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ORIGINAL PAPER

# A polyphasic method for the identification of aflatoxigenic *Aspergilla* from cashew nuts

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## Abstract
The invasion of food by toxigenic fungi is a threat to public health. This study aimed at enumerating the microbial profile, detection of aflatoxin producing genes and quantification of the levels of aflatoxin contamination of cashew nuts meant for human consumption. A polyphasic method of analysis using newly formulated β-Cyclodextrin Neutral Red Desiccated coconut agar (β-CDNRDCA) and Yeast Extract Sucrose agar (YES) with Thin Layer Chromatography (TLC), Polymerase Chain Reaction (PCR) and High Performance Liquid Chromatographic (HPLC) method was adopted in determining the aflatoxigenic potential of the isolates, the presence of aflatoxin biosynthetic gene (*aflM, aflD, aflR, aflJ omt-A*) and estimation of the total aflatoxin content of the nuts. The fungal counts ranged from 2.0 to 2.4 $\log_{10}$cfu/g and sixty-three fungal isolates belonging to 18 genera and 34 species were isolated. The *Aspergillus* spp. were the most frequently isolated (50.79%) while *Trichoderma spp*. (1.59%) were the least. and fluorescence production was enhanced on the newly formulated β-CDNRDCA by the aflatoxigenic species. The *aflD* gene was amplified in all the isolates while *aflM, aflR and aflJ* gene were each amplified in 77.77% of the isolates and *omt-A* gene in 70.37%. The aflatoxin content of the nuts ranged from 0.03 to 0.77 µg/kg and were below the 4 µg/kg EU recommended limit for total aflatoxins. The present work confirms that a single method of analysis may not be sufficient to screen for the presence of aflatoxins in foods, as with a combination of different methods.

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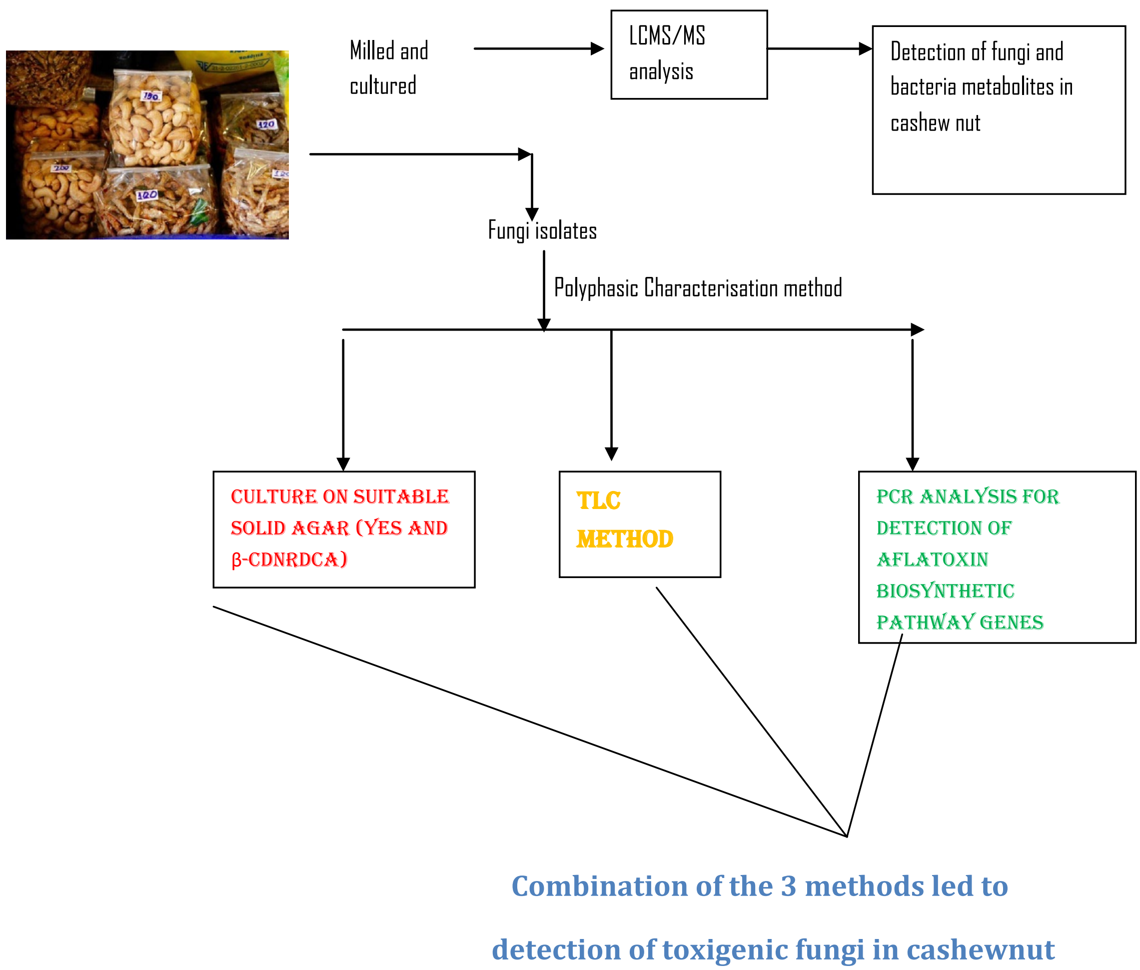
**Graphical abstract**




**Keywords** Cashew nut · *Aspergillus* · Aflatoxin · Biosynthetic gene · Aflatoxigenic fungi · Polymerase chain reaction · Polyphasic

## Introduction

Fungi are ubiquitous organisms whose spores may be dispersed by wind, insects and floods (Egan et al. 2014). They are capable of growing on simple and complex food products producing various metabolites. Recently, more than hundreds of thousands of fungal species were classified as natural contaminants of agricultural and food products (Nleya et al. 2018).

*Aspergillus* spp., which is one of the most important genera of micro-fungi play important roles in various fields of concern such as human, plants, and animals as spoilage agents of food commodities or as producers of toxic secondary metabolites. In addition, they are used for food fermentations and in industrial bioprocesses (Ghorai et al. 2009).

Among the *Aspergillus* spp. the section *Flavi* has been a major concern to the industry in the last five decades, as it contains species that produce a group of highly toxigenic compounds; the aflatoxins (Rodrigues et al. 2011).

Aflatoxin contamination of crops is a serious food and feed safety issue worldwide and causes significant economic losses yearly. Aflatoxins ($B_1$, $B_2$, $G_1$, and $G_2$) are secondary metabolites of fungal origin produced mainly by the closely related fungi, *A. flavus, A. parasiticus* and *A. nomius* (Nleya et al. 2018). As a result of the toxicity and impact of aflatoxins on health as potential liver toxins and carcinogens, the tolerated amount of aflatoxins in foods and feeds is closely monitored and regulated in several countries. In most countries, the maximum tolerated levels for aflatoxin $B_1$ in foods ranged from 0 to 20 µg/kg (Ojuri et al. 2018; Onyeke et al. 2017). It is therefore expedient to monitor the level and presence of aflatoxigenic fungi and aflatoxins in food and feeds.

