxin producing gene across regions. (b) Polyphasic methods for toxigenicity test.

3.6. Fungal Metabolites and Mycotoxins in Maize Samples

3.6.1. Analysis of Liquid Chromatography Mass Spectrometry Mass Tandem (LCMS/MS) for Fungal Metabolites in Maize Samples

Aflatoxin was not detected in any of the maize samples analyzed using the LCM-S/MS. However, other detected metabolitesviz: 3-Nitropropionic acid; Sterigmatocystin; seco-sterigmatocystin; averufin; Kojic acid; and orsellinic acid. The limit of detection (LOD) ranged between 0.04 µg/kg (averufin and seco-sterigmatocystin) and 21 µg/kg (kojic acid), while limit of quantification (LOQ) ranged from 0.13 µg/kg (averufin) to 68 µg/kg (kojic acid). Percentage recovery ranged from 75.5% (seco-sterigmatocystin) to 168.8% (orsellinic acid) (Table 6). At least 36.59% of maize samples had one or more of the metabolites mentioned above while 20.33% of the samples had traceable amounts of kojic acid, but below limit of quantification (data not shown).

In all the regions, orsellinic acid had the highest percentage of occurrence (29.27%; concentration range between 235.76 and 829.60 µg/kg), followed by kojic acid (5.69%; concentration range of 65.61 to 587.51 µg/kg) while the rest were found in only one sample (0.81%). Fifty percent (50%) of maize samples from WR contained at least, one of these metabolites while about twenty-two percent (22.03%) of maize samples from ER had these metabolites (Table 7). 3-Nitropropionic acid was not detected in maize samples from ER, metabolites such as sterigmatocystin and seco-sterigmatocystin were also detected in maize from WR. 3-Nitropropionic acid (15.70 µg/kg), Sterigmatocystin (1.69 µg/kg), seco-sterigmatocystin (0.36 µg/kg) and averufin (0.48 µg/kg) were detected in only one sample.

Table 6. LCMS/MS detected metabolites in maize and their frequency of occurrence.

Analytes	LOQ (µg/kg)	LOD (µg/kg)	Recovery (%)	Frequency of occurrence	
				WR (n = 64)	ER (n = 59)
				32 (50%)	13 (22.03%)
Kojic acid	68	21	78.6	4 (6.25%)	3 (5.08%)
3-Nitropropionic acid	2.5	0.75	87.8	1 (1.56%)	0
Sterigmatocystin	0.25	0.08	91.3	0	1 (1.69%)
Seco-sterigmatocystin	0.14	0.04	75.5	0	1 (1.69%)
Averufin	0.13	0.04	104.1	0	1 (1.69%)
Orsellinic acid	60	20	168.8	27 (42.19%)	9 (15.25%)
Aflatoxin G$_1$	-	-	-	-	-
Aflatoxin G$_2$	-	-	-	-	-
Aflatoxin B$_1$	-	-	-	-	-
Aflatoxin B$_2$	-	-	-	-	-

Table 7. Mean aflatoxin concentrations and frequency of distribution of toxins in maize from different regions (HPLC method).

	Western Region				Eastern Region		
Sample	B_2	B_1	Total Aflatoxin (µg/kg)	Sample	B_2	B_1	Total Aflatoxin (µg/kg)
Mean	0.45	3.02	3.53	Mean	0.25	19.01	19.26
Range	0 - 17	0 - 65	0 - 65	Range	0 - 12.9	0 - 1069.8	0 - 1082.7

	Frequency of distribution				
	G_2	G_1	B_2	B_1	Total
WR (n = 64)	0	0	9 (14.06%)	16 (25%)	22 (34.38%)
ER (n = 59)	0	0	3 (5.08%)	11 (18.64%)	12 (20.34%)
SA (n = 123)	0	0	12 (9.76%)	27 (21.95%)	34 (27.64%)

3.6.2. Detection of Aflatoxins in Maize Samples Using High Performance Liquid Chromatography

Standard curves were constructed by calculating the ratio of the peak areas for each aflatoxin standard samples spiked before and after extraction at three levels of 25, 50 and 100 µg/ kg for all aflatoxins analyzed (Suppl. Figure 1). The toxins (G_2, G_1, B_2 and B_1) were eluted at 6 - 7, 7 - 8, 8 - 9 and 10 - 11 minutes respectively. The aflatoxin content of the maize samples was measured in µg/kg.

