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Occurrence of mycoflora, their association and production of aflatoxin B_1 in groundnuts

Ncube J., Ndlovu E., Musarandega L. and Maphosa M*

Department of Crop and Soil Sciences, Lupane State University, Box 170, Lupane, Zimbabwe.

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Groundnut (*Arachis hypogaea* L.) is an important food crop in Africa which is a source of nutrients and income in rural areas of Zimbabwe. It is considered to be a crop highly susceptible to aflatoxin contamination. Accordingly, the objectives of this study were to understand the presence of mycoflora, their association and the level of contamination by aflatoxins of groundnut from various markets in Zimbabwe. Thirty groundnut samples were purchased randomly from Bulawayo (Shashe and Main market), Gweru (Kudzanayi and Kombayi markets) and Harare (Mbare and Highfield markets). Identification of various fungi was determined using the cultural method on Czapek Dox Agar. Fungi belonging to genera *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus* were isolated and characterised from six groundnut markets. *Rhizopus* species was the most dominant and negatively associated with other fungi species which is attributed to differences in environmental requirements or competition. *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus parasiticus* were identified in groundnut samples with *A. flavus* being the dominant and found in all markets. The range of AFB_1 in groundnut samples analysed using a semi-quantitative immunochromatographic technique was within the safe limits for human consumption according to existing Zimbabwe (5 ppb) regulation. The presence of aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) in groundnuts, however, means there is potential for aflatoxin production and fungal proliferation when conditions are favourable.

Key words: Aflatoxigenic, *Arachis hypogaea, Aspergillus* species, mycology.

INTRODUCTION

The impact of aflatoxin contamination on agricultural commodities is immense and production losses and trade has been severely affected. In developing countries due to lack of storage infrastructure, poor harvesting and handling techniques and lack of effective monitoring mechanisms aflatoxins occur frequently in various agricultural commodities (Negash, 2018). One of these crops is groundnut that is widely grown in semi-arid areas and prone to aflatoxin contamination. There is low production of groundnut in African countries because of unreliable rains, lack of inputs, use of retained seed, poor agronomic practices, pests and diseases (Ajeigbe et al., 2015; SNV, 2012). Among other challenges is the frequent recurrence of droughts and variable rainfall patterns. Drought increases the probability of aflatoxin contamination on groundnut at any stage of production

*Corresponding author. E-mail: mmaphosa@lsu.a.czw.

cycle and thereafter affecting international exports from Africa. Warm climate and high humidity experienced in most tropical countries predispose crops to aflatoxin contamination (Pazderka and Emmott, 2010).

Groundnut is an important crop in the livelihoods of many Zimbabweans and comes second after maize. Majority of farmers grow groundnut during rainy season which is a risk factor during harvest and post-harvest handling that predisposes the crop to aflatoxin contamination. The ideal environmental conditions for aflatoxin production in stored seed are high temperatures up to 45°C, high humidity 65 to 90%, moisture in excess of 9%, damage by insect pests, rodents and lack of genetic inheritance in the host plant (Okello et al*.*, 2010; Ncube and Maphosa, 2020).

Dube and Mtetwa (2015) showed that the majority of stakeholders involved in the groundnut value chain in Mutare, Zimbabwe had never heard about aflatoxins. Several researchers showed that most commodities were contaminated by aflatoxins in Zimbabwe especially groundnuts and maize (Nleya et al., 2018). Dried traditional foods in Zimbabwe were found contaminated by the aflatoxigenic fungus from Bulawayo markets (Dangwa et al., 2014). Similarly, Mupunga et al*.* (2014) observed that groundnuts from Bulawayo markets in Zimbabwe were contaminated with high levels of aflatoxin. Dube and Maphosa (2014) reported aflatoxin prevalence in groundnut samples collected from seven districts of the Matebeleland provinces in Zimbabwe. Aflatoxin results for groundnuts from Bulawayo showed that 17% of the samples were contaminated with total aflatoxins ranging from 6.6 to 622.1 ppb (Mupunga, 2013). Accordingly, there is need for more research and understanding the prevalence of the aflatoxigenic fungi and current levels of aflatoxin contamination of groundnut on the market. There has been no attempt to identify which fungal species are more prevalent in groundnut as well as their association. Accordingly, the knowledge of aflatoxigenic fungal diversity, their association and prevalence of aflatoxin contamination will lead to informed breeding for resistance and aflatoxigenic management.

