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Current status of mycotoxigenic fungi in cereal grains in the central region of Botswana: a mini survey

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ABSTRACT

Mycotoxins are secondary metabolites produced by fungi that can contaminate food, both human and animal feed at all stages of the food chain. A number of factors play a role in the proliferation of mycotoxins such as climate, humidity, harvest and storage conditions. This study was looking at the occurrence and identification of the fungi obtained from the cereal grains in the central district of Botswana. Samples collected were yellow maize (18), white maize (4), millet (10), cowpeas (11), sorghum (11) and china peas (1) each weighing about 500 g. Upon the arrival of samples, water activities of the samples were obtained. Seeds were sterilized in sodium hypochlorite, to be plated onto PDA for fungal extraction. The polymerase Chain reaction was used for the identification of the fungi and samples were sent to Inqaba laboratories for sequencing. The results showed that yellow maize was contaminated by *Fusarium*, *A. niger* and *A. flavus*; white maize was contaminated by *F. proliferatum*, *F. fujikuroi* and *Gibberella moniliformis*; red sorghum was contaminated by *A. flavus*, *A.oryzae*, *Penicillium*, *Alternaria* and *Chaetomium muelleri*; millet was contaminated by *Epicoccum sorghinum* and curvularia branchyspora and cowpeas were contaminated by *Aspergillus* and *Alternaria* species. Overall the most contaminated cereal product was millet, yellow maize, white maize, cowpeas and red sorghum at 40%, 37%, 27%, 10% and 4% respectively.

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1. Introduction

The climatic and environmental conditions in Southern Africa are characterised by high temperatures, humidity, uneven rainfall and constant occurrences of drought. These conditions are suitable for mycotoxigenic fungal growth and proliferation (1-4). Seventy percent of Botswana's landmass is occupied by the Kalahari Desert, leaving the country with limited supplies of water, high temperatures and dry seasons. The country's climate which is semi-arid aggravates the limited physical water supply (5) and also offers conducive environment for fungal growth, resulting in high risk of mycotoxigenic contamination in grains produced. Mycotoxins are poisonous secondary metabolites produced by certain moulds. These toxins are invincible, tasteless, chemically-stable and present one of the greatest challenges to food safety. Mycotoxins contaminate a wide variety of agro-products and are capable of causing disease and death in humans and animals when ingested (6). Vegetal matter is the major protein source for the world, at 57% (7) and cereal grains make the major portion of this, most especially for developing countries and low and middle income countries (LMCs) where the risk of foodborne diseases is more severe (8-10), of which Botswana is one. Most of these cereals are eaten processed into different products such as soft porridge,

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maize meal. It is known that the typical processing techniques for cereals do not get rid of toxins, if contaminated (11-13). Therefore, contamination of these products by mycotoxins poses a serious health risk to a large portion of the population, especially immuno-compromised. It has been reported that cereal grains are susceptible to contamination by five most important fungal metabolites namely *aflatoxins* (AFB), *fumonisins* (FB), *zearalenone* (ZEN), *deoxynivalenol* (DON) and *ochratoxin* A (OTA) (14). These most critical mycotoxins are also classified because of their high prevalence, serious adverse health effects and high economic cost (15-17).

In as much as mycotoxin contamination have been found to be a major concern in many countries in Southern Africa, very few studies have been conducted on dietary mycotoxins in this region compared to other regions worldwide, this is primarily because of lack of resources such as advanced laboratory equipment, inadequate research funds, capacity, expertise, and limited surveillance systems. Monitoring and enforcement of regulatory standards are rare or non-existent(18).

In Botswana the only mycotoxin related legislation concerns *aflatoxin* levels mostly produced by some species of the genus *Aspergillus*. There is little information on the incidence of other mycotoxins and their public health impact in the country. It is imperative that assessment of the risks associated with these hazards be conducted to support additional mycotoxin legislation.

The objective of this study was to sample a variety of unprocessed grains from central storage facilities in Botswana; to screen these samples for mycotoxin contamination, to isolate and identify any mycotoxigenic fungi from samples to gain insight into the types of mycotoxins and mycotoxigenic fungi that are problematic in the region.

2. Materials and methods

2.1. Sample collection

Fifty five (55) samples consisting of yellow maize (18); white maize (4), millet (10), cowpeas (11), sorghum (11) and china peas (1) each weighing about 500 g were collected from the different regions of Botswana. Samples were placed and sealed in plastic bags to avoid contamination and extraneous humidity. Upon arrival samples were kept frozen. Samples were collected from grains destined for human consumption.