Aflatoxins G_1 and G_2 were absent in all analyzed samples as revealed by HPLC technique. However, aflatoxins B_1 and B_2 were found in both regions in varying amounts. The ranges for aflatoxin B_1 concentration (0 - 65 and 0 - 10.70 µg/kg) was higher than AFB_2 (0 - 17 µg/kg to 0 - 12.9 µg/kg) across WR and ER. The ER had a mean value of aflatoxins B_1 of 1.04 µg/kg compared to the WR with a mean value of 3.11 µg/kg. The same trend was observed for AFB_2 with WR having a higher mean value of 0.05 µg/kg and ER with a mean value of 0.98 µg/kg. Thus, the mean total aflatoxin was higher in WR (3.59 µg/kg) compared to ER (1.09 µg/kg) (Table 6). There was no significant difference between aflatoxins B_2 and B_1 in ER and WR. The WR recorded a higher percentage contamination of samples for both aflatoxins B_1 (25%) and B_2 (14.06%) compare to aflatoxins B_1 (18.64%) and B_2 (5.08%) from ER (Table 6).

4. Discussion

Moisture content of grain at post-harvest and during storage is critical in the quality of the product after storage. A moisture content of 13% is the maximum moisture content required for grain storage [46]. Analysis of moisture content in maize samples in this study revealed a lower range of mean moisture content (7.38% and 8.84%) in samples from both regions. Previous authors have also reported similar moisture content rangesof 10.23% - 14.12% and 11% - 13% in maize grain from regions in Southern Africa [47] [48]. Contamination by fungi and production of mycotoxin in grain can happen in the field or during storage,

hence, fungi are classified as field and/or storage microbes. *Aspergillus* species are both field and storage fungi [49]. Therefore, with such low moisture, the fungal load observed and contamination by aflatoxin in maize in this study could have been due to invasion in the field and proliferation during storage [50] [51]. For example, Kimanya, De Meulenaer [52] found levels of aflatoxin up to 21,667 μg/kg in freshly harvested maize before sorting and 1758 μg/kg in the same stocks of maize after sorting and stored for 5 months. Moisture content was found to have no significant effect on contamination by aflatoxin in maize. Studies have shown that moisture below 13% has little or no influence on the growth of fungi and mycotoxin production during storage.

The three main genera of fungi known for producing mycotoxins; *Aspergillus*, *Fusarium*, and *Penicillium* [53] were found in maize grown in SA. Although this study focused on*Aspergillus* spp., fungal spp. belonging to eight genera (*Fusarium*, *Aspergillus*, *Penicillium*, *Rasamsonia*, *Talaromyces*, *Paecilomyces*, *Byssochlamys* and *Verticillium)* were found in maize from the different agro-climatic regions of SA. Out of the eight genera, four distinct genera (*Talaromyces*, *Paecilomyces*, *Byssochlamys* and *Verticillium)* were not found in maize from the Eastern Regions. From this study the mycotoxin producing genera were more in the WR than the ER. This result corroborate earlier studies in SA maize where similar genera of microbes were isolated in their maize [54] [55]. The latter author reported incidence of mycotoxin-producing genera of *Aspergillus*, *Penicillium* and *Fusarium* in maize produced in Tunisia and Egypt; *Fusarium* spp. 95.3%, *Aspergillus* 87.5% and *Penicillium* 64.0% [55]. The geographic location of sample could be the primary factor affecting composition of microbiota in grains [56].

Out of the thirteen *Aspergillus* species isolated in this study, *A. flavus* (47.15%) was the most predominant followed by *A. niger* (4.69%) and *A. fumigatus* (4.69%), which contradicts the reports of Egbuta [57] and Chilaka, De Kock [54] who found higher occurrences of *A. fumigatus* (45%) than *A. flavus* (43%) in SA commercial maize (73.4%) and Nigeria (66.7%) respectively. The high incidence of *A. flavus* in both regions compared to other members of *Aspergillus* section *flavi* group (*A. parasiticus*, *A. mnutus* etc.) could be due to previous reports that *Aspergillus flavus* are more prevalent during times of high heat and drought [23]. Also, *A. flavus* are natural habitant of soil, hay, decaying vegetation, and grains acting as the reservoir of inoculums for infection of kernels in the field. *Aspergillus flavus* possess a higher adaptability to growth substrates in a range of environments (field and storage fungi) and can produce spores that remain viable even under extremely strict conditions [1] [58] [59]. Fungi that thrive in a particular area are strongly determined by the prevailing climatic conditions [56] [60]. The findings of this study corroborate those of Kankolongo, Hell [61] in Zambian maize in which *A. flavus* and *A. niger* were the most prevalent fungal isolates of maize grains.