MATERIALS AND METHODS

Collection of groundnut samples

Thirty samples of groundnuts were purchased from six open markets in three cities of Zimbabwe, Bulawayo (Shashe and Bulawayo main markets), Harare (Mbare and Highfield markets) and Gweru (Kombayi and Kudzanayi markets). These markets were purposively selected because they receive groundnuts from most places of the country. Five groundnut vendors were randomly selected from each market and raw, shelled 1 kg groundnut samples were collected per vendor. Samples collected represented the whole consignment and was done by collecting groundnuts from different sections of the sack. Collected samples were packaged in polyethylene bags, sealed, labelled and transported to Lupane State University Laboratory and kept in a cool refrigerator at 4°C.

Preparation of media

Czapek Dox Agar (CDA) (Thermoscientific, UK) was used to isolate and identify the fungi and was prepared according to manufacturer's instruction.

Fungal isolation

Isolation of fungi from groundnuts was done under laboratory conditions using Czapek dox agar. Groundnut samples were surface sterilised for one minute using 80% ethanol and air dried for 30 min. Sterilized and unsterilized samples were plated on petri-dishes (10 kernels per petri dish) containing CDA. Ten dried seeds were placed in each petri-dish and replicated three times. Plates were incubated at 25°C for 72 h and replicated three times in completely randomised design. After incubation, morphological and growth characteristics were observed under a microscope. Colony characteristics (colour, shape) that grew and number of seeds infected with the same type of fungus were recorded. The individual isolates were transferred to new CDA plates in order to obtain pure cultures. Inoculation was done using flame sterilised inoculating needle dipped into a spore formed of the suspected fungi. The Petri-dish containing the CDA medium was spot inoculated and incubated at 25°C for 72 h in an incubator. Slides were made for examination of morphological characters under a microscope and colony characteristics were recorded (Cappuccino and Sherman, 1999).

Characterisation and identification of fungi

Identification was done using microscopic and macroscopic examination based on colony and morphological characteristics of pure cultures of the isolates. The micro morphological characteristics has been used for fungal identification including the shape of conidia heads, serration, the number of branching points between vesicle and philiades (uniseriate or biseriate), stripes (colour, shape, texture, and dimensions), vesicles shape and diameter, presence of metulae and conidia (Samson et al., 2014; Nyongesa et al., 2015). The *Aspergillus* consists of swollen conidiophore tips forming vesicle with philiades and metulae with chains of conidia. However, conidiophore tip of *Penicillium* lacks vesicles and has a number of metulae followed by philiades (Campos, 2019). The macro morphological features were used for identification of species based on the colony colour and texture (yellow green for *Aspergillus flavus* and dark or nearly ivy green for *Aspergillus parasiticus*) using the identification keys illustrated in a manual (Cappuccino and Sherman, 1999). However, the following characteristics of colonies were also considered; colony growth rates, texture, colour of mycelia, colony reverses and degree of sporulation (Samson et al., 2014; Nyongesa et al., 2015). Based on macro and micro morphological characters, the following species were identified, *A. flavus, Aspergillus fumigators*, *Aspergillus niger*, *A. parasiticus*, *Mucor* species, *Penicillium* species, and *Rhizopus* species. Different *Aspergillus* species with varying morphologies were identified. Macro and micro morphological characteristics were used for fungal identification from genus to species level together with taxonomic keys (Cappuccino and Sherman, 1999; Samson et al., 2014; Nyongesa et al., 2015).

Semi-quantitative detection of AFB_1 using immunochromatographic technique

AFB_1 Rapid Test based on competitive lateral flow immunochromatographic assay (Krska and Molinelli, 2009) was performed as a semi-quantitative step to identify whether aflatoxins

