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# Effect of sorghum (*Sorghum bicolor* L. Moench) grain conditions on occurrence of mycotoxin-producing fungi

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## Abstract

**Background:** Sorghum is a staple cereal crop that is well adapted to arid and semi-arid lands (ASALs). It has a potential of assuring food security and livelihoods in the ASALs. The objective of this study was to determine the effect of sorghum grain conditions on occurrence of mycotoxin-producing fungi.

**Methods:** Two kilograms of sorghum grains were sampled from the breeder's crop at Egerton University research field and at a farmer's field at *Kampi Ya Moto*. Sorghum was sampled at dough stage and at physiological maturity. Sorghum grains sampled at dough stage were divided into three sets. Set one was immediately examined for fungi; set two was sun dried for 21 h and set three was stored for a fortnight before being examined for fungi. Grains were plated on potato dextrose agar medium and incubated at  $25 \pm 2$  °C for 7 days. Fungal colonies growing were sub-cultured and identified using a microscope and a standard mycological catalogue based on micro- and macro-morphological features.

**Result:** Identified fungi were *Aspergillus, Penicillium* and *Fusarium* species. Aflatoxins were detected in 37 samples using reverse-phased HPLC at a wavelength of 365 nm. The aflatoxin B1, B2, G1 and G2 were detected in 10.81, 5.41, 18.92 and 32.43 % of the samples, respectively. There was no aflatoxin detected on 32.42 % samples. These results would contribute to reduced risk of mycotoxin-producing fungi in sorghum grain, minimize grain losses and improve grain quality among smallholder farmers in sorghum growing areas.

**Conclusion:** Aspergillus, Fusarium and Penicillium species of fungi do occur in sorghum grain both in the field and in the store.

Keywords: Fungi, Mycotoxin, Sorghum bicolor

## Background

Sorghum grains are often contaminated by moulds. They are ideal substrates for mould growth when poorly dried and stored [1], and is a serious biotic constraint in sorghum production areas. Many of these fungi are facultative parasites or saprophytic fungi. They contribute to pre- and post-harvest deterioration of grains [2]. Grain infection occurs at the base near the pedicel interfering with grain filling and causes premature formation of black layer. This leads to development of smaller seeds

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A major concern associated with grain mould is the production of mycotoxins which are harmful to both humans and animals [4]. The species associated with mycotoxin production on contaminated sorghum grains



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are Aspergillus sp. Fusarium sp. and Penicillium sp. [4]. Aspergillus sp. produces aflatoxins B1, B2, G1 and G2, while Fusarium sp. produces mycotoxins, fumonisins, trichothecenes and zearalenone. Penicillium sp. produces mycotoxin isofumigaclavine. Mycotoxin contamination may occur while the grain is still in the field, soon after harvesting and during storage [5]. The Food and Agriculture Organization (FAO) estimates that 25 % of the world's food crops are affected by mycotoxin. In an attempt to harmonize the current tolerance to aflatoxin, World Health Organization (WHO) and FAO proposed maximum limits of 15  $\mu$ g/kg [6]. The objective of this study was to determine mould and mycotoxin contamination of sorghum grain obtained from farmers and freshly harvested from the field.

## Methods

## Collection of sorghum samples during farm survey

Surveys were carried out in major sorghum growing regions in Kenya. These were Bondo and Siaya subcounties in Siaya county, Kibwezi and Kathonzweni sub-counties in Makueni county and Rongai and Njoro sub-counties in Nakuru county. During the survey, a total of 88 samples were collected from farmers' storage facilities. One thousand grams of each sample was placed in a sterile khaki sampling paper bags and kept at 4 °C in the laboratory until the time of analysis.

### Preparation of culturing medium

The medium for fungi isolation was potato dextrose agar (PDA). The medium was prepared according to the manufacturer's recommendations. Thirty-nine grams of PDA powder was suspended in one litre of distilled water in a conical flask. The medium was heated on a hot plate and stirred till the mixture dissolved completely. It was heated for the powder to completely dissolve. The medium was sterilized by autoclaving (model WACS-1100) at 121 °C for 15 min and then allowed to cool to about 45 °C. The cooled medium was aseptically poured into petri dishes in a laminar flow hood (model LCB-0153B-A2) and allowed to solidify.

