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Biodiversity of *Aspergillus* Species and Aflatoxin Contamination in Stored Cereals and Legumes in Cameroon

Landry Thierry Voukeng Dongmo [a,b], Hermine Mahot [c], Sylver Landry Lontsi Dida [a], Fabrice Christian Gbaporo Gbaporo [a], Patrice Ngatsi Zemko [a], Laurence Ngadjui Ngata [a], Nathalie Mbega Sanama [a], Zachée Ambang [a*] and Onana Boyomo [b]

[a] Laboratory of Biotechnologies and Environment, Phytopathology and Plant Protection Research Unit, Faculty of Science, University of Yaounde I, Cameroon.
[b] Department of Microbiology, Laboratory of Microbiology, Faculty of Science, University of Yaoundé I, Cameroon.
[c] Laboratory of Biological control and Applied Microbiology, IRAD-Yaoundé, Cameroon.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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**Corresponding author: E-mail: zachambang@yahoo.fr;*

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ABSTRACT

In cereal and legume grains, the presence of *Aspergillus* spp. and their aflatoxins induce huge economic losses, health problems and environmental problems during storage. Thanks to their mycotoxins, Aspergillus, Penicillium and Fusarium cause losses of up to 25% of agricultural production worldwide, and 50% in developing countries. The aim of this work is to identify *Aspergillus* spp. isolated from maize, soybean, rice and peanut grains in storage, while examining their aflatoxin contamination levels. To this end, 25 composite samples of each grain were systematically collected from the five agro-ecological zones (AEZ) of Cameroon. Morphological characterization of *Aspergillus* spp. isolates was carried out using various specific culture media. Aflatoxin determination was carried out using standard aflatoxin extraction and ELISA strip quantification. The results show that, of the 100 composite samples collected, 99% were contaminated by a wide variety of molds, with a prevalence of 334/745 or 44.83% of *Aspergillus* spp. Of the *Aspergillus* spp. isolates obtained, 21.85% were derived from peanuts, 27.84% from rice, 21.56% from maize and 28.74% from soybeans. *Aspergillus* prevalence varied with agro-ecological zones (AEZs), and the most affected zones were AEZ V with 31.73% *Aspergillus* spp. isolates, and AEZ III with 28.14% *Aspergillus* spp. isolates. *Aspergillus* diversity according to AEZs shows that section *Flavi* is the most represented with 33% of isolates, successively followed by sections *Nigri, Fumigati, Terrei, Candidi* and *Circumdati* with 27%, 16%, 6%, 6% and 6% respectively. Examination of the total aflatoxin contamination levels of the various grains showed that, irrespective of the foodstuffs and AEZs, aflatoxin contamination was 100%. However, the highest mean concentration of aflatoxin was recorded in AEZ V (9.63 µg/kg). By commodity, samples of maize (9.46 µg/kg) followed by rice (8.92 µg/kg) showed the highest aflatoxin levels in Cameroon. In the end, all the foodstuffs showed a very high diversity of Aspergillus, and all the aflatoxin levels exceeded the regulatory thresholds in force.

Keywords: Variety; Aspergillus spp; aflatoxins; various grains; storage.

1. INTRODUCTION

In developing countries, food security remains one of the most important sustainable development objectives. In Cameroon, the production and storage of foodstuffs such as maize, soybean, rice and peanuts is one of the means used to achieve this objective. Due to their nutritional importance, these commodities represent the bulk of the diet of humans and animals (Cruz et al. 2019). Throughout the Central African region, and particularly in Cameroon, inadequate environmental conditions and storage practices provide optimal conditions for mold growth and mycotoxin accumulation in these foodstuffs (Ngoko et al. 2001). The development of mold on foodstuffs can have a number of implications, leading to food spoilage (change in appearance, taste, cost, smell, etc.). Among the most frequently incriminated molds, the *Aspergillus* genus is by far the most prevalent.

Aspergillus is a cosmopolitan group of molds first described by Antonio Micheli in 1729 (Ainsworth 1976, Klich 2007). The identification of *Aspergillus* was initially based on morphological characteristics grouping macro and micro characteristcis that provided a general concept of the different species (McClenny 2005) later, molecular features provided more precise species information (Hong et al. 2013). The genus *Aspergillus* belongs to the phylum *Ascomycetes*, class *Eurotiomycetes*, order *Eurotiales*, and family *Eurotiaeae* (El Khoury et al. 2011, Samson et al. 2014). Identification based on macro-characteristics includes colony color and texture, presence of exudates and sclerotia formation. Micro-characteristics include conidial head shape, seriation, vesicle shape and diameter, stipe length, width, texture and color, conidial size, shape, texture and color, sclerotia size for those that formed it (Klich et al. 2002). *Aspergillus* are classified into sections on the basis of seriation, and the shape of the conidial head, which is globose or star-shaped (Samson et al. 2004a).

This fungal genus is particularly feared for the aflatoxins it produces. Indeed, aflatoxins are a major concern for human and animal health, and especially for the significant economic losses they cause (Bryden et al. 2012, Oguz 2012). Recent studies have shown that aflatoxin exposure through various sources is linked to growth disorders in humans and animals (Bryden

2007, Shuaib et al. 2010, Khlangwiset et al. 2011). Aflatoxins are liver toxins known to inhibit nucleic acid and protein synthesis (Sumit et al. 2010). Reduced immune function, hepatotoxicosis, hemorrhage, teratogenesis and mutagenesis are associated with aflatoxicosis (Otim et al. 2005). Rodrigues et al., (2011) surveyed the presence of mycotoxins in animal feed in the Middle East, West and Central Africa, including Israel, Jordan, Lebanon, Syria Yemen Nigeria, Sudan, Egypt, Algeria, Kenya, Ghana and South Africa, revealing that 98% of ingredients used in feed formulation are positive for aflatoxins. In Cameroon, Ngoko et al., (2008), Ngoko (2001) also found that foodstuffs are also highly susceptible to fungal infections, which tend to increase with storage time. Since then, there is a need to renew the database on different *Aspergillus* spp. inventories of cereals and legumes in storage in the different agro-ecological zones of Cameroon and their related mycotoxin. Therefore, the objective of this study was to identify *Aspergillus* spp. isolated from stored maize, soybean, rice and groundnut while examining aflatoxin contamination in the five agro-ecological zones of Cameroon.

2. MATERIALS AND METHODS

2.1 Study Areas

The study was carried out in Cameroon's five agro-ecological zones (AEZs I, II, III, IV and V). Agroecological Zone I (AEZ I), is the Sudano-Sahelian zone. The climate is characterized by monomodal rainfall, and an average temperature of 31 ˚C with a maximum temperature between 40 and 45˚C. The high Guinean savannah zone (AEZ II) is characterized by a Guinean-type climate with bimodal rainfall. Average temperatures range from 20 to 26˚C. The Western Highlands zone (AEZ III) is characterized by a Cameroonian-type climate marked by two seasons of unequal length, with average temperatures below 19 ˚C and abundant rainfall. The Dense Rainforest zone with monomodal rainfall (AEZ IV) is characterized by a very humid and hot Cameroonian climate, a variant of the equatorial climate. Temperatures vary between 22 and 29 ˚C. The bimodal rainforest zone (AEZ V), characterized by a Guinean-type climate with average temperatures of 25 ˚C and rainfall of 1500-2000 mm per year (SAILD 2021). Fivesites per AEZ were sampled per agroecological zone, i.e. a total of 25 sites for the five (Fig. 1).

2.2 Grain Sampling

Cereal and legume grains were collected in the five agro-ecological zones of Cameroon. In each agroecological zone, maize, rice, soybean and peanut grain samples were collected in five localities. In each locality, an average of 25 samples of each commodity were collected and pooled into a composite sample for a total of five samples of each grain per agroecological zone. Sampling was carried out in accordance with Commission Regulation (EC) N°401/2006 of February 23, 2006. Five kg of samples of each grain or legume were taken from 50 or 100 kg bags (i.e. 100 g) at 5 different points in each of the 10 or 20 bags in different corners of the store. At the end of sampling, a total of 100 composite samples of 100 kg each were collected from the five agro-ecological zones. These were placed in sterile BIOHAZARD plastic sampling bags and transported to the laboratory for analysis.

2.3 Seeds Mositure Content

The modified protocol of ISTA (2005) was used here, but with a lower temperature to measure the evolution of water loss correlated with seed weight loss. Indeed, The relative humidity of the seeds was determined from a 100 g sample of each specimen placed in the oven (The mark is TIJDNTDO and the Serial number is YPO-480) at 75°C and maintained for 3 minutes. The samples were removed from the oven and reweighed. The operation is repeated until the sample weight no longer varies. Finally, the grains are left to cool, then weighed to obtain the dry weight. The moisture content of the grains (in %) was calculated according to the formula used by ISTA protocol.

$$\%\ \text{relative humidity} = \frac{\text{initial grain weight} - \text{final grain weight x100}}{\text{initial grain weight}}$$

2.4 Determination of Aflatoxin content in Grains

2.4.1 Aflatoxin extraction

For the extraction of aflatoxins (AFs), twenty (20) grams of each sample were ground in a SILVER CREST blender (Model AMM-02) and introduced into 100 mL of a 9:1 acetonitrile-potassium chloride mixture containing 4% potassium chloride. The pH of this mixture was adjusted to 1.5 using a chloridric acid solution, and the mixture was stirred with an orbital shaker.