The analytical equipment required to accurately detect toxins in food commodities are expensive and also require expertise which precludes its use in developing nations. Hence, the need for the adoption of inexpensive, readily available and reliable cultural methods for detection of aflatoxins in commodities (Atanda et al. 2011). Furthermore, the application of molecular techniques to distinguish between aflatoxin producing and non-producing strains of *A. flavus* and related species, through the correlation of the presence or absence of one or more genes involved in the AF biosynthetic pathway and the ability/inability to produce AFs is fast replacing the conventional cultural methods in most modern laboratories of the world (Rodrigues 2011). Thus the the combination of cultural, chromatographic and molecular methods for the detection of aflatoxigenic fungi is a better approach as the cultural method can be useful in pre-screening large number of *Aspergillus* spp. This minimizes wastage of resources and also helps to identify non-aflatoxigenic spp. which can be used as potential bio-control agents in agricultural fields.

Due to the high nutrient profile of cashew nuts it is highly susceptible to microbial attack at pre/post harvest level (Adetunji et al. 2018) thus the incidence of aflatoxigenic fungi and aflatoxins have been reported from different countries; Nigeria (Adebajo and Diyaolu 2003; Ezekiel et al. 2012; Sombie et al. 2018), Brazil (Milhome et al. 2014), Kenya (Ndung'u et al. 2013), Turkey (Yilmaz and Aluc 2014a) but information on the microbial profile, aflatoxigenic potential of Aspergilli spp. and aflatoxin contamination of cashew nuts sold in South Africa is not available in literature.

Thus this work aimed to enumerate the microbial profile, identify and characterize the aflatoxigenic fungi isolated from roasted cashew nuts, detect the aflatoxin producing genes and quantify the levels of aflatoxin contamination of the nuts in Mafikeng, South Africa through a polyphasic approach in order to know the risk level of the cashew nut consumers in the province.

## Materials and methods

### Sample location

Sampling of the nuts was carried out in Mafikeng, North West Province, South Africa in July, 2017.

Mafikeng is the capital city of the North-West Province of South Africa. It is located on the coordinate 25°51′S 25°38′E, close to South Africa's border with Botswana and is 1400 km (870 mi) Northeast of Cape Town and 260 km (160 mi) West of Johannesburg. It is built on the open veld at an elevation of 1500 m (4921 ft), by the banks of the Upper Molopo River. In 2011, it has a population of 15,117 with population density of 620/km$^2$. The town has an average annual rainfall of 300–700 mm, while the summer temperatures ranges between 22 and 34 °C with average winter temperature of 16 °C (range 2–20 °C) in a single day.

### Sampling and sample preparation

One hundred grams of 12 different brands of cashew nuts; roasted and salted (n = 8), roasted and non salted (n = 3) and raw (n = 1) were purchased in triplicates from each of the supermarkets in Mafikeng town, to make a total of 36 samples (100 g each). The sampling plan was a function of the availability of the cashew nut brands at the various super markets. The triplicates of each brand of sample (300 g) were pooled together to form 12 homogenized composite samples. The composited nuts were further divided by the processes of quartering and each of the quartered samples further subdivided into two equal parts; one-part of the sample was direct plated while the remaining part was milled in a Waring blender (IKA, Model M20, Germany) and stored at 4 °C for further analyses.

### Reagents and chemicals

Methanol, Potassium bromide, Nitric acid, sodium hydroxide were purchased from local dealers in South Africa while Aflatoxin Standards (B1, B2, G1, G2 and Total Aflatoxins) were purchased in crystalline form from Sigma (Sigma, St. Louis, MO, USA).

### Fungal colony counts

Moulds incident in the milled samples were isolated by the direct plate technique as reported by Ezekiel et al. (2014). One gram of each sample was suspended in 9 ml of sterile distilled water and mixed for 2 min by hand inversion. A, 0.1 ml aliquot of the suspension was spread plated in triplicates on Potato Dextrose Agar (PDA) and incubated at 28 °C for 48 h after which the colonies in each plate were counted and recorded as the fungal load per sample and the Colony Forming Unit (CFU/g) calculated for each sample.

### Isolation of sample-borne mycoflora

The direct seed plating method as described by Adetunji et al. (2018) was adopted for the isolation of the endophytic fungi in the cashew nuts. Three-four seeds of the nut samples (10–15 g) were surface sterilized by 2% (V/V) sodium hypochlorite solution in a sterile conical flask for 1 min and then washed with three changes of sterile distilled water for 2 min to remove the toxic activity of chemical agents on the samples. Four half cotyledons of the cashew nuts were placed aseptically at equidistance in Petri dishes containing molten PDA incorporated with 0.01% (W/V) chloramphenicol to inhibit bacteria growth. Three replicates were made and the plates were incubated in the dark at 25 °C for 7 days. Each fungal colony was again carefully transferred into sterile solid PDA plates for final purification at 25 °C for 5 days prior to DNA extraction of the fungal isolates. The fungal colonies were identified molecularly by PCR analysis and subsequent sequencing. Due to the high frequency of the black Aspergilli in the isolated organisms, they were grouped into ten classes based on their morphological appearances on PDA plates prior to DNA extraction.

The isolation frequency and relative density of each species was calculated as described by (Saleemi et al. 2012):

$$\text{Frequency}\ (\%) = \frac{\text{Number of samples contaminated with a specie or genus}}{\text{Total number of samples}} \times 100$$

$$\text{Relative Density}(\%) = \frac{\text{Number of isolates of species or genus}}{\text{Total number of isolates}} \times 100$$

## Molecular characterization of fungal isolates

### Extraction of genomic DNA

The extraction of the genomic DNA of the fungal isolates was done with the Zymo Research kit (Zymo-Research fungal/Bacterial Soil Microbe DNA, D6005, USA) supplied by Bio lab, South Africa, according to the manufacturer's instructions.

Briefly; a loopful of fungal spores from 5-day old cultures was scooped into the Bashing Bead™ lysis tubes and 750 µl of the lysis solution added to the tubes. The tubes containing the cultures were beaten in a bead beater (Inqaba Biotech mode No, SI D258, USA) at the maximum speed for 14 min and centrifuged in a micro-centrifuge at 10,000×*g* for 1 min. Four hundred microlitre of the supernatant was transferred into a collection filter tube which was centrifuged at 10,000×*g* for 1 min after which 1200 µl of the binding buffer was added to enhance the binding of the DNA to the filter column. The mixture was further centrifuged in the column in the collection tube and the flow through discarded. About 200 µl and 500 µl of pre-washed and washed buffers were added separately to each column respectively and centrifuged at the same conditions. Thereafter, 100 µl of the DNA elution buffer was added to the column after washing and centrifuged to elute the DNA.

### PCR amplification of genomic DNA

The amplification of the Internal Transcribed Spacer Region (ITS rDNA) of the fungal isolates from the cashew nuts was carried out with the Polymerase Chain Reaction (PCR) using the universal ITS 1 (TCC GTA GGT GAA CCT GCG G) and ITS 4 region primers (TCC TCC GCT TAT TGA TAT GC). These primers were commercially synthesized by Inqaba biotechnical Industrial (Pty) Ltd (Pretoria, South Africa).

The PCR amplicons were analyzed by electrophoresis on 1% (w/v) agarose gel to confirm the expected size of the amplicons (670 bp) and visualized using Chemi Doc Image Analyzer (Sambrook and Russell 2001).

### DNA sequencing

The sequencing of the purified PCR products were done at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa with PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit using the dideoxy chain termination method and electrophoresed with a model ABI PRISM® 3500XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) by following the manufacturer's instructions.

