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Changes in Fungal Population and Aflatoxin Levels and Assessment of Major Aflatoxin Types in Stored Peanuts (*Arachis hypogaea* Linnaeus)

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Abstract

Peanut kernels of Homabay Local, Valencia Red, ICGV-SM 12991 and ICGV-SM 99568 cultivars were stored for six months in jute, polypropylene and polyethylene bags to assess the effect of storage bags, temperature and R.H. on fungal population and aflatoxin contamination. Moisture content (M.C.), fungal population and aflatoxin levels were determined before storage and after every 30 days during storage. Isolates of *Aspergillus flavus* and *A. parasiticus* were assayed for production of aflatoxin B1, B2, G1 and G2. The correlation between MC, population of *A. flavus* and *A. parasiticus* and aflatoxin levels in peanuts was also determined. Six fungal pathogens were commonly isolated from the peanut samples and occurred as follows in decreasing order: *Penicillium* spp. (106.6 CFU/g), *A. flavus* L-strain (4.8 CFU/g), *A. flavus* S-strain (2.9 CFU/g), *A. niger* (2.6 CFU/g), *A. parasiticus* (1.7 CFU/g) and *A. tamarii* (0.2 CFU/g). The overall population of *A. flavus* L-strain was 66% higher than that of *A. flavus* S-strain. Ninety one percent of *A. flavus* and *A. parasiticus* isolates produced at least one of the four aflatoxin types assayed, with 36% producing aflatoxin B1. Total aflatoxin levels ranged from 0 - 47.8 µg/kg with samples stored in polyethylene and jute bags being the most and least contaminated, respectively. Eighty nine percent and 97% of the peanut samples met the EU (≤ 4 µg/kg) and Kenyan (≤ 10 µg/kg) regulatory standards for total aflatoxin, respectively. Peanuts should be adequately dried to safe moisture level and immediately packaged in a container - preferably jute bags - which will not promote critical increases in fungal population and aflatoxin contamination.

Keywords: aflatoxin, *Aspergillus* spp., bag types, peanuts

1. Introduction

Crops in tropical production areas throughout the world often become contaminated with aflatoxins, which are toxic fungal metabolites (Shephard, 2008). It is estimated that more than five billion people in developing countries are at risk of chronic exposure to aflatoxins through contaminated foods (Strosnider et al., 2006). Fungal growth and aflatoxin contamination of peanuts and other foodstuffs remains a challenge in developing countries where agricultural and food processing systems are not properly designed to handle food safety risks. The challenge is exacerbated by suboptimal postharvest conditions including handling, storage and processing (Wu & Khlangwiset, 2010).

Nyanza and Western provinces are the leading production regions of peanuts in Kenya with lower contribution from Eastern and Rift Valley provinces (United States Agency for International Development [USAID], 2010). Of the 25,098 hectares planted with peanuts in 2008, Nyanza accounted for 75.6%, Western for 16.3%, Eastern for 4.1% and Rift Valley for 4% of the total area. Nyanza and Western provinces account for 89% of peanuts output while 11% is produced in the rest of the country.

Peanuts and maize are the most susceptible crops to aflatoxin contamination (Wu & Khlangwiset, 2010). The two crops and their products are widely consumed in Kenya. Production of peanuts in Kenya in 2010 was 19,000 tons (FAOSTAT, 2012), with an estimated per capita consumption of 0.5 kg/ca (USAID, 2010). On the other hand, production of maize in the country is estimated at 3.14 million MT with a per capita consumption of 98 kg contributing about 35% of the daily dietary energy consumption (United States Department of Agriculture [USDA], 2013). Based on these statistics, high consumption of peanut and maize products in the country increases the risk of aflatoxin exposure to consumers. Aflatoxin contamination can occur at pre- and post-harvest stages (Waliyar et al., 2005). During storage, peanuts are prone to various types of deterioration some of which are attributed to growth of storage fungi which cause decrease of germination ability, loss in kernel weight, discoloration of kernels, heating and mustiness, chemical and nutritional changes, and mycotoxin contamination (Malaker, Mian, Bhuiyah, Akanda, & Reza, 2008).

Aflatoxin is a group of naturally occurring carcinogenic compounds which are mainly produced by *Aspergillus flavus* (Link), *A. parasiticus* (Speare) and *A. nomius* (Kurtzman et al.) (Strosnider et al., 2006). Aflatoxin B1 (AFB1) - the most toxic of the aflatoxins - has been classified by the International Agency for Research on Cancer (IARC) as a group 1 human carcinogen (IARC, 1993). Consumption of aflatoxin contaminated foods affects human health causing aflatoxicosis, cancer, stunted growth in children and/or immune suppression (Shephard, 2008; Wu, 2010) among others. The worst aflatoxicosis outbreak recorded in the world occurred in Kenya in 2004 where 317 cases of aflatoxin poisoning and 125 deaths were reported (Centers for Disease Control and Prevention [CDC], 2004). Other documented fatal aflatoxicosis outbreaks reported in Kenya are: 20 cases in 1981 (Ngindu et al., 1982); 80 cases and 30 deaths in 2005; and 9 deaths in 2006 (Wagacha & Muthomi, 2008). The health concerns associated with aflatoxin (and other mycotoxins) have led many countries to set standards on acceptable limits of total aflatoxins as well as different aflatoxin types. The upper limit set by European Union for total aflatoxin in peanuts is 4 μg/kg and 2 μg/kg for AFB1 (Commission of the European Communities, 2006), while the corresponding limits by the Kenya Bureau of Standards (KEBS) are 10 and 5 μg/kg, respectively (KEBS, 2007).

