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Isolation of *Aspergillus flavus* strains from Bambara groundnut (*Vigna subterranea* (L.) Verdcourt) seeds and screening for the production of aflatoxin B1 and B2

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ABSTRACT

In Burkina Faso, Bambara groundnut is the second most important food legume after cowpea, especially in rural areas. However, this crop, due to certain conditions of production, storage or preservation is susceptible to be contaminated by potential mycotoxin producing fungi. The aim of this study was to evaluate the aflatoxinogenic capacity of *Aspergillus flavus* isolates isolated from Bambara groundnut seeds produced in Burkina Faso. Thus, 198 isolates of fungi belonging to section Flavi were isolated from 99 samples of Bambara groundnut seeds collected in the three agro-ecological zones of Burkina Faso. From the 198 isolates, 28 were identified as *Aspergillus flavus*. Then, we investigated their potential for aflatoxin B_1 (AFB_1) and B_2 (AFB_2) production by growing them on rice at 25°C for 7 days. AFB_1 and AFB_2 were extracted and quantified by liquid chromatography–mass spectrometry (LC-MS/MS). All of the strains (28) produced AFB_1 at concentrations ranging from 46.987 µg kg^{-1} to 1080.320 µg kg^{-1}. However, AFB_2 was produced by 26 strains with concentrations ranging from 47.200 µg kg^{-1} to 1760.240 µg kg^{-1}. Globally, most of the tested strains produced higher levels of AFB_1 than AFB_2. The results obtained point out the risk associated with post-harvest fungi and the need for the development of storage technics to control them, for food security.
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Keywords: *Aspergillus flavus*, Aflatoxin, liquid chromatography-mass spectrometry, Bambara groundnut, Burkina Faso.

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INTRODUCTION

Bambara groundnut (*Vigna subterranea* L. Verdc.) is the third most important food legume in Africa after groundnut and cowpea, both in terms of consumption and cultivation (Olukolu et al., 2012). In Burkina Faso, it is the second most important food legume after cowpeas, consumed by populations in rural and urban areas (Ouoba et al., 2016). Unfortunately, preventing post-harvest losses of this legume is a major challenge for farmers (Ouoba et al., 2016). Indeed, the storage conditions have negative impacts on their quality and quantity due to the actions of insects and fungi that can occur during storage, shipment, and marketing in shops (Olagunju et al., 2018; Assoumane Issa et al., 2020). Specifically, fungal contamination of crops can cause considerable economic losses through the reduction of their organoleptic and nutritional qualities (Olagunju et al., 2018). *Aspergillus* is one of the three most important fungal genera involved in food spoilage and the production of mycotoxins, the others being *Fusarium* and *Penicillium* (Marin et al., 2013). Mycotoxin contamination by toxigenic *Aspergillus* commonly occurs because of high moisture content during storage and the presence of insects and mites. Contamination with *Aspergillus* and subsequent production of aflatoxins during storage is considered one of the most serious health problems throughout the world (Gong et al., 2019). Various mycotoxins have been identified in food contaminated by *Aspergillus* species, the most important being aflatoxins and ochratoxin A (Daou et al., 2021). Aflatoxins B_1, B_2, G_1, and G_2 are the most toxic and carcinogenic naturally occurring mycotoxins (Benkerroum, 2020). Aflatoxin B_1 (AFB_1), which is the most toxic form, has been classified by the International Agency for Research on Cancer (IARC) as a group 1 human carcinogen (IARC, 2012). AFB_1 and Aflatoxin B_2 (AFB_2) are produced by several members of *Aspergillus* section Flavi during the infection of crops (Benkerroum, 2020). *Aspergillus flavus* is often the most frequently encountered aflatoxin-producing species (Ouattara-Sourabie et al., 2011).

To contribute to a better knowledge of their action, samples of Bambara groundnut seeds were collected in the three agroclimatic zones of Burkina Faso to isolate strains of *Aspergillus flavus*. Then, tests were conducted to evaluate the mycotoxigenic potential of these strains.

