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Isolation of *Aspergillus* section *Flavi* and determination of aflatoxins in Bambara groundnut sold in Cotonou main markets (Benin)

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Abstract

Legumes such as cowpea (*Vigna ungiculata*) and Bambara groundnut (*Vigna subterranean*) are the most sought in Benin and in the West Africa sub-region. These legumes are an important protein source because of their high content of essentials amino acids. But, like many dried agricultural and food products, they are subject to contamination by molds and mycotoxins during storage.

In order to assess the contamination of Bambara groundnuts, by the most dangerous and widespread mycotoxins in Africa (aflatoxins), especially in Benin, this study was conducted in Cotonou (Benin) main markets. Thirty-five (35) samples of Bambara groundnuts were collected from six (6) different markets in Cotonou. The fungi are isolated and identified morphologically on different media after direct culture. The species of *Aspergillus* section *Flavi* aflatoxigenic potential are evaluated and the contamination of samples by aflatoxins was determined by Thin Layer Chromatography (TLC).

The results of this study showed that the average water content of Bambara groundnuts samples ranged from 4.85 to 9.35%. The main species of mold isolated are *Mucor spp.*, *Rhizopus spp.*, *Alternaria sp.* and *Aspergillus spp*. Forty-six (46) strains of *Aspergillus* section *Flavi* were isolated on Potatoes Dextrose Agar (PDA). All of them have been identified as aflatoxigenic strains and were divided into different groups according to their morphological appearance and secondary metabolites. Twenty- two samples (62.85%) of Bambara groundnuts were contaminated by aflatoxins.

This study showed that Bambara groundnuts which sold in the main markets of Cotonou constitute a health risk for populations.

Keywords: Bambara groundnut, *Vigna subterranean*, *Aspergillus flavus*, *aflatoxins*.

1. INTRODUCTION

To have a balanced diet, humans need nourishing food that contains essential nutrients. The nutrients needed by humans are proteins that span structure (growth, tissue repairs), while fats and carbohydrates generally provide the energy needs (Lagnika *et al.*, 2017; Mathieu-Daude *et al.*, 2001). Foods rich in these elements are cereals and legumes such as cowpea and Bambara groundnut that are consumed in Africa.

Although the environment has all elements necessary for organism survival, various health problems jeopardize people's health, especially in developing countries such as Benin (Zinzindohoue, 2012). Indeed foodstuffs (cereals, tubers, legumes, vegetables, etc.) (Dudler, 2005) are subject to various contaminations among which are those caused by mycotoxins. These are produced by toxigenic molds of the genus *Aspergillus (A)*, *Penicillium (P)* and *Fusarium (F)*(Zinedine and Idrissi, 2007). Aflatoxin B1 is the most dangerous because of its toxicity. Aflatoxins pose serious health hazards to humans and domestic animals because they frequently contaminate agricultural commodities. In 1993, Bayman *et al* showed that aflatoxin produced by *Aspergillus flavus* (*A. flavus*), *Aspergillus parasiticus* (*A. parasiticus*) and *Aspergillus nomius* (*A. nomius*) are highly teratogenic, mutagenic and carcinogenic in animals and in human. In 2007, Njapau *et al.* reported the death of about one hundred and twenty-five people after consuming contaminated corn with aflatoxins in Kenya. Aflatoxins produced by *A. flavus* and *A. parasiticus* contaminate foods such as rice, corn, peanuts, spices etc (Cho et *al., 2008* ; Gnonlonfin *et al.* 2012; Jalili and Jinap, 2012; Serra *et al*., 2002).