### Sequence analysis

Finch TV software version 1.4.0 was used for the analysis of Chromatograms, (sense and antisense) resulting from sequencing reaction for good quality sequence assurance. The resulting chromatographs were edited using BioEdit Sequence Alignment Editor (Hall 2004) after which, the resulting consensus ITSrDNA sequences obtained 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 the probable organisms in question (Altschul and Koonin 1998). The sequences were later deposited in the GenBank for accession number allocation.

## Aflatoxigenicity test of fungal isolates

### Determination of aflatoxigenic potential of *Aspergillus* isolates in culture media

The culture media used in these assays were Yeast Extract Sucrose Agar (yeast extract; 20 g, sucrose; 150 g, agar; 20 g and $MgSO_4$; 0.5 g) as reported by Criseo (2001) and β-Cyclodextrin Neutral Red Desiccated coconut agar (β-CDNRDCA). The Neutral Red Desiccated coconut agar (NRDCA) was prepared as described by Atanda et al. (2011) and enhanced with 0.3% β-cyclodextrin (W/V) following the method of previous authors (Abbas et al. 2004; Fente et al. 2001; Ordaz et al. 2003) who recommended the addition of 0.3–3% (W/V) cyclodextrin to agar to enhance their fluorescence production. A preliminary test by us had shown that there was no significant difference in the intensity of fluorescence between the 0.3 and 3% (W/V) β-cyclo-dextrins respectively (data not shown). Mycelia plugs (6 mm diameter) of the *Aspergillus* isolates were inoculated unto the centre of 9 mm Petri dishes containing different test media in triplicates and the isolates incubated unilluminated at 28 °C for 3–14 days. The isolates from the supplemented NRDCA plates were examined between the 3rd and 5th day (Atanda et al. 2011) for the presence of fluorescence of agar surrounding the growing *Aspergilla* colonies under UV light (365 nm), which was expressed by positive or negative signs, while the YES agar plates were checked on the 7th and 14th day for aflatoxin producing ability by the ammonium hydroxide vapour-induced colour change test as described previously (Jefremova et al. 2016). Briefly, 2 ml of concentrated ammonium hydroxide solution (W/V, 25%, 17.03 m) was placed on the inside of the lid of the inverted Petri dish containing the isolate on YES agar and left for 5–10 min to observe the color change at the reverse side of the agar. Plates that tested negative were re-examined on the 14th day for colour change.

### Detection of aflatoxin producing genes of *Aspergilla*

The DNA of suspected *Aspergilla* were examined for the presence of five important aflatoxin producing genes (*aflR, aflJ, aflM, aflD* and *omt-A*) present in the aflatoxin biosynthesis pathway by PCR using previously reported primer sets (Rashid et al. 2009). The genes, their primer sequences and their product sizes (Table 1) were selected from already reported data (Rashid et al. 2009). The PCR reagents and primers were supplied by Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa and the polymer chain reactions were carried out in PCR Thermal Cycler (Applied Biosystems).

**Optimization of polymerase chain reaction** Polymerase Chain Reaction conditions were optimized separately for the target genes. A reaction volume of 25 µl, containing: 8.5 µl nuclease-free water, 12.5 µl PCR Master Mix, 1 µl of oligonucleotide forward and reverse primers (10 µm) and 2 µL template DNA mixed in the PCR tubes (Montso et al. 2014) were used. The thermal cycle conditions are also shown in Table 1 with varying annealing temperatures ranging from 58 to 75 °C for the five genes respectively. The PCR amplified products were checked on 1% gel by electrophoresis and visualized under Gel documentation system for electrophoretic bands at the various base pair regions for each gene.

## Qualitative determination of aflatoxin producing ability of isolates by thin layer chromatographic method

The extraction of aflatoxin from the isolates was carried out according to Midorikawa et al (Midorikawa et al. 2008; Yin et al. 2009) with some modifications: the 7-day old *Aspergillus* isolates on YES agar were divided into two equal halves using sterile surgical blades (one-half for the ammonia test and the other half for TLC analysis), the first half of the agar containing the isolates were scooped into 50 ml centrifuge tubes and 15–20 ml of 70% methanol–water (70:30) added and kept on a shaker for 30 min, after which they was centrifuged at 5000 rpm for 5 min and the extracts decanted into clean tubes. The extracts were evaporated to dryness by air blowing in the dark evolution chamber. The residues were reconstituted with 500 µl of 100% methanol. A, 20 µl volume of each of the reconstituted extract was 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.

## Quantitative determination of aflatoxins by high performance liquid chromatography

### Extraction of aflatoxins from the nuts

The extraction of the milled cashew samples was carried out with the EASI-EXTRACT AFLATOXIN (R-BIOPHARM RHONE LTD) immunoaffinity column kits. The aflatoxin standard solution (25 µg/ml) was prepared in acetonitrile–water (98:2 V/V). The working standard solution was prepared daily from the aflatoxin standard solution (Stroka et al. 2000).

The extraction was carried out as described in the product's extraction kit. Briefly; 5 g sodium chloride was added to 50 g of each sample in a 500 ml beaker and 100 ml of distilled water added and blended at high speed for 1 min, followed by the addition of 150 ml of 100% methanol to the mixture, which was blended for another 2 min. The mixture was then filtered through Whatman No. 4 filter paper for 10 min in order to ensure that the toxins in the mixture are completely eluted and the pH adjusted to 7.4 using 2 M sodium hydroxide solution. A, 5 ml aliquot of the filtrate was diluted with 5 ml of phosphate buffered saline (PBS) solution and 20 ml of the diluted filtrate (equivalent to 1 g of sample) was passed through the column at a flow rate of 2 ml/min. The toxin was then eluted from the column at a

**Table 1** Optimization conditions for polymerase chain reaction

| Primer name | Target gene | Sequence 5′–3′ | PCR conditions | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Product size | Pre-denaturation | Denaturation | Annealing | Elongation | Final elongation |
| Nor 1 | *Nor (aflD)* | ACCGCTACG CCGGCA CTCTCG GCAC | 400 bp | 94 °C—10 min | 94 °C—1 min | 65 °C—1 min | 72 °C—2 min (33 cycles) | 72 °C—5 min (1 cycle) |
| Nor 2 | | GTTGGCCGC CAGCTT CGACAC TCCG | | | | | | |
| Ver1 | *Ver (aflM)* | GCCGCAGGC CGCGGA GAAAGT GGT | 537 bp | 95 °C—4 mins | 95 °C—1 min | 58 °C—1 min | 72°C—30 s (30 cycles) | 72°C—10 mins (1 cycle) |
| Ver 2 | | GGGGATATA CTCCCG CGACAC AGCC | | | | | | |
| Omt1 | *Omt-A* | GTGGACGGA CCTAGT CCGACA TCAC | 797 bp | 94 °C—5 min | 94 °C—1 min | 75 °C—2 mins | 72 °C—2 min (33 cycles) | 10 min (1 cycle) |
| Omt 2 | | GTCGGCGCC ACGCAC TGGGTT GGGG | | | | | | |
| *AflR 1* | *AflR* | TATCTCCCC CCGGGC ATCTCC CGG | 1032 bp | 95 °C—4 min | 95 °C—1 min | 60 °C—1 min | 72°C—30 s (30 cycles) | 72°C—10 mins (1 cycle) |
| *AflR 2* | | CCGTCAGAC AGCCAC TGGACA CGG | | | | | | |
| *AflJ F* | AflJ | TGAATCCGT ACCCTT TGAGG- | 737 | 95 °C—10 min | 95 °C—50 s | 58 °C—50 s | 72 °C—2 min (30 cycles) | 72 °C—10 min (1 cycle) |
| *AflJ R* | | GGAATGGGA TGGAGA TGAGA | | | | | | |

flow rate of 1 drop per sec with 1.5 ml of 100% methanol (HPLC grade) and 1.5 ml of water collected unto an amber glass vial. A, 100 µl of the eluent of each sample and aflatoxin standards (25, 2.5, 0.25. 0.0025, 0.00025 µg/ml) were subsequently injected into the HPLC system.