MATERIALS AND METHODS

Collection of Bambara groundnut seeds samples

Samples of Bambara groundnut seeds used to isolate the strains of *Aspergillus* section Flavi were collected from the three agro-ecological zones (Sahelian, Sudano-Sahelian, and Sudanian zones) of Burkina Faso. The locations of sample collection sites were randomly selected in each of the three agro-ecological zones from a list initially drawn up according to the accessibility, the level of Bambara groundnut production, and the need to cover the study area (Ouili et al., 2022). Forty-seven (47) locations were surveyed throughout the country: 9 locations in the Sahelian zone, 25 locations in the Sudano-Sahelian zone, and 13 locations in the Sudanese zone. A total of 99 samples were collected, 25 were from the Sahelian zone, 51 from the Sudano-Sahelian zone, and 23 from the Sudanian zone (Ouili et al., 2022).

Isolation and morphological identification of *Aspergillus flavus* isolates

The standard blotter method described by Mathur and Kongsdal (2003) was adopted with slight modifications, for the isolation of microorganisms. Two hundred seeds from each sample were placed on moistened blotters in Petri dishes at the rate of 10 seeds per dish and incubated for 7 days at 20 - 25°C under alternating cycles of 12 h near Ultraviolet Light and 12 h darkness. Then, the individual seeds were examined for the presence of fungi under a stereomicroscope (MOTIC SMZ-140-N2LED, Spain). Any visible *Aspergillus flavus*-like mycelial growth or spores characterized by greenish, white, yellow, yellow-brown, or shades of green coloration was considered as the initial isolation criterion. Then, successive subcultures were performed

on Potato Dextrose Agar (PDA) medium in order to purify the developed fungi. Sub-culturing was carried out by placing a fragment of the mycelium in the center of a new Petri dish using a sterilized loop. The purified isolates were kept on PDA at 4°C.

PDA media was also used for morphological identification. Macroscopic features of the isolates including colony growth, color, texture, spores, and reverse color were observed after 10 days of incubation (Klich, 2002; Samson et al., 2010).

For microscopic evaluation, a fragment of mycelium was collected from the fungus and placed on a slide; a drop of methylene blue was added before covering with a coverslip. Microscopic features such as conidiophores, vesicles, metules, phialides, shape, and texture of spores were observed under a microscope (MOTIC SFC-18, Hong Kong, Asia) at 10, 40, and 100 magnifications. Several identification keys were used including those described by Klich (2002) and Samson et al. (2010).

Screening for aflatoxins production

In a 250 mL flask, 21 mL of distilled water was added to 25 g of rice (final moisture 50%). Each flask was sterilized at 121°C for 20 min. The rice was then inoculated with 2 mL of a suspension of spores (10^8 spores/mL) of each isolate (Roussos, 2006). The cultures were incubated at 25°C for 7 days for growth and sporulation. After that, the flasks were heated at 70°C for 24 h (in order to destroy the fungal spores) and then, dried at 80°C for 24 h. A rice sample processed without inoculation was also used as negative control. Each test was conducted in triplicates.

Detection and quantification of aflatoxins

Extraction of aflatoxins

Ten grams (10 g) of each fermented substrate (pasteurized and dried rice) were ground to a fine powder and mixed with 40 mL of an Acetonitril-water solution (60/40 v/v) using a stir bar at 10, 000 x g for 2 min. Then, the mixture was centrifuged at 2, 800 x g for 10 min. The supernatant was collected and stored at - 20°C for 24 h. From the clarified supernatant, 4 mL were taken and diluted with 44 mL of a phosphate-buffered saline (PBS, pH 7.4). The PBS solution was previously prepared by dissolving in 1 L of distilled water, the following compounds: KCl 0.2 g, KH_2PO_4 0.2 g, Na_2HPO_4 1.16 g, NaCl 8 g. The pH was adjusted to 7.4 with HCl (0.1 M) or NaOH (0.1 M) (Daradimos et al., 2000). Then, the extracts were filtered through a Whatman no.4 filter paper before passing through an immunoaffinity kit AflaStar columns provided by Romer Labs.