2.2. Seed sterilization and plating

Grain seeds were first thawed to room temperature prior to sterilization. Seed sterilization was done according to the method Joshaghani et al. 2013 with few modifications (19). A solution of 2.5% of sodium hypochloride was used in the sterilization of cereal grains. About 30 cereal grains were placed in conical flasks and 100 ml of the sterilising solution was used for rinsing the seeds. This was done by swirling for 2 min and the process was repeated three times with the last rinsing in 100 ml of sterile distilled water. The rinsed seeds were blot dried on sterile paper towel. The sterilized cereal grains were placed on potato detroxose agar (PDA) (Oxoid, England) plates, about 12-15 seeds per plate. PDA was prepared by suspending 39 g of the agar in a litre of distilled water, the solution was boiled and sterilized at 121° C for 15 min, cooled and poured into plates to solidify. Inoculated plates were incubated at 25°C for 2-5 days aerobically for fungal growth. Following growth, individual fungi was sub-cultured onto PDA plates to obtain pure cultures; plates were further incubated at 25°C for 2-5 days.

2.3. Percentage contamination and identification using microscopy

Following initial culture, each plate was examined for the type of mould contaminating the grains. Mould colour was the growth characteristic used as the primary differentiating factor. The proportion of seeds contaminated by each mould type was noted and used to determine the percentage contamination. The fungi was scraped off from the agar and placed on a microscopic slide, a drop of methylene blue dye was used to stain the fungi and a cover slip was placed on top. This was observed under the microscope at 10X and 100X using the compound light microscope (Leica, Wetzlar, Germany).

2.4. DNA extraction

Deoxyribonucleotide (DNA) was extracted from purified fungal isolates. DNA extraction was done using Fungal/Bacterial DNA extraction kit (Zymo Research Corporation, California, USA). Purified isolates (about 70 mg) from PDA media were scrapped into a 1.5 ml ZR Bashing BeadTM lysis tube, 750 µl of DNA lysing solution was added. Fungal cells were lysed using a cell disruptor genie bead beater fitted with a 2 ml tube holder assembly (Scientific industries

inc.,USA), processed at maximum speed for 5 min. Lysed samples were centrifuged at 10,000 rpm for 1 min and the supernatant was transferred to a zymo-spin IV spin filter in a collection tube and centrifuged at 7,000 rpm for 1 min. The filtrate was added to fungal DNA binding buffer and centrifuged in a Zymo-spin IIc column placed in a collection tube at 10,000 × g for 1 min. This was followed by washing of DNA in the column using a pre-wash buffer and wash buffer. Fungal DNA was finally eluted with 100 µl of DNA elution buffer and centrifuged at 10,000× g for 30 min. Eluted DNA was stored at -20°C for further analysis. The concentration of the DNA was determined before storage.

2.5 Polymerase Chain Reaction (PCR)

Amplification of DNA was done using ITS-1/ITS-4 primer pairs for forward and reverse amplification as described by Egbuta et.al 2014, with few modifications. Primers were commissioned at Inqaba Biotechnical Industrial (Pty) Ltd, South Africa. The final PCR solution for each sample consisted of 25 µl of 2 x PCR master mix, 1µl of each 10 µM primer, 2 µl of DNA, and constituted to a final volume of 50 µl with nuclease free water. Polymerase chain reaction was performed using Applied Biosystems Veriti 96 well thermocycler (Waltham, Massachusetts). Cycling conditions were set as :Pre dwelling at 95°C for 3 min, 35 cycles denaturation at 95°C for 1 min, annealing at 58°C for 45 s, extension at 72°C for 1 min 30 s, post dwelling at 72°C for 10 min and held at 4°C until samples were retrieved. Gel imaging was done using gel imaging system Syngene Lasec (Cape Town, South Africa).Samples were then sent to at Inqaba Biotechnical Industrial (Pty) Ltd, Pretoria, South Africa, for sequencing.

