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(RESEARCH ARTICLE)

Isolation and detection of mycotoxin producing fungi species from dry stored groundnut sold in Keffi, Nigeria

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Abstract

Isolation and detection of mycotoxins producing fungi from stored dry groundnut sold in Keffi. One hundred (100) dry groundnuts (*Arachis hypogaea*) was collected from different dry groundnut storage shops Keffi Metropolis in a sterilized tube and transported to Microbiology Laboratory of Nasarawa State University, Keffi. Fungi species were isolated using standard microbiological methods. The percentage occurrence of fungi was 68.3 %. The highest fungi was isolated from Angwan Lambu (66.6 %) and lowest was from Old market (91.6%). The fungi species isolated in this study were *Penicillium digitatum, Phaecocromonium. Parasiticum, Aspergillus oryzae, Aspergillus flavus* and *Fusarium graminearum*. The highest fungi isolated from Angwan Lambu were *Aspergillus niger* (33.3%) and lowest was *Penicillium digitatum* (8.3 %). From High court the highest were *Penicillium digitatum, Aspergillus flavu* and *Fusarium graminearum* (16.6 %). From Angwan Waje the highest were *Aspergillus niger* and *Aspergillus oryzae*(16.6 %). From Old market the highest was *Aspergillus oryzae* (33.3 %) and lowest was *Fusarium sp* (8.3 %). The mycotoxins detected were cyclopiazonic acid, fumonisins, aflatoxin, ochratoxin, and patulin. *Aspergillus niger* screened 4 produce mycotoxin were 3 produced Ochratoxin and 1 produced Fumonisins. *Penicillium digitatum* screened 3 for mycotoxins 2 produced Patulin and 1 produced aflatoxin. *Aspergillus oryzae 1 produce* citrinin. 3 *Aspergillus flavus* isolated 2 produced both aflatoxin, ochratoxin and 1 cyclopiazonic acid and 6 *Fusarium graminearum* screened 4 were observed to produce mytcotoxins; 1 produce Patulin, 1 Aflatoxin and 2 produced Fumonisins. Different fungi species isolated in this study are mycotoxin producing fungi there is need to improve on methods of drying and storage of groundnut in the study area.

Keywords: Fungi; Mycotoxin; Dry groundnut; Storage; Isolation

1. Introduction

Fungi are ubiquitous plant pathogens that are major spoilage agents of foods and feed stuffs. The infection of plants by various fungi not only results in reduction in crop yield and quality with significant economic losses but also contamination of grains with poisonous fungal secondary metabolites called mycotoxins [1].

The ingestion of such mycotoxin contaminated grains by animals and human beings has enormous public health significance, because these toxins are capable of causing diseases in man and animals [2]. Although the involvement of fungi and their toxins in causing diseases to man and animal's dates back to the period when the Dead Sea Scrolls were written it seems the evidence of their historic occurrence and impact were not obvious until the Middle Ages, when ergot alkaloids poisoning outbreaks in Europe were responsible for the death of thousands of people. Subsequently, between 1940s and 1950s a lethal human disease caused by *Fusarium* toxins and referred to as 'Alimentary Toxic Aleukia' was reported in Russia [3]. Similarly, in 1938 in Japan, *Penicillium* species were responsible for the coloring of rice that erratically led to the fatal human cardiac syndrome called 'yellow rice disease' [4].

The livestock industry was also affected as seen by the devastation of the New Zealand sheep industry by Facial eczema a fungal infection caused by *Pithomyces chartarum* in 1822. Other deadly animal syndromes arising from fungal

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infections and termed differently as equine leukoencephalomalacia, stachybotryotoxicosis, red mould diseases and red clover disease, vulvovaginitis and mouldy corn toxicosis plagued the world. In spite of these episodes' little attention was paid to fungal diseases [5]. This investigation focus on isolation and detection of mycotoxin producing fungi from stored dry groundnut sold in Keffi, Nigeria

2. Material and methods

2.1. Methods

2.1.1. Study area

This study was carried out in Nasarawa State University Keffi. Keffi is approximately 68km away from the Federal Capital Territory (FCT) Abuja and 128km away from the state capital (Lafia). Keffi is situated at Longitude $8^0$5'E Latitude $7^0$5'N and is 850 meters above sea level [6].

2.2. Sample collection

One hundred (100) samples of Dry groundnut (*Arachis hypogaea*) was collected from different dry groundnut storage shops Keffi Metropolis in a sterilized tube and transported to Microbiology Laboratory of Nasarawa State University, Keffi.

