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Comparative Analysis of Mycotoxigenic Fungi and Mycotoxins Contaminating Soya Bean Seeds and Processed Soya Bean from Nigerian Markets

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Abstract

Concern for food safety has continued to grow worldwide including the issue of mycotoxin contamination of food products from farm to fork. In this regard, soya bean seeds and processed soya bean powder bought from some Nigerian markets were screened for fungal and mycotoxin contamination. Fungal identification was done by both conventional and molecular methods after samples were cultured on potato dextrose agar (PDA), ohio agricultural experimental station agar (OAESA), malt extract agar (MEA) and czapek yeast agar (CYA). Mycotoxin analysis by thin layer chromatography and high performance liquid chromatography was done after extraction and clean-up by multi-mycotoxin extraction procedure and solid phase extraction (SPE) isolute strong ion exchange (SAX) columns. Results from the analysis showed that soya bean seeds had higher incidences of fungal species such as *Alternaria* (52.4%) and *Aspergillus flavus* (42.9%). Mycotoxins detected include aflatoxins, ochratoxin A and fumonisin B with highest concentration of 3.430 µg/g, 0.125 µg/g and 4.286 µg/g respectively, which were below regulatory limits. The study showed that there was co-occurrence of aflatoxins and fumonisin B_1 in both sample types and though these values are low, should not be ignored as a result of health risks associated with exposure to these compounds.

Keywords

Aflatoxins, Ochratoxin A, Fumonisin B_1, Legumes, *Aspergillus*, *Fusarium*

1. Introduction

Rich in nutrients, soya bean is a good source of protein and dietary fiber and has been reported to be the only vegetable with complete protein having the ability to lower LDL (bad cholesterol) levels [1]. Although native to East Asia, this legume is also cultivated in West Africa and Nigeria in particular [2]. Cultivation of soya bean in Nigeria started in the 1900s at a small scale in the northern part of the country, and spread fast to other parts of the country [3]. Planting of this crop is usually in June/July, during the peak of the rainy seasons and harvesting in October/November of the same year [2].

Due to the hot and humid climate of most regions in West-Africa, including Nigeria, soya bean can be exposed to harsh weather conditions such as high temperatures and humidity, which are some of the conditions for fungi contamination and mycotoxin production by fungi on food commodities [4] [5] [6]. There are reports of mycotoxin contamination of many food commodities [7] [8] [9] including seeds and legumes [10] [11] [12], which soya bean belongs to. These mycotoxins are known to have very adverse health effects including carcinogenic, immunosuppressive, mutagenic and cytotoxic effects [4] [13]. Some of the major occurring mycotoxins have been classified as carcinogens by the International agency for Research on cancer with aflatoxins being one of such mycotoxins [14] [15] [16].

In Nigeria, soya bean is consumed in different forms-cooked, roasted or as processed powder. As a result of its high protein content, this food crop is usually processed into soya bean powder and soya bean milk, used as a supplement to foods including weaning foods for babies and children. Processing soya bean seeds into powder includes drying, dehulling, fermenting and milling of the seeds [17]. Mycotoxins which are chemical compounds produced by fungi species, are able to withstand very high temperatures and pressure such that, they are not easily degraded or destroyed by cooking or baking [5] [18]. Despite their ability to withstand heat, it is reported that mycotoxin contamination in food commodities can be reduced by some practices such as dehulling, sorting, and cleaning [19] [20].

Considering the high nutritional content of soya bean and its ability to lower LDL levels in the body, most health organizations including the American Diabetes Association and the American Heart Association have recommended legumes as a major food group that can contribute to prevent diseases and promote good health [1] [21]. Considering these recommendations, the quality of legumes with regards to mycotoxin contamination has to be verified. A wide variety of food crops and commodities have been investigated for mycotoxin and fungi contamination over the years but little or nothing has been done in regards to soya bean in Nigeria despite its dietary importance and popularity. This study aims to investigate the occurrence of major mycotoxins—aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FUMs), deoxynivalenol (DON) and zearalenone (ZEA) in soya bean seeds and processed soya bean powder; as well as determine the occurrence of filamentous fungi in the food commodities, in order to compare the degree of contamination in seeds and in processed products.