3. Results

Figure 1. Maize seeds contaminated by different mould on PDA media with pure isolated cultures

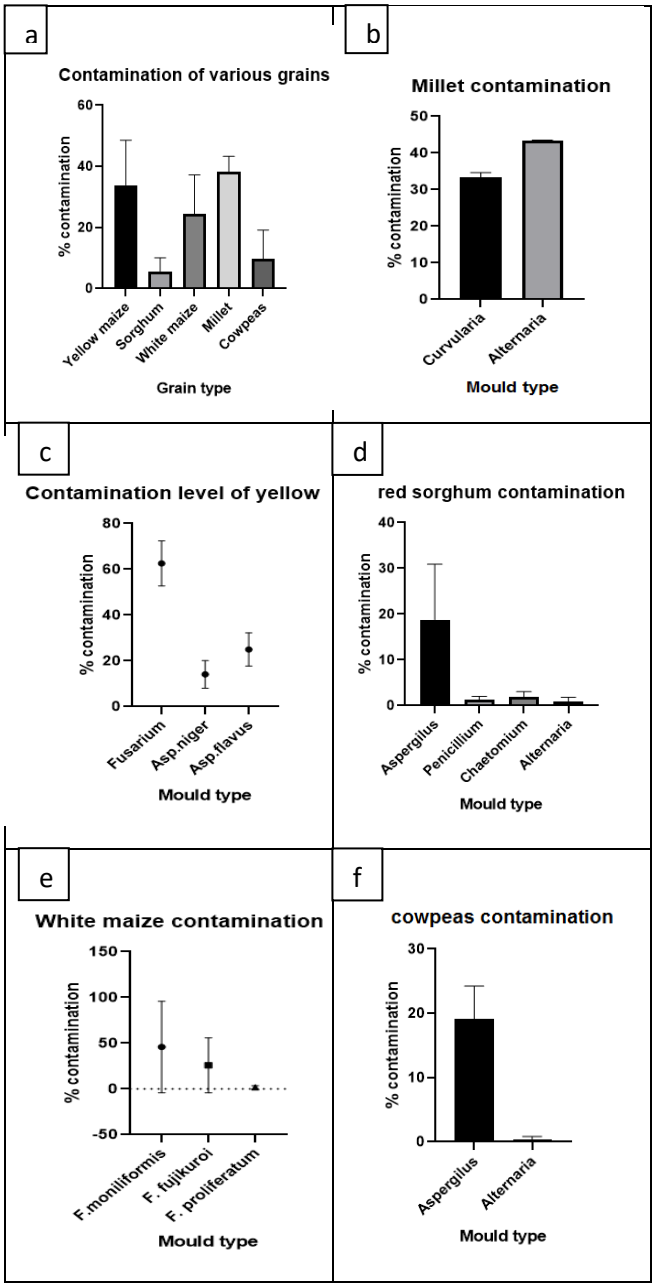

Figure 2. (a-f): Comparison of contamination rates between cereal grains

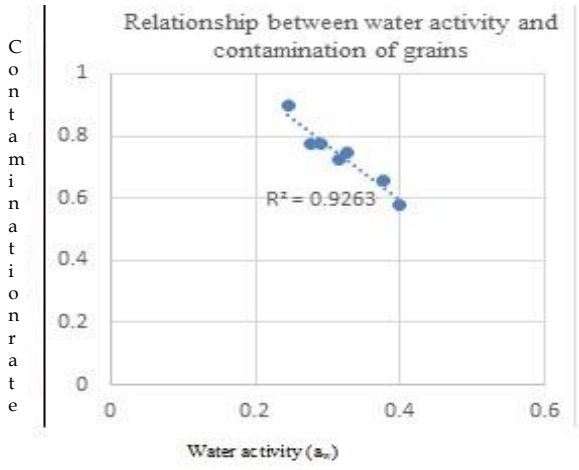

Figure 3. Ranges of water activity obtained from cereal grains.

4. Discussion

From the results figure 2(a) millet was the most contaminated cereal grain at 40%, followed by yellow maize at 37%, white maize at 27%, cowpeas at 10% and lastly sorghum at 4%.

Figure 2(b) shows that millet was contaminated by *C .branchyspora* and *Alternaria*, with *Alternaria* being the most contaminant at 44%. This finding was congruent with Hertveldt, 2015 who also found millet to be the most contaminated cereal grain in a study that compared millet and sorghum. (14) Found millet to be contaminated mostly by *Alternaria* and *Aspergillus* species (14).

The highest contaminant in yellow maize was *Fusarium* at 62%, *A. flavus* (22%) and *A. niger* (18%) as shown by figure 2(c). White maize, figure 2(e) was contaminated by different species of *Fusarium*, the most being *F. moniliformis* at 48%. According to (6), throughout its growth, harvest, transport and storage, maize is susceptible to fungal infections from *Fusarium* and *Aspergillus* species and consequent contamination with their mycotoxins, principally fumonisins and aflatoxins (20,21).