### Isolation of fungi from cultured sorghum grains

For fungal isolation, 1000 seeds of each sorghum sample were surface sterilized in 2.5 % sodium hypochlorite solution for 1 min and then rinsed twice with sterile distilled water. The grains were then placed in 70 % ethanol for 30 s and then rinsed in sterile water. From each sample, 300 grains were placed in the medium. Each sample was replicated three times. Petri dishes were placed in a completely randomized design in an incubator (model PIN30 (201) at temperature of  $25 \pm 2$  °C for 7 days. At the end of this period, the resulting fungal colonies were

individually sub-cultured onto fresh potato dextrose agar.

### Identification of fungi isolated from sorghum grains

Observations on colony colour were made. Each fungal colony was examined under light microscope. Vegetative and fruiting bodies of the isolates of fungal species were identified using mycological keys and manuals [7–9].

### Sampling from field-grown sorghum crop

Sorghum grains were sampled from breeder's crop varieties growing in farmer's field at *Kampi Ya Moto* and Egerton University research field. Five samples were collected from Egerton University, while nine samples were collected from *Kampi Ya Moto* (Table 1). Three plants were sampled from two inner rows in each plot at dough stage and physiological maturity. Samples were placed in sterile khaki paper bag and kept at 4 °C. Samples collected at dough stage were split into three sets as: (1) set I was subjected to immediate examination for fungi, (2) set II was sun dried for 3 days (7 h per day), then examined for fungi and (3) Set III was stored for 2 weeks then examined for fungi.

## Detection of strains of aflatoxin using high-performance liquid chromatography (HPLC)

Based on the preliminary result on the occurrence of fungi on sorghum grains (Tables 1 and 2), a sub-sample of the 23 accessions were selected. The samples were analysed for aflatoxin using a method of Gnonlonfin et al. [10]. One hundred grams of each sample was milled and sieved using a 20-mesh sieve to obtain fine flour. Ten grams of each sample was obtained and placed in a conical flask containing 1 g of sodium chloride and 25 ml of extraction solution [methanol/water (80/20, v/v)]. The mixture was shaken at 250 rpm for 10 min using an orbital shaker (model Heidolph unimax 2010) followed by centrifugation (Model 6000 Centurion) at 4000 rpm at 5 °C for 5 min. The extract was filtered through a Whatman No. 1 filter. After filtration, 10 ml of the filtrate was diluted with 40 ml of distilled water. Thereafter, 10 ml was passed through aflatest immunoaffinity column (VICAM, Watertown) fitted on a solid phase manifold at a flow rate of 1 drop/s. The immuno affinity column was washed with 15 ml water. Aflatoxins were eluted with three millilitre methanol into a 4 ml amber vial and then stored at 4 °C until analysis. The experiment was repeated four times.

After preparation of samples, aflatoxins were analysed using reverse-phased isocratic HPLC (model surveyor PDA detector, auto sampler plus and pressure pump) [10]. The mobile phase was water/methanol/acetonitrile (2500/550/550) at flow rate of 2.0 ml/min. Aflatoxin extract

Sample accession	Sampling site	Sorghum variety	Grain conditions	Fusarium sp.	Aspergillus sp.	Penicillium sp.
EU04	Egerton	Ndamoga	IM	+	+	+
EU04	Egerton	Ndamoga	PM	+	+	_
EU07	Egerton	IS9203	PM	+	_	_
EU08	Egerton	Cyhure	PM	+	_	+
EU010	Egerton	IS25561	PM	+	+	_
EU010	Egerton	IS25561	Stored	+	_	+
KYM	KYM	Cyhure 55	IM	_	+	+
KYM02	KYM	Cyhure 55	PM	+	+	+
KYM03	KYM	IESV93042SH	Stored	_	+	_
KYM04	KYM	Kipkelion#2	Stored	+	+	+
KYM04	KYM	Kipkelion#2	Sun dried	+	_	_
KYM05	KYM	Nyiragikoli	Sun dried	+	_	_
KYM06	KYM	IS8884	Sun dried	+	_	_
KYM07	KYM	Muhimpundu	Sun dried	+	_	_
KYM10	KYM	Kipkelion #1	PM	+	_	_
KYM10	KYM	Kipkelion #1	Sun dried	+	+	_

Table 1 Occurrence of fungi in sorghum grain conditions from fields at Egerton University (EU) and Kampi Ya Moto (KYM)

+ Fungi present, – fungi absent

PM physiological maturity, IM immediate observation after harvesting

was injected at 10  $\mu$ l. The fluorescence detector was set at excitation of 365 nm and emission of 440 nm. The postcolumn derivatization was performed using photochemical reactor for enhanced detection. The data were collected and processed using HP chem station (Darmstadt, Germany) for LC software. Determination of aflatoxin by HPLC was based on retention time in minutes as G1 (10.6 min), G2 (9.6 min), B1 (13.9 min) and B2 (12.9 min) [11].