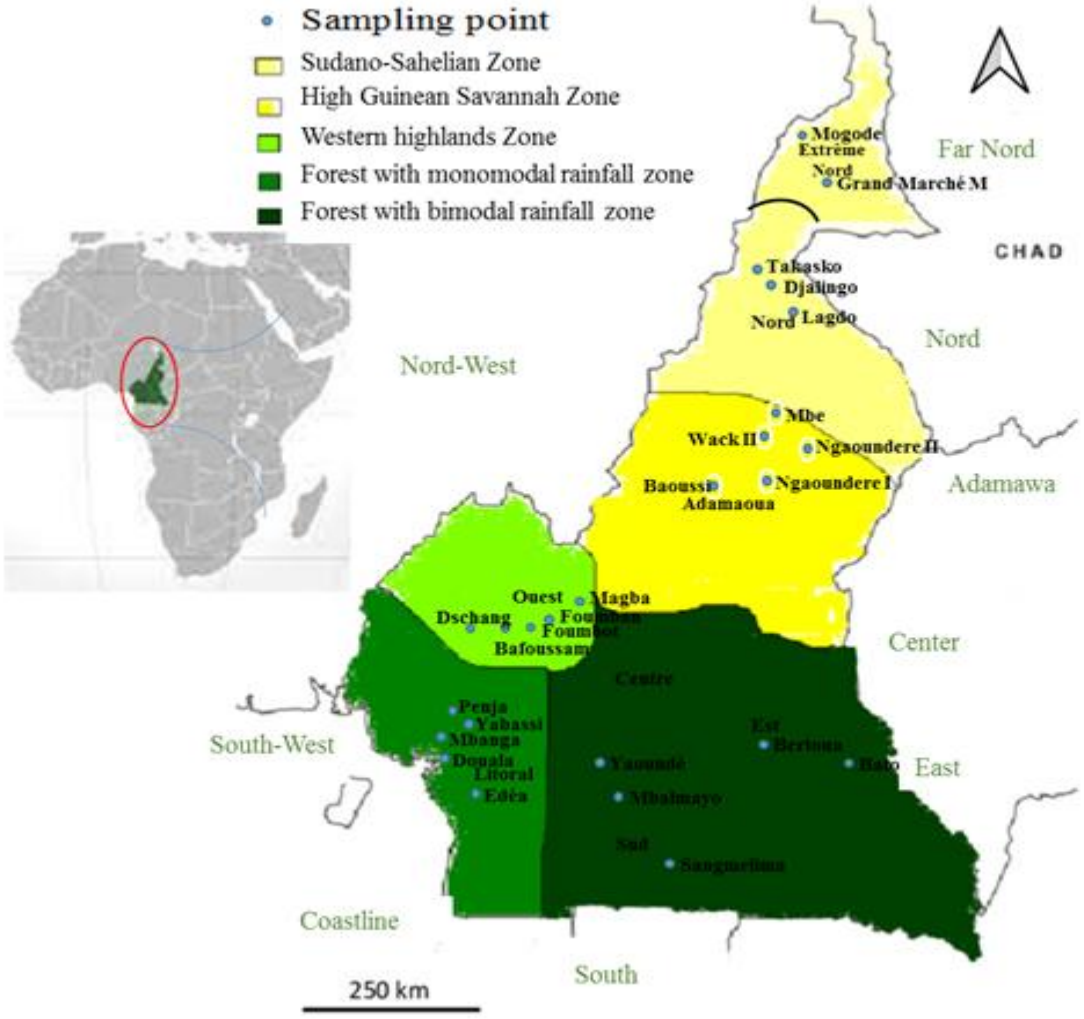

Fig. 1. Sampling sites

(ENDEAVOR 5000) for 20 minutes. Finally, the grindate was filtered under vacuum on Whatman paper No. 4 (Nguyen et al. 2016).

2.4.2 Aflatoxin purification

In a 500 mL beaker, 50 mL filtrate and 100 mL hexane were introduced for degreasing and the mixture was stirred with an orbital shaker (ENDEAVOR 5000) for 10 minutes, then left to stand and the upper phase consisting of hexane was removed using a German rotary evaporator Heidolph. The operation is repeated a second time by adding 50 mL hexane and the lower (aqueous) phase is recovered. Next, 50 mL of chloroform is added to the aqueous phase, and the whole is stirred for 20 minutes and left to stand to observe a two-phase separation. The lower phase (chloroform) is recovered. This step is then repeated twice on the same aqueous phase, this time adding 2 x 25 mL chloroform as extraction solvent. The different phases were then combined and the chloroform removed at 40°C under vacuum in a rotary evaporator. After this step, 2 mL of methanol are added to the residue, and the solution is stirred and filtered through filter paper. In the final step, the filtrate is dried under nitrogen gas and the residues dissolved in 500 µL of methanol (Nguyen et al. 2016). This solution is used for the ELISA assay of AFs.

2.4.3 Aflatoxin determination by ELISA technique

Aflatoxin levels in the various filtrates were determined using an enzyme-linked immunosorbent assay (RIDAQUICK SCAN®) in accordance with the manufacturer's instructions and the protocols defined by the laboratory. For

around 80 samples, aflatoxin levels were measured by Myco-Foss on ELISA strips.

2.5 Mold Caracterisation

2.5.1 Mold isolation

Ten (10) g of the surface of each sample is disinfected in 10% bleach, followed by 90% ethanol, for one minute. After rinsing twice with sterile distilled water, the grains are dried with sterile filter paper and seeded under aseptic conditions, using sterile forceps, in Petri dishes containing sterile potato dextrose agar (PDA) medium and sterile coco extract agar (CEA) medium (5 petri dishes for each medium). The combination is incubated at 28 ± 4°C for 4 to 6 days (Samson 1991, Mathew et al. 2011).

The infection rate of the grains and the number of samples contaminated by the different genera and species were recorded. The degree of contamination is expressed as a percentage of contaminated grains and samples according to the following formula Mathew et al. 2011).

$$\%PC = \frac{NGC}{NTG}$$

%GC = Percentage of contaminated Grains;
NGC = Number of contaminated Grains;
NTG = Total number of Grains.

2.5.2 Identification of molds on culture media

Macroscopic and microscopic characterization was used to identify mods. Macroscopic examination were carried out on pures cultures observed with the naked eye or a magnifying glass. Culture characteristics studied were colony color (obverse and reverse) and variation over time, color and color change of the medium, surface texture and odor (Guiraud 1998, Samson et al. 20004b). In addition to PDA medium, dichloran 18% glycerol agar (DG18); Czapeck agar (CZA); corn yerst agar (CYA); malt extract agar (MEA) and coco extract agar (CEA) were used for specific mold identification. Cultural characteristics on CYA, CZA and MEA media were compared with those described by Samson et al., (2007). Microscopic examination was carried out using fresh preparations. Observation was carried out at x100 and x400 magnification. Isolated molds were identified according to the following microscopic criteria: hyphae with or without septation, conidiophore characteristics, morphology and structure of spores or conidia, diameter of conidia and diameter of vesicles (Guiraud 1998, Samson et al. 2004a).

2.6 Statistical Analysis of Data

The raw data obtained in the course of this work were organized using Microsoft Excel version 2016. Data were subjected to analysis of variance (ANOVA) using R software version 4.2.2. Data normality (Shapiro-Wilk test, $P > 0.05$) and variance homogeneity (Levene test ; $P > 0.05$) were checked. The Kruskal-Wallis test was used to compare means when the data did not conform to the normal distribution, and in the opposite case, the Tukey HSD test was used ($P < 0.05$). Principal component analysis (PCA) between physical parameters and grain aflatoxin content was performed to see the existing relationship using R software.

3. RESULTS

3.1 Total Aflatoxin Levels in Sampled Grains

All foodstuffs collected in the five AEZs were contaminated with aflatoxins. However, what ever the types of considered grains the aflatoxin content average was higher in AEZ V (9.63 ± 5.13µg/kg) and lower in AEZ IV (5.94 ± 0.77µg/kg). The highest total aflatoxin content were 14.06 ± 0,57µg/kg for soybeans, 16.33 ± 2,76µg/kg for maize, 17.26 ± 0,45µg/kg for rice and 10.60 ± 0.52 µg/kg for peanuts grains respectively in AEZ II, AEZ I and in AEZ V. While the lowest values of aflatoxin content were 4.86 ± 0.23 µg/kg for soybeans, 5.00 ± 0.00 µg/kg for maize, 5.06 ± 0.12 µg/kg for rice and 5.50 ± 0.50 µg/kg for peanuts grains in AEZs I, V, IV and II respectively (Table 1).

Our result showed that aflatoxin content in each grain type were significantly different from one AEZ to another. Also, in each of the five AEZ, there was a significant difference between the aflatoxin content evaluated in the four grains type.

3.2 Variation of Seeds Mositure Content According to Agro-Ecological Zones

Grain relative humidity (RH) varies according to foodstuffs and AEZ. For rice, moisture content varies from 13.7% (AEZ I) to 16.96% (AEZ V). In maize, it varies from 13.46% (AEZ IV) to 15.23% (AEZ I). Similarly, the rate varies from 13.03%

(AEZ V) to 14.7 (AEZ II) in soybeans and from 13.16% (AEZ III) to 14.86% (AEZ V) in groundnuts. In terms of AEZs, the highest mositure content was obtained in AEZ V (14.72), followed by AEZ III (14.65). On the other hand, the lowest mositure content value was recorded in AEZ IV (13.72). It should be noted, however, that there were significant differences in mositure content between the AEZs for any grains. Simlarly, there is no significant difference between foodstuffs within the same AEZ, except in zons 3 and 5. However, Rice (16.96%) followed by maize (16.33%) had the highest levels (Table 2).