Red sorghum figure 2(d), was mostly contaminated by *Aspergillus* species at 18%, followed by *Penicillium, Chaetomium muelleri, A. alternata* at 0.5%. Previous studies by Doko et al. 1996, Siame et al. 1998, Nkwe et al. 2005, reported only *Fusarium* species as contaminating sorghum and millet in Botswana, in a study that looked at 40 randomly selected cereals and cereal based commodities collected from Botswana, Kenya, Malawi, Mozambique and South Africa, Tanzania, Uganda, Zambia and Zimbabwe (22-24).

The current study also suggests that contamination by *Aspergillus* species may have occurred at an earlier stage, in the history of the sample, where conditions were better suited for such growth.

Cowpeas figure 2(f), was contaminated by *Aspergillus* and *Alternaria* species with *Aspergillus* being the most contaminant at 18%. *Alternaria* was the least contaminant. Kritzinger, 2000 who did a study on storage fungi and mycotoxins associated with cowpea also found *Aspergillus* and *Alternaria* species to be the ones contaminating cowpeas. However, in the latter study the highest contaminant was *Alternaria* followed by *Aspergillus*.

According Magan, 2004 and Milani 2013, a number of factors have been found to play a role in mycotoxin contamination such as biological factors, harvesting, storage, processing and climate. Environmental conditions that include temperature and moisture content also play a role (26,27).

Mannaa 2017 confirms that fungi that spoil grains can be divided into two discreet groups and an intermediate group, depending on the different stages of crop growth, harvest and storage conditions (28). The first group is the field fungi that affect the grains while still in the field before harvesting. Magan 2004 states that implicit factors such as interactions with insects and mites, fungal strains and spore load, microbial ecosystems and intrinsic factors such as water activity, nature of substrate, nutrient composition contribute to this fungal contamination in the fields (26). It usually includes the species of the genera *Alternaria* and *Fusarium* species.

The second group is the storage fungi where contamination of grains occurs during harvesting and increases during storage. Intrinsic factors such as moisture content and processing factors like rapidity of drying, rewetting and blending of grain and temperature are the major contributors to this contamination type. Contamination may be present in lower levels before harvesting, or may start during harvesting season and escalate in numbers during storage when environmental conditions allow. The typical fungi in this group are *Aspergillus* and *Penicillium* genera (26).

Last is the intermediate group. In this group, the fungi grow when the water activity (aw) remains high during storage. It comprises of the genera *Cladosporium*, *Fusarium* and *Trichoderma*. It is notable that *Fusarium* occurs in both the field as well as the intermediate (storage) fungi groups (26).

Hertveldt 2015 states that, cereal grains are susceptable to contamination by five most important fungal metabolites namely aflatoxins (AFB), fumonisins (FB), zearalenone (ZEN), deoxynivalenol (DON) and ochratoxin A (OTA). These most critical mycotoxins are so classified because of their high prevalence, serious adverse health effects (15,16) and high economic cost (17).

The grains used in this study showed a spread of water activities (figure3) from 0.24 to 0.4 aw as seen in the graph above. It was found that increased water activity had a strong negative co-relation with contamination rate. These results contrast with Giorni et al. 2008 who found that with increased water activity, there was an increase in fungal growth (29). It is notable that in the current study, the water activity range studied is 0.2 to 0.4 while Giorni et al. 2008 investigated water activity ranges of 0.95 to 0.98 twice what was found in the current study. Furthermore, as none of the grains in the current study had visible contamination, it is likely that all the contaminants had sporulated and were not actively growing at this depressed water activity until enrichment on growth media. As such, the results are not necessarily mutually exclusive. The main point to take away from this finding is that contaminant fungi will be found even on very low water activity foods, likely as spores which may vegetate and produce toxins as soon as conditions favour.

5. Conclusion

Cereal grains in Botswana showed worrying contamination levels between 10-40% for cowpeas, maize and millet while only sorghum had low (4%) contamination. These levels portend the risk that consumers face from mycotoxins. Mitigation factors to reduce these levels of fungal contamination on cereals ought to be studied so as to protect the harvest. Moreover, emerging techniques to transform food-borne mycotoxins into harmless products using biotechnologies must also be pursued. However, it must be borne in mind that conditions conducive for fungal growth and development in stored grains are not always ideal for mycotoxin production. A lot of dynamics play a role for production of toxins. The aw, temperature, interactions with other organisms like insects, chemical preservatives and storage time should be considered, and not be taken in isolation. All these dynamics come together during storage (28).

Conflict of interest

The authors declared that they have no conflict of interest.

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