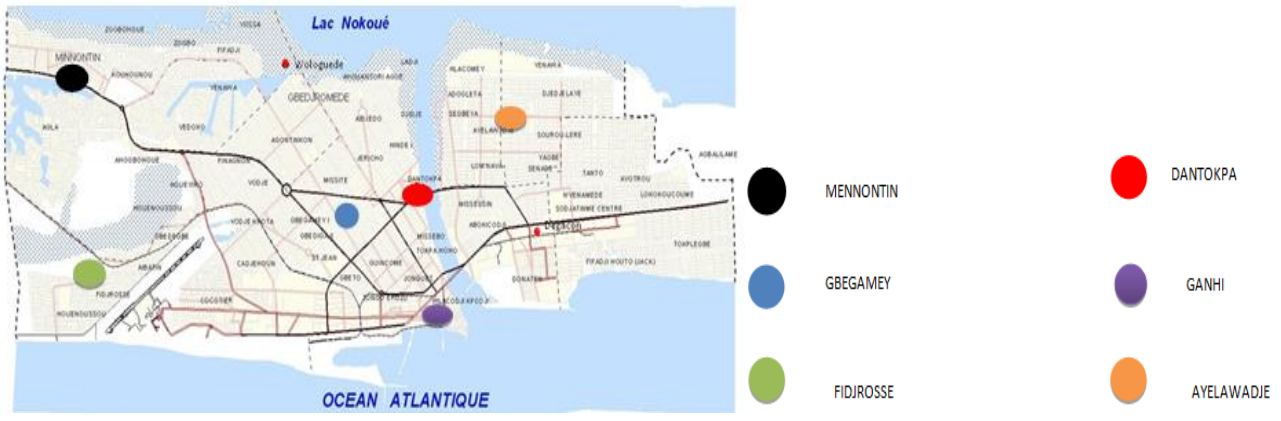
Recognition of the need to control aflatoxin contamination of food and feed grains has elicited various approaches from researchers to eliminate this toxin from maize and other susceptible crops. The approach to enhance host resistance through conventional means has gained renewed attention following the discovery of natural resistance to *A. flavus* infection and aflatoxin production in many crops like maize (Brown *et al*., 2001) cassava (Adjovi *et al., 2015*; Adjovi *et al., i2014;* Gnonlonfin *et al.*, 2008) and cowpea (Houssou *et al.*, 2009). In this last product, Houssou *et al.* have proposed the presence of protein which confers this property to cowpea. This study focused on the research of aflatoxins and *Aspergillus* section *Flavi* on Bambara groundnut (*Vigna subterranean*), a legume of the same genus with cowpea. The aim is to investigate if Bambara groundnuts stored and sold in Benin are contaminated by *Aspergillus* section *Flavi* aflatoxinogenic's strains and aflatoxins or have a resistance property as cowpea.

2. MATERIALS AND METHODS

Sampling

Thirty-five (35) samples of Bambara groundnut (*V. subterranea*) were collected from six (6) main markets in Cotonou (Gbegamey, Kindonou, Dantokpa, Fijrossè, Akpakpa, and Ganhi) (figure 1). Sampling was done from different traders in order to have heterogeneous samples. Samples were transported to the laboratory and stored at 4°C for different analysis.

Figure 1: Sampling



Water content

Water content is determined by AOAC (Association of Official Analytical Chemist) method 934.01 AOAC. 5 g of Bambara groundnut (*V. subterranea*) are ground in a blender and dehydrated at 70 ° C. After 20 hours, the mass of Bambara groundnut (*V. subterranea*) is measured. Then, moisture is determined in the percentage of wet mass.

Mycological analysis

The direct plating method described by Ravi Kiran et *al*. (2005) was used with some modifications. Ten seeds of each sample of Bambara nuts are surface sterilized with 10% bleach (potassium hypochlorite solution) for 1 min and rinsed twice with sterile distilled water. Three seeds of Bambara nuts were placed on Dichloran Chloramphenicol 18% Agar (DG 18) (Oxoid Ltd, Hampshire, UK) in Petri dishes and incubated at 25 ºC for 7 days. Triplicate of each sample analysis are done. After this, each Fungi colony are transplanted on the center of Potatoes Dextrose Agar (PDA) and incubated at 30°C for 7 days.

***Aspergillus* section *Flavi* identification**

The total number of fungal isolated from each product was recorded. Identification of *Aspergillus* section *Flavi* was firstly based on morphology. Macroscopic and microscopic features of each colony were used. A loop full of pure isolate cultured on potatoes dextrose agar (PDA) was sub-cultured on malt extract agar (MEA), Yeast Extract Agar (YES) and Aspergillus Flavus Parasiticus Agar (AFPA) (Oxoid Ltd., Hampshire, UK) for identification. The MEA and YES plates were incubated at 25°C for 7 days. The identification of fungi was based on gross morphology and microscopic features such as spores and fruiting structures using Pitt and Hocking (2009) descriptions.

Detection of aflatoxigenic strains

- **Extraction**

Aflatoxins were extracted from 7 days cultures of Aspergillus Section Flavi strains on malt extract agar medium. Each culture are extracted with methanol/water (80/20, V / V). The extracts are dry at 60°C. The dry extract obtained is taken up in methanol 400µl.

- **Aflatoxins detection**

Aflatoxins spot were detected by Thin Layer Chromatography.10 µL of extracts and standard of Aflatoxins (Sigma Aldrich) were deposited on the fluorescent chromatographic plate (1.05553. DC-Alufolien Kieselgel 60). Migration solvent is ether / methanol / water (96: 3: 1, v / v / v). The plates placed in the solvent system were dried and observed at UV 365nm.