Aflatoxins purification by immunoaffinity

The AflaStar kits (Romer Labs Inc. 1301 Stylemaster Drive, Union, MO 63084-1156, USA), specific to AFs B_1, B_2, G_1, and G_2 were used. The aflatoxins were purified by passing 40 mL of each extract through an immunoaffinity column (AflaStar$^{(r)}$ R - Immunoaffinity Columns) at a flow rate of 2 mL/min. As the sample passes through the column, the antibodies selectively bound to the different aflatoxins and form an anti-body-antigen complex. The column was then washed with 20 mL of PBS (flow rate 5 mL/min) in order to eliminate the compounds not fixed to the column. Then, the aflatoxins bound to the column were eluted by the addition of 2 mL of methanol at a flow rate of 1.2 mL/min.

Identification and quantification of aflatoxins

Standard preparation

For the standard preparation, each of the standard reagents, aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2) was dissolved in acetonitrile at 1 mg/mL and was stored at 4°C in the dark until use. To prepare the working standard for LC/MS analysis, each Aflatoxin (AF) stock solution was equally pipetted and transferred to a vial, and it was then diluted with the mobile phase. The final concentration of each AF was 1 ng/mL

Chemicals

AFB_1 and AFB_2 (Sigma Aldrich, Tokyo, Japan) standards (purity ≥99%) were used. High Performance Liquid Chromatography (HPLC) grade acetonitrile and methanol and ammonium acetate were obtained from Wako Chemical (Osaka, Japan).

Water was purified in-house with a Milli-Q system (Millipore, Tokyo, Japan).

Instrumentation

The eluate was analyzed using an LC-MS/MS system which consisted of an Agilent 1290 series vacuum degasser, binary pump, automatic well plate changer, thermostatically controlled column compartment, Agilent 6400 triple quadrupole mass spectrometer with an electrospray ionization source. The mobile phase was a time-programmed gradient using water (eluent A) and methanol (eluent B). Both contained 10 mM ammonium formate and 0.1% formic acid. The chromatographic run started with 95% A and 5% B. A linear decrease in eluent A occurred with 100% eluent B in 5 min.

The quantitative determination was carried out using MRM (Multiple Reaction Monitoring) mode. The parameters for MRM transitions are shown in Table 2.

Statistical Analysis

The results were expressed as mean ± SD from the triplicate analysis. The differences in aflatoxin mean concentrations in the fermented rice were compared by Analysis of variance (ANOVA) using XLSTAT-Pro 7.5.2 software. Interpretation of values was performed using the Fisher test at probability level p = 5%.

RESULTS

Isolation and identification of fungal isolates

Based on the greenish coloration of the colonies, 198 isolates (42 isolates from Sahelian zone, 85 isolates from Sudano-Sahelian zone and 71 strains from Sudanian zone) were isolated from the samples of Bambara groundnut. The fungal growth was observed on PDA medium (Table 1). The *Aspergillus flavus* - like isolates were morphologically identified based on the yellow-green conidial color, and the globose to sub-globose vesicles. The colonies of the isolates of *Aspergillus flavus* exhibited a greenish color that spread radially from the point of inoculation. As the colony progressively spread, it became slightly raised with the center changing into floccose and rough. On the reverse, the colonies presented clear exudates and cream color. The sporulation started after five days from the center and progressed radially covering the surface of the colony. Then, it presented a white border surrounding the sporulating colony. The microscopic features of these fungal isolates showed that the conidia had a globose shape ranging between 250 µm and 450 µm in diameter with thin walls and rough texture. The conidiophores had a rough texture and thick walls; they were non-pigmented and unbranched. On the basis of these macroscopic and microscopic characteristics, only 28 isolates (out of the 198) were considered as *Aspergillus flavus* and used for the following tests. Among these 28 isolates, 06 derived from the Sahelian zone, 10 from the Sudano-Sahelian zone, and 12 from the Sudanian zone (Figure 1).

Potential of the *Aspergillus flavus* strains for the production of aflatoxins B_1 and B_2

All of the 28 *Aspergillus flavus* isolates tested (100%) produced aflatoxin B_1 when they were grown on rice. The average quantities of aflatoxin B_1 varied from 46.987 µg kg^{-1} to 1080.320 µg kg^{-1}. The highest aflatoxin B_1 content was produced by isolate AVBF26 while the lowest quantity was produced by isolate AVBF24 (Table 2).