2. Materials and Methods

2.1. Solvents and Standards

All solvents used in this study were of analytical grade and procured from Sigma-Aldrich Co., South Africa. Mycotoxin standards-AFs, OTA, DON, ZEA, FBs and NIV were procured from Sigma-Aldrich Co., South Africa.

2.2. Sampling

Forty-one (41) samples comprising of twenty-one (21) soya bean seed samples and twenty (20) processed soya bean samples were purchased from different markets in Lagos, Nigeria, put in sterile plastic bags, sealed and transported to the laboratory in the University of Johannesburg, Doornfontein campus. Soya bean seeds were milled using a laboratory miller (IKA M20, Merck, Darmstadt, Germany) and stored at −20˚C prior to further analysis.

2.3. Mycological Screening of Samples

Samples were screened in triplicates for fungal contamination using potato dextrose agar (PDA), ohio agricultural experimental station agar (OAESA), czapek yeast extract agar (CYA) and malt extract agar (MEA). Following the methods applied by Egbuta *et al.* [22] and Phoku *et al.* [23], six (6) serial dilutions of each sample were first cultured on PDA and OEASA to determine colony formation units. Fungal colonies were further sub-cultured on PDA, MEA and CYA. Fungi species were identified using conventional and molecular methods. Percentage incidence of fungal isolates in both types of samples was calculated thus:

$$\left(\text{Number of positive samples} \div \text{total number of samples}\right) \times 100$$

For molecular identification of fungi species, DNA was extracted from representative samples of isolates and amplified using universal primers FF2 (GTT AAA AAG CTC GTA GTT GAA C) and FR1 (CTC TAA TCT GTC AAT CCT TAT T) [24]. To amplify the ITS region on the DNA extracted, a polymerase chain reaction (PCR) was done using a thermocycler following set PCR conditions. Amplicons (PCR products) were sent to Inqaba biotec laboratory, where they were cleaned and sequenced using a SpectruMedix model SCE 2410 automated DNA sequencer (SpectruMedix, State College, PA). Sequences obtained were cleaned using chromaslite and Bio-Edit software and blasted on NCBI.

2.4. Mycotoxin Extraction

Following the methods of Njobeh *et al.* [25] and Egbuta *et al.* [26], extraction and clean-up of mycotoxins (AFs, OTA, ZEA, DON and NIV) from all food samples was done. Two fractions of mycotoxin extracts–Neutral and Acid fractions were extracted from each sample and stored at −8˚C for further analysis. Solid phase extraction (SPE) isolute strong ion exchange (SAX) columns (International Sorbent Technology, UK) was used to extract, and clean-up fumonisins from food samples following the method

of Shepherd *et al.* [27] with some modifications. Fumonisin extracts were dried and stored at 4˚C for further analysis.

2.5. Thin Layer Chromatography and High Performance Liquid Chromatography

To determine the occurrence of mycotoxins in the extracts, TLC and HPLC analyses were implemented. For TLC, extracts were spotted on aluminium backed silica gel TLC plates alongside standards and run in specific mobile phases (DEI—Dichloromethane/Ethyl Acetate/Propan-2-ol (90:5:5 v/v), TEF—Toluene/Ethyl Acetate/Formic Acid (6:3:1 v/v), BWA-Butanol/Water/Acetic acid (12:5:3 v/v/v), DA—Dichloromethane/Acetone (90:10 v/v)). Retention factor (RF) values for AFs and OTA extracts were determined after viewing plates using Ultra Violet light in a fluorescence analysis cabinet CM-10A model (Spectronics Corporation, NewYork, USA). Other mycotoxins (DON, ZEA, and FB1) RF values were determined after plates were sprayed with derivatising agents.