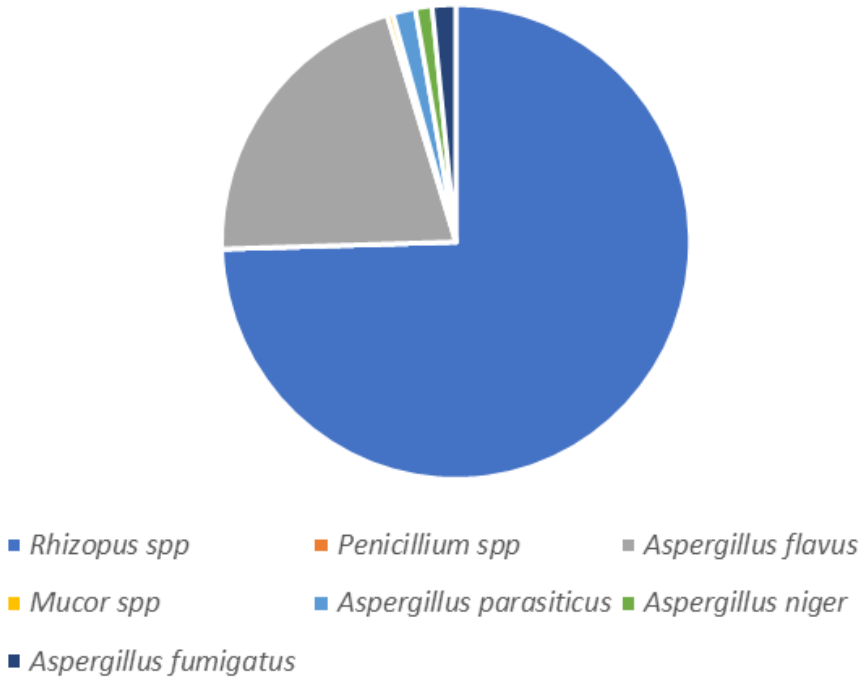

Figure 1. Fungal diversity isolated from groundnut in the major markets of Zimbabwe.

AFB_1 were present. This method has a lower limit of 5 ppb for AFB_1. Thirty samples of groundnuts were brought to the laboratory and each sample was mixed thoroughly to achieve complete homogenization. About 10 g of groundnuts were picked from each sample and grounded to the particle size of the instant coffee using the pastel and mortar. A 2 g groundnut sample, 2 ml of pure water, 8 ml of ethyl acetate were added into a 15 ml centrifugal tube and mixed for 10 minutes. After emulsification, the sample was centrifuged for 5 minutes at 4000 rpm. About 4 ml of supernatant (ethyl acetate layer) was transferred into a 250 ml beaker and evaporated to near dryness and diluted in water. The results of AFB_1 test were interpreted within 5–10 minutes after placing the droplets into the assay hole (Krska and Molinelli, 2009).

Data collection

Genus and species of fungi found growing on the surface of groundnuts samples collected from six groundnut markets were recorded together with their frequency of occurrence (Algabr et al., 2018). The percentage frequency of occurrence was calculated using the following formula:

% Frequency = Number of seeds on which fungi are growing / Total number of seeds × 100

Experimental design and statistical analysis

The experimental design used in this research is a 6×2 factorial replicated three times arranged in Completely Randomised Design (CRD). The two factors were divided into two categories which are markets and sterilisation. Principal component analysis was used to detect fungal species association and level of importance. All data were analysed using Genstat 13 (Payne et al., 2010).

RESULTS

Incidence of fungal contamination in groundnuts

Prevalence of fungal species significantly differed ($p<0.05$) across the six markets. *Rhizopus* species were the most prevalent fungus followed by *A. flavus* (Figure 1).

A. flavus was found in both sterilised and unsterilised groundnut from the six markets (Table 1). *Rhizopus* spp. were isolated from both non-sterilised and surface sterilised samples of five groundnut markets with a higher percentage of non-sterilised than in sterilised groundnut samples. *Penicillium* species were found in Harare Mbare market and isolated in 0.6% of surface sterilised groundnuts. Bulawayo Shashe market was the only market were *Mucor* spp. (4%) was found in non-sterilised groundnuts.

Principal component analysis of fungal distribution in groundnut samples of six markets of Zimbabwe

The principal component biplot analysis showed high correlations between *A. flavus* and *A. fumigatus* and *A. parasiticus* and *Penicillium* spp. as evidenced by the same direction dimension vectors and small angles between them (Figure 2). *A. flavus* and *A. parasiticus* were the most prominent fungal species as shown by the long vector. *Rhizopus* was negatively associated with all

Table 1. Fungal species (%) isolated from groundnuts using CDA method.