### Method validation for HPLC analysis for aflatoxin determination

The total aflatoxin content of the samples was quantified with a high-performance liquid chromatography column, using a Shimadzu liquid chromatograph (Kyoto, Japan) fitted with a fluorescence detector. The operating conditions for the HPLC system are shown in Table 2. The HPLC method used was validated by determining its linearity, accuracy and sensitivity. The linearity was determined by construction of calibration curves from standards of AFB1, AFB2, AFG2, AFG1, total aflatoxin ($AF_{tot}$) and from extract of blank samples of previously analysed cashew nuts that did not contain any of the aflatoxins. Linear range was examined at 5 different concentrations of each standard from 0.0025 µg/ml to 25 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 were applied to the calibration curves. Matrix effect (ME) was calculated for each analyte by comparing the slope of the standard calibration curve with the

**Table 2** Optimization conditions for high performance liquid chromatographic analysis

| Apparatus/activity | Condition |
|---|---|
| Derivatisation | KOBRA Cell at 100 µA setting |
| Guard Catridge | Inertsil ODS-3 |
| Analytical | Inertsil ODS-3V |
| Column | 5 µm, 4.6 mm × 150 mm (Hichrom) or equivqlent |
| Mobile | Water:methanol (60:40 v/v; modified to 55:45 v/v). Add 119 mg of potassium bromide and 350 µl 4 M nitric acid to 1 l of mobile phase |
| Pump flow rate | 1.0 ml/min |
| Florescence Detector | Excitation: 362 nm<br>Emission: 425 nm ($B_1$ and $B_2$)<br>455 nm ($G_1$ and $G_2$) |
| Column heater | Maintain guard and analytical columns at 40 °C |
| Injector | Auto sampler/reodyne valve |
| Injector Volume | 100 µl |
| Elution order | $G_2$, $G_1$, $B_2$, $B_1$ |

matrix-matched calibration curve for the same concentration levels.

The sensitivity of the methodology or system used was evaluated by limit of detection (LOD) and limit of quantification (LOQ), which were 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 was 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 (AFB1, AFB2, AFG1, AFG2 AFtot).

Quantification of the toxins was performed by measuring peak areas, the retention time and comparing them with the relevant standard calibration curves (AOAC 2007).

### Statistical analyses

Statistical Analyses were performed using SPSS for windows version 26 (SPSS Inc., Chicago, Illinois). One way Analysis of Variance (ANOVA) and Tukey's HSD test at 5% significance level was used to compare the means for fungal counts, the frequency of organisms in the cashew nuts and the aflatoxin concentration of the cashew nuts.

## Results

### Fungal counts and distribution of fungal isolates in cashew nuts

Seventy-five percent (75%) of the cashew nuts assayed on Potato Dextrose Agar were contaminated with fungi and the fungal counts ranged from 2.0 to 2.4 $\log_{10}$cfu/g with a mean of 2.2 $\log_{10}$cfu/g (Table 3). A total of 63 Isolates were identified by amplification of the ITS region of the fungi isolates and sequencing of the PCR amplicons of the isolates (Table 4). The sequenced isolates were submitted to the Genbank for allocation of accession number to the organisms (Table 4). The isolated fungi were categorized to five major genera (*Aspergillus, Penicillium, Alternaria, Curvularia, Trichoderma*) and other minor fungal genera. The *Aspergill*a were the most frequently isolated (50.79%), spp. followed by the summation of other minor fungi (33.33%) and *Penicillia* (6.34) while the *Trichoderma spp*.(1.59%) were the least frequent organisms. The *Aspergillus* genera had the highest relative density (50.79) based on the total no of fungal isolates contaminating the cashew nuts and *A*.

**Table 3** Fungal contamination of cashew nuts

| Sample code | Sample description | Fungal count $\text{Log}_{10}$(cfu/g) |
|---|---|---|
| A | Roasted and non-salted | –[a] |
| B | Roasted and salted | 2.3 |
| C | Roasted and salted | 2.2 |
| D | Roasted and salted | 2.0 |
| E | Roasted and salted | 2.0 |
| F | Roasted and salted | 2.3 |
| G | Roasted and salted | 0 |
| H | Roasted and non-salted | 0 |
| I | Roasted and salted | 2.0 |
| J | Roasted and Salted | 2.3 |
| K | Raw cashew nut | 2.4 |
| L | Roasted and non-salted | 2.3 |
| Mean fungal count | | 2.2 |

[a]Samples with no fungal growth were excluded from the mean calculation

**Table 4** Fungal profile of cashew nuts

| Sample identity | Fungal identity | Accession No. | % Similarity with other Isolate at gene bank |
|---|---|---|---|
| Seq01A | *Aspergillus nomius* | MG596623 | 99 |
| Seq02A | *Aspergillus flavus* | MG596624 | 99 |
| Seq03A | *Aspergillus oryzae* | MG596625 | 99 |
| Seq04A | *Aspergillus flavus* | MG576092 | 99 |
| Seq05A | *Aspergillus flavus* | MG596660 | 100 |
| Seq06A | *Aspergillus flavus* | MG576093 | 99 |
| Seq07A | *Aspergillus nomius* | MG576094 | 99 |
| Seq08A | *Aspergillus oryzae* | MG596626 | 99 |
| Seq09A | *Aspergillus flavus* | MG576095 | 99 |
| Seq10A | *Aspergillus nomius* | MG596627 | 99 |
| Seq11A | *Aspergillus oryzae* | MG576096 | 99 |
| Seq12A | *Aspergillus oryzae* | MG576097 | 99 |
| Seq13A | *Aspergillus oryzae* | MG596628 | 99 |
| Seq14A | *Aspergillus parvisclerotigenus* | MG576098 | 99 |
| Seq15A | *Aspergillus oryzae* | MG596629 | 99 |
| Seq16A | *Aspergillus flavus* | MG596630 | 99 |
| Seq17A | *Aspergillus flavus* | MG576099 | 99 |
| Seq18A | *Byssochlamys spectabilis* | MG596631 | 99 |
| Seq19A | *Byssochlamys spectabilis* | MG576100 | 99 |
| Seq20A | *Aspergillus fumigatus* | MG576101 | 99 |
| Seq21A | *Aspergillus fumigatus* | MG576101 | 99 |
| Seq22A | *Aspergillus fumigatus* | MG596632 | 99 |
| Seq23A | *Aspergillus fumigatus* | MG596633 | 99 |
| Seq24A | *Aspergillus fumigatus* | MG576101 | 99 |
| Seq25A | *Penicillium guanacastense* | MG576102 | 99 |
| Seq26A | *Penicillium meleagrinum* | NA | 99 |
| Seq27A | *Aspergillus sydowii* | MG596634 | 99 |
| Seq28A | *Penicillium crustosum* | MG596635 | 99 |
| Seq29A | *Talaromyces funiculosus* | MG576103 | 99 |
| Seq30A | *Curvularia australiensis* | MG596636 | 99 |
| Seq31A | *Trichoderma longibrachiatum* | MG596637 | 100 |
| Seq32A | *Talaromyces funiculosus* | MG576103 | 99 |
| Seq33A | *Rhizopus stolonifer* | NA | 99 |
| Seq34A | *Aspergillus ochraceus* | MG576104 | 100 |
| Seq35A | *Aspergillus awamori* | MG576105 | 99 |
| Seq36A | *Aspergillus niger* | MG596641 | 100 |
| Seq37A | *Aspergillus niger* | MG596638 | 100 |
| Seq38A | *Aspergillus tubingensis* | MG596639 | 99 |
| Seq39A | *Alternaria alternata* | MG596640 | 100 |
| Seq40A | *Aspergillus niger* | MG596641 | 100 |
| Seq42A | *Aspergillus awamori* | MG576106 | 99 |
| Seq43A | *Aspergillus niger* | MG576107 | 99 |
| Seq44A | *Aspergillus niger* | MG596642 | 99 |
| Seq45A | *Aspergillus niger* | MG596643 | 99 |
| Seq46A | *Alternaria alternata* | MG576108 | 99 |
| Seq47A | *Cochliobolus lunatus* | MG596644 | 99 |
| Seq48A | *Curvularia lunata* | MG576109 | 99 |
| Seq49A | *Alternaria alternata* | MG596645 | 99 |
| Seq50A | *Curvularia lunata* | MG576110 | 99 |

