IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)
e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 13, Issue 1 Ver. I (January. 2019), PP 27-32
www.iosrjournals.org

Storage Fungi Invasion and Aflatoxin Contamination Periods in Egusi Kernels: An Assessment

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Abstract: *Egusi is infected with many fungi including Aspergillus which produces aflatoxin in crops before or after harvest and during storage. Field infection of Aspergillus has not been reported in Nigerian egusi, yet stored egusi have aflatoxin contamination (AC) problem. This study was undertaken to assess fungi invasion and aflatoxin formation periods in egusi and determine critical stage in egusi value chain when AC occurs and hence apply adequate control measures. Freshly harvested egusi seeds/kernels 3kg each were evaluated for fungi contamination and AC under laboratory shelf storage for 24 weeks. Subsamples (20g) were bi-weekly and analysed for aflatoxins (µgkg^{-1}) at sampling and after poor storage using standard analytical procedures. Fungi isolation and identification were done using standard laboratory procedures. Experimental design is completely randomized design with three replicates. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05.}$ Sclerotium rolfsii, Trichoderma viride, Botryodiplodia theobromae, Rhizopus, Penicillium, Fusarium and M. phaseolina were found on freshly harvested egusi. Sclerotium recording highest mean (5.2/10 seeds), followed by Fusarium (3.0/10 seeds) at harvest. A. flavus was detected four weeks after storage; which increased with storage time. Fungi earlier detected thrived on seeds for eight weeks, they died out thereafter. Aflatoxin was not detected in egusi kernels up to 4 months of storage but were detected in poorly-stored shelled (13.2-654.7µg/kg) and unshelled (9.8-3643.5 µg/kg) egusi from 4th week upwards. Aspergillus and AC in egusi is purely a post-harvest problem. When properly dried, adequately handled before and during storage AC can be absolutely controlled in egusi.*

Key words: *Aflatoxin formation, Aspergillus contamination, egusi seed, poor storage*

Date of Submission: 01-01-2019 Date of acceptance: 15-01-2019

I. Introduction

Egusi (melon) *Colocynthis citrullus* L. of the Cucurbitaceae family is produced in abundance in southwest, southeast and central parts of Nigeria (Van der Vossen *et al*., 2004). It is a food crop in many sub-Saharan African countries where it is used mainly as a soup additive which is rich in oils. It is cultivated as an increasingly important cash crop (Van der Vossen *et al*., 2004; Brisibe *et al.*, 2011).

Egusi is a valuable export commodity to sell to people who have emigrated from Africa (Van der Vossen *et al*., 2004). Egusi production in Nigeria amounted to 347,000 tons from 361,000 ha, Cameroon produced 57,000 tons, Sudan 46,000 tons, Congo 40,000 tons, Central African Republic 23,000 tonnes and Chad 20,000 tonnes (FAO, 2003). Outside Africa, China is an important producer with a production of 25,000 tonnes. An estimated 5000–7000 tons is traded from Nigeria to other West African countries. Sudan exports about 27,000 tons, mainly to Arab countries (FAO, 2003; Van der Vossen *et al*., 2004). The largest producers of egusi seed are West and Central Africa whose climates are vulnerable to aflatoxin contamination.

A number of important diseases affecting egusi have been reported. These include bacterial, viral and fungal diseases such as *Fusarium* wilt anthracnose, gummy stem blight and powdery mildew. In Nigeria in particular, *Aspergillus* spp is not among the field diseases reported in egusi (Bankole, 1993; Van der Vossen *et al*., 2004; Kehinde, 2008, 2011)

Field fungi occur before harvest and can be detected by routine inspection. Field diseases do not continue to increase in storage if grain is stored at the proper moisture content and temperature. Their numbers decrease with increasing storage; they gradually die during storage and by six months, they would have completely disappeared (Bankole, 1993).

Storage fungi are usually not present to any serious extent before harvest. Small quantities of spores of storage fungi may be present on grain going into storage or may be present on spilled grain present in harvest, handling and storage equipment or structures. This small amount of inoculum can increase rapidly leading to

significant grain infection under improper storage conditions (http://agebb.missouri.edu/storage/disease/sgfungi.htm).