Market	BS	BS	BM	BM	GK	GK	GKo	GKo	HM	HM	HH	HH
State of seed	n-s	s-s	n-s	s-s	n-s	s-s	n-s	s-s	n-s	s-s	n-s	s-s
Rhizopus spp.	75	67	-	-	66.6	40.6	82.6	80	90	84	68.3	80.6
Penicillium spp.	-	-	-	-	-	-	-	-	-	0.6	-	-
Mucor spp.	4	-	-	-	-	-	-	-	-	-	-	-
Aspergillus flavus	27	11	26	18	38	33.3	30	26	10	8	7.3	6
Aspergillus parasiticus	2	-	3	-	-	-	-	-	-	0.6	3	0.6
Aspergillus fumigatus	0.7	-	8	2.7	1.3	1.3	-	-	0.6	-	-	-
Aspergillus niger	0.67	-	2.6	-	0.6	-	-	-	-	-	-	2

BS - Bulawayo Shashe, BM - Bulawayo Main, GK - Gweru Kudzanayi, GKo - Gweru Kombayi, HM - Harare Mbare, HH- Harare Highfield, n-s - not sterilised, s-s - surface sterilised.

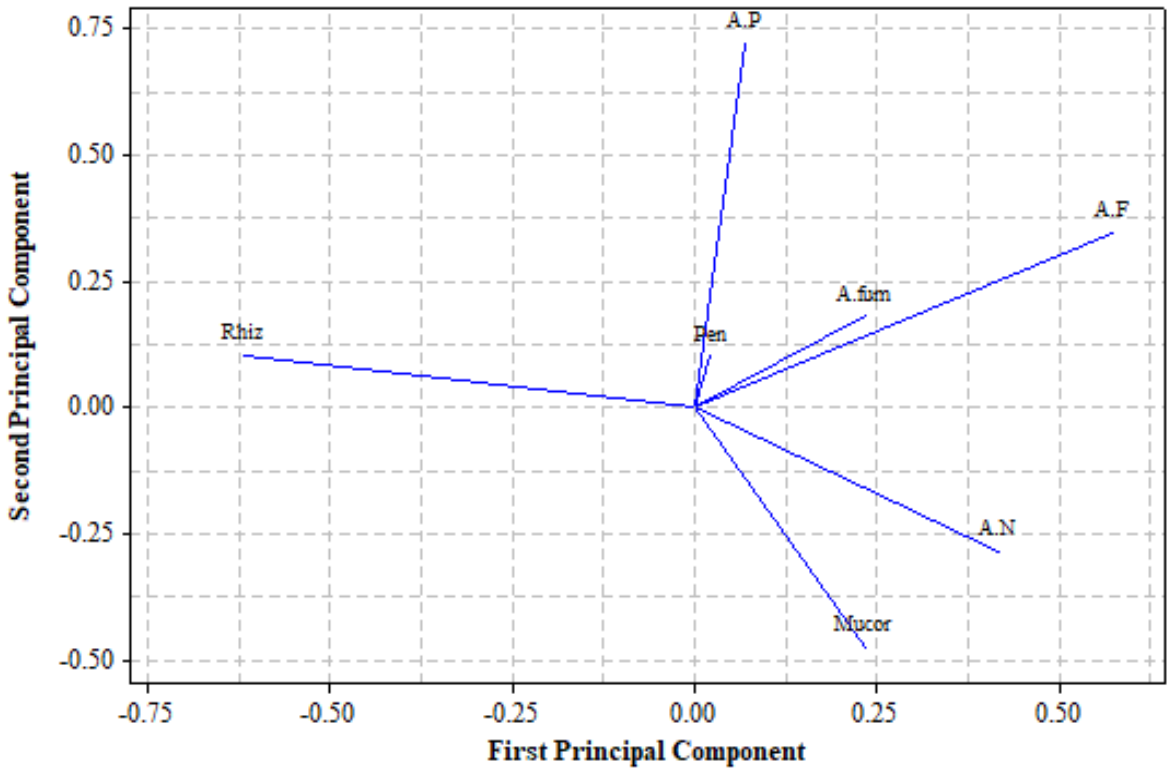

Figure 2. Biplot analysis of six fungal species observed from 30 groundnut samples collected from six major markets of Zimbabwe.
Rhiz - *Rhizopus*, Pen - *Penicillium*, A.fum - *A. fumigatus*, A.F - *A. flavus*, A.N - *A. niger*, -, A.P - *A. parasiticus*.

the other aflatoxigenic fungi with negative vector loadings (Figure 2).