**Table 4** (continued)

| Sample identity | Fungal identity | Accession No. | % Similarity with other Isolate at gene bank |
|---|---|---|---|
| Seq51A | *Exserohilum rostratum* | MG596646 | 99 |
| Seq52A | *Epicoccum sorghinum* | MG576111 | 99 |
| Seq53A | *Ovatospora unipora* | MG576112 | 99 |
| Seq56A | *Periconia macrospinosa* | MG576113 | 99 |
| Seq57A | *Aspergillus ochraceus* | MG596647 | 99 |
| Seq58A | *Chaetomium globosum* | MG576114 | 99 |
| Seq60A | *Aspergillus terreus* | MG576115 | 99 |
| Seq61A | *Lecanicillium aphanocladii* | MG596648 | 99 |
| Seq62A | *Monascus purpureus* | MG576116 | 99 |
| Seq64A | *Arthrinium hyphopodii* | NA | 92 |
| Seq74A | *Aspergillus terreus* | MG596654 | 99 |
| Seq77A | *Aspergillus minisclerotigenes* | MG596655 | 99 |
| Seq78A | *Pithomyces sacchari* | MG596656 | 99 |
| Seq79A | *Aspergillus fumigatus* | MG596657 | 100 |
| Seq80A | *Penicillium citrinum* | MG596658 | 99 |

*NA* Not Assigned Accession Number yet

*niger* and *A. fumigatus* had the highest (21.87) and least densities (6.25%) when considering the total number of *Aspergillus* isolates (Table 5).

## Aflatoxigenicity test of fungal isolates

### Aflatoxigenic potential of *Aspergillus* isolates in culture media

Only 14.8% (4 out of 27) of the tested *Aspergillu*s isolates were positive on both the enhanced β-cyclodextrin NRDCA and the ammonium hydroxide vapour-induced colour change on the YES medium (Table 6). The positive isolates on the NRDCA showed yellow pigmentation at the reverse side of the plates with blue or blue-green ring of fluorescence of agar around the isolates on the obverse and reverse side of plates under the long wave UV light (365 nm). The aflatoxin production potential of the isolates were also confirmed on YES agar by colour change of the agar from golden yellow to pink, deep brown, or plum red colouration depending on the intensity of the aflatoxins produced. The non-*Aspergilli* isolates such as *Trichoderma, Talaromyces* and *Curvularia*. spp. were negative indicating the specificity of the test for aflatoxins (Table 6).

### Aflatoxin producing genes of *Aspergilla*

The molecular pattern of the *Aspergilla* are shown in Fig. 1a–e and Table 6. Deoxyribonucleic acid (DNA) fragments of *aflR, aflJ, omt-A, aflM* and *aflD* genes were

**Table 5** Distribution of fungal isolates of cashew nuts

| Fungi isolate | No of sample infected | Isolation frequency (%) based on total no of samples (n = 12) | No. of isolate | %Relative density of total isolate (n = 63) | %Relative density of *Aspergilla* (n = 32) |
|---|---|---|---|---|---|
| *Aspergillus* spp. | | | 32 | 50.79 | |
| *A. flavus* | 6 | 50 | 9 | 14.28 | 18.75 |
| *A. fumigates* | 2 | 16.66 | 2 | 3.17 | 6.25 |
| *A. oryzea* | 3 | 25 | 3 | 4.76 | 9.37 |
| *A. niger* | 7 | 58.33 | 13 | 20.63 | 21.87 |
| Other *Aspergillus* spp. | 4 | 33.33 | 5 | 7.9 | 12.5 |
| *Penicillium* spp. | 4 | 33.33 | 4 | 6.34 | |
| *Alternaria* spp. | 2 | 16.66 | 2 | 3.17 | |
| *Curvularia* spp. | 3 | 25 | 3 | 4.76 | |
| *Trichoderma* spp. | 1 | 8.33 | 1 | 1.59 | |
| Other fungi | 8 | 66.66 | 21 | 33.33 | |