Suboptimal postharvest conditions including handling, storage and processing have been suspect in playing a major role in aflatoxin accumulation in food crops within developing countries (Wu & Khlangwiset, 2010). Most households and traders in Kenya prefer polypropylene and polyethylene bags for packaging and storing peanuts with less than 1% of the traders storing their products in the recommended jute bags (Mutegi et al., 2013). The packaging/storage material used influences microbial and aflatoxin contamination of stored peanuts (Mutegi et al., 2013) and maize (Hell, Cardwell, Setamou, & Poehling, 2000).

The aflatoxin producing moulds grow on peanut kernels at high moisture level ≥ 7% in the presence of temperatures ranging from 21 °C to 37 °C. Fungal growth in storage facilities is also favored by high relative humidity (R.H.) above 83 - 85% (Christensen, Mirocha, & Meronuck, 1977). However, peanuts are stable at 70% R.H., between 7 - 9% moisture content (M.C.), at which conditions fungal growth is arrested (Indian Council of Agricultural Research [ICAR], 1987). When peanuts absorb moisture from the environment or when the environmental R.H. exceeds the equilibrium R.H. of the kernels, fungal growth occurs (Hayma, 2003).

Peanuts in Kenya are frequently infected by *A. flavus* and *A. parasiticus* (Mutegi, Ngugi, Hendriks, & Jones, 2012; Wagacha, Mutegi, Karanja, Kimani, & Christie, 2013) and this infection can occur at all stages in the peanut value chain (Waliyar et al., 2005). Determination of the level of fungal infection of a foodstuff as well as establishing the occurrence of common species is important as the fungal population and diversity could give an indication of the food quality and the presence and types of mycotoxins (Suanthie, Cousin, & Woloshuk, 2009). Aflatoxin producing fungi occur in three sections of the genus *Aspergillus*, but section *Flavi* contains the greatest number of potential aflatoxin producers (Pildain et al., 2008). Based on morphological, genetic and physiological criteria, *A. flavus*, the main aflatoxin producing species, can be divided into two morphotypes, the S and L strains (Cotty, 1994). The S-strain produces high levels of B-aflatoxins with some strains producing both B- and G-aflatoxins (Barros, Torres, Rodriguez, & Chulze, 2006; Cardwell & Cotty, 2002), while the L-strain produces less B-aflatoxins (Barros et al., 2006; Probst, Bandyopadhyay, Price, & Cotty, 2011). *Aspergillus parasiticus* and *A. nomius* produce both B- and G-aflatoxins (Cardwell & Cotty, 2002).

The objectives of this study were to i) assess the effect of storage conditions on fungal population and aflatoxin contamination of peanuts; ii) assess the effect of storage/packaging bags - commonly used in households and markets in Kenya - on fungal population and aflatoxin contamination of peanuts; iii) determine the aflatoxin types produced by isolates of *A. flavus* and *A. parasiticus* from peanut kernels.

2. Materials and Methods

2.1 Storage Conditions and Their Rationale

This study simulated the average temperature and relative humidity (R. H.) conditions of Nairobi and Homabay districts of Kenya. Homabay district in Nyanza province is a leading producer of peanuts in Kenya (Ministry of Agriculture, 2004; Mutegi et al., 2012) while Nairobi is a major market outlet of peanuts sourced from within Kenya and other countries, and has both large and small scale peanut processing enterprises. Both Homabay and Nairobi have a high demand for raw peanuts and peanut products.

The temperature and R.H. were maintained at two levels - 19 °C and 64% R.H.; and 24 °C and 56% R.H. - being average conditions in Nairobi and Homabay districts of Kenya, respectively (Kenya Meteorological Department, 2010). The annual temperature and R.H. data during 2009 for Nairobi (Kenya Meteorological Department Headquarters) and Homabay (Kisumu Meteorological Station) were obtained from the Kenya Meteorological Department, which helped guide in the choice of temperature and R.H. for the storage experiment. Controls entailed storage of peanuts at ambient temperature (22 ± 3 °C) and R.H (55 ± 5%) at the Microbiology laboratory, Department of Food Science and Nutrition, University of Nairobi.

2.2 Storage Bags and Peanut Varieties

The storage containers used in the study were jute, polypropylene and polyethylene bags. Households and traders in Kenya commonly use polypropylene and polyethylene bags to store peanuts (Mutegi et al., 2013), while jute bags are recommended for storage (Kennedy & Devereau, 1994). Visually clean peanut seeds of two local varieties (Homabay Local and Valencia Red) and two improved varieties (ICGV-SM 12991 and ICGV-SM 99568) were purchased from traders in western Kenya, the leading peanut producing region in the country. One and a half kilogram sample of each peanut variety was packed into each storage bag and replicated twice. The containers were incubated at three temperature and R.H. levels (19 °C and 64% R.H.; 24 °C and 56% R.H.; and ambient temperature - 22 ± 3 °C, and R.H. - 55 ± 5%). The experiment was run for a period of six months from April to September, 2011.

2.3 Sampling

Sampling entailed thoroughly mixing the 1.5kg sample and drawing a 100 g sub-sample. The sub-sample was sub-divided into two equal portions of 50g where one portion was analyzed for rancidity (data not shown) and M.C., and the other for fungal infection and total aflatoxin level. Sampling was done for six months with an initial sampling before storage of the peanuts. The sub-samples taken for analysis were not returned to the storage containers.

2.4 Determination of Moisture Content

Moisture content of peanut kernels was determined using the oven drying method. The kernels were ground in a kitchen coffee grinder (Coffee Grinding Mill, Armco Kenya Ltd, Nairobi, Kenya). Two grams of the ground sample were placed on an aluminium dish, which was placed in a dry air oven (Memmert ULM 500, Büchenbach, Germany). The samples were dried at 105°C for 3 hours and the net weight of the dried sample determined. Each sample was replicated twice and the M.C. calculated as follows:

$$\text{Moisture content (\% weight basis)} = \frac{M_0 - M_1}{M_0} \text{ X } 100$$

Where: M0 - initial weight, in grams of test portion; M1 - final weight, in grams of dried test portion.