Many groups of fungi are known to contaminate egusi seeds during storage, reduce seed storability, quality and above all deposit toxic metabolites (mycotoxins) in the seeds. Seed-borne fungi are also responsible for deterioration of food reserves in the seeds. Fungal deterioration of seeds occurs in form of seed rot, sclerotization of seed, and seed discolourization (Shetty, 1992).The most common storage fungi are species of *Aspergillus* and *Penicillium* (http://agebb.missouri.edu/storage/disease/sgfungi.htm). These fungi are widely distributed and almost always present.

Most storage fungi are also known to produce a large number of metabolites in seeds, some of which are toxic to humans. Various studies have shown that egusi is contaminated with many pathogens of fungal origin during storage. Fungi of the group *Aspergillus*, *Rhizopus*, *Penicillium* amongst others have been reported as seed pathogens of Nigerian stored egusi (Chiejina, 2006, Somorin and Bankole, 2010).

Aspergillus spp have not been reported as field disease of melon in Nigeria. Therefore, *Aspergillus* infection in melon and production of aflatoxin contamination usually occurs at the postharvest stage. Aflatoxins are naturally occurring mycotoxins produced by mainly *Aspergillus flavus* (Link) and *Aspergillus parasiticus* (Speare). Aflatoxins are produced as secondary metabolites of these fungi under conditions which favour their growth (Othman and AL-Delamiy, 2012). Aflatoxins are among the most carcinogenic substances known that have many adverse economic, biological and health effects. Aflatoxins are not destroyed in almost all the processes and cooking methods employed in food production.

Aflatoxin contaminations culminate in reduced crop value and diminished health of humans and domestic animals that consume the contaminated crops (Wu and Khlangwiset, 2010). Aflatoxin has been classified as a class one human carcinogen and is now a well-recognized public health hazard. Therefore, the monitoring of aflatoxin contamination points in agricultural products should continue to receive high priority (Strosnider *et al*., 2006; Reddy *et al*., 2010). More so, a greater percentage of egusi seeds produced in Nigeria are not allowed beyond Nigerian borders due to aflatoxin contamination. There is therefore the need to determine the critical stage in egusi value chain when aflatoxin contamination occurs as one of the key steps for proper management of aflatoxin production in egusi seeds. The objectives of this study therefore are to evaluate storage fungi invasion and aflatoxin contamination periods in the egusi kernels.

II. Materials And Methods

a. Determination of Appearance of Storage Fungi and Aflatoxin-producing Strains Period in Egusi kernels

Egusi seeds were purchased from a contracted farmer immediately after harvesting for this study. Three kilograms (3 kg) of clean freshly harvested sun dried unshelled egusi seeds were stored in clean woven plastic sacks. Also 3 kg of aseptically dehusked/shelled seeds/kernels were store in separate woven plastic sacks. Both were placed on the laboratory shelf and monitored for fungal contaminants and aflatoxin contamination. Fungal contaminants were detected by agar plate method. Ten seeds/kernels were plated on each 9 cm Petri dish in triplicates. Different fungal colonies that emerged were counted and identified by making slides from fungal colonies; mounting on the Olympus BX 51M reflected light optical microscope (www.labx.com) and using mycological reference books and research articles (Alexopoulus *et al*. 1996; Barnett and Hunter, 1999; Alexopoulos e*t al.*, 2002; Samson *et al*., 2004).

2.2 Aflatoxin Contamination Period in Egusi kernels

Twenty grams (20g) subsamples of each egusi seeds and kernels were collected bi-weekly and analysed for aflatoxins immediately after sampling and after poor storage conditions using the methods of Atehnkeng *et al*, 2008. Poor storage condition was mimicked by wetting the seeds/kernels with few drops sterile water, and then allowed to stay for four days before aflatoxin analysis (Atehnkeng *et al*., 2014). The experiments were laid out I completely randomized design in three replicates. Each of the above experiments was repeated twice.