## Results

## Morphological features of fungi identified in sorghum grain conditions

Aspergillus species colonies on potato dextrose agar (PDA) were grey–green to dark green rapidly growing, densely matted, heavy rapidly growing and spreading. Morphology of conidiophores was  $10-20 \,\mu$ m in diameter just below the apex. Phialides measured  $6-10 \times 3-5 \,\mu$ m in diameter. Conidia were typically spherical to subspherical, spiny,  $3-6 \,\mu$ m in diameter and oval to pear-shaped. Colonies growing on agar were colourless to brownish (Fig. 1).

*Fusarium* species; macro conidia and micro conidia were septate and measured  $3-5 \times 10-13 \ \mu\text{m}$  in diameter. Macro conidia were stout, distinctly septate, had a curved ventral and dorsal surface. Micro conidia were spindle shaped. Conidiophores were unbranched and branched monophialides with 240–350  $\mu\text{m}$  in diameter. Colony colour was white to orange with the latter colour being common. Plate reverse was that of the upper surface (Fig. 2).

*Penicillium* species; the colony morphology was bluegreen. Plate reverse was yellow. Conidiophores arose from aerial hyphae. Conidial chains were borne on phialides. Conidia were globose. Metulae were of relatively uniform length,  $12-15 \mu m$  long and a terminal vesiculate of approximately 5  $\mu m$  diameter. Phialides were ampulliform,  $8-12 \mu m$  long. Conidia were relatively spherical,  $2.0-3.0 \mu m$  in diameter borne in long, well-defined columns one per metula arranged in a characteristic whorl on each conidiophore (Fig. 3).

# Occurrences of mould fungi in sampled stored sorghum grains from farmers' storage facilities

Results presented in Table 2 indicated that 35/88 (39.77 %) of the samples were infected with *Aspergillus* species, 29/88 (32.95 %), with *Fusarium* species and 12/88 (13.64 %) of the samples were infected with *Penicillium* species.

There were no fungi contaminant in 35 samples, while sorghum sample accession Si022 (Nyakotoyo) was the only sample that had been infected with *Aspergillus*, *Fusarium* and *Penicillium* species, the three mould fungi species.

# Occurrence of mould fungi on field samples sorghum grains

The results presented in Table 1 indicate that the most predominant species was *Fusarium* sp. All samples except KYM03 were contaminated with *Fusarium sp.* (92.9 %). *Aspergillus* sp was isolated in five samples

 Table 2 Occurrence of fungi in sorghum grain from farmers' storage facilities

Sample accession	Variety	Fusarium sp.	<i>Aspergillus</i> sp.	Penicillium sp.
Bon009	Ochuti	+	+	_
Bon012	Andiwo	+	+	_
Bon015 <sup>a</sup>	Gadam	-	_	-
Bon020 <sup>a</sup>	Local	-	-	_
Bon021	Nyakidi	+	+	_
Bon023 <sup>a</sup>	Nyakotoyo	-	-	_
Bon026	Ochuti	+	+	_
Bon028	Seredo	+	+	_
Bon029 <sup>a</sup>	Seredo	+	_	_
Bon057	Seredo	-	+	-
Bon058	Seredo	-	+	_
Bon083 <sup>a</sup>	Rakwar	-	_	_
Bon084 <sup>a</sup>	Seredo	-	_	_
Bon086ª	Sila	_	_	_
Bon087	Serena	-	+	_
Ka059 <sup>a</sup>	Gadam	-	_	_
Ka060	Local	+	_	+
Ka061ª	Local	-	_	_
Ka062 <sup>a</sup>	Local	-	_	_
Ka063 <sup>a</sup>	Seredo	-	-	_
Kib064	Seredo	+	_	_
Kib065ª	Seredo	-	_	_
Kib066	Seredo	_	+	_
Kib067 <sup>a</sup>	Seredo	-	_	-
Kib068	Seredo	-	+	-
Kib069	Gadam	-	+	_
Kib070	Gadam	+	+	_
Kib071	Gadam	-	+	-
Kib072	Local	+	+	-
Kib073	Local	+	+	—
Kib074	Gadam	-	+	_
Kib075	Gadam	-	+	_
Kib076	Gadam	-	+	—
Kib077	Gadam	+	+	-
Kib078 <sup>a</sup>	Serena	_	_	-
Kib079	Serena	+	+	-
Kib080 <sup>a</sup>	Gadam	-	_	_
Kib081	Seredo	+	+	_
Nj030	EU26	+	_	+
Nj031	EU26	+	-	+
Nj032	EU26	+	-	+
Nj033	EU26	-	+	+
Nj034	EU26	-	_	+
Nj035	EU26	-	_	+
Nj036	Local	+	_	-
Nj037	Local	_	_	+
Nj038	Seredo	-	-	+
Nj039	Seredo	_	_	_

