Occurrence of mycotoxin-producing molds isolated from stored peanut grains from different markets in Brazzaville, Congo.

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Abstract- The objective of this study was to evaluate the microbial quality of stored peanut grains. A total of twelve (12) samples of stored peanut grains without pods were randomly collected from the warehouses of the Total, Texaco and PK markets in the city of Brazzaville, Congo. A microbiological analysis of the mycoflora of the stored peanut grains and their capacity to produce mycotoxins was performed. The identification results showed the presence of a diversity of molds including *Aspergillus* (57%), *Alternaria* (2%), *Cladosporium* (5%), *Fusarium* (4%), *Mucor* (9%), *Penicillium* (9%), *Phoma* (1%), *Rhizopus* (7%), *Scedosporium* (6%), some of which are incriminated in mycotoxins production. Some isolates of molds of the genus *Aspergillus*, *Penicillium* and *Fusarium* showed a potential for mycotoxin production on CAM agar (27%) and others on YES broth coupled with thin layer chromatography (60%). At the end of this analytical study, the stored peanut grains from the different markets in Brazzaville were all contaminated with molds, some of which are toxigenic, which is a global health problem.

Keywords- Microbial quality, peanut, molds, mycotoxins

I. INTRODUCTION

Peanuts are among the most widely consumed food grains in the world in their various forms, grains, oil, porridge, flour, cake and paste. In Africa, peanuts are grown locally, but some of them are imported, stored in warehouses and sold in the various markets in the cities [1]. In order to be able to consume these food grains in the long term, throughout the year, it is necessary to store them in warehouses [2], but unfortunately, in these warehouses, which are often makeshift, without any sanitary agents, the proliferation of microorganisms is favoured, among which are storage molds, which are one of the agents of deterioration that contribute to altering the marketable and hygienic quality of the stored grains [3].

The majority of molds that most often contaminate stored peanut grains are likely to be toxigenic, harmful to the health of consumers, as they have the capacity to produce mycotoxins [4]. Mycotoxins are secondary metabolites mainly produced by molds belonging to the species *Aspergillus*, *Penicillium* and *Fusarium*, which have adverse health effects on consumers, animals and humans, including hepatic, carcinogenic, immunosuppressive, teratogenic [5] and infertility effects [6].

Numerous minor and major cases leading to death from food poisoning related to the consumption of stored food grains sold in markets have already been reported in most Central, West and East African countries [7][8][9][10].

In the Republic of Congo, peanuts are also highly valued by the population as part of daily life. Peanuts are most often bought in warehouses located in the markets of the big cities. However, poor conditions in the warehouses can influence the quality of the peanuts sold in the market warehouses as they favour the presence of toxic molds that can harm the health of consumers. It should be remembered that contamination of peanuts can occur at any stage, from the field to the warehouse and even during the processing of peanuts into by-products. This shows the need to analyse the mycoflora of peanuts stored in the different markets of Brazzaville to have an aspect on the level of microbiological qualities in order to evaluate their sanitary impacts, it is thus in this register that this study falls.

II. MATERIAL AND METHODS

2.1. Study area

The different warehouses of the three most popular large markets in the city of Brazzaville located in the different districts were selected as sampling locations. The laboratory aspect of this research was carried out at the Laboratory of Cellular and Molecular Biology, Faculty of Science and Technology, Marien Ngouabi University, Brazzaville, Congo.

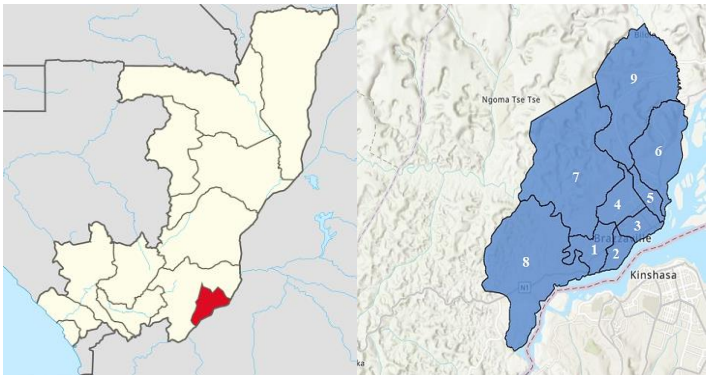


Figure 1: Districts of Brazzaville (Congo Republic)
[1] Makelekele, [2] Bacongo, [3] Poto-Poto, [4] Moungali, [5] Ouenze, [6] Talangai, [7] M'filou-Ngamaba, [8] Madibou, [9] Djiri

2.2. Sampling

A total of twelve (12) samples of stored peanut grains, each weighing 500g, were collected from warehouses in different markets in Brazzaville, Congo, over a three-month period (October, November, December) in 2020. The samples were packed in sealed plastic bags, labelled and transported to the laboratory where they were analysed.