3.3 Correlation between Seeds Mositure Content, Temperature and total Aflatoxin Content of Grains

The correlation test between grain relative humidity, storage temperature and total aflatoxin content in grains shows a positive correlation between relative humidity and total aflatoxin content. Total aflatoxin content in the various commodities is strongly correlated with grain relative humidity, with correlation values of 0.92 for rice, 0.77 for maize, 0.95 for Peanut and 0.80 for soybeans. These results also show a positive correlation between total aflatoxin content and grain storage temperature, as is the case for rice (0.70), Peanut (0.63) and a little corn (0.45). In the case of soybeans, the low negative correlation (-0.13) suggests that this parameter indirectly influences the total aflatoxin content of grains. (Fig. 2).

3.4 Principal Component Analysis between Studied Parameters

Principal component analysis (PCA) of relative humidity, temperature and total aflatoxin content as a function of AEZ indicates a good representation of the variables and a good rate of information restitution on axes 1 and 2, with restitution rates of 99.8% for rice, 94% for maize, 96.6% for Peanut and 94.8% for soybeans. Axis 1 of the PCA contributes 89.9% for rice, 66.3% for maize, 84.9% for Peanut and 59.9% for soybeans to the variations in the system studied, and is represented by total aflatoxin content and relative grain moisture. As a result, the latter measures the direct link between the two variables in relation to grain storage temperature for each commodity. Moreover, these results show that in some AEZs, these variables are more represented than in others. Axis 2 of the PCA contributes 9.9% for rice, 27.7% for maize, 13.7% for groundnuts and 34.9% for soybeans to the variations in the system studied, and is represented by total aflatoxin content and grain storage temperature. This shows the level of direct linkage between the two variables in relation to grain relative humidity for each commodity. (Fig. 3).

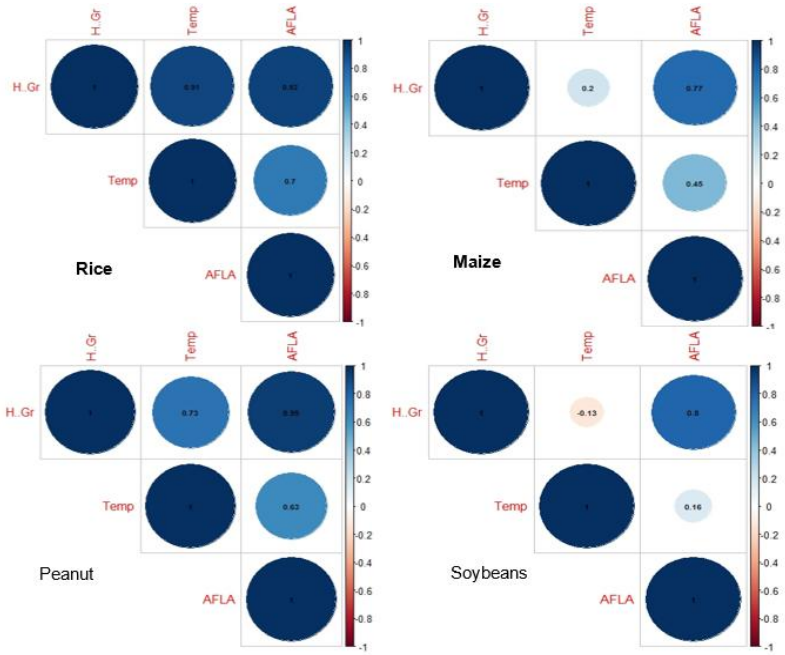

Fig. 2. Correlation between mositure content, temperature and total aflatoxin content of grain

Table 1. Comparison of average aflatoxin levels in the various foodstuffs sampled according to agro-ecological zones

Foodstuff	AEZ I	AEZ II	AEZ III	AEZ IV	AEZ V	*P-value*
soybeans	4.86 ± 0.23^{b,C}	14.06 ± 2.76^{a,A}	6.50 ± 0.43^{b,A}	6.20 ± 1.10^{b,A}	5.17 ± 0.20^{b,A}	0.05
maize	16.33 ± 0.57^{a,A}	6.73 ± 0.25^{c,A}	12.39 ± 0.18^{b,A}	6.36 ± 0.68^{c,A}	5.50 ± 0.50^{c,A}	0.014
Rice	6.60 ± 0.26^{c,B}	7.47 ± 0.50b^{c,A}	8.00 ± 0.52^{b,A}	5.06 ± 0,12^{d,A}	17.26 ± 0.45^{a,A}	0.011
Peanut	5.73 ± 0.30b^{c,BC}	5.00 ± 0.00^{c,A}	5.36 ± 0.55b^{c,A}	6.06 ± 0.12^{b,A}	10.60 ± 0.52^{a,A}	0.017
P-value	0.015	0.12	0.15	0.16	0.62	

AEZ= Agro-Ecological Zone. Numbers in the same row followed by the same lower-case letter are not significantly different at the 5% level. The same applies to numbers in the same colunn followerd by the same capital letter

Table 2. Comparison of seeds mositure content by agroecological zone

AEZs	AEZ I	AEZ II	AEZ III	AEZ IV	AEZ V	p-value
Rice	13.7±0.17^{c,B}	15.21±0.99^{b,A}	15.1±0.36^{b,B}	13.37±0.14^{c,A}	16.96±0.15^{a,A}	0.0122
Maize	15.23±0.35^{a,A}	13.9±0.1^{b,A}	16.33±0.15^{a,A}	13.46±0.5^{b,A}	14.03±0.68^{b,BC}	0.0227
Soybeans	13.7±0.17^{ab,B}	14.7±1.01^{a,A}	14.0±0.1^{a,C}	14.14±0.7^{a,A}	13.03±0.15^{b,C}	0.0601
Peanut	13.67±0.38^{c,B}	13.48±0.07^{bc,A}	13.16±0.21^{c,D}	13.9±0.1^{ab,A}	14.86±0.32^{a,B}	0.0218
p-value	0.08162	0.407	0.01556	0.3176	0.01879	

AEZ= Agro-Ecological Zone. Numbers in the same row followed by the same lower-case letter are not significantly different at the 5% level. The same applies to numbers in the same colunn followerd by the same capital letter.

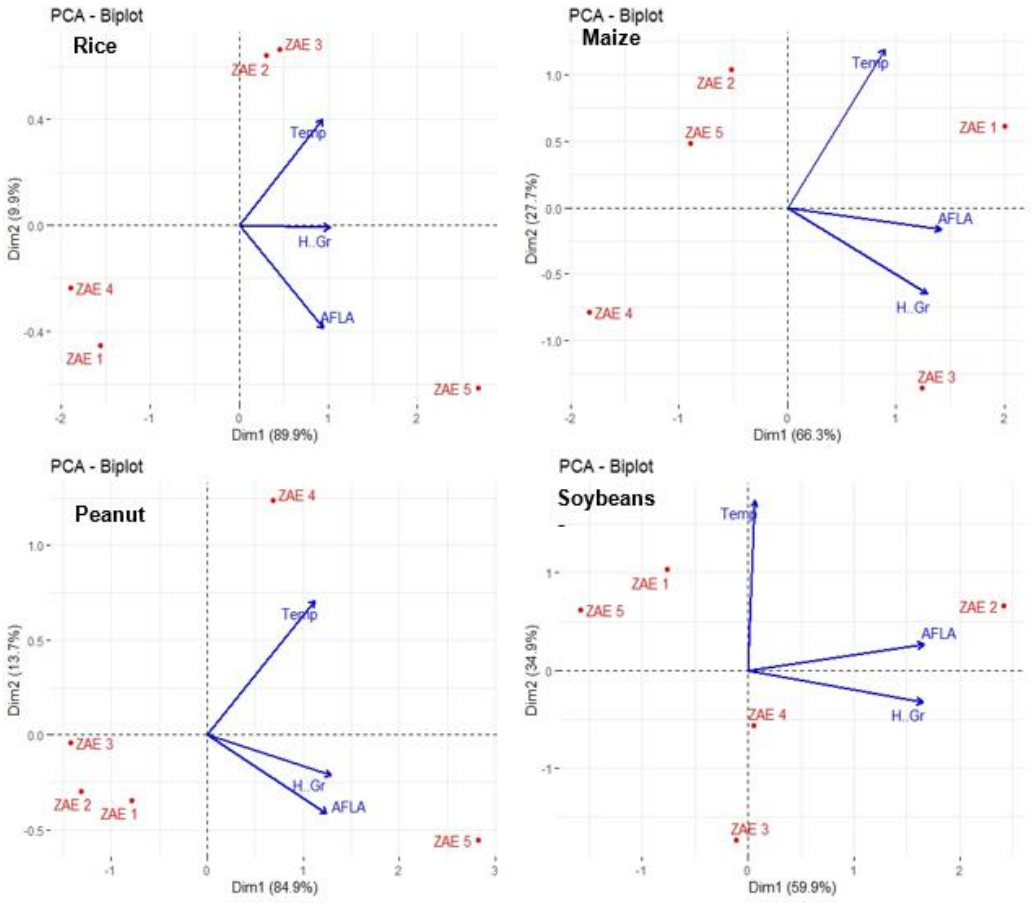

Fig. 3. Principal component analysis between total aflatoxin levels, mositure content, storage temperature and ZAEs (ZAE= agroécological zone)

3.5 Grain Contamination Rates by *Aspergillus* spp.

The rate of moulds contamination of foodstuffs has generally been estimated at 98.75%. more specifically, this contamination rate is 100% for Maize, rice ans peanuts. Soybean, on the other hand, recorded a contamination rate of 95% (Table 3).