2.3 Data analysis

All data collected were analyzed using the SAS (version 9.4, SAS Institute, Cary, NC) and descriptive statistics. Data on aflatoxin levels in egusi kernel and fungal incidence were summarized and analyzed using analysis of variance of the SAS (version 9.4, SAS Institute, Cary, NC). Means were compared using least significant difference test (LSD) at P = 0.05 procedure in SAS (SAS Institute Inc., Cary, NC, USA) to compare the differences among the means of samples drawn in different weeks. Prior to analysis, logarithmic transformation was done on mean values of aflatoxin concentration data using $\log_{10}$ (µg of aflatoxin per gram of ground egusi+1) to stabilize variances between data collected before analysis. However, aflatoxin analysis was done using the original values.

III. Results

3.1 Storage fungi invasion period in Egusi seeds/kernels

Sclerotium rolfsii (Curzi), *Trichoderma viride (*Pers.), *B. theobromae, Rhizopus* sp., *Penicillium* sp., *Fusarium* sp. and *M. phaseolina* were found on freshly harvested sundried shelled egusi seeds with *Sclerotium rolfsii* recording the highest mean of 5.2 per 10 seeds followed by *Fusarium* sp (3.0 per 10 seeds) immediately after processing (Table 1). *A. flavus* was first detected four weeks after the samples were placed on the shelf while *A. niger, A. tamarii, Fusarium* spp and *Penicillium* spp were noticed from the second week. *Fusarium* spp and *Rhizopus* spp reduced appreciably as the weeks progressed while *Aspergillus* species increased with the storage time. *S. rolfsii, B. theobromae, Fusarium* spp and *M. phaseolina* only thrived on the seeds within the first eight weeks, but died out thereafter.

In the second trial, *S. rolfsii* and *Rhizopus* sp. were isolated from freshly harvested egusi seeds with *Rhizopus* sp. recording the highest mean of 3.5 per 10 seeds while *S. rolfsii* had a mean of 1.0 per 10 seeds immediately after processing (Table 2).

Table 1. Fungal invasion period in egusi kernels (shelled) (first trial).

						Fungal species/10 seeds				
Weeks	Mc	Af	Nig	Tam	Rhz	Scl	Fus	Pen	Bot	Mac
0	13.0	0.0	0.0	0.0	8.4	5.2	3.0	0.0	0.0	1.0
2	9.6	0.0	2.3	5.3	8.5	0.0	2.7	0.7	0.0	0.0
4	9.4	1.0	2.5	0.0	8.3	4.3	6.7	0.0	0.0	0.0
6	9.3	2.0	8.5	1.3	0.9	0.0	0.0	0.0	0.0	0.0
8	9.4	3.3	9.5	0.5	4.0	0.0	0.0	0.0	7.2	0.0
10	9.6	3.0	9.2	0.0	4.5	0.0	1.0	0.0	0.0	0.0
12	9.4	4.0	9.5	0.5	1.5	0.0	0.5	0.0	0.0	0.0
14	9.5	3.5	9.3	3.0	0.5	0.0	0.0	0.0	0.0	0.0
16	9.5	4.0	10.0	2.0	1.5	0.0	0.0	0.0	0.0	0.0
18	9.4	3.0	7.7	2.0	1.0	0.0	0.0	0.0	0.0	0.0
20	9.7	6.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	9.9	7.0	10.0	2.0	3.0	0.5	1.0	0.0	0.0	0.0
24	9.8	6.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LSD (0.05)		2.9	2.3	1.9	1.9	2.1	1.6	0.3	5.2	0.0

Mc= moisture content, Af= *Aspergillus flavus,* Nig = *Aspergillus niger,* Tam = *Aspergillus tamarii,* Fus =*Fusarium solani,* Rhz = *Rhozopus* sp., Pen =*Penicillium* sp., Bot = *Botryodiplodia theobromae,* Scl = *Sclerotium rolfsii.*

Table 2. Fungal invasion period in egusi kernels (shelled) (second trial)