High performance liquid chromatography (HPLC) analysis involved use of a hplc instrument—HPLC Spectra Physics SCM400 SYSTEM (Waters, Milford, MA, USA), Shimadzu Corporation (Kyoto, Japan) LC-20AB liquid chromatograph equipped with CBM-20A communication bus module, LC-20AB degasser, CTO-20A column oven, Nova-Pak 4 mm C18 reversed phase analytical column (250 × 4.6 mm, 5 µm), SIL-20A auto sampler, RF-10AxL fluorescence detector, RID-10A refractive index detector and SPD-M20A photodiode array detector linked to LC solutions version 1.22 Software Release. Different mobile phases were used for each mycotoxin using different detection methods following the method used by Makun *et al.* [28] in their study. Flow rate of mobile phase was at 1 ml per minute and volume of sample injected per analysis was 40 µl.

2.6. Statistical Analysis

Using Microsoft excel 10, results were analyzed to determine percentage incidence, mean values, standard deviation and concentration ranges for fungal contamination and mycotoxin contamination in samples.

3. Results

3.1. Fungal Analysis

Screening of soya bean samples represented in **Table 1** shows the occurrence of different species of filamentous fungi with high incidence of *Alternaria* species (52.4%), which is followed by *Aspergillus flavus* at a percentage incidence of 42.9%. In contrast, processed soya bean samples were contaminated with less fungi species with the highest incidence of *Fusarium subglutinans* (20%), followed by *Aspergillus parasiticus* (15%) and *Fusarium verticillioides* (15%). Total colony formation units recorded in **Table 1** showed that *Alternaria* had highest value in both sample types.

3.2. Thin Layer Chromatography Detection of Mycotoxins in Soya Bean Samples

Mycotoxin analysis by thin layer chromatography detected some major mycotoxins in

Table 1. Incidence of filamentous fungi species in soya bean seeds and processed soya bean samples.

Filamentous fungi species	Soya Bean seeds (n = 21)		Processed soya bean (n = 20)	
	Percentage incidence	Total colony formation unit	Percentage incidence	Total colony formation unit
Acremonium strictum	9.5	3.3×10^6	0	0
Alternaria	52.4	22.4×10^6	0	0
A. alliaceus	4.8	0.8×10^6	0	0
A. caespitosus	4.8	0.8×10^6	0	0
A. candidus	19.1	7.7×10^6	0	0
A. carbonarius	9.5	4.1×10^6	0	0
A. flavus	42.9	18.2×10^6	5.0	1.7×10^6
A. fumigatus	14.2	6.3×10^6	10.0	4.1×10^6
A. niger	14.2	5.9×10^6	0	0
A. niveus	4.8	0.5×10^6	0	0
A. oryzae	28.6	9.1×10^6	0	0
A. parasiticus	33.3	11.7×10^6	15.0	6.8×10^6
A. terreus	4.8	0.7×10^6	0	0
Cladosporium	38.1	15.7×10^6	5.0	1.1×10^6
C. pallescens	9.5	4.8×10^6	0	0
E. regulosa	28.6	8.8×10^6	0	0
E. amstelodami	9.5	3.9×10^6	0	0
F. proliferatum	19.1	8.0×10^6	0	0
F. subglutinans	14.2	6.1×10^6	20.0	7.2×10^6
F. verticilliodes	23.8	8.6×10^6	15.0	6.3×10^6
P. fulvus	4.8	0.7×10^6	0	0
P. aethiopicum	4.8	0.5×10^6	0	0
P. aurantiogreosum	14.2	5.8×10^6	0	0
P. glabrum	19.1	8.1×10^6	0	0
P. olsonii	4.8	0.7×10^6	0	0
P. polonicum	9.5	4.2×10^6	0	0
Rhizopus	4.8	0.6×10^6	0	0

n—number of samples.

extracts from soya bean seeds and processed soya bean samples. Figure 1 shows aluminium backed silica gel TLC plates of samples positive for aflatoxins and fumonisin B_1. Incidence of mycotoxins detected by TLC represented in Table 2, showed highest occurrence of aflatoxins in comparison with other mycotoxins detected.