3.6 Prevalences of *Aspergillus* spp. Isolates on Foodstuffs from Different Agro-Ecological Zones (AEZ)

A total of 745 mold isolates was derrived from the collected samples. The microscopic and macroscopic characteristics of the isolates enabled them to be grouped into 21 genera, namely: *Aspergillus, Metharizium, Fusarium, Rhizopus, Penicillium, Trichoderma, Alternaria, Apophysomyces, Botrytis, Basidiobolus, Paecilomyces, Conidiobolus, Bipolaris, Mucor, Cunningamella, Curvularia, Epicoccum, Gliocladium, Lichteimia, Pithomyces* and *Syncephalastrum*. The genus *Apergillus* was predominant, with a prevalence of 44.83%. Of the *Aspergillus* isolates obtained, 21.85% were from peanut, 27.84% from rice, 21.56% from maize and 28.74% from soybean. The number of *Aspergillus* also varied not only between grain types within an AEZ, but also between AEZ within grain types. But in general, the frequency of appearance of *Aspergillus* has been higher in the AEZ III, while it was lowest in the AEZ IV (Table 4).

3.7 Prevalence of Average Numbers of *Aspergillus* spp. Isolates by Agro-Ecological Zone

Analysis of the prevalence of *Aspergillus* spp. by AEZ revealed a significant difference (p-value =3.719e-07) between AEZ V and all other AEZs except AEZ III. AEZ V is the most attacked (5.4), followed by AEZ III (4.7). AEZ IV recorded the lowest prevalence (1.95). (Fig.4).

Table 3. Moulds contamination of grains

Commodities	Number of samples	Number of contaminated samples	Contamination rate (%)
Maize	25	25	100
Soybean	25	24	96
Rice	25	25	100
Peanuts	25	25	100
Total	100	99	99

Table 4. Distribution of 745 mold isolates by agro-ecological zone

Agro-ecological zone	Foods commodities	Number of mold isolates	Number of isolates of *Aspergillus* spp.	Frequency of appearance
Zone I	Peanut	14	04	2.41 %
	Soybeans	31	10	32.25%
	Rice	35	18	10.84 %
	Maize	19	8	42.11 %
	Total	99	40	40.4 %
Zone II	Peanut	42	19	11.44 %
	Soybeans	27	18	66.66%
	Rice	13	03	1.81 %
	Maize	33	15	45.45 %
	Total	115	55	47.82%
Zone III	Peanut	49	24	14.46 %
	Soybeans	32	20	75%
	Rice	70	26	15.66 %
	Maize	36	24	66.66%
	Total	187	94	50.26%
Zone IV	Peanut	22	06	3.61 %
	Soybeans	20	8	40 %
	Rice	30	13	7.83 %
	Maize	33	12	36.36%
	Total	105	39	37.14%
Zone V	Peanut	38	20	52.63 %
	Soybeans	75	40	53.33%
	Rice	76	33	43.42 %
	Maize	50	13	26 %
	Total	239	106	44.35%
Total		745	334	44.83%

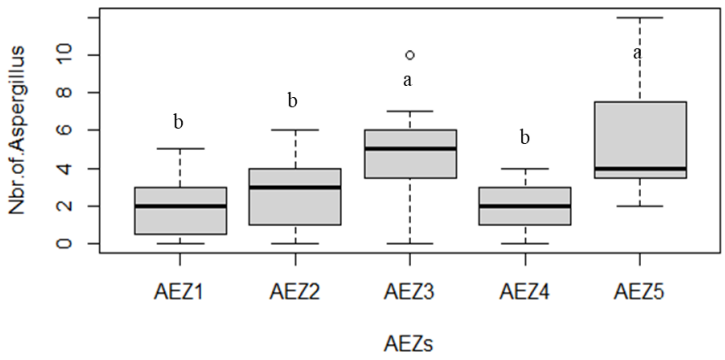

Fig. 4. Prevalence and average number of *Aspergillus* spp. isolates by agro-ecological zone (AEZ)

Table 5. Prevalence of the average number of *Aspergillus* spp. isolates in foodstuff by agro-ecological zone (AEZ)

	Number of Aspergillus in 10 grains					
Foodstuff	**AEZ1**	**AEZ2**	**AEZ3**	**AEZ4**	**AEZ5**	***P-value***
Maize	2.13 ±1.50^{b,B}	3.33 ±1.8^{b,A}	5.13 ±1.30^{a,A}	2.73 ±1.48^{b,A}	2.93 ±0.96^{b,B}	0.0001
Soybean	2.27 ±1.58^{b,B}	3.87 ±1.06^{b,A}	4.33 ±3.56^{b,A}	2.07 ±1.03^{b,AB}	8.33 ±3.5^{a,A}	9.222e-07
Rice	3.93 ±1.16^{c,A}	0.93 ±0.70^{d,B}	5.53 ±1.60^{b,A}	2.93 ±0.70^{c,A}	7.33 ±1.05^{a,A}	2.486e-12
groundnut	1.13 ±0.92^{b,B}	4.13 ±1.85^{a,A}	5.13 ±0.92^{a,A}	1.60 ±0.74^{b,B}	4.33 ±0.82^{a,B}	4.525e-10
P-value	6.016e-05	2.757e-06	0.18	0.0008	2.222e-08	

AEZ= Agro-Ecological Zone. Numbers in the same row followed by the same lower-case letter are not significantly different at the 5% level. The same applies to numbers in the same colunn followerd by the same capital letter

3.8 Prevalence of Average Numbers of *Aspergillus* spp. Isolates by Foodstuff and Agro-Ecological Zone (AEZ)

All foodstuffs collected in the five AEZs were attacked by molds. However, the prevalence of the average number of *Aspergillus* spp. isolates showed a significant difference between foodstuff and AEZ. The highest number of *Aspergillus* spp. isolates (8.00) was recorded on soybeans from AEZ V. In maize, the number of *Aspergillus* spp. isolates is highest in AEZ III (4.8) and lowest in AEZ II (1.6). In rice, the number was higher in AEZ V (7) than in AEZ II (0.6). The number of *Aspergillus* spp. isolates in peanuts was 4.8 in AEZ III and 4 in AEZ V. Overall, *Aspergillus* spp. prevalence in Cameroonwas highest and approximately similar in soybean (3.85) rice (3.84) and groundnut (3.72) (Table 5).

3.9 Characterization of *Aspergillus* Spp. Biodiversity in the Five Agro-Ecological Zones of Cameroon

Macroscopic and microscopic characterization of *Aspergillus* from different specific media revealed the presence of 18 morphotypes which were grouped into 7 sections, including section *Flavi* ; section *Nigri* ; section *Fumigati*; section *Circumdati* ; section *Candidi* ; section *Nidulent* and section Terrei.

3.9.1 *Flavi* section

They are characterized by yellow-green to brown conidial heads and sclerotia. On CYA, colonies are granular, flat, light yellow-green. Heads are biseriate, with occasional phialides on the vesicle. Conidiophores are hyaline and rough. Conidia are globose to subglobose (Table 6). This section regroup amoung *Aspergillus* species found : *A. parasiticus B, A. flavus, A. parasiticus A, A. nomius, A. oryzae, A tamarri* (Fig. 5, 6, 7, 8, 9, 10).

3.9.2 *Nigri* section

Nigri section of *Aspergillus* are characterized by black or almost black, biserial conidial heads 3mm in diameter and 15 to 20µm long. The vesicles are spherical to pyriform and the stipes smooth and hyaline. On CYA, colonies are smooth, flat, dark brown to black. Heads are biseriate with some phialides on the metules. Conidiophores are hyaline and rough. Conidia were globose to subglobose (Table 6). Figs. 11, 12, 13, 14 and 15 represente *Aspergillus* found in this study and belonged to *Nigri* section.

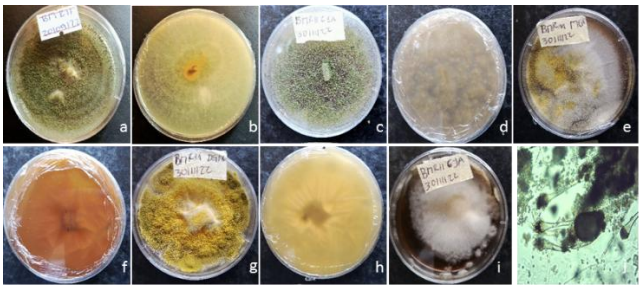

Fig. 5. Isolate BMR11, on PDA (a & b), on CEA (c & d), on MEA (e & f), on DG18 (g & h), on CYA (i) ; microscopy (j) *Aspergillus parasiticus B*

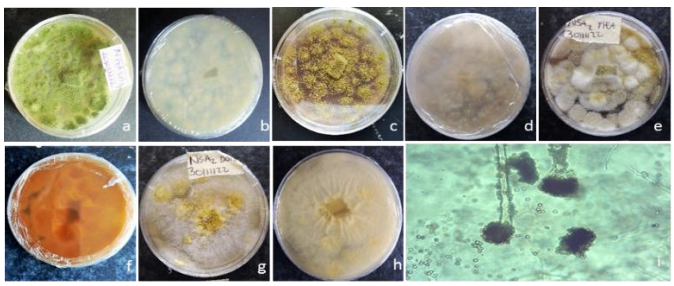

Fig. 6. Isolate NSA2, on PDA (a & b), on CEA (c & d), on MEA (e & f), on DG18 (g & h) ; microscopy (i) *Aspergillus flavus*