However, 92.85% of the *Aspergillus flavus* isolates (26 isolates out of 28) were able to produce aflatoxins B_2. The isolates AVBF42 and AVBF23 did not produce aflatoxin B_2 when grown on rice. The average quantities of aflatoxin B_2 varied from 47.200 µg kg^{-1} to 1760.240 µg kg^{-1} (Table 3). The highest aflatoxin B_2 content was produced by isolate AVBF37 while the lowest quantity was produced by strain AVBF52. Out of 28 *Aspergillus flavus* strains tested, 19 produced higher levels of AFB_1 than AFB_2 in rice culture (Figure 2). Only nine isolates that is AVBF66, AVBF37, AVBF8, AVBF17, AVBF22, AVBF6, AVBF24, AVBF31 and AVBF68 produced more AFB_2 (283.680, 1760.24, 137.30, 972.85, 627.70, 144.45, 56.08, 199.14 and 231.57 µg kg^{-1} respectively) than AFB_1 (83.973, 320.53, 110.74, 325.86, 582.90,

59.30, 46.98, 70.88 and 49.57 µg kg^{-1} respectively). The non-inoculated sample, used as negative control was free from any trace of AFs (B_1 or B_2).

The results of the analysis of variance (ANOVA) performed on the means of AFs produced by the different *Aspergillus flavus* isolates tested showed that:

-The tested isolates exhibited significant differences in the capabilities of (i) AFB_1 and (ii) AFB_2 production (Table 3).

-There is a significant difference between the means of AFB_1 and AFB_2 produced by each isolate except isolate AVBF8 (Figure 2).

Table 1: Mobile phases in the chromatography run.

Time	A (%)	B (%)
0	95	5
5	0	100
6	0	100

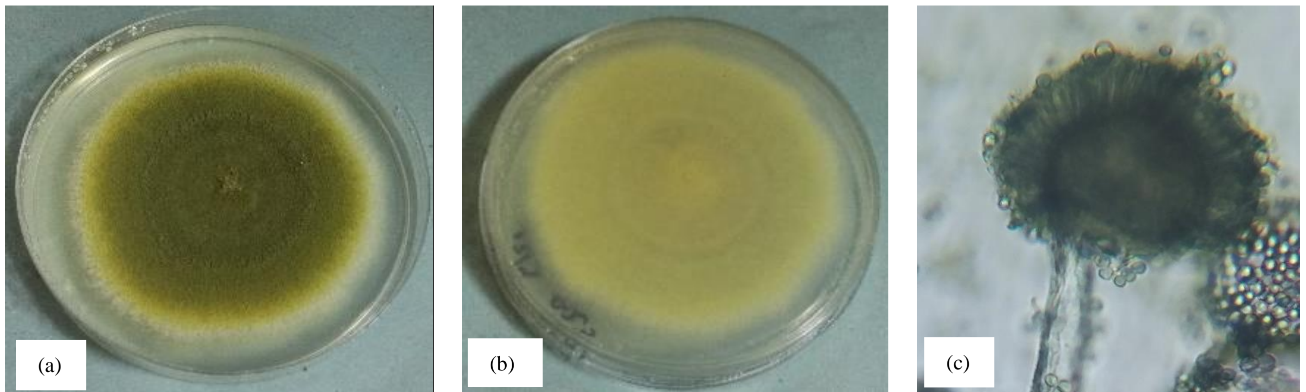

Figure 1. Macroscopic features of *Aspergillus flavus* isolate 26 colony showing the greenish conidia encircled with a white border (a); the reverse cream color of the colony (b); and the aspergillary head showing the biseriate with philiades (c).

Table 2: Data acquisition parameters of MRM transitions for each aflatoxin.