In this study, 27.64% of the *A. flavus* isolates were aflatoxins producers where

10.57 of the isolates produced aflatoxins concentration above the South Africa's set standard (5 μg/kg). Gruber-Dorninger, Jenkins [62] found that only 0.7% of the 282 maize samples collected in South Africa had aflatoxins above the set standard for SA, and concluded that aflatoxins showed a low prevalence in feed samples from South Africa and detected levels were mostly unproblematic. On the contrary, AFB_1 were reported in 70% of the 40 maize samples in an assessment conducted in 2010 from two feed mills in KwaZulu-Natal [54]. In addition, the results of this study contradicts those of Meyer, Skhosana [63] who in a multi-mycotoxin long-term monitoring period (2014-2018) study, reported specifically that no aflatoxin was found in SA commercial maize. Meyer and collaborators further explained that total aflatoxin contamination were reported in three white maize samples from areas in the North West province in the 2014-2015 production seasons. In the summers of 2010, 2011, 2012 especially 2015, South Africa suffered severe droughts [62] [64] [65] that led to low crop yields in South Africa, for instance, there was a 30% reduction in maize production in 2015 (from 14,250,000 tons in 2014 to 9,955,000 tons) when SA experienced very low rainfall that amounted to 141 mm from 1991 to 2014 [62]. In already hot climates, more frequent drought may result in higher mycotoxin production [66]. Numerous studies agree on the main role of drought and high temperatures in higher aflatoxin production in maize [23] [67] [68].

Aspergillus flavus were found in 61% of the maize samples from both regions where about 28% did produce aflatoxins. Fungal growth and AFs production in cereals depends on temperature, moisture, etc. [69]. The non-production of aflatoxins could be attributed to: high temperatures and water stress which reduces production of AFB_1, despite the growth of *A. flavus* under these conditions [70], then 2015 been a year of drought and very low rainfall (141 mm). Interactions between water activity and temperature have prominent effects on *Aspergillus* spp. and production of aflatoxin [71] [72]. These authors studied the effect of interactions of temperature and water activity (aw) on the biosynthetic regulatory gene (aflR) expression and production of AFB_1 by *A. flavus* in maize. They observed the greatest growth of *A. flavus* at 30°C/0.99 aw with no growth at 20°C/0.90 aw [71] [72]. Based on the investigation by Battilani, Toscano [73] on the possible emergence of AFB_1 in cereals as a result of climate change, they projected that for every 2°C increase in temperature, there is an increase in AFs risk. In addition, Shooshtari, Mohammadi [74] found that a mutation including substitution of some bases and many other different physiological conditions affecting biosynthesis of aflatoxin, does affect production of aflatoxin. Furthermore, isolated spp. are genetically different and the nucleotide sequence is not associated with the mentioned primer. For example, the ITS molecular marker identified most of the *Aspergillus flavus* as *Aspergillus oryzea*. *A. oryzae* is a domesticated variant of *A. flavus* [75], which could be another reason aflatoxins were not produced.

Though there are regulatory limits for aflatoxins in only 15 African countries, the regulations vary widely among these countries. Exposure to aflatoxin in food

is a significant risk factor [76], as they are teratogenic and mutagenic and chronic exposure to aflatoxins have resulted in reduced immune activities [77], malnutrition [78] and growth impairment [79]. All South Africans consume maize in one form or another due to its use as an ingredient in different food products (maize meal, grits, corn flakes and snacks) [80] [81]. Maizeconsumed by livestock and poultry, also end up in the human food chain, through meat products, dairy products, cheeses and eggs. Therefore, SA maize quality has a direct impact on the health of humans and animals who consume the products. To find the problem attributable to aflatoxin contamination of food, both detected concentrations and consumption habits mustn't be ignored [82]. The mean value of total aflatoxins concentration in this study ranged between 1.09 and 3.59 µg/kg for the regions, which is below the recommended daily standards for human consumption in South Africa and USA [62] [83].