2.3. Isolation of fungi

The dry groundnut was disinfected with ethanol and washed with sterilized water two times and the fungi was isolated from dry groundnut using direct inoculation method as described by Zaki *et al.,*[7]. The Dry groundnut was inoculated directly to the Potatoes Dextrose, Sabouraud Dextrose Agar and Malt Extract Agar plates and incubated at room temperature for 4days. The fungi grown was sub-cultured to obtain pure colonies and stored in slant for further identification.

2.3.1. .Identification of the fungi

Identification of fungi isolates was carried out using a method described by Ekeleme *et al.,* [8]. Identification was based on fungi standard procedure using cultural, morphological and microscopic characteristics. The cultural characteristics were determined by their appearance on culture plates while the morphological features were determined microscopically using lactophenol cotton blue staining technique. A drop of Lactophenol blue was put on free glass slide and mounting pin was used to pick fungal and mix with the lactophenol blue and view under x10 and x100 objective lens of microscope morphology characteristics was compared with standard fungi chart.

2.3.2. Preparation of Substrate for Mycotoxin Production

Groundnut substrates were prepared using a method described by Amadi and Adeniyi, [9]. Groundnut was purchased from Keffi Metropolis and grind into powder form using clean grinding machine and sieve. Five hundred gram (500g) powder form was added into 4 liter of distilled water to form a homogenous mixture and placed at 4ºC for further use.

Preparation of Inoculum for Fermentation

Preparation of inoculum for mycotoxin production was carried out as described by Makut and Ekeleme [10]. Seven milliliter (7 ml) of peptone water and glass beads was prepared and autoclaved at 5.0 lbs/in2 pressure at 121 °C for 15 min and three milliliter (3ml) of tween 80 was added into medium. Five milliliter (5 ml) of the medium containing of 3 % tween 80, peptone water and glass beads was transferred into four (4) days' slant culture of fungi, shaken thoroughly until spores were dislodged and the spore suspension was incubated at room temperature for 6 hours and stored for use.

2.4. Production of Mycotoxin

Medium for mycotoxin production was formulated using a method described by Ekeleme et al. [8]. One hundred millimeter medium containing (g/l): soybean cake and groundnut cake 2.5 g each, NH_4Cl 0.4 g, KH_2P O40.1 g, $MgSO_4$,$7H_2O$ 0.025 g] was added into glucose 120 g but was modified by using groundnut substrate, M [groundnut substrate (20 g/L), $(NH4)_2SO_4$ 1.5 g, and KH_2PO_4 4.1 g,] was added into mineral salt medium containing ZnSO4.7H2O0.09 g, $CuSO_4.5H_2O$0.1 g, $MnSO_4$ and 5 g/l $MgSO_4$0.4 g was added in 50ml of water in a conical flasks. The flasks were plugged with cotton and autoclaved at 15 psi for 15 min. after cooling the 5ml of prepared fungi inoculum was spread on the media and incubate for 5days.

2.4.1. Mycotoxin detection

Sample preparation

Ten milliliters of the growth medium was mixed with 20 mL of 25:75 (v/v) water/methanol and was shake for 30 min. After centrifugation at 8500rmp for 15 min, 5 mL of the supernatant was transferred to a 15mL glass tube and evaporated under water bath at 50 ℃. The dry residue was reconstituted with 0.25 mL of a 95:5 (v/v) water/methanol mixture and centrifuged for 10 min at 17,000 rmp; the supernatant was used directly for the analysis. Samples, in which the concentration exceeded the highest level of calibration, was diluted and re-injected.

2.4.2. Instrumentation for Mycotoxin Analysis

Detection and quantification of mycotoxins was performed with high-performance liquid chromatography coupled with tandem mass-spectrometry (LC/MS/MS). Chromatographic separation was carried out using Nexera X2 UHPLC (Shimadzu, Tokyo, Japan) equipped with 100 _ 2.1 mm,2.6 _m Kinetex C18 column, (Phenomenex, Torrance, CA, USA). The column will be maintained at 40°Cand the injection volume will be 2µL. The mobile phase consisted of 2.5 mM ammonium acetate acidified with 0.1% acetic acid (A), and methanol (B). The methanol (B) concentration was raised gradually from5% to 95% within 8 min, brought back to the initial conditions at 9 min, and allowed to stabilize for3 min. The mobile phase was delivered at a flow rate of 0.4 mL/min. The LC system was coupled withAPI 6500 hybrid triple quadrupole/linear ion trap mass spectrometer (Sciex, Concord, ON, Canada), equipped with a turbo-ion electrospray (ESI) ion source.

3. Results

The percentage occurrence of fungi isolated from stored groundnuts is as given in Table 1. The percentage occurrence show that the total occurrence was 68.3 %, were Angwan Lambu had 66.6 %, High court and Angwan Waje had 50.0 %, new market had 83.3 % and Old market had 91.6% respectively.