Aflatoxin	Retention time (min)	Fragmentor voltage (V)	Molecular weight	Precursor ion (m/z)	Product ion	Collision energy (V)
B_1	4.68	130	312.1	313.1	285.1	20
					241.0	35
					269.1	25
B_2	4.7	130	314.1	315.1	287.1	25
					259.1	25
					243.0	40

Table 3: Production of AFB_1 and AFB_2 by *Aspergillus flavus* strains cultivated on rice at 25°C for 7 days.

Agro-ecological zones	***Aspergillus flavus* isolates code**	**Average AFB_2 (µg kg^{-1})**	**Average AFB_1 (µg kg^{-1})**
Sahelian zone	AVBF67	128.667 ^{hij}	894.160 ^{b}
Sahelian zone	AVBF66	283.680 ^{d}	83.973 ^{l}
Sahelian zone	AVBF66'	94.560 ^{ijkl}	861.947 ^{b}
Sahelian zone	AVBF54	94.880 ^{ijkl}	719.360 ^{cde}
Sahelian zone	AVBF50	91.547 ^{ijklm}	793.387 ^{bc}
Sahelian zone	AVBF80	51.973 ^{lm}	611.573 ^{efgh}
Sudano-sahelian zone	AVBF10	96.160 ^{ijkl}	899.493 ^{b}
Sudano-sahelian zone	AVBF42	0.000 ^{n}	578.320 ^{gh}
Sudano-sahelian zone	AVBF36	72.507 ^{klm}	773.387 ^{bcd}
Sudano-sahelian zone	AVBF37	1760.240 ^{a}	320.533 ^{jk}
Sudano-sahelian zone	AVBF52	47.200 ^{m}	642.400 ^{defg}
Sudano-sahelian zone	AVBF93	104.347 ^{hijk}	897.760 ^{b}
Sudano-sahelian zone	AVBF43	81.413 ^{klm}	712.907 ^{cdef}
Sudano-sahelian zone	AVBF2	66.933 ^{klm}	567.920 ^{gh}
Sudano-sahelian zone	AVBF3	87.307 ^{jklm}	416.053 ^{ij}
Sudano-sahelian zone	AVBF8	137.307 ^{hi}	110.747 ^{l}
Sudanian zone	AVBF26	134.880 ^{hi}	1080.320 ^{a}
Sudanian zone	AVBF26'	75.280 ^{klm}	811.733 ^{bc}
Sudanian zone	AVBF27	136.213 ^{hi}	777.787 ^{bc}
Sudanian zone	AVBF28	183.547 ^{fg}	790.667 ^{bc}
Sudanian zone	AVBF17	972.853 ^{b}	325.867 ^{jk}
Sudanian zone	AVBF22	627.707 ^{c}	582.907 ^{fgh}
Sudanian zone	AVBF6	144.453 ^{gh}	59.307 ^{l}
Sudanian zone	AVBF6'	128.960 ^{hij}	686.373 ^{cdefg}
Sudanian zone	AVBF24	56.080 ^{lm}	46.987 ^{l}
Sudanian zone	AVBF31	199.14 ^{ef}	70.88 ^{k}
Sudanian zone	AVBF68	231.573 ^{e}	49.573 ^{l}
Sudanian zone	AVBF23	0.000 ^{n}	483.573 ^{hi}

Values in the same column with different letter superscripts are significantly different at the level of 5%.