Maize from both regions in SA contained AFs and traceable amounts of other metabolites using HPLC and LCMS/MS techniques. HPLC detected AFs while LCMS/MS did not detect any aflatoxins in the same sample rather detected a number of other metabolites, some of which are precursors of aflatoxins. The reason might be that, while HPLC is vastly used for chromatography fingerprinting of the constituents, where standards (AFB$_1$, AFB$_2$, AFG$_1$ and AFG$_2$) solvent calibration thereby reprogramming the HPLC for the targeted aflatoxins. Unfortunately, these standards did not exist for the co-metabolites, therefore could not be picked up by the HPLC method. On the other hand, LC-MS/MS is the most successful interface and a powerful approach for identification of the unknown constituents [84]. The reason might be that LC-MS/MS is effective in quantifying trace level contaminants in food and feed as well as a range of parent compounds and their metabolites [85], many of which are easy to ionise and give good sensitivity often with crude sample preparation. This is because most of these metabolites (averufin, sterigmatocystin and seco-sterigmatocystin) are active precursors of AFB1 formation [86]. Using AF standards enhanced the HPLC method specificity. The results of this study corroborate those of Meyer, Skhosana [63] who detected no aflatoxins in maize samples using the LCMS/MS technique. In this study, we detected 6 metabolites in some maize samples using LCMS/MS compared to the study conducted in Nigeria by [44] where they detected about 65 metabolites in maize samples grown in Nigeria using the same method. Thirteen mycotoxin regulated metabolites in South Africa [63]; maize samples from SA used in this study contained about 23% of the regulated metabolites (aflatoxins B$_1$ and B$_2$ and 3-Nitropropionic acid). Apart from the AFB$_1$ and AFB$_2$ detected by the HPLC technique, the LCMS/MS detected metabolites such as: averufin, sterigmatocystin, seco-sterigmatocystin, 3-Nitropropionic acid (3NPA), Kojic acid and Orsellinic acid.

Among the metabolites found in the maize samples, averufin, sterigmatocystin and seco-sterigmatocystin were found only in one sample from the ER. Averufin, sterigmatocystin and seco-sterigmatocystin metabolites are active precur-

sors for AFB$_1$ formation [86]. Averufin (AVF) is a non-toxic early precursor of aflatoxin by a blocked mutant of *Aspergillus parasiticus*. Early steps in the synthesis of AFB1 involves the conversion of norsolorinic acid (NOR) to averantin (AVN), which is oxidized to 59-hydroxyaverantin (HAVN) and then to averufin (AVF). Yabe, Ando [87] found that HAVN is an intermediate in the enzymatic conversion of AVN to AVF. Yu, Chang [88] found that the *avnA* gene encodes a cytochrome P-450-type monooxygenase, necessary for converting AVN to HAVN. However, the enzyme involved in the oxidation of HAVN was not identified.

Sterigmatocystin is a metabolite produced mainly by *Aspergillus* fungi, and is an intermediate in the biosynthesis of aflatoxin B$_1$. Sterigmatocystin is a potentially health hazardous mycotoxin, produced mainly by *Aspergillus* species and is usually detected in food and feed as a natural contaminant [89] [90], recent data indicates that exposure to sterigmatocystin can also occur through direct skin contact or inhalation. As a precursor of aflatoxin biosynthesis with hepatotoxic action, sterigmatocystin is classified as group 2B carcinogen by IARC [91] [92] [93] due to its association with immunotoxic, immune modulatory activities as well as mutagenic effects. The structure and bioactivity of sterigmatocystin are similar to those of aflatoxin; however, its toxicity is weaker compared to aflatoxin and is observed in the liver and the lung [94]. Suggestions are that sterigmatocystin is 1/10 as potent as aflatoxin B1 with a 10 day LD50 of 120 mg/kg in rats [95] [96]. The IARC-classification of sterigmatocystin is group 2B, which means it is carcinogenic in other species and possibly carcinogenic to humans, however, a definitive link between human exposure and cancer has not been proved.

The 3-Nitropropionic acid, one of the regulated metabolites detected by the LCMS/MS method, was found only in one sample (074/M/2015) from the WR contaminated with *A. flavus*. 3NPA is a natural potent environmental toxin and mitochondrial inhibitor [96], toxic to both humans and animals. 3NPA, a metabolite produced by a number of fungi, including *Aspergillus* species and widely found in food such as cereals and sugar cane, among others [97] and caused mostly by extreme weather, stressed crop growth and storage conditions [98], which can further rise under global warming conditions [99]. This might be the reason 3NPA was found only in WR, where weather conditions are extreme. Acute encephalopathy can develop to dystonia in humans exposed to low doses of 3-NPA [100]. 3NPA induces neurodegeneration in the caudate putamen in humans and experimental animals, resembling Huntington's disease so, 3NPA poisoning primarily used as an animal model of selective neurodegeneration [101] [102] where toxin-treated experimental animals showed decreased motor performance [103]. Also, 3-NPA inhibits succinate dehydrogenase, a key enzyme for oxidative energy production [104], and causing ATP levels in the brain to fall. This effect develops fast and is not limited to the sites of morphological damage [101]. Since the nervous system requires lots of energy to work, mitochondrial damage is probably reflected in the electrical activity of the brain.