2.5 Microbiological Assays

Isolation of fungal species was carried out on Modified Dichloran Rose Bengal (MDRB) agar, using the protocol by Horn and Dorner (1998). From each of the 50g ground sub-samples, 2 replicates of 2.5 g each were placed in calibrated centrifuge tubes, into which 10 mL of 2% water agar solution was added and mixed thoroughly. A 0.2 mL of the suspension was pipetted, spread onto MDRB plates, and incubated for 7 days at 30 °C, after which the colonies were identified and classified. Colony counts of *A. flavus* L-strain, *A. flavus* S-strain, *A. parasiticus*, *A. tamari* (Kita), *A. niger* (van Tieghem), *Penicillium* spp. and other fungal species were recorded.

Pure colonies on MDRB agar medium were sub-cultured onto the Czapek yeast extract agar (CYA; 1 g K_2HPO_4, 10 mL Czapek concentrate, 5 g powdered yeast extract, 30 g sucrose, 15 g agar), whose pH was adjusted to 7.2 and the plates incubated at 30 °C for 7 days. Species of *Aspergillus* section *Flavi* were identified based on colony colour, texture and conidial morphology characteristics (Klich, 2002), and by comparison with reference strains obtained from Dr. Bruce Horn (USDA National Peanut Research Laboratory, Dawson, Georgia, USA).

2.6 Screening Isolates of A. flavus and A. parasiticus for Aflatoxin Production

A total of 250 isolates of *A. flavus* and *A. parasiticus* from the peanuts were screened for production of aflatoxin B1, B2, G1 and G2. The screening was done in high sucrose yeast extract (YES) liquid medium (Horn & Dorner, 1998). Conidia from single spore colonies of *A. flavus* (S- and L-strains) and *A. parasiticus* were inoculated into 6 mL vials containing 2 mL YES medium. Inoculated vials were incubated in the dark at 30 °C for 7 days, with intermittent shaking using a Stuart® vortex shaker (Bibby Scientific Limited, Staffordshire, UK). After incubation, 2 mL of chloroform was pipetted into each vial, the mixture vortexed for 60 seconds and left to stand overnight in a fume hood. Using a micro-pipette, 5 µL of the chloroform extract was spotted on silica gel 60 thin layer chromatography (TLC) plates (EMD Chemicals Inc., Gibbstown, New Jersey, USA), along with analytical grade standards of aflatoxin B1, B2, G1 and G2 (ICRISAT, Patancheru, India). Previously selected toxigenic strains were used as positive controls. The plates were developed in chloroform, acetone and distilled water, in the ratio of 88:12:1.5 respectively, until the solvent covered about 90% of the plate length. The plates were transferred to a darkroom and scored for the presence or absence of the four aflatoxin types under UV-light.

2.7 Analysis of Peanut Samples for Aflatoxin Levels

A 20g sub-sample was drawn from the 50g sample from each storage bag. The powder was triturated in a blender in 100 mL of 70% methanol (70 mL absolute methanol in 30 mL distilled water, v/v) containing 0.5% potassium chloride (w/v) until thoroughly mixed. The extract was transferred to a conical flask and shaken for 30 min at 250 rpm. The extract was then filtered through Whatman No. 1 filter paper and diluted 1:10 in phosphate buffered saline containing 500 µL/L Tween-20 (PBS-Tween) and analyzed for aflatoxin contamination with an indirect Competitive Enzyme-Linked Immunosorbent Assay (ELISA) as described by Waliyar et al. (2005). This method has a detection limit of 0.5 µg/kg.

2.8 Data Analyses

Data on fungal population and aflatoxin levels were subjected to analysis of variance (ANOVA) using PROC ANOVA procedure of Genstat Discovery 2 statistical software (Version 13, Lawes Agricultural Trust, Rothamsted Experimental Station, 2006) and means compared using Fisher's protected LSD test at 5% significance level. For the fungal isolation data, infection frequency from each plate was pooled and mean frequency determined. Percentage data that were skewed were transformed using arcsine $\sqrt{p/100}$ while other skewed data were transformed to $\log_{10}$ for data analysis and separation of means. Pearson correlation coefficient (SPSS version 16) was used to establish the correlations between different parameters.

For aflatoxin data, samples were grouped into three categories based on their aflatoxin content: samples with:≤ 4 µg/kg, > 4 µg/kg to ≤ 10 µg/kg, and >10 µg/kg. The ≤ 4 µg/kg category represents the European Union (EU) regulatory limit for total aflatoxin; peanuts in the second category would be rejected in the EU but would be accepted under the KEBS limits, while nuts in the third category would be rejected under the KEBS standards.

3. Results

3.1 Moisture Content of Peanut Kernels

There were significant ($p \leq 0.05$) differences in M.C. of peanut samples stored under different temperature and R.H. conditions and storage bags as presented in details in Mutegi et al. (In press). Moisture content of the samples varied from 3.3 to 6.9% and significantly ($p \leq 0.05$) decreased gradually from 5.6% before storage to 4.9% after four months of storage after which it stabilized. Overall ranking of M.C. in peanuts in different containers was as follows, in increasing levels: polypropylene (5.1%), polyethylene (5.2%) and jute bag (5.3%). The mean M.C. of peanuts stored under different temperature and R.H. conditions was as follows: 24 °C and 56% R.H. (5.0%), 22 ± 3 °C and 55 ± 5% R.H. (5.2%), and 19°C and 64% R.H. (5.4%). The M.C. of the four varieties varied significantly ($p \leq 0.05$) and was in increasing order: ICGV-SM 12991 (5.1%), Homabay Local (5.2%), ICGV-SM 99568 (5.2%), and Valencia Red (5.3%), respectively.