**Table 6** Aflatoxigenicity test of fungal isolates

| S/No. | Aspergilla | Name of isolate | Conventional method | | | Aflatoxin biosynthetic gene[d] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | β-CDNRDCA (3rd day)[a] | Ammonium vapor test[b] | TLC test[c] | *aflR* | *aflJ* | *aflM* | *aflD* | *omt-A* |
| 1 | 1A | *Aspergillus nomius* | +++ | + | + | + | + | + | + | + |
| 2 | 3A | *Aspergillus oryzae* | X | X | X | + | + | + | + | X |
| 3 | 4A | *Aspergillus flavus* | X | X | X | + | + | + | + | + |
| 4 | 5A | *Aspergillus flavus* | +++ | + | + | + | + | + | + | + |
| 5 | 6A | *Aspergillus flavus* | X | X | X | + | + | + | + | + |
| 6 | 7A | *Aspergillus nomius* | X | X | X | + | + | + | + | X |
| 7 | 8A | *Aspergillus oryzae* | ++ | + | + | + | + | + | + | + |
| 8 | 9A | *Aspergillus flavus* | X | X | X | + | + | + | + | + |
| 9 | 10A | *Aspergillus nomius* | X | X | + | + | + | + | + | + |
| 10 | 11A | *Aspergillus oryzae* | X | X | X | + | + | + | + | + |
| 11 | 12A | *Aspergillus oryzae* | X | X | X | + | + | + | + | + |
| 12 | 13A | *Aspergillus oryzae* | X | X | X | + | + | + | + | + |
| 13 | 14A | *Aspergillus parvisclerotigenus* | X | X | X | + | + | + | + | + |
| 14 | 15A | *Aspergillus oryzae* | X | X | X | + | + | + | + | + |
| 15 | 16A | *Aspergillus flavus* | X | X | X | + | + | + | + | + |
| 16 | 17A | *Aspergillus flavus* | X | X | X | + | + | + | + | + |
| 17 | 18A | *Byssochlamys spectabilis* | X | X | X | + | + | + | + | + |
| 18 | 19A | *Paecilomyces variotii* | X | X | X | + | + | + | + | + |
| 19 | 20A | *Aspergillus fumigatus* | X | X | X | X | X | X | + | X |
| 20 | 22A | *Aspergillus fumigatus* | X | X | X | + | + | + | + | + |
| 21 | 30A | *Curvularia australiensis* | X | X | X | X | X | X | + | X |
| 22 | 31A | *Trichoderma longibrachiatum* | X | X | X | X | X | X | + | X |
| 23 | 32A | *Talaromyces funiculosus* | X | X | X | X | X | X | + | X |
| 24 | 37A | *Aspergillus niger* | X | X | X | X | X | X | + | X |
| 25 | 38A | *Aspergillus tubingensis* | X | X | X | X | X | X | + | X |
| 26 | 61A | *Lecanicillium aphanocladii* | X | X | X | + | + | + | + | + |
| 27 | 77A | *Aspergillus minisclerotigenes* | +++ | + | + | + | + | + | + | + |

*β-CDNRDCA* beta cyclo dextrin neutral red desiccated coconut agar

[a]+++ yellow pigment and deep blue fluorescence under UV, [a]++ yellow pigment and blue fluorescence under UV, [a]X no pigment, no fluorescence, [b]+ reddish brown coloration, [b]X no color change, [c]+ positive for aflatoxin production, [c]X negative for aflatoxin production, [d]+ gene is present, [d]X gene is absent

visualized at 1032, 737, 797, 537 and 400 bp respectively. However, some unexpected genes with band sizes of below 500 bp and 1000 bp as against the expected 537 bp of *aflM* gene were detected in two isolates (Seq 20A and 32A) when the genes were amplified with the Ver-A primer. The Nor-1 (*aflD*) gene was also amplified in all the isolates of both *Aspergill*a and the non *Aspergilla* (100%) while Ver (*aflM*), *aflR and aflJ* gene were each amplified in only 77.77% of the organisms and *omt-A* gene in 70.37% (Table 6).

All the *Aspergill*a with the exception of *Aspergillus fumigatus*, Aspergillus oryzea and *Aspergillus nomius* contained the five aflatoxin biosynthetic genes tested. The aflatoxin biosynthetic genes were also found in all the *Byssochlamys spectabilis* isolates (Table 6).

## Detection of aflatoxin production by TLC

The Thin Layer Chromatographic (TLC) test revealed that 18.52% (5 of 27) of the fungal isolates on both the supplemented NRDCA and YES agar tested positive for the presence of aflatoxins on TLC plates (Merck KGaA, Darmstadt Germany). The positive isolates for the TLC analysis produced a bright blue fluorescence on TLC plates under 365 nm long wave UV light.

## Aflatoxin content of cashew nuts

The operating conditions for the HPLC system are shown in Table 2. The standard calibration curves showed good linearity with $R^2$ values ranging from 0.9994 to 1 and the

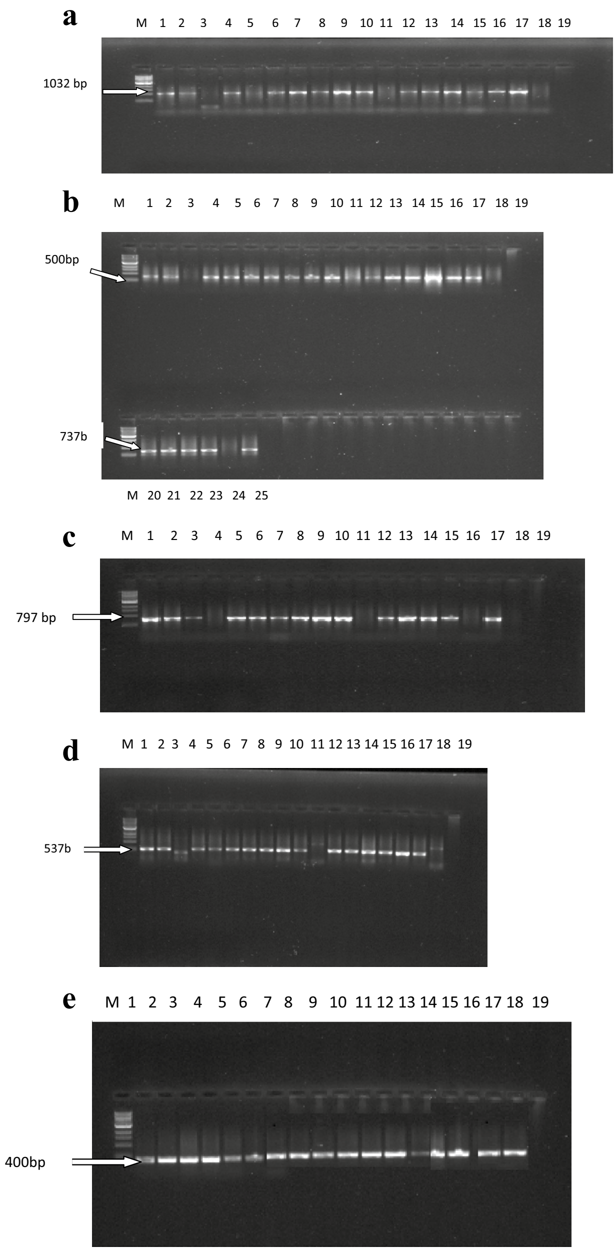



**Fig. 1** **a** Gel Electrophoretic pattern for PCR products expressing *aflR* Gene at 1032 bp region. M: 1 kb DNA ladder; Lane 3, 11, 18: negative isolates, lane 19: negative control, lane 1, 2, 4, 6–10, 12–17: positive isolates. **b** Gel electrophoretic pattern for PCR products expressing *aflJ* gene at 737 bp region. M: 1 kb DNA ladder; Lane 3, 18, 24: negative isolates, Lane 19: negative control, Lane 1, 2, 4–17, 20–23, 25: positive isolates. **c** gel electrophoretic pattern for PCR products expressing *omt-A* gene at 797 bp. M: 1 kb DNA ladder; Lane 4, 11, 16, 18: negative isolates, Lane 19: negative control, Lane 1–3, 5–10, 12–15, 17: amplification of *omt-A* gene in positive isolates. **d** Gel electrophoretic pattern for PCR products expressing *aflM* gene (Ver) at 537 bp. M: 1 kb DNA ladder; Lane 3, 11, 18: amplification not at expected band size, Lane 19: negative control, Lane 1–2, 4–10, 12–17: positive isolates. **e** Gel electrophoretic pattern for PCR products expressing *aflD* Gene (Nor-1) at 400 bp region. M: 1 kb DNA ladder; Lane 19: negative control, Lane 1–18: amplification of *aflR* gene in positive isolates

**Table 7** Aflatoxin contamination of cashew nuts

| Sample code | Sample description | Total aflatoxins (µg/kg) |
|---|---|---|
| A | Roasted and non-salted | 0.15 |
| B | Roasted and salted | 0.03 |
| C | Roasted and salted | 0.14 |
| D | Roasted and salted | 0.25 |
| E | Roasted and salted | 0.15 |
| F | Roasted and salted | 0.15 |
| G | Roasted and salted | 0.77 |
| H | Roasted and non-salted | 0.21 |
| I | Roasted and salted | 0.38 |
| J | Roasted and salted | 0.18 |
| K | Raw cashew nut | 0.14 |
| L | Roasted and non-salted | 0.21 |

limit of detection (LOD) and limit of quantification (LOQ) were 0.01 and 0.02 µg/kg respectively with 85% percentage recovery. The toxins ($G_2$, $G_1$, $B_2$ and $B_1$) were eluted at 5.5–6.5, 6.5–8.0, 8.0–9.5 and 10.0–11.5 min respectively.