## Table 2 continued

Sample accession	Variety	Fusarium sp.	Aspergillus sp.	Penicillium sp.
Nj040	Seredo	_	_	+
Ron045 <sup>a</sup>	Local	_	_	_
Ron046 <sup>a</sup>	Local	_	_	_
Ron047 <sup>a</sup>	Local	_	_	_
Ron048 <sup>a</sup>	Local	_	_	_
Bon088ª	Local	_	_	_
Ron049 <sup>a</sup>	Local	_	_	_
Ron050 <sup>a</sup>	Local	_	_	_
Ron051 <sup>a</sup>	Local	_	_	_
Ron052 <sup>a</sup>	Local	_	_	_
Ron053 <sup>a</sup>	Local	_	_	_
Ron054 <sup>a</sup>	Local	_	_	_
Ron055 <sup>a</sup>	Local	_	_	_
Ron056 <sup>a</sup>	Local	_	_	_
Ron41	EU26	+	_	+
Ron42 <sup>a</sup>	EU26	_	_	_
Ron43 <sup>a</sup>	EU26	_	_	_
Ron44 <sup>a</sup>	Gadam	_	_	_
Si001	Local	+	_	_
Si002 <sup>a</sup>	Local	_	_	_
Si003 <sup>a</sup>	Local	_	_	_
Si004 <sup>a</sup>	Local	_	_	_
Si005	Nyaisife	_	+	_
Si006	Nyaodakuru	_	+	_
Si007	Nyauranga	+	+	_
Si008	Ochuti	+	+	_
Si010	Ofunjo	_	+	_
Si011	Ofunjo	+	+	_
Si013	Gadam	+	-	_
Si014	Gadam	+	+	_
Si016	Gopari	_	+	_
Si017 <sup>a</sup>	Nyakabala	_	_	_
Si018	Nyakabala	_	+	_
Si019	Nyakabala	+	+	_
Si022 <sup>b</sup>	Nyakotoyo	+	+	+
Si024	Nyakwadida	_	+	_
Si025 <sup>a</sup>	Nyaloka	_	_	_
Si027	Ochuti	+	_	_
Si082	Othuwa	_	+	_
Si085	Oloro	+	_	—

+ Fungi present, – fungi absent

Bon Bondo sub-county, Ka Kathonzweni sub-county, Kib Kibwezi sub-county, Nj Njoro sub-county, Ron Rongai sub-county, Si Siaya sub-county

<sup>a</sup> Sample with no contaminant

<sup>b</sup> Sample with three fungal species

(35.7 %), while *Penicillium* sp was isolated from two samples (14.3 %) namely EU08 and KYM04. All the three fungal species were isolated from KYM04.



Fig. 1 Aspergillus species. a Colonies on plate upper surface. b Colonies on back of plate



nies on *back* of plate



### Aflatoxin in sorghum grains

Results presented in Table 3 indicate aflatoxin B1, B2, G1 and G2 which were detected using HPLC method. Aflatoxin B1 was detected in 10.81 % of the samples analysed. Aflatoxin B2 was detected in 5.41 %. Aflatoxin G1 was detected in 18.92 % of the samples, while aflatoxin G2 was detected in 32.42 % sorgum samples. There was no aflatoxin in 32.42 % of the samples. Three of the B1 aflatoxin and G2 were detected in farmers' stored sorghum grains sample represented by Bon012, Bon058 and Kib073 and only in EU10 in freshly harvested grains. B2 was detected in freshly harvested sorghum grains represented by KYM04 and KYM10.