3.2 Incidence of Fungal Species Associated With Peanuts

Six fungal pathogens were commonly isolated from the peanut samples (Table 1). Incidence of the fungal species was significantly ($p \leq 0.05$) different and in decreasing order: *Penicillium* spp. (106.6 CFU/g), *A. flavus* L-strain (4.8 CFU/g), *A. flavus* S-strain (2.9 CFU/g), *A. niger* (2.6 CFU/g), *A. parasiticus* (1.7 CFU/g) and *A. tamari* (0.2 CFU/g). The population of *Penicillium* spp. was consistently high at the three temperature and R.H. regimes. Other frequently isolated moulds included *Fusarium, Trichoderma* and *Eurotium* spp. The peanut variety did not influence the diversity of fungal species isolated, but had a significant ($p \leq 0.05$ effect on the total population of fungal pathogens with mean infection of the varieties ranked in the following decreasing order: Homabay Local (71.9 CFU/g), ICGV-SM 99568 (21.1 CFU/g), Valencia Red (17.2 CFU/g) and ICGV-SM 12991 (13.5 CFU/g).

However, the type of storage bag as well as storage temperature and R.H. did not significantly ($p \geq 0.05$) influence the total fungal population.

Population of the main aflatoxin producing fungi - *A. flavus* (S and L strains) and *A. parasiticus* - was influenced by the variety, storage bag, and temperature and R.H. (Table 2). However, there was no consistent pattern on how these conditions influenced the population of the moulds. Within each month, there was no significant ($p \geq 0.05$) variation in the population of the three fungal species except at the sixth month of storage. Overall, the population of *A. flavus* L-strain was 66% higher than that of *A. flavus* S-strain.

Peanut variety, storage temperature and R.H. significantly ($p \leq 0.05$) influenced the population of *A. flavus* (S and L strains) and *A. parasiticus*, whereas the type of storage bag did not have a significant ($p \geq 0.05$) effect (Figure 1). The highest and lowest population of the three pathogens was recorded when peanuts were stored at 19 °C and 64% R.H. (mean = 3.5 CFU/g), and 24 °C and 56% R.H. (mean = 2.8 CFU/g), respectively. Population of the three pathogens in different peanut varieties was ranked as follows: Homabay Local (5.6 CFU/g), ICGV-SM 12991 (2.8), ICGV-SM 99568 (2.1) and Valencia Red (1.9 CFU/g).

Table 1. Mean colony forming units [CFU/g peanuts] of fungal species isolated from kernels of four peanut varieties stored in different bag types at different temperatures and relative humidity for six months

Temp. [°C]	R.H.[a] [%]	Bag Type	Variety	AF[S]	AF[L]	AP	AN	AT	PEN	Others[d]
19	64	Jute	Homabay Local	15.2	5.7	1.4	1.2	0.0	64.8	272.1
			Valencia Red	0.2	5.5	2.4	1.7	0.0	10.2	59.8
			ICGV-SM 12991	0.0	8.3	0.0	0.2	0.0	5.5	84.3
			ICGV-SM 99568	3.1	10.7	0.0	0.7	0.0	36.0	36.0
		Polypro-pyrene	Homabay Local	8.6	5.2	2.4	1.4	0.0	640.5	212.6
			Valencia Red	3.8	7.4	0.0	1.9	0.0	14.8	45.7
			ICGV-SM 12991	0.2	0.7	0.0	1.2	0.0	13.8	62.6
			ICGV-SM 99568	0.7	0.7	1.2	2.4	0.2	41.2	91.9
		Poly-ethylene	Homabay Local	1.7	14.8	0.5	0.7	0.0	79.8	169.5
			Valencia Red	0.0	1.9	2.4	5.5	0.2	216.4	57.1
			ICGV-SM 12991	16.2	23.3	0.0	0.0	0.0	37.1	75.5
			ICGV-SM 99568	0.0	0.7	0.5	2.9	0.0	73.6	60.5
			Mean	4.1	5.4	0.9	1.6	0.0	102.8	102.3
24	56	Jute	Homabay Local	4.3	9.5	0.0	1.4	0.0	654.3	202.6
			Valencia Red	5.0	12.6	0.0	6.2	2.4	44.3	54.8
			ICGV-SM 12991	0.0	2.9	0.0	0.2	0.0	28.6	49.0
			ICGV-SM 99568	0.0	0.2	0.0	1.0	0.0	191.4	43.3
		Polypro-pyrene	Homabay Local	2.1	4.5	0.0	1.0	0.0	240.2	97.9
			Valencia Red	0.0	1.2	0.2	3.1	0.0	42.4	67.6
			ICGV-SM 12991	0.0	1.0	2.4	0.2	0.0	54.8	64.3
			ICGV-SM 99568	0.7	12.6	0.0	14.8	0.0	63.8	90.0
		Poly-ethylene	Homabay Local	7.1	9.8	4.8	12.4	0.0	234.0	102.0
			Valencia Red	0.0	3.8	0.0	5.5	0.0	55.7	26.4
			ICGV-SM 12991	0.0	10.0	0.0	0.5	0.0	10.7	61.4
			ICGV-SM 99568	2.9	3.1	0.0	1.9	0.0	44.8	87.4
			Mean	1.8	5.9	0.6	4.0	0.2	138.8	78.9
RT[b]	AR.H.[c]	Jute	Homabay Local	3.3	7.6	7.4	1.7	0.0	186.9	121.4
			Valencia Red	0.2	0.7	0.0	2.1	0.0	37.4	35.0
			ICGV-SM 12991	0.2	7.1	0.0	1.0	0.0	28.6	61.7
			ICGV-SM 99568	2.4	2.6	0.0	1.4	0.2	19.0	130.7
		Polypro-pyrene	Homabay Local	16.0	4.0	2.4	3.6	0.0	150.5	124.3
			Valencia Red	0.5	1.0	0.0	3.1	0.2	32.4	101.4
			ICGV-SM 12991	0.0	0.5	2.4	1.4	0.0	5.2	51.4
			ICGV-SM 99568	0.0	2.4	24.3	2.6	0.2	16.7	56.2
		Poly-ethylene	Homabay Local	3.6	7.9	2.4	0.7	2.4	386.9	162.1
			Valencia Red	0.0	0.2	2.4	5.2	0.0	20.7	72.9
			ICGV-SM 12991	0.0	0.0	0.0	0.2	0.0	4.8	91.4
			ICGV-SM 99568	5.2	1.0	0.5	2.9	0.0	51.2	95.7
			Mean	2.6	2.9	3.5	2.2	0.3	78.4	92.0
			Grand mean	2.9	4.8	1.7	2.6	0.2	106.6	91.1
			Significance	***	**	**	**	ns	***	***