Table 2 shows the cultural characteristics of fungi species isolated. The fungi isolated were *Aspergillus niger* colonies Initial growth was white, later becoming black with pale yellow on the reverse side. *Penicillium digitatum* the color of the colonies were grey green, the conidia were smooth to finely roughened surface and well defined columns. *Aspergillus flavus* Colonies are granular, flat, with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate, later splitting to form loose columns biseriate. *Fusarium graminearum* colonies grow slowly, surface usually orange to deep apricot due to confluent conidial slime, aerial mycelium sometimes floccose and whitish.

The percentage occurrence of fungi species in different location is as given in Table 3. The highest percentage isolated from Angwan Lambu was *Aspergillus niger* (33.3 %) followed by *Aspergillus flavu* (16.6 %) and the least was *Penicillium digitatum* (8.3 %). From High court the highest were *Penicillium digitatum, Aspergillus flavu* and *Fusarium graminearum* (16.6%) followed by *Aspergillus niger* and *Aspergillus oryzae* (8.33 %). From Angwan Waje the highest were *Aspergillus niger* and *Aspergillus oryzae* (16.6 %) and the least were *Phaecocromonium. Parasiticum, Aspergillus flavus* and *Fusarium graminearum* (8.3 %). New market the highest was *Aspergillus niger* (33.3 %) followed by *Aspergillus flavus* and *Fusarium graminearum* (25.0 %), *Phaecocromonium. Parasiticum* (16.6 %) and least were *Penicillium digitatum* and *Aspergillus oryzae* (8.3 %). From Old market the highest was *Aspergillus oryzae* (33.3 %) followed by *Aspergillus niger* (16.6 %), and the lowest were *Penicillium digitatum, Aspergillus flavu* and *Fusarium graminearum* (8.3 %) respectively.

Table 1 Percentage occurrence of fungi isolated from stored groundnuts

Location	No. sample	No. isolated (%)
Angwan Lambu	12	8(66.6)
High court	12	6(50.0)
Angwan Waje	12	6(50.0)
New market	12	10(83.3)
Old market	12	11(91.6)
Total	60	41(68.3)

Table 2 Cultural and Microscopic characteristics of fungi species isolated from stored groundnuts

Cultural characteristics	**Microscopic characteristics**	**Inference**
Initial growth is white, later becoming black with pale yellow on the reverse side	Ball likeconidiophores and hyphae is septate	*Aspergillus niger*
Slow growing, the color of the colonies were grey green, the conidia were smooth to finely roughened surface and well defined columns	Mycelium gives rise to simple and long conidiophores which branch at about two thirds of the way to the apex, like broom. conidia appear as tiny, spore-like structures	*Penicillium digitatum*
Colonies are granular, flat, with radial grooves, yellow at first but quickly becoming bright to dark yellow-green with age. Conidial heads are typically radiate, later splitting to form loose columns biseriate	The conidiophores is biseriate with philiades radiating from all sides, the globose conidia with varying sizes that are slightly roughened and unbranched conidiophore which is nonseptate, rough, and hyaline	*Aspergillus flavus*
Colonies growing slowly; surface usually orange to deep apricot due to confluent conidial slime; aerial mycelium sometimes floccose and whitish	Conidiophores loosely branched, with short and swollen phialides, maroconidia curved and pointed at the apex septate microconidia absent	*Fusarium graminearum*

Table 3 Percentage occurrence of fungi species in different locations

Fungi	**No. sample**	**Location isolated (%)**					
		Angwan Lambu	**High court**	**Angwan Waje**	**New market**	**Old market**	**Total**
Aspergillus niger	12	4 (33.3)	1(8.33)	2(16.6)	3(25.0)	2(16.6)	12(100)
Phaecocromonium. Parasiticum	12	0(0.0)	0(0.0)	1(8.33)	2(16.6)	0(0.0)	3(25.0)
Penicillium digitatum	12	1(8.33)	2(16.6)	0(0.0)	1(8.33)	1(8.33)	5(41.6)
Aspergillus oryzae	12	0(0.0)	1(8.33)	2(16.6)	1(8.33)	4(33.3)	8(66.6)
Aspergillus flavu	12	2(16.6)	2(16.6)	1(8.33)	3(25.0)	1(8.33)	9 (75.0)
Fusarium graminearum	12	0(0.0)	1(8.33)	1(8.33)	3(25.0)	1(8.33)	6(50.0)