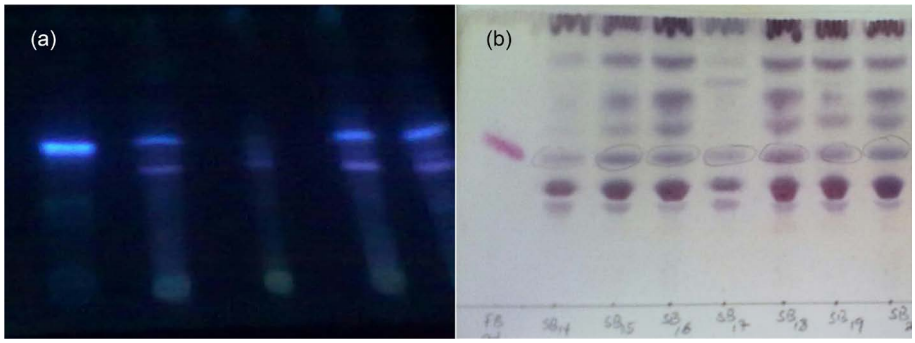

Figure 1. Aluminium backed-silica gel thin layer chromatography plates showing aflatoxins (a) and fumonisin B_1 (b) in samples analysed for mycotoxin contamination.

Table 2. Detection of mycotoxins by thin layer chromatography.

Mycotoxins	% Incidence	
	Soya bean seeds	Processed soya bean powder
Aflatoxins	71.4	25
Fumonisin B_1	52.4	70
Ochratoxin A	0	0

3.3. High Performance Liquid Chromatography Detection of Mycotoxins in Soya Bean Samples

Quantification of mycotoxins in samples by HPLC showed the occurrence of mycotoxins at concentrations which were beyond the limit of detection by TLC. Different concentration ranges for aflatoxins B_1, B_2, G_1 and G_2; ochratoxin A and fumonisin B_1 indicated in Table 3 shows that soya bean seed samples were contaminated with as high as 2.27 µg/g of FB_1, 3.43 µg/g of total aflatoxins and 0.05 µg/g of OTA. Processed soya bean samples were less contaminated with aflatoxins in comparison with soya bean samples, whereas, they were contaminated with higher levels of FB_1 up to 4.28 µg/g. It was also observed that all samples of both soya bean seeds and powder soya bean, were contaminated with FB_1 with a percentage incidence of 100 for both sample types.

A further analysis of general mean for the three mycotoxins detected by hplc in both sample types (Figure 2), shows that there was a prevalence of aflatoxins in soya bean seed samples, whereas, with fumonisin B_1, processed soya bean samples were more contaminated. For ochratoxin A, there was little difference in levels of contamination across both sample types.

It was also observed that despite the occurrence of zearalenone and deoxynivalenol producing fungi species, these mycotoxins were not detected in samples.

4. Discussion

Mycological and mycotoxin analyses of soya bean seeds and processed soya bean indicated that different filamentous fungi and some major mycotoxins were present in the samples. One of the reasons for this result, is the chemical composition of soya bean

Table 3. Incidence and concentration range of mycotoxins detected by high performance liquid chromatography.

Mycotoxins	Soya bean seeds		Processed soya bean powder	
	percentage incidence	concentration range (µg/g)	percentage incidence	concentration range (µg/g)
Aflatoxins	100	0.111 - 3.430	45	0.000 - 0.813
Fumonisin B_1	100	0.033 - 2.270	100	0.182 - 4.286
Ochratoxin A	23.8	0.000 - 0.051	40	0.000 - 0.125