Detection of Aflatoxins in Bambara groundnuts

Aflatoxins were extracted and analyzed according to the method of Bankole and Mabekoje (2004). 5 g of Bambara were ground, mixed with twenty-five (25) ml of methanol / water (85: 15v / v) and stirred for three (3) minutes. The mixture was filtered through Whatman filter paper N° 1. Twenty (20) ml of the filtrate were mixed with twenty (20) ml of sodium chloride (10% NaCl) and shaken well. The mixture was transferred to a separatory funnel and with 10 ml of hexane. The hexane layer was discarded and the aqueous phase was recovered. 25 ml of chloroform was added to the aqueous phase. The Chloroform phase was rejected. The extract obtained was filtered through Whatman N°1 containing 2.5 g of anhydrous sodium thiosulfate. The filtrate obtained was evaporated with a rotary evaporator (RE 300). The eluate was dried down at 60°C and then reconstituted in 50 µL of methanol and stored at 4°C until analysis. Analysis of extracts obtained from the Bambara groundnut (*V. subterranean*) is done as previously described by Thin layer chromatography.

Statistical analysis

Data were analyzed with ANOVA for multiple comparisons among means. Correlation and regression coefficients were calculated using SPSS for Windows version 18.0 (SPSS, Chicago, IL).

3. RESULTS

Water content

Average water content is recorded in the table below. These averages vary from 4.85% to 9.35% (Table 1).

Table 1: Average of sample water content

Sites	Akpakpa	Gbégamey	Dantokpa	Ganhi	Fidjrossè	Kindonou
Moy %	5,85	5,85	6,40	8,45	9,35	4,85
Ecart types	0,003	0,016	00	0,002	0,002	0,004

Mycological analysis

Bambara groundnut or *V. subterranea* are more contaminated by species of *Aspergillus spp*, *Rhyzopus spp*, *Mucor spp*, and *Alternaria sp*. The prevalence of each genus are respectively:

In Aspergillus species, two main dominant species contaminate Bambara samples, *A. niger* (incidence 67.38) followed by *A. flavus* (27.22). The incidence of this last species varies between 5.28% in Kindonou's samples and 31.87% in Fidjrosse's samples.

Samples from Fidjrosse's market were the most contaminated with *A. flavus*. The ANOVA Turkey made an error rate of 5% has allowed us to find a significant difference between the samples from Fidjrosse and those from Dantokpa, Ganhi, Gbegamey, Akpakpa, and Kindonou. Samples from Kindonou seem the least contaminated.

Forty-six (46) strains isolated have been morphologically identified as strains of *Aspergillus* section *Flavi* and two as *Aspergillus* section *Tamarii*. The repartition of *Aspergillus* section *Flavi* strains by sight is as follows: Dantokpa (12), Akpakpa (10), Ganhi (8), Fidjrosse (7), Gbegamey (6) and Kindonou (5).

Detection of Aspergillus section Flavi isolated nature

The analysis of media extract revealed that all of the 46 strains isolated are aflatoxigenic. The aflatoxinogenics strains were classified according to the different types of aflatoxin they produce in Table 2.

The culture on AFPA media show that all strains are aspergillic acid producers (Table 2).

These results show that the Bambara groundnut (*V. subterranea*) sold in the city of Cotonou is contaminated with strains of aflatoxinogenic *Flavi* section. Samples from Akpakpa markets Dantokpa and Fidjrosse are more contaminated than from Ganhi, Gbegamey and Kindonou.

Aflatoxins detection in Bambara groundnuts samples

The results of the qualitative analysis of collected samples carried out show that 22 of the 35 samples are infected by aflatoxins. The results are shown in Table 3 following.

Table 2: Type patterns of *Aspergillus* section *Flavi* strains based on aflatoxins, aspergillic acid and sclerotia production

Types	**Sclerotia**	**Aflatoxins**	**Aspergillic acid**	**Related species**	**Number of strains**
I	None	AFB1	+	*A.flavus*	13
II	None	AFB1, AFB2, AFG1, AFG2	+	*A.parasiticus*, *A.bombicys*, *A.novoparasiticus*, *A.arachidichola*, *A.pseudocaelatus*	4
III	Large and abundant	AFB1	+	*A.togoensis*	5
IV	Large and abundant	AFB1, AFB2	+	*A.flavus*, *A.pseudonomius*, *A.pseudotamarii*	5
V	Large and little	AFB1, AFB2, AFG1, AFG2	+	*A.transmontanensis*, *A.sergii*, *A.toxicarius*	8
VI	Small and little	AFB1	+	*flavus clade*	1
VII	Small and little	AFB1, AFB2, AFG1, AFG2	+	*A.minisclerotigenes*, *A.parvisclerotigenus*	10