Four principal components were important (eigen value >1) and explained 68% of the total variation. The first principal component explained 23% of the variation and was strongly positively associated with *A. flavus* and negatively associated with *Rhizopus* (Tables 2 and 3).

In the second, third and fourth principal components explaining 38%, 53% and 68% of variance respectively *A. parasiticus* had the highest positive loading (Table 2). These results suggest the high prevalence of the two fungal species, that is, *A. flavus* and *A. parasiticus*.

The results obtained in thirty groundnut samples collected from all six major markets of Zimbabwe, showed that AFB_1 was not detected after analysis. This showed that AFB_1 level was below the cut-off point of 5 ppb (Table 4). The results of each sample were recorded as positive or negative.

Table 2. Eigen analysis of the correlation matrix of six fungal species occurring in 30 groundnut samples collected from main markets in Zimbabwe.

Eigenvalue	1.5918	1.0866	1.0607	1.0114	0.9157	0.7584	0.5754
Proportion	0.227	0.155	0.152	0.144	0.131	0.108	0.082
Cumulative	0.227	0.383	0.534	0.679	0.809	0.918	1.000

Table 3. Rotated component matrix of six fungal species from 30 groundnut samples collected from main markets in Zimbabwe.

Fungal species	PC1	PC2	PC3	PC4
Rhizopus spp.	-0.618	0.104	0.074	-0.012
Penicillium spp.	0.023	0.101	-0.027	0.971
Mucor spp.	0.233	-0.476	0.344	0.036
Aspergillus flavus	0.574	0.347	0.012	0.075
Aspergillus parasiticus	0.069	0.720	0.501	0.146
Aspergillus niger	0.416	-0.285	0.245	-0.088
Aspergillus fumigatus	0.235	0.181	-0.751	-0.145

Table 4. Semi-quantitative detection of AFB_1 (cut-off point of 5 ppb) in groundnuts from major markets of Zimbabwe.

Source/Market	AFB1 (≥5 ppb)
Bulawayo Shashe	Not detected
Bulawayo Main	Not detected
Gweru Kudzanayi	Not detected
Gweru Kombayi	Not detected
Harare Highfield	Not detected
Harare Mbare	Not detected

DISCUSSION

Occurrence of mycoflora in six selected groundnut markets in Zimbabwe

Different types of fungi were found in groundnuts sourced from six selected markets of three major cities of Zimbabwe which are Bulawayo Shashe, Bulawayo Main, Gweru Kombayi, Gweru Kudzanayi, Harare Mbare and Harare Highfield. The presence of *Aspergillus* spp. (*A. flavus, A. parasiticus, A. niger, A. fumigatus*), *Mucor* spp., *Penicillium* spp. and *Rhizopus* spp. in groundnuts have also been reported (Mupunga, 2013; Njoroge et al., 2016). Abuga (2014) isolated *Mucor, Penicillium, Rhizopus* and *Aspergillus* spp. with different frequencies on groundnut seeds. Other studies done in Kenya, Nigeria and Yemen also revealed the occurrence of *Aspergillus, Mucor, Rhizopus* and *Penicillium* spp. in groundnuts and other products (Menza and Muturi, 2018; Salau et al., 2017; Tobin-west et al., 2018). This suggests that conditions that favour one fungal species may support another species as well (Bayman et al., 2002). Results of the present study revealed higher fungi occurrence on groundnut from Bulawayo Shashe market and the lowest was found in Gweru Kombayi market. The variations could be caused by poor storage, poor sanitation and handling by the vendors though groundnuts are prone to aflatoxin contamination (Tobin-west et al., 2018). Groundnuts from all the six markets were stored in uncovered sacks exhibiting them to consumers thereby being exposed to airborne fungal spores.