## Discussion

Three common mould fungi were isolated in sorghum grains which were collected from farmers' stores and also from freshly harvested grains. Aspergillus species was predominant in sorghum from farmers' stores (Table 2), while Fusarium species was predominant in freshly harvested grains (Table 1). Aspergillus species are of particular concern because of their effects on human health. Aflatoxins have both carcinogenic and hepatotoxic actions. Depending on the duration and level of exposure, dietary exposure to aflatoxins is a major risk factor for hepatocellular carcinoma, particularly in areas where hepatitis B virus infection is endemic. Ingestion of higher doses of aflatoxin can result in acute aflatoxicosis, which manifests as hepatotoxicity [12]. An outbreak of aflatoxicosis from contaminated food has been documented in Kenya, India, and Thailand [13]. In April 2004, an outbreak of hepatotoxicity was identified among people living in Kenya's eastern and central parts. Epidemiologic investigations determined the cause of aflatoxin poisoning as ingestion of contaminated maize (corn). In that year, 317 cases were reported with 125 deaths as a result of aflatoxicosis [14]. The infestation and infection of Fusarium in cereals are of great concern worldwide as plant pathogens and producers of mycotoxins. Fusarium species occurring in cereal grains have inhibitory effects of trichothecenes on eukaryotic cells [15]. Toxins secreted by this fungus in grains have been found to be associated with disruption of normal cell function by inhibiting RNA, DNA, protein synthesis, and cell division [15, 16]. Penicilium species are of particular public health importance because they have both carcinogenic and hepatotoxic actions. It depends on the duration and level of exposure [17].

The isolation of fungal species belonging to the genera *Aspergillus, Fusarium* and *Penicilium* species was in conformity with the findings of Monica et al. [17] who reported the isolation of the same fungal species. The predominance of *Aspergillus* species from farmers' storage facilities observed in this study conforms to reports elsewhere [17] and [18]. It also concurs with the findings of stored rice grains in Nigeria [7]. *Aspergillus* species dominates on cereals in the tropics [19]. It grows at high moisture content and more rapidly than the *Fusarium* and *Penicillium* species as these latter two will take a longer time to sporulate. *Aspergillus* species infection in sorghum grains is a serious problem in a crop grown under rainfed conditions. *Aspergillus* species infection of the crop occurs in the field before harvest [20].

The results presented in Table 2 indicated that 35/88 (39.77 %) of the samples were infected with *Aspergillus* species. Sorghum grains infestation by micro-organisms is a common and widespread phenomenon which has been widely reported. *Aspergillus* species was

Sample accession Grain condition		Retention time (min)	Fungi	Aflatoxin detected
Bon012	Farmers store	14.0	A. sp	B1
Bon015	Farmers store	10.6	A. sp	G1
Si018	Farmers store	9.7	A. sp	G2
Si019	Farmers store	10.5	A. sp	G1
Bon020	Farmers store	_	-	-
Bon023	Farmers store	10.6	A. sp	G1
Bon026	Farmers store	9.5	A. sp	G2
Si027	Farmers store	_	-	-
Bon029	Farmers store	9.7	A. sp	G2
Bon057	Farmers store	9.6	A. sp	G2
Bon058	Farmers store	13.9	A. sp	B1
Kib068	Farmers store	9.6	A. sp	G2
Kib070	Farmers store	9.7	A. sp	G2
Kib072	Farmers store	9.7	A. sp	G2
Kib073	Farmers store	14.0	A. sp	B1
Kib074	Farmers store	_	-	-
Nj030	Farmers store	9.7	A. sp	G2
Nj038	Farmers store	10.5	A. sp	G1
Nj039	Farmers store	_	-	-
Ron041	Farmers store	_	-	-
Si004	Farmers store	10.4	A. sp	G1
Si017	Farmers store	_	-	-
Si085	Farmers store	_	-	-
EU04	PM	9.7	A. sp.	G2
EU07	PM	_	-	-
EU08	PM	_	-	-
EU10	PM	14.0	A. sp.	B1
EU10	Stored	9.6	A. sp.	G2
KYM02	PM	9.6	A. sp.	G2
KYM03	Stored	10.5	A. sp.	G1
KYM04	Stored	12.1	A. sp.	B2
KYM04	Sun dried	_	-	-
KYM05	Sun dried	_	-	-
KYM06	Sun dried	_	-	-
KYM07	Sun dried	10.6	A. sp.	G1
KYM10	PM	12.1	A. sp.	B2
KYM10	Sun dried	9.6	A. sp.	G2