[a] - Relative humidity; [b] - Room temperature (22 ± 3°C), [c] - Ambient R.H. (55 ± 5%); [d] - Other fungal pathogens - *Fusarium* spp., *Trichoderma* spp., *Eurotium* spp., unidentified fungal species.

AF[S] - *A. flavus* S-strain, AF[L] - *A. flavus* L-strain, AP - *A. parasiticus*, AA - *A. alliaceaus*, AC - *A. caelatus*, AN - *A. niger*, AT - *A. tamarii*, PEN - *Penicillium* spp.

, * - Significant at 5% and 1% levels, respectively; ns - not significant ($p \geq 0.05$).

Table 2. Colony forming units [CFU/g peanuts] of *A. flavus* [S and L strains) and *A. parasiticus* isolated from kernels of four peanut varieties stored in different bag types at different temperatures and relative humidity for six months

Temp. [°C]	R.H.[a] [%]	Bag type	Variety	Time [months]							Sig.
				0[d]	1	2	3	4	5	6	
19	64	Jute	HL	2.2	4.4	4.4	5.6	2.2	27.8	5.6	ns
			VR	0.6	0.0	0.0	12.8	5.6	0.0	0.0	**
			12991	0.0	0.0	0.0	0.0	19.4	0.0	0.0	***
			99568	7.2	0.0	0.0	12.2	12.8	0.0	0.0	**
		PP	HL	3.9	0.6	18.3	12.2	0.6	1.1	1.1	**
			VR	7.2	0.0	0.0	7.2	6.1	0.0	5.6	ns
			12991	0.0	1.1	0.0	1.1	0.0	0.0	0.0	ns
			99568	2.2	0.0	0.6	1.1	2.2	0.0	0.0	**
		PE	HL	17.8	2.2	2.8	11.7	3.9	1.1	0.0	ns
			VR	0.0	0.0	1.7	5.6	2.8	0.0	0.0	ns
			12991	0.0	0.0	45.0	0.0	1.1	0.0	0.0	***
			99568	0.0	1.1	0.0	0.0	1.1	0.6	0.0	ns
24	56	Jute	HL	3.9	1.7	0.6	11.7	6.7	1.7	6.1	ns
			VR	0.0	0.0	0.0	1.7	0.0	39.4	0.0	***
			12991	0.6	0.0	0.0	0.6	5.6	0.0	0.0	ns
			99568	0.6	0.0	0.0	0.0	0.0	0.0	0.0	ns
		PP	HL	1.1	7.8	1.1	1.7	1.1	2.8	0.0	ns
			VR	2.2	0.0	0.6	0.0	0.6	0.0	0.0	ns
			12991	0.0	0.0	1.1	1.1	5.6	0.0	0.0	ns
			99568	1.1	0.0	5.6	0.6	22.8	0.6	0.6	**
		PE	HL	12.2	5.6	1.7	8.3	14.4	6.7	1.7	ns
			VR	1.1	0.0	0.6	7.2	0.0	0.0	0.0	**
			12991	0.0	0.0	0.0	23.3	0.0	0.0	0.0	**
			99568	5.6	0.6	0.0	6.1	0.6	1.1	0.0	ns
RT[b]	AR.H.[c]	Jute	HL	6.1	0.6	9.4	12.8	13.3	0.0	0.6	**
			VR	0.0	0.0	0.6	1.1	0.0	0.6	0.0	ns
			12991	0.0	0.0	5.6	11.1	0.6	0.0	0.0	ns
			99568	0.0	0.0	5.6	6.1	0.0	0.0	0.0	ns
		PP	HL	1.1	1.1	7.2	6.7	8.3	5.6	22.2	ns
			VR	0.6	0.6	0.0	2.2	0.0	0.0	0.0	ns
			12991	0.6	0.0	0.0	0.6	0.0	5.6	0.0	ns
			99568	0.6	0.0	55.6	5.6	0.6	0.0	0.0	ns
		PE	HL	15.0	1.1	0.6	1.1	6.1	0.0	8.3	**
			VR	0.0	0.0	0.0	0.6	5.6	0.0	0.0	ns
			12991	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ns
			99568	5.6	5.0	3.3	0.6	1.1	0.0	0.0	ns
			Mean	2.7	1.2	5.0	5.0	4.2	2.6	1.4	
			Sig.	ns	ns	ns	ns	ns	ns	**	

[a] - Relative humidity; [b] - Room temperature (22 ± 3°C), [c] - Ambient R.H. (55 ± 5%), [d] - Before storage; PP - Polypropyrene, PE - Polyethylene; HL - Homabay Local, VR -Valencia Red, 12991 - ICGV-SM 12991, 99568 -ICGV-SM 99568.