(Ito et al., 2001; White and Hill, 1943; Saito and Tsuruta, 1993;(Frisvad, Skouboe and Samson, 2005) (M. B. Pildain *et al.*, 2008; Rank *et al.*, 2011; Varga, Frisvad and R. A. Samson, 2011; Soares *et al.*, 2012; Adjovi *et al.*, 2014)

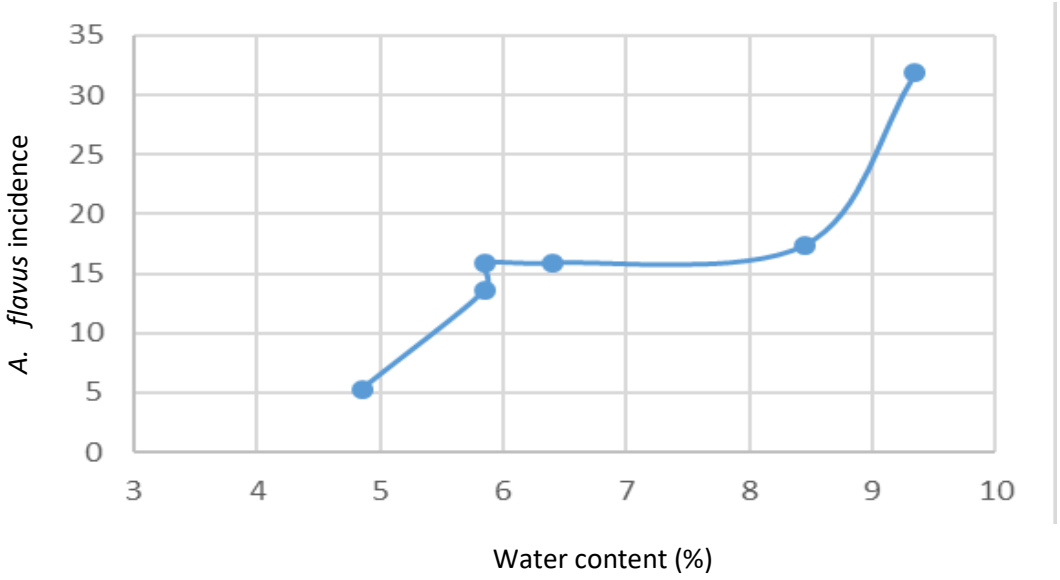
Table 3: Aflatoxins contaminant in Bambara groundnuts (*V. subterranea*) in Cotonou sample

Sites	Samples	AFB1	AFB2	AFG1	AFG2
	E_1A	-	-	-	-
	E_2A	-	-	-	-
Akpakpa	E_3A	-	-	-	-
	E_4A	+	+	+	-
	E_5A	+	+	+	-
	E_1F	+	-	+	-
	E_2F	+	+	+	-
Fidjrossè	E_3F	+	-	+	-
	E_4F	+	-	+	-
	E_5F	+	+	+	-
	E_6F	+	-	-	-
	E_1G	+	-	-	-
	E_2G	-	-	-	-
Gbégamey	E_3G	+	+	-	-
	E_4G	-	-	-	-
	E_5G	+	-	-	-
	E_6G	-	-	-	-
	E_1K	+	+	+	-
	E_2K	+	-	-	+
Kindonou	E_3K	+	-	-	-

4. DISCUSSION

Usually, foods are contaminated by different microorganisms like molds (Marzia et *al*., 2014). This contamination is often favored by the high water activity of the substrate, but also by other factors such as the nature of the substrate and temperature (Yu and Ehrlich, 2011). However, it has been proven that foods with low water content are also contaminated by mold (Schatzki et *al*., 2002). These findings demonstrate that *A. flavus* infection is not only due to water activity but also of inappropriate post-harvest handling methods used by farmers and other value chain actors. This is the case of the seeds of Bambara groundnut (*V. subterranea*) that have low water activity (4.85% to 9.35%) but are nevertheless highly contaminated by fungi. Similar results were found by Schatzki *et al*. (2002) and Houssou *et al*. (2009) who worked respectively on cowpea and groundnut (legume) whose water content ranged respectively from 6, 27-8% and 8, 8-12.3% and have high fungi contamination. Figure 2 shows the correlation between Bambara groundnut water content and *Aspergillus* section *Flavi* contamination.