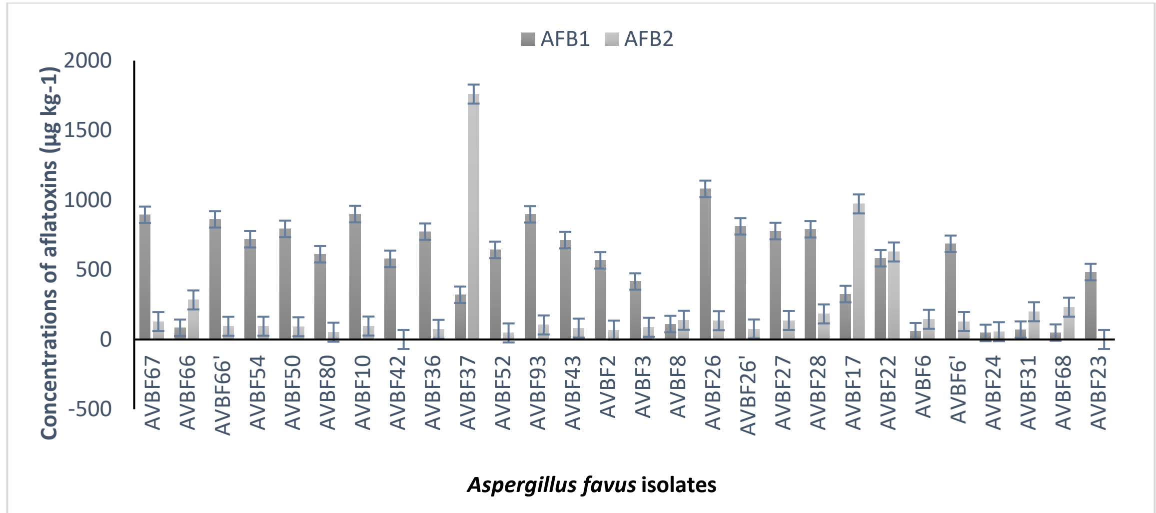

Figure 2: Levels of aflatoxin B_1 compared to aflatoxin B_2 produced by the tested *Aspergillus flavus* isolates.

DISCUSSION

Several isolates of *Aspergillus flavus* were isolated from the seeds of Bambara groundnut. The colonies were cream-colored on the reverse side, woolly in texture with a flaky center. The vesicles were subglobose to globose with bisecting phialides. This is in agreement with the descriptions made by Hedayati et al. (2007). These features were typical of the descriptive taxonomic keys provided by Klich (2002) and in compliance with the features of *Aspergillus flavus* previously documented by Thathana et al. (2017). Contamination from the field, during post-harvest operations, or storage could explain the presence of *Aspergillus flavus* isolates in the tested seeds. According to Baddi et al. (2021), inappropriate harvesting, drying, and storage practices contribute to the development of fungi, mainly the genus *Aspergillus*.

This study revealed varying distribution of *Aspergillus flavus* in the three agro-ecological zones of Burkina Faso (21.42% in the Sahelian zone, 35.71% in the Sudano-Sahelian zone, and 42.85% in the Sudanian zone). Rainfall patterns could be the cause of the substantially higher infection rates in the Sudanian and Sudano-Sahelian zones than in the Sahelian zone. The rainy season in the Sudanian and Sudano-Sahelian zones of Burkina Faso last up to six months, making the weather essentially cloudy. This complicates the drying of the harvested pods, the sun being the main source of energy. Bambara groundnut pods that have not dried completely are prone to fungal contamination. Indeed, Manizan et al. (2018) and Daou et al. (2021) have reported that insufficiently dried seeds are prone to fungal growth, aflatoxin accumulation, and rotting during storage. In addition, insufficient or low light intensity in these areas lengthens the time required for adequate drying of the pods. The long drying periods on open surfaces expose the pods to spoilage by small ruminants, rodents and poultry (Njoroge et al., 2019). These animals can also be a source of contamination of crops with aflatoxigenic fungi. In West Africa, isolation of *Aspergillus flavus* was reported from Bambara groundnut seeds in Nigeria (Isadeha and Time, 2018). Several studies have also reported the presence of *Aspergillus flavus* in Bambara groundnut seeds in South Africa (Shabangu, 2009; Olagunju et al., 2018). Fungal contamination constitutes a major concern in Bambara groundnut preservation and trading. In fact, these fungi can significantly reduce the

nutritional value of the seeds (Kayode et al., 2021). In addition to reducing seed quality, some fungi can produce mycotoxins. *Aspergillus flavus* is one of these fungi as it can produce AFB_1 and AFB_2.

