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Mycoflora and occurrence of aflatoxin in dried vegetables in Benin, Mali and Togo, West Africa

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ABSTRACT

Fungal infection and aflatoxin contamination was evaluated on 180 samples of dried vegetables such as okra, hot chilli, tomato, melon seeds, onion and baobab leaves from Benin, Togo and Mali collected in September to October 2006. These products are dried to preserve them for lean periods and decrease their perishability. Fungal contamination was evaluated after plating on selective media with a total of 561 fungal isolates identified, ranging from 18 in tomato and 218 in baobab leaves. Baobab leaves, followed by hot chilli and okra showed high incidence of fungal contamination compared to the other dried vegetables, while shelled melon seeds, onion leaves and dried tomato had lower levels of fungal contamination. Species of *Aspergillus* were dominant on all marketed dried vegetables irrespective of country. Mycotoxin assessment by Reversed-Phase High Performance Liquid Chromatography showed that only okra and 3.2 µg/kg on hot pepper. This is the first time that mycotoxigenic fungi and resultant toxins were found on dried vegetable products sampled from African markets. Previous reports have mostly highlighted the risk of mycotoxin exposure from staple crops in Africa, but such risks now need to be evaluated for other products such as dried vegetables.

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1. Introduction

Vegetable consumption provides a valuable contribution to the nutrition of rural and urban populations in West Africa. Numerous studies have recorded uses and consumption patterns of vegetables (Gakou et al., 1994; Humphry et al., 1993; Mertz et al., 2001), but few studies have reported on dried vegetables. Drying of foods is practiced in Africa to make the products more durable and preserve them for food insecure periods. Drying is mainly done on an artisanal scale or through small-scale industrial units. Dried products can be infected with fungi and other contaminants either already present on the primary product, or during the drying process that takes place under unhygienic conditions; further spoilage can occur during storage, handling and transport till sale (Mandeel, 2005). Crops or products that are susceptible to fungal growth can also be contaminated with mycotoxins (Bankole and Mabekoje, 2004; Garbutt, 1997). Mycotoxins are hazardous to consumers' health and affect food quality leading to economic losses including loss of commercial value (Bhat and Vashanti, 1999; Moss, 1998; Otzuki et al., 2001).

Reports of microbial contamination of dried vegetable products are rare. In Yemen, Alghalibi and Shater (2004) have investigated the mycoflora and mycotoxin contamination of some dried fruits including raisins, dates and figs; 23 fungal species belonging to 15 genera were isolated from these dried fruits; Aspergillus niger, A. flavus, A. fumigatus, A. ochraceus, Penicillium chrysogenum and Rhizopus stolonifer were the most prominent isolates. Moreover, dried fruits were contaminated by aflatoxin B₁, with raisins having the highest levels ranging from 130 to 350 µg/kg. In India, aflatoxin B₁ production in dried chillies (Capsicum annum L.) kept in cold stores was reported, with mean levels of 5.5 µg/kg (Ravi Kiran et al., 2005). Lugauskas et al. (2005) investigated toxin producing fungi in fresh fruits sampled in Lithuania. P. expansum, Sclerotinia sclerotiorum, Alternaria alternata, and P. italicum were the prevalent fungal species, while P. expansum, A. niger, R. oryzae and P. italicum dominated on dried fruits. On smoked paprika sampled from Spain, Aspergillus, Cladosporium, Penicillium and Fusarium were the most commonly isolated fungal genera with total counts of more than 4 log Colony Forming Unit per g (CFU/g) (Martin et al., 2005). Spices are highly susceptible to mycotoxin development with average contamination levels of 0.09, 0.63, 2.88 and 0.03 μ g/kg of aflatoxin B₁ detected in black pepper, ginger, red paprika and cumin, respectively (McKee, 1995). In red paprika, Zinedine et al. (2006) reported higher levels of 9.68 µg/kg aflatoxin B₁. In Bahrain, red chilli and black pepper showed high levels of fungal infestation with 1580 and 1120 CFU/g, respectively (Mandeel, 2005). On dry 'tatase' peppers (Capsicum annum L.) seven mould species were isolated with A. niger,

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A. flavus and *Geotrichum candidum* being the dominant ones, but no aflatoxins were detected (Atanda et al., 1990). Researchers have investigated the mycoflora and nutritional value of shelled melon seeds (*Citrulus vulgaris* Schard.) in Nigeria and studied potential drying options that will enhance product quality (Bankole et al., 2005). Aflatoxin B₁ was detected at levels above 5 μ g/kg in 32.2% of the samples, while only 3.5% contained toxin above the 20 μ g/kg Nigerian tolerance level for food (Bankole et al., 2006).