Figure 2: Correlation between Bambara groundnut contamination by *A. flavus* strains and the water content



In this study, Bambara groundnut collected in Cotonou's main market is mainly contaminated by genus *Mucor*, *Rhizopus*, *Aternaria* and *Aspergillus*. Usually, Bambara groundnut (*V. subterranea*) is harvested, sun-dried and sold without treatment, which can promote the contamination by soil molds and storage conditions contribute to storage's fungi development. Hot and humid climates, prevalent in the tropics promote fungi that infect the crop in the field and/or immediately after harvest during storage (IARC, 2002). Also, Houssou *et al.* (2009) reported that among the factors contributing to contamination by molds of the genus *Aspergillus*, storage plays an important role. This observation is confirmed by(Seetha *et al.*, 2018) who have noted the contamination of fresh Bambara groundnut by fungi like *Aspergillus flavus* which increase during storage. Virtually all of our samples are contaminated by molds of the genus *Aspergillus* with an average of CFU up to 94.68% and 50 strains isolated. Among this genus, the species *A. niger* had the most prevalence (67.38%) followed by the section *Flavi* (prevalence of 27.22%) with 46 strains identified and the last *Aspergillus* section *Tamarii* with 2 strains. The prevalence of *Aspergillus* section *Flavi* in this work is less than in a study in Durban which shows

the high presence of *Aspergillus* section *Flavi* in 64.7% of the Bambara groundnut sample (Olagunju *et al.*, 2018).

The identification of species and aflatoxigenic nature of *Aspergillus* section *Flavi* isolated on Bambara groundnuts in Benin, reveal that most of them produce sclerotia and all of the 46 isolates, produce aflatoxins (Table 2). Strains have been regrouped into seven types based on aflatoxins type produced, sclerotia size and aspergillic acid production. It's known that all of *Aspergillus* of section *Flavi* is not *Asperillus flavus* var. *flavus* which is characterized by the production of aflatoxins B1 only (Varga *et al.*, 2011). Many authors have shown evidence that *A. flavus sensu lato* may consist of several species (Geiser *et al.*, 1998); (B. Pildain *et al.*, 2008); (Soares *et al.*, 2012)). Diversity in the population of *Aspergillus* section *Flavi* exists with respect to their size of sclerotia (Kiewnick *et al.*, 2005). S-strain (those producing small sclerotia) produce high amounts of aflatoxins and L-strains with large sclerotia (Cotty and Cardwell, 1999; Kiewnick *et al.*, 2005). Chemotaxonomy permits also identification of *Aspergillus* section *Flavi* species. The group in section Flavi is composed of (i) an *A. flavus* clade containing *A. flavus, A. parvisclerotigenus and A. minisclerotigenes,* (ii) an *A. parasiticus* clade containing *A. parasiticus, A. arachidicola, A. transmontanensis* and *A. sergii* and (iii) *A. mottae* as a sibling to the clade containing both *A. parasiticus* and *A. flavus* (Soares *et al.*, 2012). In this work, types called type I, III, IV, VI and VII can be identified as *Aspergillus flavus* clade *flavus,* with type III as *A. togoensis* causes of the similitudes like described by (Rank *et al.*, 2011) who produce AFB1 only and large sclerotia; type II and V can be related to *A. parasiticus* clade.

The presence of aflatoxins have been revealed in Bambara groundnuts produced and sold in Cotonou mains markets. In this work, 22 of the 35 samples collected (62.86%) were infected by aflatoxins. Maringe *et al.* (2017) have found 3 out of 89 (3.4%) Bambara nut samples contaminated by aflatoxins in South Africa which is very small compared to our results. Another study of (Seetha *et al.*, 2018)shows the contamination of fresh and stored Bambara groundnut by aflatoxins in central Tanzania.

This result confirms and shows that Bambara groundnut (*V. subterranean*), does not contain aflatoxins inhibitory substances and appears to be a real danger in Benin such as peanuts, corn, and cereal as favorable substrates for aflatoxins production.

CONCLUSION

At the end of our study on the evaluation of the contamination of the Bambara groundnut (*Vigna subterranae*) in Benin, it appears that the Bambara groundnut (*Vigna subterranae*) produced and sold in Benin is subject to contamination by molds of the genus *Mucor*, *Rhizopus*, *Alternaria*, and *Aspergillus*. Many strains of *Aspergillus* section *Flavi* (46) of different clade isolated have been identified as aflatoxinogenic. 62.86% of the collected samples were found contaminated with different types of aflatoxins.

This study is another work to reveal the importance of the health hazard posed by the consumption of food contaminated by mycotoxins in Benin.

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