The aflatoxin content of the nuts were not significantly different ($p \leq 0.5$) and ranged from 0.03 to 0.77 µg/kg with roasted and salted cashew nuts from location B and G having the least (0.03 µg/kg) and highest (0.77 µg/kg) aflatoxin concentrations respectively (Table 7).

## Discussion

The low fungal counts of the nuts may be due to the presence of anacardic acid in the nuts which suppressed the growth of microorganisms (Oluwafemi et al. 2009) in the nuts. A similar report of low fungal count ($1–9 \times 10^3$ and $1–4.5 \times 10^2$ cfu/g) was reported for plain and salted roasted cashew nuts from Erbil market in Iraq (Abdulla 2013) and selected locations in Lagos, Nigeria (Adetunji et al. 2018). The authors further reported that salted and roasted cashew nuts from Iraq recorded the least fungal count of $3 \times 10^3$, in contrast, no significant differences were recorded in the fungal counts of salted and non-salted cashew nuts in this report. Furthermore, higher fungal counts of $1.8–163 \times 10^2$ cfu/g (Adebajo and Diyaolu 2003), $1.0–52.0 \times 10^5$ cfu/g (Adeniyi and Adedeji 2015) $1.0–14 \times 10^4$ cfu/g (Oluwafemi et al. 2009) and were previously reported for cashew nuts from various locations of Nigeria. The geographical and climatic variations across the different countries may be responsible for the differences in the microbial counts.

The predominance of *A. niger*, *A. flavus* and *Penicillium* spp. in the cashew nuts corroborated the report of previous authors who reported the prevalence of the afore mentioned organisms in their cashew nuts (Adebajo and Diyaolu 2003; El-Samawaty et al. 2014). In addition, Adeniyi and Adedeji (2015) reported on the presence of *Fusarium* spp. in cashew nuts from Nigeria, El-Samawaty et al. (2014) also documented the presence of *Fusarium* spp. from cashew nuts from Saudi Arabia. However, we did not isolate *Fusarium* species in our present study as with Adetunji et al. (2018), Adebajo and Diyaolu (2003) and Abdulla (2013).

Some fungal isolates such as *Exserohilum rostratum, Epicoccum sorghinum, Ovatospora unipora, Periconia macrospinosa, Lecanicillium aphanocladii, Monascus purpureus, Arthrinium hyphopodii, Chaetomium globosum* that are not commonly isolated from foods were found in the cashew nuts. *Exserohilum* is a dematiaceous fungi, mostly found in the upper surface of the soil, commonly associated with foliar plant diseases and rarely with human and animal phaeohyphomycosis (Sharma et al. 2014). Though *E. rostratum* is a rare cause of infection in people, the species can cause a spectrum of diseases including allergic fungal sinusitis, skin and subcutaneous infections, invasive diseases and occasionally keratomycosis; an inflammation of the cornea (Joseph et al. 2012). The fungus was implicated in the 2012 nationwide outbreak of fungal meningitis in the United States caused by contaminated corticosteroid injections from the New England Compounding Center (NECC) in Framingham, Massachusetts (Sharma et al. 2014). *Epicoccum sorghinum* (Sacc.) also known as *Phoma sorghina* is a fungus associated with spoilage of sorghum. The presence of this pathogen in sorghum leads to reduced crop yield, seed viability and kernel weight loss and on the long run significant economic losses (Forbes et al. 1992). The fungus produces tenuazonic acid (TA), a mycotoxin that causes acute toxicity to organisms and therefore prevents the consumption of sorghum grains as food and feed (Åkesson et al. 2014). The draft genome of this fungus has genes involved in the TA pathway. The presence of this fungus in cashew nuts might be as a result of cross contamination from the fields.

This report further corroborate the suitability of NRDCA and YES media for the screening of aflatoxigenic fungi in foods and agricultural products (Atanda et al. 2006, 2011), Davis et al. (1987), Abbas et al. (2004) and Ezekiel et al. (2014) as all positive isolates of both media with the exception *of A. nomius* tested positive for aflatoxin production on TLC. Slow growth kinetics or environmental factors such as relative humidity, incubating temperature among other factors might influence aflatoxin producing ability of the isolate on the media. In addition, the concentration of aflatoxin released into the medium might be too low for the ammonia vapor reaction or fluorescence production under UV light In contrast to the higher sensitivity of the TLC. The addition of β-cyclodextrin to the NRDCA further enhanced the production of fluorescence on the agar (Degola et al. 2012; Suzuki and Iwahashi 2016). The bright blue fluorescence of $AFB_1$ is produced by a complex reaction between the toxin released by the fungi into the growth medium and the hydrophobic pocket of β-cyclodextrin (Degola et al. 2012). The cyclodextrins have the unique feature of trapping fluorescences in their cavities (Del Valle 2004) while the yellow pigmentation and ammonium hydroxide vapour tests are based on the production of yellow anthraquinone biosynthetic intermediates in the aflatoxin pathway (Maragos et al. 2008; Sudini et al. 2015).

It was earlier reported that the production of yellow pigmentation, fluorescence and aflatoxin production were complimentary (Atanda et al. 2006, 2011), in agreement, our findings showed a direct correlation between the cultural methods and aflatoxin production. Previous authors had linked the production of yellow pigments by aflatoxigenic fungi to Averufin; an intermediate substrate in the aflatoxin biosynthetic pathway between norsoloniric acid (NOR) and Versicolorin A (Abbas et al. 2004; Sudini et al. 2015). In addition, our work complimented the recommendation of Yazdani et al. (2010) and Fani et al. (2014) that the ammonium vapour test should be combined with pigmentation and fluorescence production test in screening for aflatoxigenic fungi as production of yellow pigmentation alone is not a reliable indicator of aflatoxin production. The authors also suggested the addition of chromatographic and molecular analysis to confirm the aflatoxin producing ability of these fungi.

The combination of these three analytical methods could therefore be a reliable and confirmatory test for presence of aflatoxins in organism or food substrates in developing or underdeveloped countries where access to molecular analytical tools are scarce or unaffordable. The utilization of TLC for detection of mycotoxins in foods or organisms had been reported by several authors (Atanda et al. 2011; Ezekiel et al. 2013) and this method still remains a reliable method of screening for mycotoxins in the laboratory, although its laborious and time consuming.