Natural occurrence of mycotoxins and fungal contamination on dried vegetables was investigated in multiple countries (Alghalibi and Shater, 2004; Begum et al., 2005; Magnoli et al., 2004; Mandeel, 2005; Nutsugah et al., 2004), but none of these studies reported on the zones surveyed in the herein presented report e.g. Mali, Togo and Benin. The objectives of this study were (1) to determine the mycoflora and mycotoxin contamination of dried vegetables from these countries, (2) to determine the processing methods and quality characteristics of these products and (3) to propose methods for reducing fungal and mycotoxin contamination.

2. Materials and methods

2.1. Sample locations

The study was conducted in the northern part of Benin and Togo, and two markets in Mali (Fig. 1). In Benin samples came from the Northern Guinea Savannah (NGS) and the Southern Savannah (SS) (Hell et al., 2003). These zones are the main producers and consumers of dried vegetable products. In each zone two main markets and three local markets were randomly selected with a total of 10 markets surveyed in Benin. In Togo, in the northern region (Central, Kara and Savannah), four markets were randomly selected, with a total of 12 markets sampled. In Mali samples were collected in the markets of Mopti and Bamako.

2.2. Dried products and their processing method

Overall, 180 samples (200 g each) of dried okra, chilli, baobab leaves, tomato, shelled melon seeds and onion leaves were examined. Thirty samples of each commodity were collected in Benin, Togo and Mali in 2006–2007. All samples were kept in a refrigerator (4 °C) till fungal and mycotoxin analysis.

During the survey, a total of 112 dried vegetable sellers were interviewed. The questionnaire determined the production process for each dried vegetable product, established the monthly income generated from the sale of dried vegetables and assessed the level of consumption of these products.

2.3. Moisture content and pH determination

Moisture content was determined by heating a specific amount of a product at 105 °C for 2 h to constant weight and reweighing (AOAC, 1995). A pH meter (Jenway[®], Dunmow, UK) measured the H+ concentration of each product.

2.4. Isolation of moulds

The method used for fungal isolation differed from one type of commodity to another depending on the form and presentation of the product e.g. dried okra, tomato and onion leaves are cut in small pieces, while chilli and melon seeds are whole, and baobab leaves are ground into powder during processing.

2.4.1. Dried okra, tomato and onion leaves

The method described by Muhammad et al. (2004) was used with some modifications. For each product, 20 pieces were surface sterilized by dipping them in 50% ethanol for 1 min and then rinsing twice with sterile distilled water. Small segments of 1 to 2 mm were cut out with a sterile scalpel and placed on potato dextrose agar (PDA) (Oxoid Ltd., Hampshire, UK) in Petri dishes and incubated at 25 °C in alternating 12-h periods of fluorescence light and dark during 5 days.

2.4.2. Chilli and shelled melon seeds

The direct plating method described by Ravi Kiran et al. (2005) was used with some modifications. Fifty pods of dry chilli or melon seeds were surface sterilized with 10% sodium hypochlorite solution for 1 min and rinsed twice with sterile distilled water. The seeds were placed on Dichloran Chloranphenicol 18% Agar (DG 18) (Oxoid Ltd, Hampshire, UK) in Petri dishes and incubated in conditions described above.

2.4.3. Baobab leaves

Fungal genera were enumerated using the dilution plate method described by Pitt and Hocking (1997). Ground samples (10 g each) were thoroughly mixed with 90 ml of sterile water containing 0.1% peptone water to make the 10^{-1} dilution. Further serial dilutions to 10^{-4} dilution were made with 0.1% peptone water. Aliquots (1.0 ml) of each dilution were then transferred to Petri dishes containing Dichloran Chloramphenicol 18% Agar (DG 18) (Oxoid Ltd., Hampshire, UK) and incubated under previously described conditions.