, * - Significant at 5% and 1% levels, respectively; ns - not significant ($p \geq 0.05$).

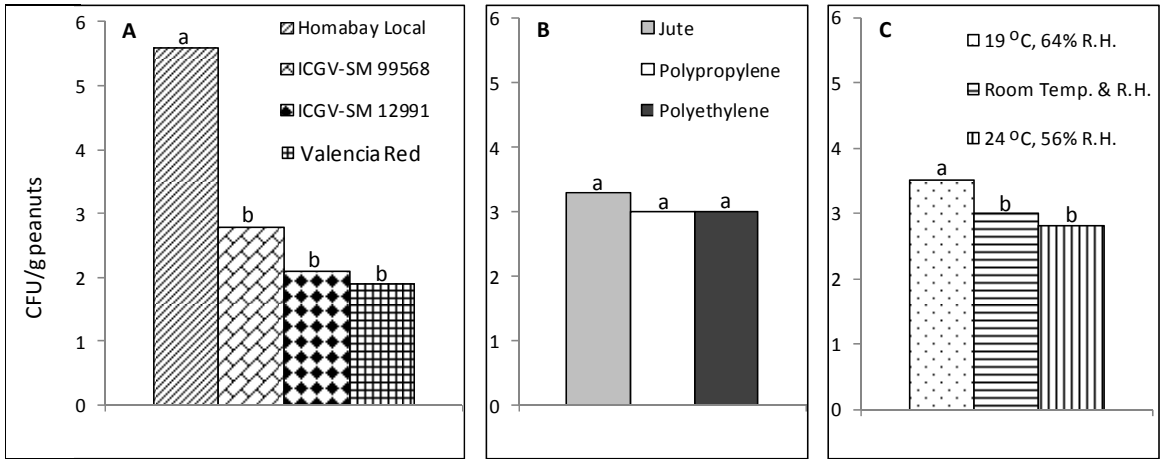

Figure 1. Mean colony forming units [CFU/g peanuts] of *A. flavus* [S and L strains) and *A. parasiticus* isolated from kernels of four peanut varieties (A), stored in different bag types (B), at different temperature and relative humidity levels (C) for six months. Bar graphs for each parameter accompanied by the same letter are not significantly ($p \geq 0.05$) different

3.3 Aflatoxin types Associated With A. flavus and A. parasiticus Isolates

Out of the 255 isolates of *A. flavus* (S- and L- strains) and *A. parasiticus* assayed, 91% were aflatoxigenic and produced at least one of the aflatoxin types, B1, B2, G1 or G2. The most common aflatoxin type was AFG2, with a mean incidence of 89% (Table 3). Aflatoxin B2 (60%) and AFB1 (36%) were however detected in higher incidences compared to AFG1 (31%). Isolates of *A. flavus* L- strain mainly produced AFB1 (39%) while AFB2 (67%) and AFG1 (42%) were the main aflatoxin types produced by isolates of *A. flavus* S- strain and *A. parasiticus*, respectively.

Table 3. Percentage of *A. flavus* [S and L strains) and *A. parasiticus* isolates from peanuts producing different aflatoxin types

Aspergillus spp.	AFB1		AFB2		AFG1		AFG2		Total
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	[n]
A. flavus S-strain	36.1	63.9	67.2	32.8	19.7	80.3	85.2	14.8	61
A. flavus L-strain	38.5	61.5	63.7	36.3	30.8	69.2	90.1	9.9	182
A. parasiticus	33.3	66.7	50.0	50.0	41.7	58.3	91.7	8.3	12
Mean	36.0	64.0	60.3	39.7	30.7	69.3	89.0	11.0	255

AFB1 - Aflatoxin B1, AFB2 - Aflatoxin B2, AFG1 - Aflatoxin G1, AFG2 - Aflatoxin G2.

+ve, -ve, positive and negative for the different aflatoxin types, respectively.

3.4 Aflatoxin Levels in Peanut Samples

There was a significant ($p \leq 0.05$) variation in the total aflatoxin levels - which ranged from 0-47.8 µg/kg - among peanut kernels of the four varieties stored in different bag types (Table 4). Peanuts stored in polyethylene bags were 7.3% and 13.4% more contaminated than samples stored in polypropylene and jute bags, respectively. Overall, kernels of Homabay Local stored in polyethylene bags at 19 °C and 64% R.H. were the most contaminated (mean = 5.5 µg/kg) while those of Valencia Red stored at room temperature and R.H. were the least contaminated (mean = 0.3 µg/kg).

Under the different temperature-R.H-packaging material combinations, samples were assessed to determine the implications of such conditions on the safety of the nuts, based on existing regulatory thresholds. Overall, 89% of the peanut samples would have met the EU standards of a maximum of 4 µg/kg for total aflatoxin while 97% of the samples would have met the Kenyan regulatory threshold of ≤ 10 µg/kg (Table 4).