The presence of two aflatoxin biosynthetic regulatory genes (*aflR and aflJ)* and three other important genes (*omt-A, aflD* and *aflM*) located in the aflatoxin biosynthetic pathway were detected in 70% of the isolates, but the genes were only expressed in 18.57% of the isolates. The presence of these genes in the isolates calls for concern as the unexpressed fungal species are also capable of producing aflatoxins under favourable conditions. It has been reported that *aflR,omt-A, aflD* and *aflM* are the four most important genes among the 25 genes in the aflatoxin biosynthetic cluster that determine aflatoxin production (Davari et al. 2015). However, our findings revealed that the presence of these four genes with *aflJ* does not confirm aflatoxin production by the isolates. The *afl*R gene regulates the activation of the transcript of pathway genes and aflatoxin biosynthesis (Woloshuk et al. 1994) but its presence in the isolates might not necessarily lead to aflatoxin production, as the AF pathway is regulated by many mechanisms (Bok and Keller 2004; Perrin et al. 2007). Similarly, *aflJ* is also an aflatoxin biosynthetic regulatory gene like the *aflR*, the two genes are divergently transcribed but with individual promoters. It is necessary for expression of other genes in the aflatoxin cluster and also involved in the conversion of pathway intermediate products to aflatoxins (Georgianna et al. 2008). The presence of the aflatoxin regulatory (*aflR*) gene was reported in the non- aflatoxin producing *A. oryzea* and *A. sojae* species (Kusumoto et al. 1998; Lee et al. 2006), both *A. flavus and A. oryzea* are morphologically very similar and *A. oryzea* is the domesticated form of *A.flavus* (Payne et al. 2006), the morphological similarity might be responsible for the presence of the aflatoxin biosynthetic genes in the two organisms. Despite the presence of aflatoxin biosynthetic genes in all *Aspergillus oryzea* (except Isolate A8) they were found to be non-aflatoxins producers. Lee et al. (2006) further postulated that when some *A. flavus* strains possess the *A. oryzea aflR* gene, they are non-toxigenic. *A. oryzae* also have the aflatoxin biosynthetic cluster but it does not appear to be functional, this might be as a result of gene deletion, addition, mutation or frame shift which might have occurred in *A. oryzea* during domestication over the years (Tominaga et al. 2006). As *A. spergillus oryzea* is utilized in the food industry for production of fermented foods, the presence of the *aflR* gene signifies a future treat to food industries as this organism has the tendency of expressing the gene in future. The aflR protein can bind the pro-moter region of each aflatoxin synthesis gene and activate gene expression (Woloshuk et al. 1994; Trail et al. 1995; Ehrlich et al. 1999). In addition, AF biosynthesis, is controlled by many environmental factors such as light (Calvo et al. 2002), carbon source, temperature, and pH (O'Brian et al. 2007; Price 2006).

The *omt-A* gene was found to be involved in the conversion of sterigmatocystin (ST) to *o*-methylsterigmatocystin in the aflatoxin biosynthetic pathway but its presence does not indicate the production of aflatoxins. For instance, *A. nidulans* does not produce AF but possess all the genes and enzymatic steps preceding the production of sterigmatocystin (Georgianna et al. 2008). The AF and ST pathways appear to have a common biosynthetic scheme up to the formation of ST, and thus information gained from both pathways has been used to study AF regulation (Hicks et al. 2002; Yu et al. 2004). Changes in the expression of genes within the AF cluster occurs in response to environmental conditions which may be favourable or not favourable for AF biosynthesis (Georgianna et al. 2008). The *Nor-A* gene helps in conversion of norsoloniric acid [the first stable product in the aflatoxin biosynthesis pathway to averufanin (AVNN)] through the activity of the ketoreductase enzyme while *Ver-A* is the gene responsible for the conversion of Averatin (AVN) to Versicolorin A (Ryan et al. 2009; Yu et al. 2004).

All the five aflatoxin biosynthetic and regulatory genes were amplified in the non *Aspergillu*s isolate—*B. spectabilis*. The sequence of the isolate was blasted and identified in two different fungal databse (NCBI and CBS-KNAW) as *B. spectabilis* with 99% similarity with other *B. spectabilis* at the GenBank and also 99% similarity with only one *Aspergillus* spp (Accession No:KJ584845). However, *B. spectabilis* had a higher total score and maximum score (1002) as compared with the *Aspergillus* strain. *Bacillus spectabilis* belongs to the *Ascomycota, Eurotiomycetes, Eurotiales* and *Trichocomaceae* group just as the *Aspergillus*, this might explain the reason for the presence of the aflatoxin biosynthetic gene in this isolate. Furthermore, the phylogenetic neighbor-joining tree analysis showed a 30% evolutionary relationship between the *Aspergillus* spp. and the *B. spectabilis* (Data not shown).

The low concentrations of aflatoxins reported for cashew nuts in this work corroborates the findings of previous workers; 0.3 ng/g for cashew nuts from Iraq (Abdulla 2013), 0.1–6.8 ng/g and 0.1–0.4 ng/g for cashew nuts from Nigeria (Adetunji et al. 2018; Ubwa et al. 2014) and 0.5–0.84 ng/g for cashew nuts from Turkey (Yilmaz and Aluc 2014b). However, higher aflatoxin concentrations were reported for other nuts such as peanuts (17.99 ng/g) and pistachio nuts (22.02 ng/g) from Iran (Ostadrahimi et al. 2014). Aflatoxin $B_1$ which is the most potent and toxigenic of the aflatoxins is a carcinogen, mutagen and immune suppressant (Adetunji et al. 2018; Nleya et al. 2018). Aflatoxin $B_1$ had also been primarily linked to human primary liver cancer and acts synergistically with hepatitis B virus (HBV) infection (Liu and Wu 2010).

The aflatoxin concentration of the nuts were lower than the EU recommended permissible limit of 4 µg/kg for total aflatoxins (EU 2006). Despite the low concentrations of the aflatoxins, consumers of cashew nuts could be at risk of aflatoxicoses as continual consumption of foods contaminated with low doses of toxins could lead to chronic toxicities.

In conclusion, we report for the first time the microbial profile and aflatoxigenicity of fungal isolates from cashew nuts consumed in North West Province, South Africa. An improved form of NRDCA (β-cyclodextrin NRDCA) which enhanced fluorescence was also developed for the first time in this study.

We further corroborate earlier report that a single method may not be adequate to screen for the presence of aflatoxins in foods or isolates, but that a combination of polyphasic methods such as cultural, analytical, chromatographic (TLC) and molecular methods (PCR) is much better. A combined approach provides a more accurate, and sensitive method for detection of aflatoxigenic species and aflatoxins in the nuts. The results also showed a strong correlation between the presence of aflatoxin biosynthetic genes as analyzed by molecular PCR and aflatoxin detection by TLC. The presence of aflatoxin producing genes in the *Aspergillus* isolates is an indication that the organisms are potential aflatoxin producers when favourable conditions are available. This could pose a future risk of exposure to aflatoxins by the consumers of cashew nuts.

However we were not able to ascertain conclusively the aflatoxin biosynthetic genes that were responsible for aflatoxin production, as majority of the isolates that did not produce aflatoxins on TLC plates possess the aflatoxin biosynthetic genes and this calls for further research including the presence of aflatoxin biosynthetic gene in *B. spectabilis*.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no conflict of interest.

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