2.4.4. All products

The total number of fungal isolates from each product was recorded. Isolates cultured on PDA were sub-cultured on malt extract agar (MEA) (Oxoid Ltd., Hampshire, UK) for identification. The MEA plates were incubated at 25 °C for 7 days. *A. flavus* and *A. parasiticus* were distinguished from other *Aspergillus* spp. by the bright orange-yellow reverse coloration on *Aspergillus* Flavus Parasiticus Agar (AFPA) (Pitt et al., 1983). Identification of fungi was based on morphology and microscopic features such as spores and fruiting structures using the standard reference book by Pitt and Hocking (1997). For baobab leaves colonies were counted at the end of each incubation period and recorded as CFU/g.

2.5. Aflatoxin analysis

2.5.1. Extraction

Aflatoxins were extracted and analyzed as described by Muhammad et al. (2004) with some modifications. A ground sample (10 g) of each dried vegetable was mixed with 50 ml of methanol/water (85:15 v/v) and blended (Power Gen 125, Fisher Scientific, Nepean, Ont., Canada) for 3 min, filtered (Whatman No.1) and 40 ml of the filtrate mixed with 40 ml of 10% sodium chloride (NaCl). The mixture was poured into a separatory funnel and defatted with 25 ml of n-hexane. The hexane layer was discarded and the aqueous layer partitioned twice with 25 ml of chloroform (May and Baker Ltd., Dagenham, UK). The chloroform layers were pooled and dried over anhydrous sodium sulphate. The chloroform was then evaporated off on a rotary evaporator (Laborota 4000 WB, Heidolph, Germany) and the residue transferred to an amber vial with 2×0.5 ml of chloroform, evaporated off under vacuum to near dryness and stored at -20 °C until analyzed. The residue was re-dissolved in 200 µl chloroform for thin layer chromatography (TLC) and high performance liquid chromatography (HPLC).

2.5.2. Qualitative analysis of aflatoxin

The chloroform (May and Baker Ltd., Dagenham, UK) extracts were analyzed on pre-coated silica gel plate type ALUGRAM[®]SIL (Macherey-Nagel, Dfiren, Germany) for the presence of different aflatoxins (B_1, B_2, G_1 and G_2) using thin layer chromatography technique according to standard procedures described by Muhammad et al. (2004).

2.5.3. Quantitative determination and confirmation of aflatoxins

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) with fluorescence detection was used to quantify aflatoxins



Fig. 1. Sampling locations in Benin, Mali and Togo, West Africa.

(Gnonlonfin et al., 2008). Samples (20 µl) were injected into the Water[™] HPLC system with Water[™] 474 Scanning fluorescence detector (excitation wavelength 335 nm; emission wavelength 440 nm). Separation was carried out using stainless steel Supelcosil LC-C₁₈ column (150×4.6 mm, 3 µm particle size) (Supelco, Bellefonte, PA). Isocratic elution with methanol:water:acetic acid (45/55/2, v/v/v) was used at a flow rate of 1.0 ml/minute. Quantification was by comparing the peak areas with those of the aflatoxin standards (Sigma, St. Louis, MO), using the Apex Chromatography Workstation (Autochrom Inc., Milford, MA). The detection limit was 0.25 µg/kg and the recovery of the method was 80%.

2.6. Data analysis

The data were analyzed with SPSS 12.0 (SPSS, Chicago, IL). Univariate analysis of variance (Tukey test) was used to compare means of fungal occurrence and incidence and means of total aflatoxin.

Numeric data was transformed using log (x+1) and percentages were transformed using Arcsin $\sqrt{x}/100$ to normalize data.

3. Results

3.1. Survey data

Dried vegetables such as onion leaves, baobab leaves, chilli, tomato and shelled melon seeds were consumed at least 6 times per week since they are often used in sauces. Dried tomato generates the highest income for traders with an average of 68,800 FCFA (African Financial Community) per 100 kg; followed by chilli with an average of 61,363 FCFA per 100 kg, while dried onion leaves generate the lowest average income of 5428 FCFA per 100 kg (data not shown).