		Fungal species/ 10 seeds							
Weeks	Mc	Af	Nig	Tam	Rhz	Scl	Fus	Pen	Bot
0	12.9	0.0	0.0	0.0	3.5	1.0	0.0	0.0	0.0
2	9.7	1.0	2.0	3.0	9.5	0.0	1.0	0.0	0.5
4	9.5	2.5	1.5	0.5	9.5	6.5	7.0	0.0	0.0
6	9.4	1.5	2.6	1.5	0.6	0.0	0.6	0.2	0.2
8	9.4	3.7	9.0	0.5	3.5	0.0	0.0	0.0	0.0
10	9.4	1.5	9.0	0.0	5.0	0.0	1.0	0.0	0.0
12	9.5	4.0	3.0	0.0	0.5	0.0	0.5	0.0	0.0
14	9.5	4.0	10.0	2.0	0.0	0.0	0.0	0.0	0.0
16	9.5	1.5	10.0	2.5	1.0	0.0	0.0	0.0	0.0
18	9.4	2.3	2.7	2.3	1.0	0.0	1.0	0.3	0.3
20	9.5	6.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22	9.5	8.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0
24	9.3	6.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0
LSD (α=0.05)		1.3	3.2	1.5	2.0	0.7	2.6	0.2	0.3

Mc= moisture content, Af= *Aspergillus flavus,* Nig = *Aspergillus niger,* Tam = *Aspergillus tamarii,* Fus =*Fusarium solani,* Rhz = *Rhozopus* sp., Pen =*Penicillium* sp., Bot = *Botryodiplodia theobromae,* Scl = *Sclerotium rolfsii.*

In the seeds (unshelled) *Trichoderma viride, B. theobromae, Penicillium* sp. *Fusarium* sp. and *A. niger* were observed (Table 3). After one month *Trichoderma viride, B. theobromae,* and *M. phaseolina* were not observed in the samples. *A. niger and Rhizopus* sp. Colonies counted increased as time progressed.

After two months *Penicillium* sp. growth was not observed on the seeds any longer. However, the colony count of other fungal species increased as the time progressed and *A. flavus* and *A. tamarii* were detected from two weeks after the samples were placed on the shelf and their colony counts increased also with time.

Table3. Fungal invasion period in egusi seeds (unshelled) first trial.

		Fungal species/10 seeds								
Weeks	Mc	Af	Nig	Tam	Rhz	Scl	Fus	Pen	Bot	Tri
0	12.5	0.0	4.0	0.0	0.0	0.0	3.0	2.0	2.0	1.0
2	9.0	1.0	5.0	1.0	1.0	0.0	1.0	1.0	3.0	3.0
4	9.5	3.0	6.0	1.0	2.0	4.0	3.0	3.0	1.0	3.0
6	9.8	2.0	6.0	2.0	0.0	0.0	0.0	1.0	0.0	0.0
8	9.5	2.0	8.0	2.0	1.0	1.0	1.0	0.0	0.0	0.0
10	9.4	5.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12	9.3	8.0	5.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14	9.1	4.0	9.0	1.0	1.0	0.0	0.0	0.0	0.0	0.0
16	9.3	2.0	10.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
18	9.7	7.0	10.0	3.0	0.5	0.0	1.0	0.0	0.0	0.0
20	10.1	4.0	5.0	3.0	6.0	3.0	0.0	0.0	0.0	0.0
22	9.8	3.0	7.0	1.0	7.0	5.0	0.0	0.0	0.0	0.0
24	10.0	10.0	10.0	4.0	10.0	4.0	0.0	0.0	0.0	0.0
LSD (α= 0.05)		1.9	2.6	2.4	1.1	1.6	1.6	0.9	0.7	0.2

Mc= moisture content, Af= *Aspergillus flavus,* Nig = *Aspergillus niger*, Tam = *Aspergillus tamarii,* Fus =*Fusarium solani,* Rhz = *Rhozopus* sp., Pen =*Penicillium* sp., Bot = *Botryodiplodia theobromae,* Scl = *Sclerotium rolfsii,* Tri = *Trichoderma viridae.*

Table4. Fungal invasion period in egusi seeds (unshelled).