### Table 3 Aflatoxin producing strains detection in sorghum grains using high-performance liquid chromatography

A. sp Aspergillus species, PM physiological maturity, EU Egerton University, KYM Kampi Ya Moto

reported in *Vigna unguiculata* seeds in Ibadan, Nigeria [21] and in *Saccharum officinarum* seeds [22]. Storage fungi are usually not present in large quantities before harvest but are widely distributed and almost always present. Contamination occurs even with small quantities of spores in the grain as it is taken for storage after harvesting. The spores could be spread through handling, or in storage equipment or from spores already present in storage structures. Under high temperature

and moisture, the small amount of inocula can increase rapidly [23].

The most predominant fungi species in field evaluated sorghum grains was *Fusarium* which infected 13/14 (92.85 %) samples. *Fusarium* species are consistently associated with infection at early grain development stages across the agro-ecological zones [24]. The fungus is a natural contaminant in cereals and other agricultural commodities [25]. This makes *Fusarium* species an economically important genus of fungi as it causes diseases on a wide variety of plants at different developmental stages and also in plant products [26]. A widespread distribution of *Fusarium* species is attributed to the ability of the fungi to grow on a wide range of substrates and their efficient mechanism of spore dispersal [27]. The results of this study are similar to those obtained by earlier workers that show *Fusarium* sp as a dominant species isolated from maize and sorghum samples [28, 29]. Though maize and sorghum are the most infected by Fusarium, an oilseed has also been shown to be susceptible [30].

In this study, *Penicillium* species was not predominant in the sorghum grains (Tables 1, 2). This finding is similar to observations elsewhere [19] on *Penicillium* species dominating in temperate zones, and the fungus is known to take longer time to sporulate. Similar results on *Penicillium* species have been reported on stored rice in Argentina and Paraguay [31].

There were interesting observations made in Table 3 which consisted of sorghum accessions selected from the results in Tables 1 and 2. There were four categories of sorghum grains. The first category represented by Bon015 (variety Gadam) had no mould detected on cultures (Table 2) but had aflatoxin G1 (Table 3). The second category represented by the entries Bon012 (var, Andiwo), Bon026 (Ochuti) and Si019 (Nyakabala) had mould growth (Table 2) and had toxin (Table 3). The third category consisted of sorghum samples such as Kib074 (var. Gadam) with mould growth (Table 2) and no toxin was detected (Table 3). The fourth category of sorghum grains had no mould growth (Table 2) and no toxin detected as represented by entries Bon020 and Nj039 (var. Seredo). Sorghum grain with mould fungi and toxins should not be consumed by human or livestock. The Aspergillus sp was of interest as it produces aflatoxin. Four strains of aflatoxin were detected in sorghum grains using HPLC method as B1, B2, G1 and G2 (Table 3). The order of acute and chronic toxicity of the aflatoxin is B1 > G1 > B2 > G2, reflecting the role played by oxidation of the 8, 9-double bond and also the greater potency associated with the cyclopentenone ring of the B series, when compared with the six-member lactone ring of the G series [32]. Aflatoxin B1 is the most toxic of the four aflatoxins. It is considered as a group I carcinogen for humans by International Agency for Research on Cancer (IARC) [33]. Aflatoxin fungi are native to tropical, warm, arid and semi-arid regions [34]. These results are similar to a survey conducted in West Africa that revealed aflatoxin B1, B2, G1, and G2 caused by Aspergillus species contaminated peanut [35] and [36, 37] on peanut products.

### Conclusion

It is evident that *Aspergillus*, *Fusarium* and *Penicillium* species of fungi were the predominant pre- and post-harvest in sorghum grains.

### Authors' contributions

AMK: conception and design, data collection and interpretation, manuscript writing and final approval of the manuscript. EKC: data organization, critical revision and final approval of the manuscript. PFA: designing, data interpretation, critical revision and final approval of the manuscript. JOO: design, critical revision and final approval of the manuscript. All authors read and approved the final manuscript.

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#### **Compliance with ethical guidelines**

### **Competing interests**

The authors declare that they have no competing interests.

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