The processing methods used to make the different products are presented in Table 1 and steps that could facilitate contamination e.g. Critical Control Points (CCP) are highlighted in the table. The surveyed products are mostly produced during the dry season. July to

Table 1

D	escription	of th	e processing	method	for o	dried	vegetables
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Product and	processing	method	description
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Baobab leaves	Okra
- Harvesting	-Harvesting
- Sorting	-Slicing
- Removal of veins	-Addition of ash
-Sun drying (3–5 days; on mat, metal sheet)	- Sun drying (max 7 days, on mat, metal sheet)
- Crushing (using mortar)	-Crushing (using mortar)
-Sieving	-Sieving
-Storage (polyethylene bags, basins)	-Storage (polyethylene bags, basins)
Shelled melon seeds	Tomato
-Harvesting (the melon)	-Harvesting
-Mass storage for inner rotting (up to 2 months)	-Additional ripening (2–3 days)
- Cracking	-Slicing
-Seed removal and washing	-Smoking
-Sun drying (3–7 days; cemented floor, metal sheet)	-Sun drying (up to 7 days)
- Shelling	-Additional drying (room temperature)
-Additional drying	-Storage (jute or polyethylene bags)
- Storage (polyethylene or jute bags, basins)	
Chilli	Onion leaves
- Harvesting	-Harvesting
-Boiling (15–60 min)	-Drying at room temperature (covered)
- Draining	- Crushing
-Sun drying (3–7 days; on mat, metal sheet)	-Sun drying
- Storage (jute or polyethylene bags,	-Ball making
basins)	- Storage (polyethylene or jute bags, basins)

In **bold**, are steps that can lead to a higher risk of fungal contamination (Critical Control Point (CCP)).

In *italic*, are steps that are not used by all producers.

September were mentioned as the suitable period for the production of dried baobab leaves, okra, and shelled melon seeds, while dried chilli were produced from January to March.

According to the traders dried products can be stored after processing an average of 16 months for chilli (max of 26 months), 12 months for okra and onion leaves (max of 14 months), 10 months for baobab leaves (max of 14 months) and tomato (max of 26 months) while the storage period is only 7 months for shelled melon seeds (max of 14 months).

Table 2

Frequency and total isolates per fungal species; total isolates, number of fungal species, moisture content (%) and pH in different dried vegetables.

Dried vegetables		Hot chili	Baobab leaves	Shelled melon seeds	Tomato	Okra	Onion leaves	Total isolates	Total frequency of isolation
Fungal species	A. flavus	71	79	17	1	43	18	229	40.8 a
	A. niger	28	104	3	15	26	1	177	31.6 a
	A. utus	-	1	-	-	-	-	1	0.2 d
	A. parasiticus	-	1	-	-	-	-	1	0.2 d
	E. amstelodami	3	3	2	-	-	-	8	1.4 d
	P. citrinum	8	14	1	-	1	-	24	4.3 c
	F. verticilliodes	42	16	14	2	6	6	86	15.3 b
	Chaetomium spp.	-	-	-	-	3	-	3	0.5 d
	Curvularia spp.	-	-	-	-	3	-	3	0.5 d
	Colletrotichum spp.	-	-	-	-	1	-	1	0.2 d
	Macrophomina phaseolina	-	-	-	-	4	-	4	0.7 d
	Rhizopus stolonifer	13	-	-	-	9	-	22	3.9 c
	Pythium spp.	1	-	-	-	-	-	0.2	0.0 d
	Rhizoctonia solani	1	-	-	-	-	-	0.2	0.0 d
Range		0-71	0-104	0–17	0-15	0-43	0-18	0.2-229	
Total isolates		167	218	37	18	96	25	561	
Number of fungal species		8	7	5	3	9	3		
Moisture content (%)		11.1	13.0	10.4	10.2	8.0	14.3		
pH		6.0	5.9	6.7	5.7	6.2	6.0		

Means within a column followed by the same letter are not significantly different from each other (Tukey test at 5%).

Moisture content and pH of the dried vegetables are summarized in Table 2. Moisture levels ranged from 8.0% to 14.3%, with the highest levels detected in leaves (onion leaves (14.3%), baobab leaves (13.0%)), followed by seeds (shelled melon seeds (10.4%)) and dried fruits (chilli (11.1%), tomato (10.2%) and okra (8.0%)). Atmospheric humidity is generally high in Benin and Togo ranging from 60 to 80%, and Mali, a Sahelian country, has lower levels ranging from 30 to 70%.