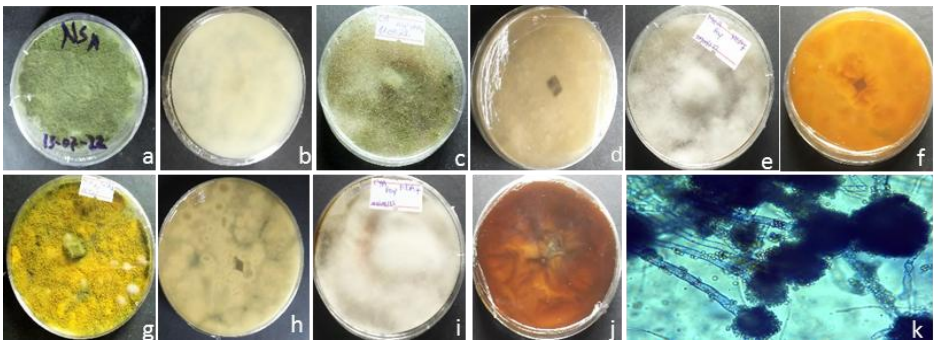

Fig. 7. Isolate NSA7, on PDA (a & b), on CEA (c & d), on MEA (e & f), on DG18 (g & h), on CYA (i & j) ; microscopy (k) *Aspergillus parasiticus A*

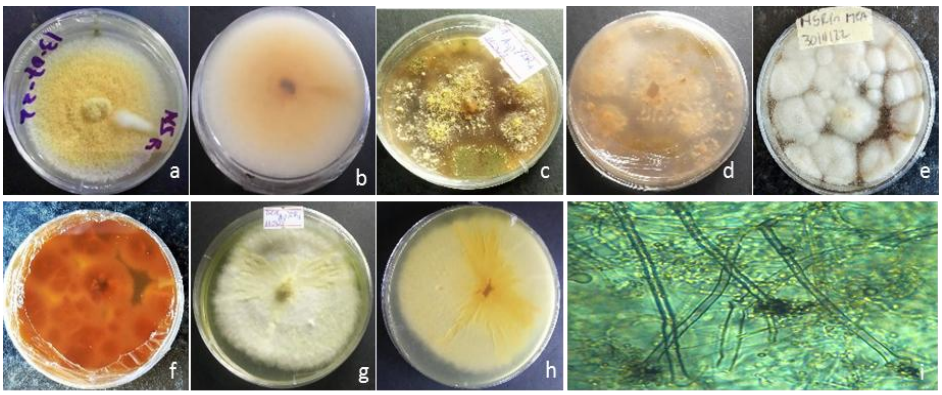

Fig. 8. Isolate NSR1, on PDA (a & b), on CEA (c & d), on DG8 (e & f), on MEA (g & h) ; microscopy (i) Aspergillus

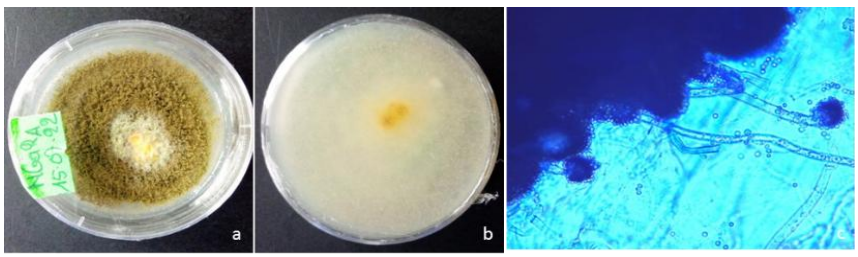

Fig. 9. Isolate NGA2A5, on PDA (a & b) ; microscopy (c) *A. oryzae*

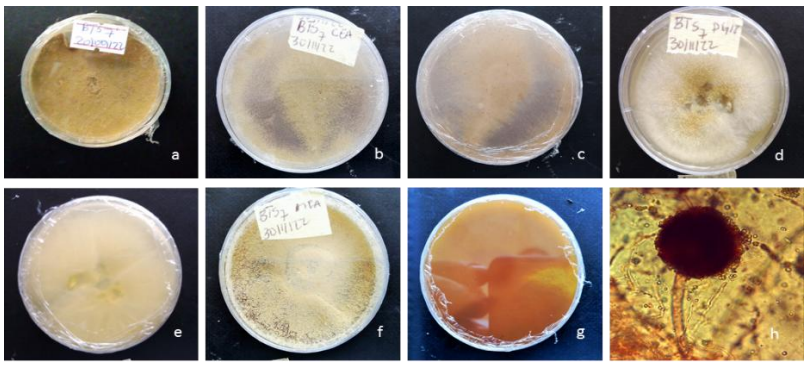

Fig. 10. Isolate BTS7, on PDA (a), on CEA (b & c), on DG18 (d & e), on MEA (f & g) ; microscopy (h) *A. tamari*

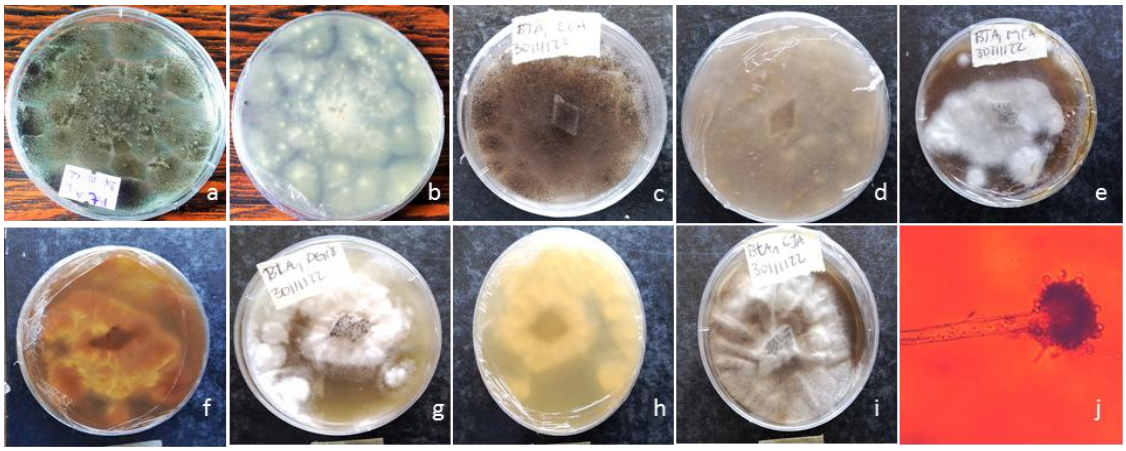

Fig. 11. Isolate BTA1, on PDA (a & b), on CEA (c & d), on MEA (e & f), on DG18 (g & h), on CYA (i) ; microscopy (j) *Aspergillus niger*

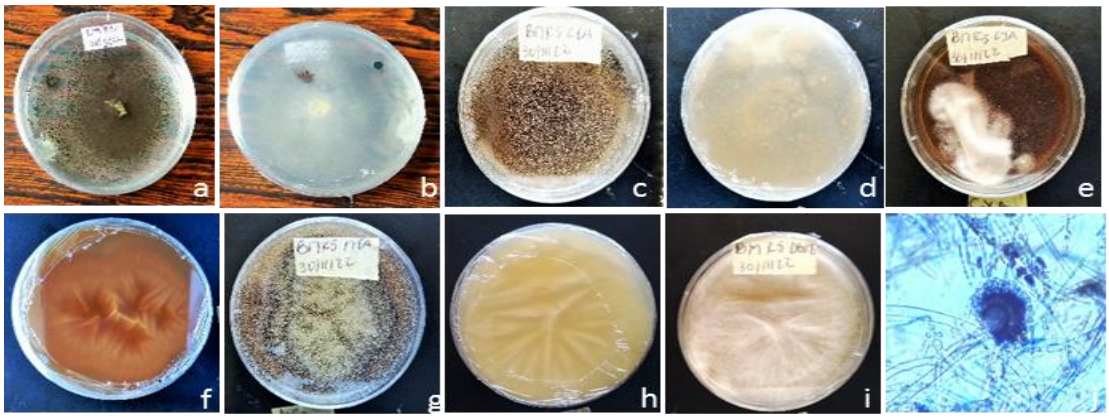

Fig. 12. Isolate BMR5, on PDA (a & b), on CEA (c & d), on CYA (e & f), on MEA (g & h), (i) ; on DG18 microscopy (j) *A. aculeatus A*

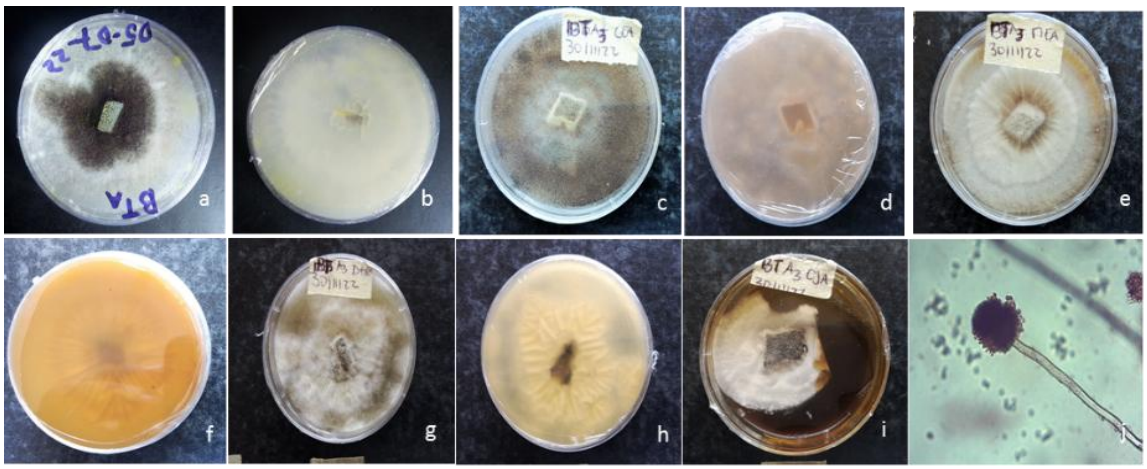

Fig. 13. Isolate BTA3, on PDA (a&b), on CEA (c&d), on MEA (e & f), on DG18 (g & h), on CYA (i) ; microscopy (j) *A. carbonarius*