Surface sterilisation was done to determine the presence of internal fungi in groundnuts and gave variable results. In all instances *A. flavus* was not affected by surface sterilisation suggesting that it was not superficial but had established itself in the groundnuts (Bayman et al., 2002). Presence of fungi was observed on both sterilised and non-sterilised groundnuts from all markets with non-sterilised having high frequency compared to sterilise suggests a high risk of aflatoxin.

Prevalence and association of *Aspergillus* spp. in selected groundnut markets

All selected groundnut markets from Bulawayo, Gweru and Harare were contaminated with at least two or more of *Aspergillus* spp., *A. flavus, A. parasiticus, A. niger* and *A. fumigatus*. In this study, *A. flavus* was identified in all groundnut markets showing that most groundnut species grown are susceptible to contamination from *Aspergillus* spp. (Salau et al., 2017). There were differences in occurrence of *Aspergillus* among Bulawayo, Harare and Gweru markets because of different weather conditions. There was high contamination from hot and dry climatic

conditions prevalent in Bulawayo and Gweru compared to low incidence of fungi to cool and wet areas of Harare. However, the risk of aflatoxin is present countrywide given that *Aspergillus* spp. mainly *A. flavus* and *A. parasiticus* are considered as aflatoxin producers (Boli et al., 2014). Previous studies revealed that *A. flavus* is mostly prevalent in foods including groundnuts and contains aflD toxigenic genes producing aflatoxin frequently in dry weather conditions (Menza and Muturi, 2018). *A. parasiticus* rarely occurred (0.3 - 6%) in these selected markets and furthermore was reportedly not a challenge on stored groundnuts (Bediako et al., 2019). *A. niger* had high occurrence in non-sterilised groundnuts from Bulawayo Main, Bulawayo Shashe and Gweru Kudzanayi while *A. fumigators* was detected in groundnuts from Bulawayo main market (8%). Bediako et al. (2019) reported that *A. niger* does not produce aflatoxins, it produces other toxins such as ochratoxin A and malformins. This study showed that some groundnuts are contaminated with *Aspergillus* and the predominant species was *A. flavus*. The presence of *Aspergillus* spp. in groundnuts exposes human to aflatoxin making it unsafe for consumption. Al-Amodi (2016) suggested that presence of these fungi causes groundnut seed to decay, reduces germination and causes damage of stored groundnuts. The widespread distribution of *A. flavus*, *A. niger*, *A. fumigatus* and *A. parasiticus* shows their importance on stored groundnuts in Zimbabwe. Even distribution of *A. flavus* across all selected markets implies that management strategies should be implemented on all markets under investigation. From this study *A. flavus* was more prevalent in the samples collected from six markets while *Mucor* and *Penicillium* spp. were less prevalent. *Rhizopus* was always negatively associated with other fungi species which can be due to differences in environmental requirements or competition. This was however contrary to Bayman et al. (2002) who observed a positive association of *A. flavus* and *A. niger* in tree nuts.

The range of AFB_1 in groundnut samples analysed in this study were within the safe limits for human consumption according to existing Zimbabwe (5 ppb) regulation (Table 2). Though groundnuts were reported as a good substrate for aflatoxins producing fungi, the results showed that none of the thirty groundnut samples from six major markets of Zimbabwe contained any detectable levels of AFB_1 (≥5 ppb). In contrast with Mupunga et al. (2014), Dangwa et al. (2014), and Siwela et al. (2011) reported high contamination levels of aflatoxins in Zimbabwean groundnuts. Previous studies on aflatoxin in groundnuts from Bulawayo showed that 2/18 contaminated samples had detectable levels of AFB_1 above 4 ppb the maximum allowable limit set by the EU and Codex Alimentarius Commission (7 ppb) (Mupunga, 2013; Kamika et al., 2014). The levels of AFB_1 were below the cut-off point of 5ppb this might be because the conditions were not conducive for aflatoxin production. Furthermore, previous studies have shown that the interaction of *A. flavus* with other fungal species decreases its aflatoxin production or degrades it (Mann and Rehm, 1976; Mislivec et al., 1988). The presence of aflatoxigenic fungi (*A. flavus* and *A. parasiticus*) in groundnuts, however, means there is potential for aflatoxin production and fungal proliferation when conditions are favourable. Though levels were low, chronic exposure to aflatoxins remains a health concern (Williams et al., 2004; Okoth and Kola, 2012).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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