The pH of the dried vegetable samples were within the acidic range, 5.7 and 6.7 in dry tomato and dry shelled melon seeds, respectively (Table 2), with most samples having pH levels around 6.0.

The fungal species recovered from the samples are listed in Table 2. A total of 561 isolates, ranging from 18 in tomato and 218 in baobab leaves, were identified during the study. Baobab leaves, followed by hot chilli and okra showed high levels of fungal count compared to the other dried vegetables, while shelled melon seeds, onion leaves and tomato had lower levels of fungal contamination.

Fourteen fungal species were isolated from the different dried vegetable samples, with 3 to 9 species per product (Table 2). The genus *Aspergillus* was most prevalent with four different species, followed by the other fungal genera with one species each (Table 2). *A. flavus, A. niger* and *Fusarium verticillioides* were found on all products. Okra yielded the widest spectrum of fungal species (9), followed by hot chilli (8) and baobab leaves (7). Tomato and onion leaves showed the lowest fungal species diversity (3). *A. flavus* was isolated from dried vegetables at the highest frequency of 40.82% followed by *A. niger* (31.55) and *F. verticillioides* (15.3), with significant differences between the fungal species (Table 2).

The Reversed-Phase High Performance Liquid Chromatography (RP-HPLC), showed that okra (3 samples) and hot chilli (1 sample) were naturally contaminated with aflatoxin B₁ and B₂. The mean aflatoxin B₁ level was 5.4 µg/kg in okra and 3.2 µg/kg in hot chilli. Aflatoxin B₂ was only present in okra at the concentration of 0.6 µg/kg. Significant differences were observed between total aflatoxin concentration of okra (6 µg/kg) and hot chilli (3.2 µg/kg) [F=11.76; p=0.000].

4. Discussion

African citizens, especially those in urban centres, have in recent years developed a heightened concern for food quality and safety, with between 20 and 60% of the population in three West African countries being aware of this problem after sensitization campaigns (James et al., 2007). Fungal and mycotoxin contamination in African food products is rarely assessed and almost never controlled, and the herein presented data shows that African populations, often exposed to mycotoxins through staple crops, are also at a high risk of being exposed to food contaminants through dried vegetables products. It has been previously reported that fungi can develop directly on the surface of vegetables and even infect the inner tissues (Lugauskas et al., 2005). The current study showed that the highest fungal levels were recorded in ground baobab leaves, hot chilli and okra. The most prevalent fungi were Aspergillus spp. (Table 2), previously isolated from similar dried vegetable products (Nutsugah et al., 2004; Ravi Kiran et al., 2005; Martin et al., 2005; Mandeel, 2005). In the current study, dried chilli had a comparable fungal species spectrum with that reported by Atanda et al. (1990), Mandeel et al. (2005) and Martin et al. (2005). Variations between the fungal contamination and those ound in the existing literature are to be expected, because of sampling variability, differences in country of origin and related environmental factors, and divergent processing and storage practices (Garcia et al., 2001, Lugauskas et al., 2005). Overall, the climatic conditions in West Africa are favourable for fungal development with high relative humidity (Marasas, 2001; Hell et al., 2003), high temperature (Velluti et al., 2000; Peterson et al., 2001) and little aeration (Fandohan et al., 2006), all conditions that accelerate fungal and mycotoxin development. In addition, in African markets, movement of people and vehicles, unloading and loading of trucks in confined spaces is common; as a result, the air in these markets abounds with dust and potentially microbial spores. The aforementioned climatic and environmental conditions are highly favourable for the propagation of fungi, especially the genera Aspergillus, Penicillium, Mucor, Rhizomucor and Rhizopus that produce and release a lot of spores (Domsch et al., 1980a).

Processing and packaging methods can have an effect on fungal contamination and levels of infestation. Chourasia (1995) reported that dried vegetables and spices stored in gunny bags and on bare ground had significantly higher incidence of fungi compared to those stored in wooden boxes, metal or glass containers. The same authors also noted that fungal infestation is compounded by insect damage in the field and during transportation. The relationship of fungal infestation and insect damage is also mentioned by Lamboni and Hell (2009). Another source of fungal infestation can be the result of inappropriate handling and storage methods, often associated with poor hygiene (Abou-Arab et al., 1999). Domsch et al. (1980b) postulated that "contamination of foodstuffs with spoilage fungi was the result of natural extraneous pollution with dust particles containing spores" during storage. This could certainly have been one of the factors that lead to the contamination of the surveyed commodities (Tables 1 and 2), since all of them were harvested, processed, stored and marketed under ambient conditions with little protection from dust and fungal spores.