Table 4. Mean proportion [%] of aflatoxin contamination level categories [µg/kg] for different peanut varieties stored at varying temperature and relative humidity conditions

Temp. [°C]	R.H.[a] [%]	Bag Type	Variety	≤4	>4-10	>10	Range	Aflatoxin level [µg/kg][d]
19	64	Jute	Homabay Local	100.0	0.0	0.0	0 - 4.0	1.5
			Valencia Red	85.7	14.3	0.0	0 - 7.2	2.0
			ICGV-SM12991	85.7	7.1	7.1	0 - 11.1	2.0
			ICGV-SM 99568	85.7	14.3	0.0	0 - 5.7	2.0
		Polypro-	Homabay Local	78.6	21.4	0.0	0 - 6.0	2.1
		Pyrene	Valencia Red	85.7	7.1	7.1	0 - 37.9	4.2
			ICGV-SM12991	92.9	7.1	0.0	0 - 4.6	1.5
			ICGV-SM 99568	78.6	21.4	0.0	0 - 5.9	1.9
		Poly-	Homabay Local	71.4	14.3	14.3	0 - 47.8	5.5
		Ethylene	Valencia Red	85.7	14.3	0.0	0 - 5.9	1.6
			ICGV-SM12991	92.9	7.1	0.0	0 - 4.2	1.3
			ICGV-SM 99568	92.9	7.1	0.0	0 - 6.3	1.5
24	56	Jute	Homabay Local	92.9	7.1	0.0	0 - 4.6	1.3
			Valencia Red	85.7	7.1	7.1	0 - 11.4	2.0
			ICGV-SM12991	92.9	7.1	0.0	0 - 4.4	1.3
			ICGV-SM 99568	100.0	0.0	0.0	0 - 3.9	1.0
		Polypro-	Homabay Local	78.6	14.3	7.1	0 - 12.0	1.9
		Pyrene	Valencia Red	100.0	0.0	0.0	0 - 2.9	1.0
			ICGV-SM12991	92.9	0.0	7.1	0 - 15.0	1.7
			ICGV-SM 99568	92.9	7.1	0.0	0 - 7.9	1.0
		Poly-	Homabay Local	85.7	14.3	0.0	0 - 7.0	2.0
		Ethylene	Valencia Red	92.9	7.1	0.0	0 - 5.8	1.4
			ICGV-SM12991	92.9	0.0	7.1	0 - 10.1	1.4
			ICGV-SM 99568	92.9	7.1	0.0	0 - 4.6	1.0
RT[b]	AR.H.[c]	Jute	Homabay Local	92.9	0.0	7.1	0 - 26.3	2.9
			Valencia Red	92.9	7.1	0.0	0 - 6.0	1.6
			ICGV-SM12991	85.7	7.1	7.1	0 - 13.7	1.8
			ICGV-SM 99568	100.0	0.0	0.0	0 - 4.0	1.1
		Polypro-	Homabay Local	71.4	14.3	14.3	0 - 15.2	3.3
		Pyrene	Valencia Red	100.0	0.0	0.0	0 - 1.8	0.3
			ICGV-SM12991	85.7	14.3	0.0	0 - 8.9	1.5
			ICGV-SM 99568	92.9	7.1	0.0	0 - 4.8	1.0
		Poly-	Homabay Local	85.7	14.3	0.0	0 - 5.5	1.9
		Ethylene	Valencia Red	92.9	7.1	0.0	0 - 4.5	1.4
			ICGV-SM 12991	78.6	7.1	14.3	0 - 15.8	2.8
			ICGV-SM 99568	85.7	14.3	0.0	0 - 5.3	1.2
			Mean	88.9	8.3	2.8	0 - 47.8	1.8

[a] - Relative humidity; [b] - Room temperature (22 ± 3°C), [c] - Ambient R.H. (55 ± 5%), [d] - Mean aflatoxin concentration.

3.5 Correlation Among Parameters Associated With Aflatoxin Contamination

Different parameters were correlated to each other with different coefficients (Table 5). Moisture content was weakly correlated (r = -0.003) to the population of *A. flavus* (S and L strains) and *A. parasiticus*. However, M.C. and total aflatoxin level were moderately positively correlated (r = 0.046). Likewise, the total aflatoxin level was positively correlated (r = 0.079) with the population of *A. flavus* (S and L strains) and *A. parasiticus*.

Table 5. Correlation matrix of different parameters associated with aflatoxin contamination of peanuts

	Moisture content	Microbial population[a]	Aflatoxin level
Moisture content			
Microbial population[a]	-0.003 **		
Aflatoxin level	0.046**	0.079**	

[a] - CFU/g peanuts of *A. flavus* (S and L strains) and *A. parasiticus*.

, * - Significant at 5% and 1% levels, respectively.

4. Discussion

Six fungal pathogens - *Penicilium* spp., *A. flavus* L-strain, *A. flavus* S-strain, *A. niger*, *A. parasiticus* and *A. tamari* - were commonly isolated from the peanut samples. Previous studies have reported isolation of diverse fungal pathogens from peanuts in Kenya (Mutegi et al. 2012; Wagacha et al., 2013). Although *A. flavus* and *A. parasiticus* are the species most frequently implicated in aflatoxin contamination (Cotty, 2006), the wide fungal species diversity poses a health risk of exposing peanut consumers to other secondary metabolites. The high incidence of *Penicillium* spp. and *A. niger* is particularly of great health concern since both produce a wide spectrum of secondary metabolites. Among mycotoxins produced by *Penicillium* spp. are ochratoxins, patulin and citrinin (O'Brien et al., 2006), while *A. niger* produces, ochratoxin A and malformins among others (Frisvad, Smedsgaard, Samson, Larsen, & Thrane, 2007; Weidenbörner, 2008). Although *Fusarium* spp. were isolated in low incidence, their infection of peanuts implies a health risk to consumers since they produce a wide range of mycotoxins such as fumonisins, trichothecenes, zearalenone and moniliformin among others (Weidenbörner, 2008).