The screening for mycotoxin production by different fungi species isolated from stored groundnut sold in Keffi is as given in Table 4,4. Out of 13 *Aspergillus niger* screened 4 produce mycotoxin were 3 produced Ochratoxin and 1 produced Fumonisins. Nine (9) of *Penicillium digitatum* screened 3 produce mycotoxins 2 produced *Penicillium* Patulin and 1 produced aflatoxin. Out of 9 *Aspergillus oryzae 1 were able to produce* citrinin mycotoxin. From 9 *Aspergillus flavus* screened 3 were observed to produce mycotoxins 2 produced aflatoxin and 1 produced fumonisins. Nine (9) *Phaecocromonium. Parasiticum* isolated were screened for mycotoxin but none were able to produce any mycotoxin. Out of 9 *Aspergillus oryzae screened only 1 produced mycotoxin namely* cyclopiazonic acid while 6 *Fusarium graminearum* screened for myctoxin production 4 were observed to produce mytcotoxins; 1 produce Patulin another1 Aflatoxin and 2 produced Fumonisins respectively.

Table 4 Screening for different mycotoxins produced by fungi species isolated

Fungi isolated	**No. Screened**	**No. positive**	**Mycotoxins produced**				
			Patulin	**Ochratoxin**	**Aflatoxin**	**Fumonisins**	**cyclopiazonic acid**
Aspergillus niger	12	4	0	3	0	1	0
Penicillium digitatum	6	3	2	0	1	0	0
Aspergillus oryzae	9	1	0	0	0	0	1
Aspergillus flavu	9	3	0	1	1	0	1
Phaecocromonium. Parasiticum	3	0	0	0	0	0	0
Fusarium graminearum	6	4	0	0	1	3	0

4. Discussion

Fungi had been known to affect groundnut seeds during the period of storage by so doing these fungi produced their secondary metabolites which are known as mycotoxins which are harmful to human and animal that consumed the infected groundnut seeds [11]. Most times atmosphere where the groundnuts are stored favors the production of these fungi secondary metabolites.

The total different species fungi isolated from stored groundnut was 68.3% higher that work reported by Tobin-West *et al.* [12] in four major markets in Port Harcourt, Nigeria, and Fagbohun and Faleye [13] as they reported 65.1% and 60.0% respectively. The occurrence of fungi was high in Old market than from any other location sampled this may be due to activities of the people storing it or from different farms the groundnut was planted and harvested because fungi is a major organisms that infect groundnut [14].

The fungi species isolated in this study were *Penicillium digitatum, Phaecocromonium. Parasiticum, Aspergillus oryzae, Aspergillus flavus* and *Fusarium graminearum.* These fungi had been identified as the major fungi species that cause groundnut rioting during storage and cause most groundnut disease in the farm [15]. From findings in this study it was observed that *Aspergillus niger, Aspergillus oryzae and Aspergillus flavus* was the highest occurring fungi which was in agreement with the findings of some other author such as [16] who reported the presence of these *Aspergillus* species on riot groundnut after harvest. Also these fungi may have been contaminated these ground right from farm or during the process of the seeds from the shell because the fungi species isolated and predominate in the soli also may be from the storage facility which may have serve as a reservoir of these fungi species as they are known to cause groundnut riot [17, 18].

It was found that these different fungi isolated from stored groundnut sold in Keffi were mycotoxins producers after growing them in groundnut flour. The mycotoxins detected were cyclopiazonic acid, fumonisins, aflatoxin, ochratoxin, and patulin which have been reported to be major mycotoxins ever detected in raw groundnut and other groundnut products as reported by Oranusi et al. [19]. It was observed that 3 Aspergillus flavus isolated produced both aflatoxin, ochratoxin and cyclopiazonic acid and the fumonisins were toxin produce by the species of Fusariu. From the molecular identification it was observed that the fungi species that were able to produced mycotoxins were highly same to those which have been reported to be mycotoxins producers in groundnuts like fusarium graminearum which have been reported to produce fumonisins, Peniciillium digitatum which produce patulin and cyclopiazonic acid.

The high rate of these fungi is a call of wake up base on the effect of mycotoxins to human and animal. These may be the cause of some illness that is related to foodborne infection or disease because it had been proven that a lot of mycotoxins cause different types of cancer, kidney infections [20], because groundnut is one of major source of food to human and animal in our society. There is need to reduce the amount of fungi contamination in groundnut start from field where it is farm to storage facilities in Keffi.

5. Conclusion

From findings of this study the following are the conclusion different fungi species were isolated from different stored groundnut sold in Keffi namely *Peniciillium digitatum, fusarium graminearum, Aspergillus flavus, Aspergillus oryzaeand Aspergillus niger* and screened for mycotoxins were observed to produce different types of mycotoxins that are known to cost different degrees of harm to human and animal when consume agriculture products that contains these different fungi and mycotoxins they produce on it.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest among of the authors

Statement of informed consent

Informed consent was obtained from all individual participants included in this study

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