Several abiotic factors influence the growth of moulds on dried vegetables; they are relative humidity and temperature together with the moisture content of the product and storage conditions (Misra, 1981; Pitt and Hocking, 1997). These factors are known to facilitate the development of the most prevalent fungi observed in this study A. flavus (Marin et al., 1998). The moisture content of the sampled commodities were higher than safe levels (Table 2), suggesting that conditions could facilitate fungal growth even after drying. Most mycotoxin producing fungal species grow at the lower limits of moisture content ranging from 12 to 13% (Aziz et al., 1998). The retailers in the surveyed countries observed that processed vegetable products were stored for a minimum of 7 months and a maximum of 26 months. Prolonged storage in poorly ventilated structures or containers, such as observed in this study, increase moisture content of the commodity, rendering the stored products more susceptible to mould growth and toxin production (Chourasia, 1995; Misra, 1981) and potentially the infestation with storage insects.

The aflatoxin analysis revealed that baobab and onion leaves had no aflatoxins. It has been commonly observed that commodities heavily contaminated with *Aspergillus* spp. revealed no aflatoxins (Trung et al., 2008). Screening spices from Egypt and Oman, Abou-Arab et al. (1999) and El-Shafie et al. (2002) did not detect aflatoxins, but isolated aflatoxigenic strains of *A. flavus*. The latter authors concluded that spices and other condiments are unsuitable substrates for aflatoxin production, due to their essential oils which may inhibit toxin production, even though there are several reports on the presence of aflatoxins in spices from divers' origins (Fazekas et al., 2005; Cho et al., 2008).

The current investigation revealed that no aflatoxins were detected in dried tomato. Previously, aflatoxins were detected on fresh tomato from Sokoto (Nigeria) market (Muhammad et al., 2004), but no report has documented toxins in dried tomatoes. On the other hand no aflatoxins were present in shelled melon seeds in this study. This is in contrast to reports from Nigeria where aflatoxin levels in melon seed ranged from 0.20 µg/kg (Ekundayo and Idzi, 1990) to a mean level of 13.7 µg/kg in samples from the Forest zone and 12.1 µg/kg in samples from the Savannah zone (Bankole and Mabekoje, 2004). Aflatoxins were only found in dried okra and hot chilli and their presence has not been previously reported, whereas Ravi Kiran et al. (2005) detected levels of 5.5 µg/kg reported in fresh stored chillies from India. Other authors have reported up to 48 µg/kg aflatoxin in chilli. Klieber (2001) found aflatoxin levels ranging from 0 to 89 µg/kg in chilli samples from divers in Australian regions. The herein presented results demonstrate the presence of aflatoxin B₁ and aflatoxin B₂ in dried okra to the extent of $5.4 \,\mu\text{g/kg}$ and $0.6 \,\mu\text{g/kg}$, respectively. This is the first report of aflatoxin contamination in okra.

This study showed that dried vegetables from markets in West Africa are contaminated with fungi than can potentially lead to mycotoxin development. We observed that dried vegetable is a good substrate for mycotoxin producing fungi and these products are exposed to environmental conditions that favour fungal development and mycotoxin formation. It has been reported that chronic ingestion of foods that are contaminated with mycotoxins can lead to much greater health risks than previously perceived (Williams et al., 2004). Evaluating the cumulative risk of mycotoxin contamination in populations in Africa needs to take into account contamination levels in whole food baskets and interactions between different types of mycotoxins, instead of focusing on the level of contamination in certain staples. Also the relative rate and amount of commodity consumed, needs to be evaluated to serve as a basis for risk assessment. On the other hand there are certain technologies that could reduce toxin contamination in the surveyed goods. These include improved processing, packaging, storage and handling practices. Many of these options need to be developed and/or adapted to African conditions. Also stakeholders in the commodity chain need to be informed about these potential options in order to improve product quality and reduce fungal and mycotoxin contamination. Ideally such products should pass strict quality control inspection before being marketed to consumers.

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