Kojic acid (5-hydroxy-2-(hydrxymethyl)-4-pyrone; KA), a non-regulated metabolite, is an organic acid secreted by several species of *Aspergillus*, especially *A. oryzae* [105]. It is multifunctional and has weak acidic property, non-hazardous biodegradation, making it an attractive and profitable skeleton for development of biologically active compounds by its derivatives [106]. Kojic acid (KA) is use as food derivative, antibiotic, antioxidant [105], a skin whitening agent, treatment of chloasma [107], antitumor agent [108] and radio protective agent [109]. This metabolite was the second highest detected in samples from both regions, while other samples had it but in unquantifiable amounts.

Orsellinic acid is a common salicylic acid unit in the biosynthesis of secondary metabolites in actinomycetes, fungi and lichens, formally isolated from chaetomium cochliodes in 1959 [110]. Orsellinic acid is a key biosynthetic intermediate of many depside metabolites in lichen and fungi. Orsellinic acid is also an important polar co-metabolite present in many fungi; however, its contribution to overall bioactivity is not well understood. Orsellinic acid is a useful standard for bioassay and analytical techniques for dereplication of common co-metabolites. This non-regulated metabolite was the most frequent metabolite detected in maize samples from both regions. Most of the metabolites detected (KA, orsellinic acid and seco-sterigmatocystin), usually colonise plant tissues and offer significant benefits to their host plants, by producing growth regulators, antimicrobials and antiviral, which are survival advantages [111].

Among the 58 *Aspergillus flavus* isolates tested if any of the five aflatoxigenic genes were present (*aflD*, *aflM*, *omtA*, *aflJ* and *aflR*), no *Aspergillus flavus* isolate had the regulatory *aflR* gene, three isolates possessed the *omt-A* gene (two isolates from WR and one from ER) eleven isolates possessed the *aflD* gene (seven from WR and four from ER) while sixteen isolates possessed the *aflJ* gene (twelve from WR and four from ER). Majority of the isolates that tested positive, possessed the *aflM* gene (twenty-one from WR and ten from ER). One isolate (from ER) possessed up to three of the five genes tested, fifteen isolates possessed two out of the five genes tested (thirteen from WR and two from ER) while twenty-eight isolates possessed, at least, one of the genes tested. 50% and 25.86% of tested isolates from WR and ER respectively tested positive for aflatoxigenicity. This study has shown that there is a clear similarity between *A. flavus* and aflatoxicity in both regions, which, agrees to earlier studies conducted using RT-PCR targeting *aflD*, *aflQ*, *aflO*, *aflP*, *aflR* and *aflS* genes [112] [113] [114] [115] [116]. Mahmoud [117] also reported a correlation between PCR amplification of four aflatoxin biosynthetic pathway genes (*aflD*, *aflM*, *aflP*, and *aflQ*), with the aflatoxins production ability of *A. flavus* isolates from stored peanuts in Egypt. Aflatoxins-producing genes present in *an Aspergillus* isolate does not necessarily confirm the isolate to be aflatoxigenic [44] [114] [118] [119]. PCR-based techniques are important to detect aflatoxigenic potential of *Aspergillus* isolates [120] [121] [122], since these genes encode the key enzymes and regulatory factors in the pathway of biosynthesis of aflatoxins.

5. Conclusion

Climate change is clear and has been increasing over the past few years and this could join a threat to food security and safety. Even though the findings of this study show that irrespective of the climatic variations of the different regions in South Africa, *Aspergillus* species and aflatoxin production is not yet a threat to South Africa's commercial food/feed industry, the frequent occurrence of drought in South Africa as experienced in 2015 is a clear sign that climatic zones that appear safe now might later lead to risk of disease and/or loss in crop production as climatic conditions change. There is, therefore, a need for long-term and continuous monitoring of *Aspergillus* species and their toxins in maize across different agro-climatic areas of South Africa (4 to 5 years) as well as other suppliers of agricultural maize and maize grown and stored by small-scale farmers. This will help simulate and model a trend that can clearly predict the long-term effects of climate change on aflatoxins in South African maize. Extensive studies will be carried out on strategies such as competitive exclusion and touch inhibition and real application be done on South African fields comparatively in the different agro-climatic regions.

Conflicts of Interest

The authors declare no conflict of interest.

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