Analysis of the aflatoxin-producing activity of *Aspergillus flavus* showed that all 28 tested isolates produced aflatoxins (Table 3). *Aspergillus flavus* is often the most frequently isolated aflatoxin-producing species from seeds (Ouattara-Sourabie al., 2011; Ky épse Dedi, Diomande 2017). Our results are consistent with this finding since all *Aspergillus flavus* isolates (100%) produced aflatoxins in rice. According to Lai et al. (2015) and Romero-Sanchez et al. (2022), rice is a favorable substrate for the development of *Aspergillus flavus* isolates and the accumulation of AFB_1 and AFB_2. The production of these mycotoxins during our study was favored by the high moisture (50%) content of the grains, resulting from their immersion in water, the aeration, and the mesophilic temperature (25°C). Environmental conditions such as high temperature and humidity increase the risk of fungal growth and mycotoxin production (Romero-Sanchez et al., 2022). Other factors that affect contamination include pH, the characteristics of the fungal strain, and the nature of the substrate (Daou et al., 2021).

Results obtained show that 67.85% (19) of toxigenic *Aspergillus flavus* isolates produced higher levels of AFB_1 than AFB_2 in rice (Table 3). This can be explained by the efficient synthesis and dominant biochemical pathway of AFB_1 compared to other Aflatoxins (Xie et al., 2018). Although aflatoxins B_1, B_2, G_1, and G_2 are synthesized from the same metabolic precursors by mycotoxigenic fungi, AFB_1 is usually the most prevalent one in cereals (Kolawole et al. 2021). These results are similar to those of Lai et al. (2015) who isolated aflatoxigenic *Aspergillus flavus* strains producing higher levels of AFB_1 than AFB_2 in rice. The generally high level of AFB_1 compared to AFB_2 in cereals is of special interest for consumer safety due to the more toxic nature of AFB_1 (Romero-Sanchez et al., 2022). This mycotoxin is hepato-carcinogenic, mutagenic, teratogenic, and suppresses the immune system (Kumar et al., 2021). In order to protect consumers' health, several countries have fixed acceptable levels of AFB_1 in foods. For example, AFB_1 concentrations that are tolerated in the European Union and China range from 5 to 20 gkg^{-1} and 0.1 to 12 gkg^{-1}, respectively (Règlement de la Commission, 2006; Tumukunde et al., 2020).

Several investigations have found that Bambara groundnut contains aflatoxins at specific levels. For instance, tests on Bambara groundnut seeds from Limpopo, South Africa, found aflatoxins in concentrations between 0.01 and 0.1 g/kg (Shabangu, 2009). Olagunju et al. (2018) showed AFB_1 levels of 0.13 to 6.90 g/kg. However, Adebiyi et al. (2020) reported lower average AFB_1 values in Bambara groundnut collected in South Africa. In samples of Bambara groundnut stored in Ethiopia, Mohammed et al. (2016) found AFB_1 values ranging from 0.0071 to 2.526 ppm. These findings confirm the presence of aflatoxigenic strains in Bambara groundnut seeds found in our study, hence the need to identify the potential sources of contamination of Bambara groundnut seeds in order to develop preventive measures. Future research efforts should include the analysis of environmental samples such as soil, water, and harvesting tools from the farms, in order to detect the producers of mycotoxins and to identify the sources of contamination of Bambara groundnut before and after harvest. This will help to prevent contamination by fungi and mycotoxins production.

Conclusion

The results of this study show that all *Aspergillus flavus* isolates isolated from Bambara groundnut produced aflatoxins, with most of them producing higher levels of AFB_1 than AFB_2. The ability of these isolates to produce such large quantities of aflatoxins is alarming. Thus, the contamination and the development of these strains in Bambara groundnut seeds should be prevented and controlled during the harvesting, processing, and storage of the seeds. Considering the health issues related to aflatoxins, in-depth research to

significantly reduce aflatoxin contamination of foodstuffs should be conducted. In addition, sensitization of small-scale producers and the public authorities could contribute to mitigating the incidence of toxigenic fungi on humans' health.

COMPETING INTERESTS

The authors declare that there is no competing interest.

AUTHORS' CONTRIBUTIONS

The authors were involved in various ways in the development of this article. YM is the principal investigator; MN, II and FN provided technical support and correction of the manuscript. ASO, MO, AO, HN, COTC, EK corrected the manuscript; directed and supervised the work. ASO conducted the lab work and drafted the manuscript.

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