		Fungal species/10 seeds								
Weeks	Mc	Af	Nig	Tam	Rhz	Scl	Fus	Pen	Bot	Tri
0	12.0	0.0	2.0	0.0	0.0	0.0	1.0	1.0	3.0	2.0
2	9.3	1.0	4.0	1.0	2.0	0.0	2.0	0.0	4.0	0.0
4	9.5	3.0	6.0	0.0	4.0	3.0	3.0	3.0	2.0	0.0
6	9.9	1.0	6.0	2.0	0.0	0.0	0.0	1.0	0.0	0.0
8	9.6	2.0	10.0	2.0	0.0	0.0	2.0	1.0	0.0	0.0
10	9.4	6.0	4.0	0.0	3.0	0.0	0.0	0.0	0.0	0.0
12	9.4	7.0	5.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0
14	9.3	3.0	9.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0
16	9.4	3.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18	9.3	6.0	9.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0
20	10.1	5.0	6.0	3.0	5.0	3.0	0.0	0.0	0.0	3.0
22	9.5	4.0	8.0	2.0	5.0	5.0	1.0	0.0	0.0	0.0
24	9.5	5.0	10.0	2.0	4.0	3.0	0.0	0.0	0.0	0.0
LSD (α= 0.05)		1.9	2.8	1.5	2.0	1.3	0.9	0.6	1.0	1.0

MC = moisture content, AF = *Aspergillus flavus,* NIG = *Aspergillus niger,* TAM = *Aspergillus tamarii,* Fus = *Fusarium solani,* Rhz = *Rhozopus* sp., Pen = *Penicillium* sp., Bot = *Botryodiplodia theobromae,* Scl = *Sclerotium rolfsii,* Tri = *Trichoderma viridae*

3.2 Appearance of Aspergillus species and aflatoxin contamination in egusi kernels

Aflatoxin was not detected in the egusi kernels for up to 4 months of storage even though *A. flavus* started appearing on the samples four weeks after being placed on the shelf. However, in the egusi kernels in which poor storage was mimicked, aflatoxin began to occur at detectable levels from 4 weeks (9.8 µg/kg) and increased at varying levels throughout the period. The mean aflatoxin levels ranged from 9.8 to 3643.5 µg/kg with an average of 642.1 µg/kg. Aflatoxin was also not detected in kernels under normal conditions of storage throughout the study period in the second experiment; but was detected in the poorly stored egusi kernels at levels ranging from 16.4 to 2526.3 µg/kg and a mean of 431.5 µg/kg (Table 5).

The mean aflatoxin levels ranged from 27.9 to 479.6 µg/kg with an average concentration of134.1 µg/kg (Table 5) in egusi seeds. However, aflatoxin levels detected in the seeds was also lower than those detected in the shelled seeds (kernels). Aflatoxin was also not detected in seeds under normal conditions of storage throughout the study period but was detected in the poorly stored egusi seeds at levels ranging from 13.2 to 654.7 µg/kg and a mean of 152.3 µg/kg (Table 5) in the second experiment.

Table5. Aflatoxin (µg/kg) production period in egusi kernels

	Aflatoxin concentration (µg/kg) in egusi kernels							Aflatoxin concentration (µg/kg) in egusi seeds						
	1st experiment				2nd experiment			1st experiment				2nd experiment		
Weeks	Mc	Afl	Af.Ps		Mc	Afl	Af.Ps	Mc	Afl	Af.S		Mc	Afl	Af.S
0	13.0	0.0	0.0		12.9	0.0	0.0	12.5	0.0	0.0		12	0.0	0.0
2	9.6	0.0	0.0		9.7	0.0	0.0	9.0	0.0	0.0		9.3	0.0	0.0
4	9.4	0.0	9.8		9.5	0.0	16.4	9.5	0.0	0.0		9.5	0.0	0.0
6	9.3	0.0	43.4		9.4	0.0	52.1	9.8	0.0	27.9		9.9	0.0	13.2
8	9.4	0.0	58.8		9.4	0.0	0.0	9.5	0.0	52.6		9.6	0.0	63.8
10	9.6	0.0	17.6		9.4	0.0	25.2	9.4	0.0	17.9		9.4	0.0	14.3