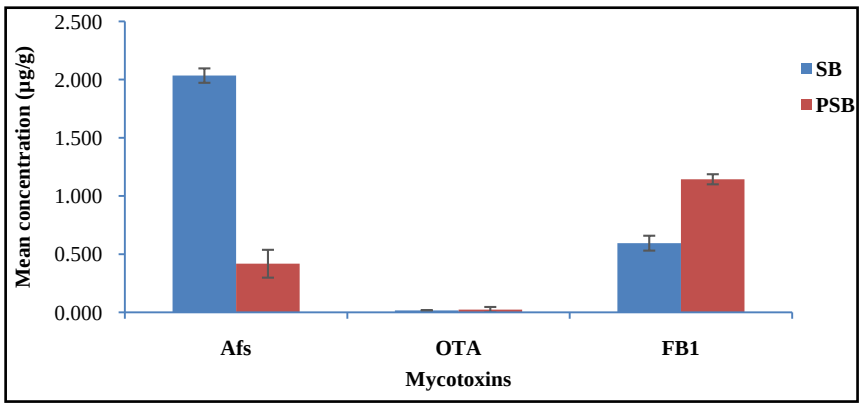

Figure 2. Comparison of mycotoxin contamination between soya bean seed (SB) and processed soya bean samples.

which makes it susceptible to microbial infection [11]. Some of the fungal species reported in this study have also been isolated from soya bean by Embaby *et al.* [29] and Bhattacharya and Raha [30]. The occurrence of more fungal species in soya bean seed samples compared with fewer species, and less incidence in processed soya bean samples, can be attributed to practices involved with processing soya bean seeds. These practices which include de-hulling, frying and milling could have contributed to reducing fungal occurrence [19] [20]. The occurrence of different filamentous fungi in seeds could be attributed to, the predominantly hot and humid climatic weather of Nigeria [5] [6] [31]. The fact that soya bean is cultivated during the rainy season which promote fungal growth could be another contributory factor. Also, the occurrence of high incidences of *Aspergillus* and *Fusarium* species could be attributed to poor storage practices, since most species of the genera are mainly storage fungi [7] [32].

However, the absence of these fungi is not a guarantee for mycotoxin free samples as was observed in the study. Although, processed soya bean samples had lower fungal incidences in most cases, mycotoxins were found contaminating processed soya bean and soya bean samples as well. Some mycotoxins such as the aflatoxins have the ability to survive degradation at very high temperatures up to 180˚C [33]. Therefore, a possible reason for mycotoxin contamination of processed samples despite the low incidence of fungal occurrence. Occurrence of ochratoxin A in both sample types were low and correlates with the low incidence of *Aspergillus niger* which is a producer of the toxin

alongside *A. ochraceus* and *P. verrucosum* [34] [35]. Although *A. niger* is mentioned as a producer of ochratoxin A, it can be classified as a low producer of the mycotoxin as compared to the other producers [34], and, this could be a possible reason for the low occurrence in samples. Mycotoxins detected in this study have also been reported to contaminate soya bean in other parts of the world including Asia and Europe [11].

All mycotoxins detected in samples screened were below both EU and Nigerian set regulatory limits of 4.0 μg/kg and 15 μg/kg respectively for aflatoxins, 5 μg/kg for OTA and 4 mg/kg for fumonisins (FDA mycotoxin regulatory guidance) [36]. This result is an indication that samples screened are considerably safe for consumption but must not be overlooked because the nutritive value of soya bean can be reduced by the presence of these contaminants [11] [29]. Also a continuous exposure to these mycotoxins even at low doses is considered a health risk to consumers [5] [13].

5. Conclusion

The study, which was done to evaluate degree of fungal and mycotoxin contamination of soya bean seeds and processed soya bean showed that, although, soya bean seeds were highly contaminated with different fungal species, both sample types were contaminated with mycotoxins. Soya bean seeds contaminated with different filamentous fungi species give an indication of poor farming and storage practices. Despite that these mycotoxins were below regulatory limits, there should be continuous efforts by all parties involved to reduce mycotoxin contamination of soya bean as well as other food products.

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Conflict of Interest

The authors hereby declare that there are no conflicts of interest.

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