Temperature (32 °C to 33 °C), R.H. (83% - 85%), water activity (0.83 to 0.97), and M.C. (>14%) are the most important factors influencing the growth of *A. flavus* in peanuts stored in enclosed facilities (Nakai et al., 2008) and aflatoxin contamination (Soler et al., 2010). Dry grains keep longer and safe from moulds because the water activity required for their growth is not met (Sanders, Shubert, & Pattee, 1982). However, high humidity conditions in many tropical countries constrain efforts to dry peanuts to acceptable moisture levels (Mestres et al., 2004), thereby increasing the risk of fungal growth and aflatoxin contamination.

Significantly higher fungal population and M.C. of kernels stored in jute bags were recorded compared to peanuts stored in polypropylene and polyethylene bags. Similar to the findings in the current study, Bulaong and Dharmaputra (2002) reported that fungal population was significantly higher in peanuts stored in jute bag than in polypropylene bag and in jute bag lined with polyethylene. Although jute bags are recommended for storage of grains, they easily absorb moisture from the environment compared to polypropylene and polyethylene bags which are non-absorptive but tend to trap heat within (Kennedy & Devereau, 1994).

Fungal pathogens isolated from peanuts have been reported to increase with increase in storage period (Bulaong & Dharmaputra, 2002). Saleemullah, Khalil and Shah (2006) reported faster growth of *Aspergillus* spp. with increase in humidity and prolonged storage of peanuts for 12-18 months compared to short storage periods for 2-3 months. In the current study, the population of *A. flavus* and *A. parasiticus* gradually increased and peaked during the third month of storage followed by a general decline, which could be attributed to decline in M.C. of the kernels as well as fungal competition.

Homabay Local was the most susceptible variety to *A. flavus* and *A. parasiticus* while Valencia Red, another local variety was least infected. Attempts to develop aflatoxin resistant varieties have been carried out in various countries (Upadhyaya, Nigam, & Thakur, 2004), but not in Kenya. These initiatives have led to the development of elite resistant varieties, which have been released as improved germplasm in Niger, Senegal and Burkina Faso (Upadhyaya, Nigam, & Thakur, 2002). However, resistance in peanuts to aflatoxin contamination under all conditions has still not been achieved and breeding efforts continue (Guo et al., 2009).

Although no consistent pattern was observed, the population of *A. flavus* (S and L strains) and *A. parasiticus*, and aflatoxin levels were influenced by the interaction of peanut variety, storage bag, and temperature and R.H. However, microbial population of the two species and aflatoxin contamination were weakly and moderately correlated to the M.C. of peanut kernels, respectively. Further studies are necessary to establish the effect of each of these parameters on the population of *A. flavus* and *A. parasiticus* and aflatoxin levels in peanuts.

The most important aflatoxin producers from a public health perspective are members of *Aspergillus* section *Flavi* and in particular *A. flavus* and *A. parasiticus*. There is phylogenetic evidence that *Aspergillus flavus sensu lato* may consist of several species (Chang, Ehrlich, & Hua, 2006). *Aspergillus flavus* S-strain produces high levels of B-aflatoxins (Garber & Cotty, 1997), with some S-strains producing both B- and G-aflatoxins (Barros et al., 2006; Cardwell & Cotty, 2002). *Aspergillus flavus* L-strain typically produces less B-aflatoxins or no aflatoxins at all (Barros et al., 2006; Garber & Cotty, 1997; Probst et al., 2011), while isolates of *A. parasiticus* can produce both B- and G-type aflatoxins (Ehrlich et al., 2004). Ability of *A. flavus* S-strain and *A. parasiticus* to produce both B- and G-type aflatoxins could possibly explain why AFG2 was detected in the highest incidence (89%). However, *A. flavus* L- and S-strains were the main producers of AFB1 and AFB2, respectively while *A. parasiticus* mainly produced AFG2, which concurs with the findings by Garber and Cotty (1997). It is worth noting that 9% of the assayed isolates did not produce any of the four aflatoxin types. This is significant because application of atoxigenic *A. flavus* L-strains has successfully reduced aflatoxin contamination of peanuts and maize through competitive exclusion of aflatoxin producers mainly the S strains (Probst et al., 2011).

Overall, 89% of the peanut samples met the EU standards for total aflatoxin (≤ 4 μg/kg) while 97% of the samples met the Kenyan regulatory threshold of ≤10 μg/kg. This implies that the storage conditions of the peanuts were fairly good. However, peanuts stored in polypropylene and polyethylene bags were 5.6% and 13.4% more contaminated with total aflatoxin than samples stored in jute bags, respectively. This could be attributed to retention of heat and moisture in polypropylene and polyethylene bags, which reduces peanuts quality, promotes fungal growth and aflatoxin contamination compared to jute bags.

5. Conclusion

Farmers and traders in Kenya should store their peanuts in jute bags as opposed to the commonly used polypropylene and polyethylene bags. Although jute bags easily absorb moisture, they are recommended because they allow good airflow compared to polypropylene and polyethylene bags which although non-absorptive trap heat within (Kennedy & Devereau, 1994), thereby promoting deterioration of peanuts quality. Besides storage of peanuts at relatively low R.H., farmers and traders should ensure that the storage container does not promote critical increases in M.C. which would otherwise be conducive for increase in fungal population and aflatoxin contamination. Introduction of subsidies in the country to encourage packaging of peanuts and other grains in jute bags which are currently more expensive than the other two bag types should be explored as a viable option. Although 97% of the samples met the Kenyan regulatory threshold of ≤ 10 μg/kg for total aflatoxin, the risk of exposure of peanut consumers in Kenya to aflatoxin still exist due to the high population of aflatoxigenic fungi with 91% of *A. flavus* and *A. parasiticus* isolates testing positive for aflatoxin production.

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