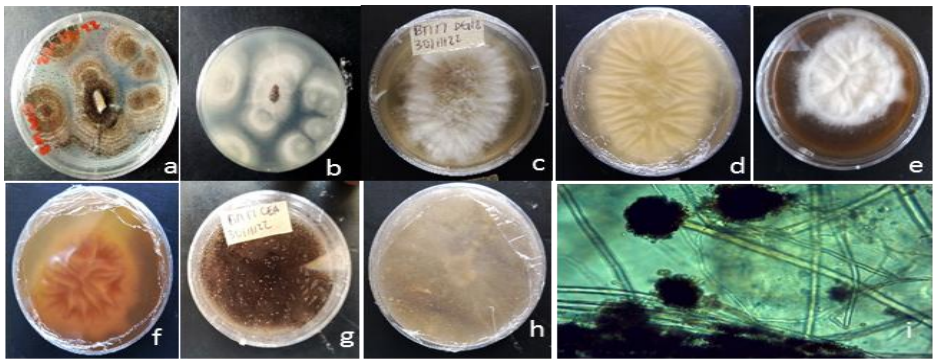

Fig. 14. Isolate NGAA2, on PDA (a & b) on DG18 (c& d), on CYA (e & f), on CEA (g & h); microscopy (i) *A. japonicus*

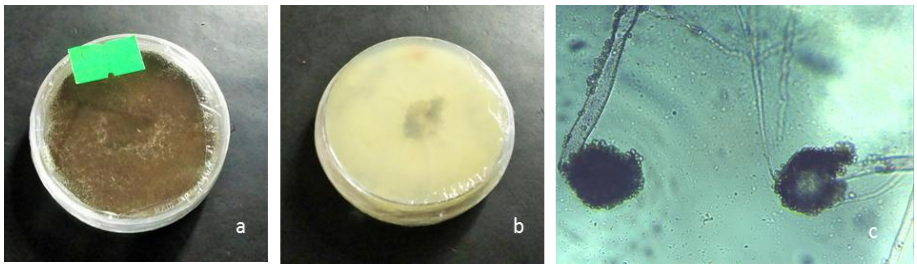

Fig. 15. Isolate MBE A5, sur PDA (a & b); microscopy (c) *A. aculeatus B*

3.9.3 *Fumigati section*

Aspergillus of the Fumigati section are characterized by blue-green, uniseriate conidial heads. The vesicles are conical with a row of phialides. Stipes are short, smooth and hyaline. On CYA, colonies are blue-green with dense, smooth, hyaline conidiophores. Heads are uniseriate. Conidiophores are hyaline and rough. Conidia are globose to subglobose and rough (Table 6). *Aspergillus* of *Fumigati* section found in this study are represented in Figs. 16, 17 and 18.

3.9.4 *Terrei* section

The *Terrei* section of *Aspergillus* are characterized by biseriate conidial heads of buff to brown color. The metules are as long as the phialides. Stipes are short, smooth and hyaline. On CYA, colonies are buff to sandy-brown with smooth, hyaline conidiophores. Heads are biseriate and compact. Conidiophores are hyaline and smooth. Conidia are globose to ellipsoidal (Table 6. Our result showed one species with the characteristic of *Terrei* section (Fig. 19).

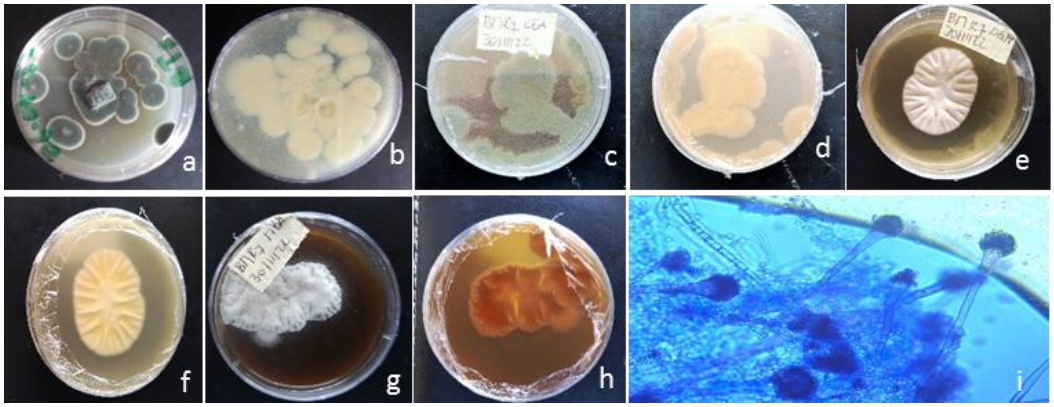

Fig. 16. Isolate BMR7 on PDA (a & b) ; on CEA (c& d) ; on DG18 (e& f) ; on MEA (g& h) ; microscopy (i) *A. fumigatus*

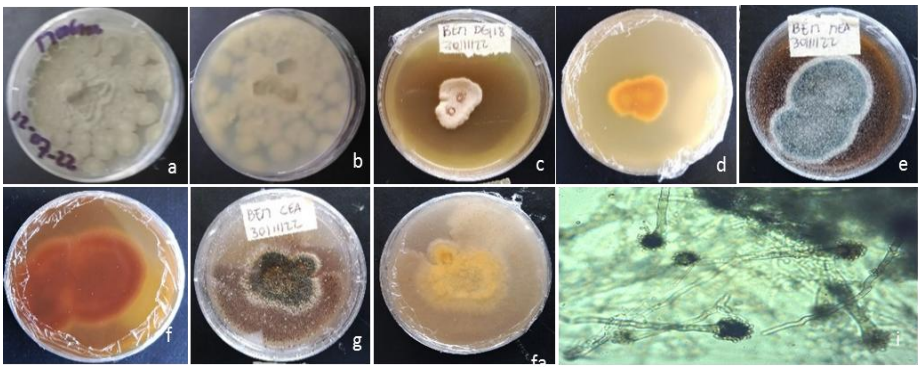

Fig. 17. Isolate BEMA3 on PDA (a & b) ; on DG18 (c & d) ; on MEA (e & f) ; on CEA (g & h); microscopy (i) *A novofumigatus*

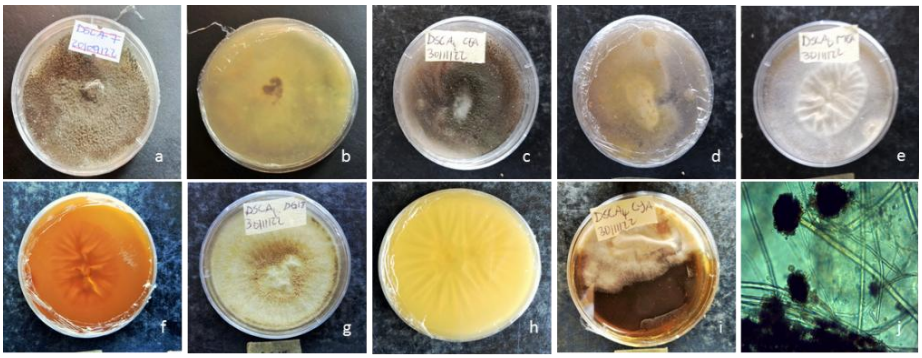

Fig. 18. Isolate DSGA7, on PDA (a & b), on CEA (c & d), on MEA (e & f), on DG18 (g&h), on CYA (i) ; microscopy (j) *A. duricaulis*

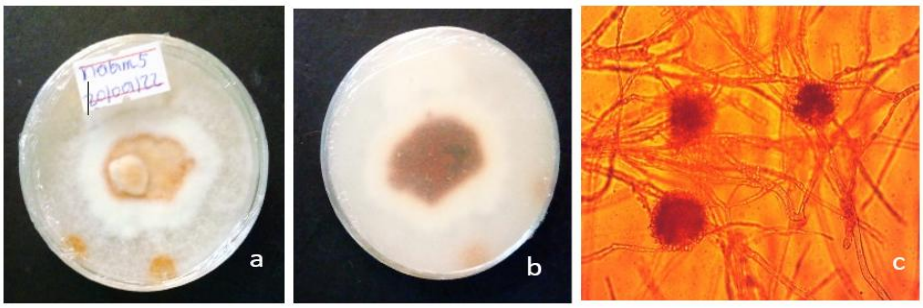

Fig. 19. Isolate MBAR5, on PDA (a & b) ; microscopy (c) *A terreus*

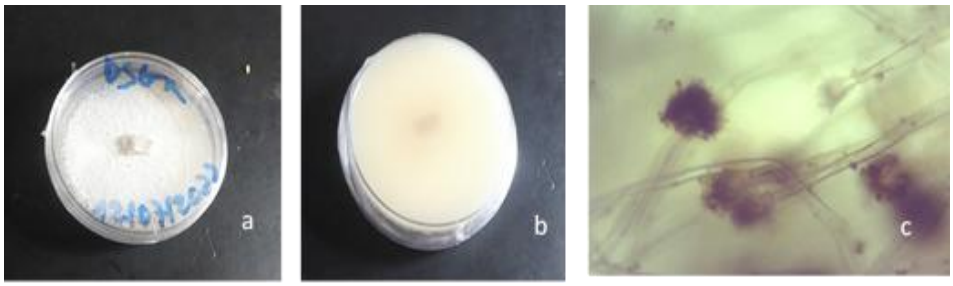

Fig. 20. Isolate DSGR2 , on PDA (a & b) ; microscopy (c) *A candidus*

3.9.5 *Candidi* section

This section is characterized by white, biserial or uniserial conidial heads. Vesicles are subglobose to globose and may be covered with phialides. Colonies were white with colorless mycelia and straw-colored undersides. Conidiophores are smooth and hyaline. Heads are biseriate or uniseriate. Conidiophores are hyaline and smooth. Conidia are globose, between 2 and 3 µm in diameter (Table 6). Our result showed one species with the characteristic of *Candidi* section (Fig. 20).