12	9.4	0.0	27.3		9.5	0.0	51.8		9.3	0.0	59.4		9.4	0.0	63.4
14	9.5	0.0	508.7		9.5	0.0	498.3		9.1	0.0	214.0		9.3	0.0	237.3
16	9.5	0.0	2665.7		9.5	0.0	2526.3		9.3	0.0	372.5		9.4	0.0	474.6
18	9.4	0.0	312.7		9.4	0.0	251.4		9.7	0.0	115.3		9.3	0.0	106.2
20	9.7	0.0	88.8		9.5	0.0	79.9		10.1	0.0	104.6		10	0.0	126.0
22	9.9	0.0	3643.5		9.5	0.0	1944.1		9.8	0.0	299.0		9.5	0.0	227.3
24	9.8	0.0	971.2		9.3	0.0	164.3		10.0	0.0	479.6		9.5	0.0	654.0
LSD (0.05)		0.0	598.8			0.0	6.2			0.0	155.0			0.0	27.0

MC = moisture content, Afl = aflatoxin level (µg/kg), Af.ps = aflaoxin content (µg/kg) after poor storage.

IV. Discussion

All the fungi identified in the freshly harvested egusi seeds in the study except *T. virida* and *A. niger* have been reported in earlier studies (Bankole *et al.,* 2004). During storage *B. theobromae* and *M. phaseolina* died out with time while *Fusarium* sp. colonies reduced appreciably. *Aspergillus* species (*A. flavus* and *A. tamarii*) were found several weeks after storage, which is similar to the findings of Bankole *et al.* (2004); while *A. niger* increased greatly during storage. This indicates that fungal contamination of kernels especially *A. flavus* are because of poor handling during processing and storage. Fungi are common environmental contaminants; they produce spores that are always present in the atmosphere and can survive unfavourable environmental conditions; this could explain their occurrence of fungi in egusi after harvesting and processing (Oranusi *et al.,* 2013). Furthermore, poor storage conditions; namely, excessive heat and moisture, pest-related crop damage, and extensive periods of time spent in storage exceeding several months is the main predisposing factor in postharvest aflatoxin accumulation in egusi seeds (Hell *et* al., 2009; Mutegi *et al*., 2010; Wu *et al*., 2011).

Shelled egusi seeds were seen to have the least contamination levels of aflatoxin after poor storage because the shells of act as a protection for fungi that penetrate the seeds. A similar result in stored podded groundnut kernels was reported in Kenya (Mutegi *et al.,* 2010). In cases where shells are broken for example through mechanical damage during processing/extraction of seeds from the pods, the chances of fungal contamination and subsequent aflatoxin production in the kernels are high. In fact, storing the seeds with their shells has been recommended as a way of reducing of aflatoxin contamination (http://www.fao.org/docrep/x5036e/x5036E1b.htm*).* Aflatoxins were not detected in the seeds for a period of 6 months storage, even though the toxigenic fungi grew on them when plated on PDA. This is because they were stored at moisture levels below 9 % that did not allow the fungi to carry out their metabolic activities. Drying seeds below the specified moisture content inhibits the activities of storage fungi regardless of how long the grains are stored (Bankole, 1993).

V. Conclusion

Aspergillus species (*A. flavus* and *A. tamarii*) were not present in freshly harvested egusi seeds but appeared several weeks after storage while *A. niger* increased greatly during storage. Fungal contamination of seeds especially *A. flavus* and the corresponding contamination with aflatoxins are as a result of poor handling during processing and storage. Storing egusi seeds for a long time with poor handling especially in the shelled form exposes them to aflatoxin contamination. The formation of aflatoxins in egusi relies mainly on contamination after harvest because undeveloped processing as well as poor storage practices are largely the post-harvest handling methods adopted in egusi production.

Acknowledgements

Authors are grateful to the Mycotoxin/Pathology unit of International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria for permitting and supporting this work to be carried out in the Mycotoxin/Pathology Laboratory of IITA.

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