3.9.6 *Nidulant* section

The *Nidulante* section is *Aspergillus* are characterized by green colonies with white and brown margins on the reverse. Biseriate conidial heads. Metules are shorter than phialides. Conidiophore stipes are short, smooth and hyaline. On PDA, colonies are dark green with white outlines. Conidiophores are smooth and hyaline. Heads are biserial. Conidia are spherical and smooth, ranging from 3 to 4µm in diameter (Table 6). Fig. 21 is *A Nidulans* belonging to *Nidulant* section and isolated in this study.

3.9.7 *Circumdati* section

Aspergillus of Circumdati section are characterized by young to ochraceus-colored, biseriate conidial heads. The vesicles are globose and completely covered by phialides. Stipes are long, rough and hyaline. On PDA, the colony is orange or cinnamon, 40-50 mm in diameter, with an orange-yellow underside. Conidiophores are rough and hyaline. Heads are biseriate and compact. Conidial heads are very large (60 to 80 µm), globose (Table 6). *Aspergillus ochraceus* found during our isolation is one of the Circumdati section (Fig. 22).

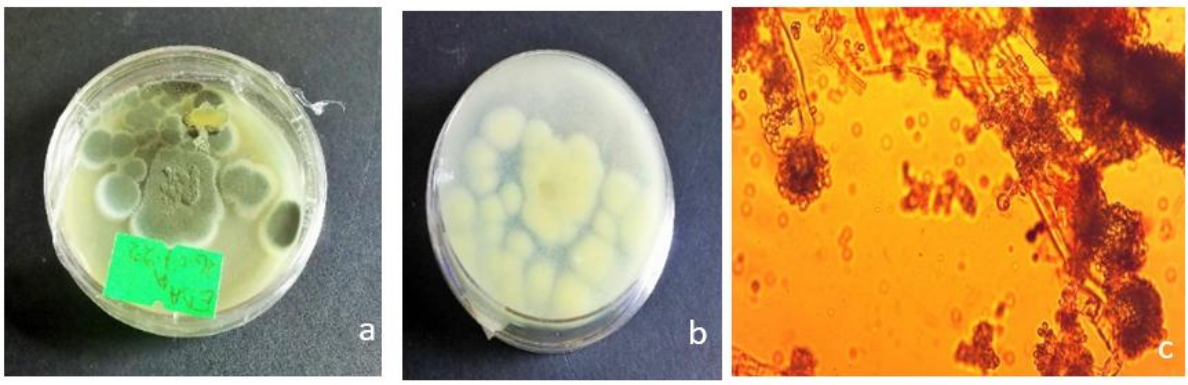

Fig. 21. Isolate EDAR12, on PDA (a & b) ; microscopy (c) *A Nidulans*

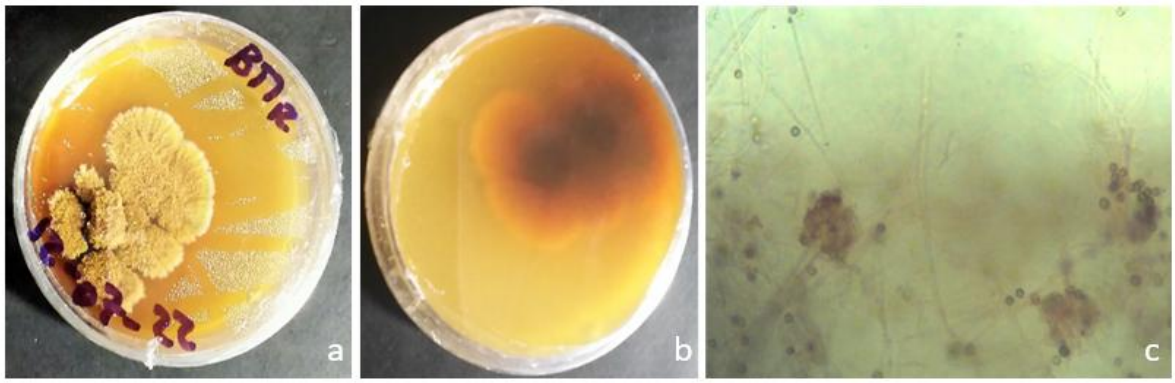

Fig. 22. Isolate BMR6, on PDA (a & b) ; microscopy (c) *A. ochraceus*

Table 6. Macrosciopic and microscopic characteristic of main *Aspegillus* isolated

Species	Colony size on PDA (mm)	Colony Texture	Conidiophore	Head type	Vesicle shape	Vesicle diameter (µm)	Conidiophore length (µm)	Color / Texture/ Shape	Conidial size (µm)
Section *Flavi*									
A. parasiticus A	25 - 40	Smooth	Unpa H	uni et bi	P/G	24 - 30	350-700	yg/r/G	4 - 5.8
A. parasiticus B	30 - 60	Rough	Unpa H	uni et bi	P/G	19 - 35	350-700	g/r/G	3 - 7
A. tamarii	45 - 55	Rough	Unpa H	bi	G	26 - 43	500-700	g/G	3 - 5
A. flavus	50 - 55	Rough	Unpa H	uni et bi	R	18 - 36	200-650	yg/r/G	3.5 - 5
A. oryzae	40-55	Rough	Unpa H	uni et bi	G	20-35	250-650	w/yg	3-5
A. nomius	45-55	Rough	Unpa H	uni	S/G	2.-40	200-500	y/w	3,5-4,5
Section *Fumigati*									
A. duricaulis	40 - 60	Smooth	Unpa H	uni	P to Clab	10 - 26	270-600	g/r/G	2 - 3.5
A. fumigatus	24 - 40	Smooth	Unpa H	uni	Spathulate to Clavate	19 - 31	250-650	g/r/G	2 - 3
A. novofumigatus (*A*)	25 - 30	Smooth	Unpa H	uni	P/R	21 - 29	300-600	g/s/G	4 - 7
Section *Nigri*									
A. carbonarius	40 - 60	Smooth	Unpa H	bi	Umbrella Shaped	41 - 60	850-2400	b/r/G	6 - 10
A. aculeatus (*A*)	37 - 49	Smooth	Unpa H	uni	G/S	48 - 74	770-2300	b/r/G	4 - 5
A. aculeatus (*B*)	50 - 60	Smooth	Unpa H	Uni	G	45 - 73	800-2000	b/r/G	4 - 5
A. niger	45 - 55	Smooth	Unpa H	bi	S/G	37 - 52	800-2500	b/r/G	4 - 6
A. japonicus	45 - 50	Smooth	Unpa H	uni	G/Elliptical	29 - 45	550-750	b/s/G	3 - 5
Section *Circumdati*									
A. ochraceaus	25 - 30	Rough	Unpa H	bi et uni	G	26 - 55	200-450	g/s/G	2.5 - 4
Section *Nodulant*									
A. nidulans	45 - 55	Smooth	Unpa H	bi	Spathulate/P	9 - 16	190-350	g/s/S	3 - 4
Section *Candidi*									
A. candidus	32 - 40	Smooth	Unpa H	uni	SG/G	17 - 30	150-300	w/s/G	2 - 3
Section *Terrei*									
A.terreus	20-40	Smooth	Unpa H	bi	G/Elliptical	18-28	120-290	w/b	1.5-2.5

The size of the colonies is after 7 days of incubation ; Unpa H = Unpartitioned Hyphae, Seriation ; uni = Uiseriate, bi = Biseriate ; P = Pyriform, G = Globose, R = Radiate, yg = Yellow Green, g = Green b = Brown, Orange, w = White, r = Rough, s = Smooth, G = Globose, E = Elliptical, S = Spherical, S/C = Short Columnar, L/C = Long Columnar, S/G = Subglobose.

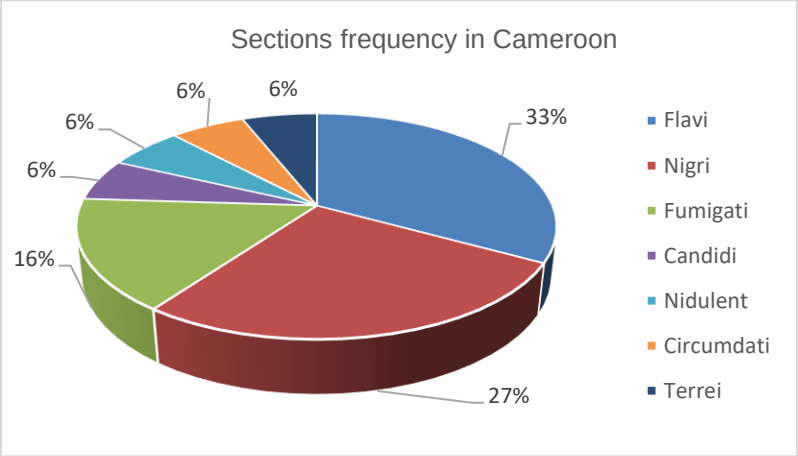

Fig. 23. Frequency of occurrence of *Aspergillus* spp. sections, all Zones combined

3.10 Frequency of occurrence of *Aspergillus* sections in Cameroon

Our results showed a variation in the frequency of the different *Aspergillus* sections. Globally whatever the AEZs takeng to account, different sections occurred in descending order Flavi section with the frequence of 33% > *Nigri* section with 27%, > *Fumigati* section with 16% then *Candidi*, *Terrei*, *Nidulent* and *Circumdati* sections with the frequence of 6%) for each one (Fig. 23).

4. DISCUSSION

Determination of aflatoxin levels in rice, maize, soybean and peanut samples from the various AEZs showed that 100% of these samples were contaminated with aflatoxins, with average levels ranging from 4.86 - 14.06 µg/kg, 5.50 - 16.33 µg/kg, 5.06 - 17.26 µg/kg, and 5 - 10.6 µg/kg respectively in soybean, maize, rice and peanut. The total aflatoxin tolerance limits for these foodstuffs, according to CODEX and EU standards, are 5 µg/kg for rice, 15 µg/kg for peanut, 15 µg/kg for maize and 5 or 10 µg/kg for soybean, depending on subsequent use. In this work, we note that only peanut strives to remain the standard if we stick to CODEX 2023. It should be noted that NJi et al., (2022) had already observed levels of aflatoxin contamination similar to, but not exceeding, average regulatory values while working on the biodiversity and distribution of aspergillus and their toxins in maize from the western and eastern regions of South Africa. similar values were also observed by Kana et al. (2013) in peanut meal intended for poultry feed formulation. In other hand, our results are lower than in Rodrigues et al. (2011) who reported that between February and October 2009, around 94% of maize in Nigeria and the border region of Cameroon was contaminated with aflatoxin, with an average concentration of 80.3 µg/kg. Our results also contrast with those of Kaaya and Kyamuhangire, (2006) who recorded 88%, 78% and 69% of aflatoxin-contaminated maize with mean levels of 30, 22 and 12.8 µg/kg respectively in the mid-altitude wet zone, mid-altitude dry zone and highland zone of Uganda. It is irrefutably clear that total aflatoxin levels varied not only by commodity, but also by AEZ. The results also show a variation in aflatoxin levels according to grain type. On the one hand, this could be explained by the fact that the composition of a substrate on which an aflatoxin-producing mold grows either positively or negatively influences aflatoxin biosynthesis. Liu et al, (2016) have shown that fatty substrates such as peanut favor the biosynthesis of aflatoxin B1 in contrast to defatted substrates such as rice. In contrast, Liu et al, (2016) showed that rice is one of the cereals in which aflatoxin levels are often the lowest. The results obtained show maize and rice samples to be the most contaminated, with no significant differences compared to soybean. These results can also be explained by the composition of rice, which is richer in sugars than peanuts. According to Achaglinkame et al., (2017) ; Wang et al., (2019), simple sugars such as glucose, sucrose, fructose and maltose favor aflatoxin production, unlike peptone, sorbose and lactose. Our study also shows that aflatoxin levels in foodstuff are correlated with temperature and with the relative humidity of the grains. The results show moisture levels of 13.7% (AEZ I) to 16.96% (AEZ V) in rice; 13.46% (AEZ IV) to 15.23% (AEZ I) in maize ; from 13.03% (AEZ V) to 14.7% (AEZ II)

in soybeans and from 13.16% (AEZ III) to 14.86% (AEZ V) in peanuts, offering a range of 13.03 to 16.96% ideal for aflatoxin production. This differs from the range (11.7 to 17.7%) obtained by Kana et al., (2013) in peanut meal intended for poultry feed formulation. On the other hand, these authors had shown that high relative humidity levels were the cause of high aflatoxin levels in peanut meal intended for animal feed formulation in three AEZ s in Cameroon. The high aflatoxin contamination of rice grains is also thought to be due to the long storage period before shipment to importing countries such as Cameroon, since most of the rice consumed by Cameroonians is imported. Variations in concentrations between AEZ can be explained by the fact that aflatoxin synthesis is influenced by climatic factors such as relative air humidity and temperature. The work of Muga et al., (2019) is highly evocative on this subject, as in their work on factors that can influence aflatoxin biosynthesis, they demonstrated that factors such as temperature, relative air humidity and CO_2 concentration significantly influence aflatoxin biosynthesis. It should also be noted that, in addition to the aforementioned factors which could explain these differences in concentrations between commodities and AEZ, there are also storage conditions and even the age of the grains (Jaibangyang et al., 2021).

Isolations showed that out of 100 grain samples, 99% reported very high mold contamination, with a predominance of *Aspergillus* spp. This high percentage had also been observed in Congo and South Africa by Ilunga et al., (2014) on peanuts and in Ethiopia by Assaye et al., (2016) on maize. on the other hand, Jaibangyang et al. (2021) observed a lower percentage than that observed in this work. Moreover, in their work, the genus Fusarium was predominant over the genus Aspergillus. our work presents the genus Aspergillus as being the predominant genus. This high presence of *Aspergillus* spp. in grains in storage in Cameroon could be explained by the fact that the climate is conducive for mold development. Indeed, Cameroon's relatively warm and humid climate is highly conducive to mold development. Yazdi et al.,(2006) have shown that mould infection of grains begins in the field and continues in storage. The prevalence of *Aspergillus* spp. was 44%. This can be explained by the fact that the total fungal flora of stored grains in tropical countries is essentially made up of highly sporulating molds, with great power of dissemination, of which *Aspergillus* is one of the most frequent genera. This predominance was also reported by Nyongesa et al., (2015). Characterization showed a predominance of isolates from section *Flavi*, followed by those from section *Nigri*, section *Fumigati*, section *Terrei* and finally section *Candidi*. Nyongesa et al., (2015), working on the diversity of *Aspergillus* spp. from maize in the field and in storage in Kenya, showed a predominance of section *Flavi* followed by section *Nigri* and *Penicillium*. They also report that each section not only has its own degree of dissemination, but also of resistance to environmental conditions and competition. Similarly, Zahng et al. (2016) put forward the argument of high capacity for dissemination and resistance to environmental conditions to explain the near ubiquity of molds of the genus Mucor in air and agricultural soils. The results obtained would find justification in this assertion, as individuals belonging to certain sections are found in almost all food samples from the various AEZs, while others appear to be specific to certain AEZs. It's also important to note that in this study, we found abundance and diversity to differ between the AEZs and localities sampled. This could be explained by the fact that climatic factors such as temperature and humidity, which are specific to each zone or region, influence both the abundance and diversity of Asperigillus in a given area. In other words, the number and activity of fungal populations change from one region to another. Moreover, in its 2021 report, the Council for Agricultural Science and Technology (CAST 2021) points out that grain infection rates depend on the year's climatic and harvesting conditions. Furthermore, according to Ngoko et al., (2001) and Lanyasunya et al. (2005), storage conditions as humidity, temperature and ventilation system have a major influence on mold development during storage in Cameroon and Kenya respectively. This study also shows that, contrary to the work of Liu et al.,(2016), which positions rice as one of the grains least likely to be colonized by molds, maize and peanuts were the least contaminated by *Aspergillus* spp. As Miller (1995) concluded, this high contamination of rice compared to groundnuts, soybeans and maize, would be due to the long storage time of rice in exporting countries before export to rice-importing countries. In fact, around 2/3 of the rice consumed in Cameroon is imported, mainly from Asia where shipments destined for export are generally stored in warehouses for a long period before taking the long road (months) to importing countries, following the First In First Out (FIFO) method.

5. CONCLUSION

Ultimately, the aim of this study was to identify *Aspergillus* spp. isolated from maize, soybean, rice and groundnut in storage, and examining their aflatoxin contamination in the five agro-ecological zones of Cameroon. The results show that 745 mold speciemens were isolated from 21 genera, namely : *Aspergillus, Metharizium, Fusarium, Rhizopus, Penicillium, Trichoderma, Alternaria, Apophysomyces, Botrytis, Basidiobolus, Paecilomyces, Conidiobolus, Bipolaris, Mucor, Cunningamella, Curvularia, Epicoccum, Gliocladium, Lichteimia, Pithomyces* and *Syncephalastrum*. The prevalence of the *Apergillus* genus was 44.83%, with 21.85% from peanuts, 27.84% from rice, 21.56% from corn and 28.74% from soybeans. Among *Aspergillus* spp. the *Flavi* section represented 33%, the *Nigri* section 27%, the *Fumigati* section 16% and the *Nidulant* (6%), *Candidi* (6%), *Terrei* (6%) and *Circumdati* (6%) sections were the least represented. The aflatoxin assay showed a contamination rate of 100%. However, AEZ V (9.63 µg/kg) recorded the highest average content, and zone IV (5.94 µg/kg) the lowest. Among foodstuffs, aflatoxin levels were highest in corn samples (9.46 µg/kg), followed by rice (8.92 µg/kg). On the other hand, aflatoxin levels were lower in peanut samples at 6.55 µg/kg. In addition, all the levels in the 04 foodstuffs were above the threshold levels stipulated in current regulations.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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