Table 1: Distribution of sample collection by source

Source	Warehouse	Number
Total market - Bacongo district (2)	W1, W2, W3, W4	4
Texaco market - Ouenze district (5)	W1, W2, W3, W4	4
PK market - M'filou-Ngamaba district (7)	W1, W2, W3, W4	4

2.3. Isolation of molds

Isolation of the molds was carried out by preparing the stock solution of 25g of crushed peanut grains sample, mixed with 225mL of sterile physiological water and followed by the method of serial decimal dilutions in test tubes. 0.1mL of each dilution obtained was deposited on the surface of Petri dishes containing Sabouraud agar and spread with a rake. The plates were then incubated at 37°C for 5 days in an oven [11]. After colony growth, purification of the mold isolates was carried out by transferring them to fresh sterile Sabouraud agar using the centre-point technique according to Nguyen [12].

2.4. Morphological identification of isolated molds

Morphological identification of molds at genus level was carried out on pure fungal cultures by phenotypic typing based on the observation of macroscopic characteristics of the thallus (shape, colour, texture) and microscopic characteristics of the filaments (septum, branching, conidia, conidiophore, sporangiospore) by comparing the data of the characteristics obtained with those of the reference books containing the identification keys of molds [13][14][15][16].

2.5. Screening of mycotoxins production

Screening for mycotoxin production capacity was performed on potentially toxigenic molds on solid and liquid media.

2.5.1. Screening of mycotoxins production on solid medium

Coconut milk agar medium (CAM) was used for the rapid screening of toxicogenic molds. 1.5g of agarose was mixed with 100mL of distilled water and sterilised. 5mL of coconut milk was aseptically added to the sterilised medium and poured into the Petri dishes. A section of young mycelium was placed in the centre of the Petri dish containing the CAM medium and incubated at 25°C for 5-7 days. Mycotoxin production was characterised by the presence of a fluorescent halo (blue, green, blue-green) around the colony revealed by the application of UV light calibrated at a wavelength of 365nm as described by Mohamed and others [17][18].

2.5.2. Screening of mycotoxins production on liquid medium

Yeast extract and sucrose (YES) liquid medium was used to screen for mycotoxin-producing molds. 0.5g of yeast extract, 1g of sodium chloride (NaCl) and 2g of sucrose were mixed with 100mL of distilled water and dispensed in 9mL volumes into test tubes and sterilised in an autoclave. After sterilisation, a portion of each fungal mycelium from 48h colonies was transferred to individual test tubes and incubated at room temperature in the dark for 14 days. The biomass formed was removed by filtering the YES medium through a Wattman filter paper. The separation of the molecular compounds was performed by thin layer chromatography (TLC). After homogenisation of the test tubes, a volume of 5mL was pipetted into falcone tubes, 5mL of chloroform was added and homogenised, then centrifuged at 12000rpm for 10min. 20µl of each fungal supernatant was placed on a silica gel chromatography plate spaced 2cm apart and 20µl of sterile YES liquid medium supernatant was used as a control. The plate was then placed in a chromatography tank, soaked in 150mL of an elution solvent mixture of chloroform and acetone (88:12) respectively as the mobile phase. After migration, the elution product was evaporated at room temperature and the plate was examined under a UV lamp at a wavelength of 365nm. The presence of the mycotoxin is reflected by a fluorescence of the spots on the chromatographic plate as described by Seifa [19].

III. RESULTS

3.1. Isolation of molds

Isolation of molds from stored peanut grains revealed mycelial thallus of different morphotypes. Eighty-two (82) molds of different morphotypes were selected, coded and purified for identification.

Figure 2: Mold isolated on Sabouraud agar (2 days)

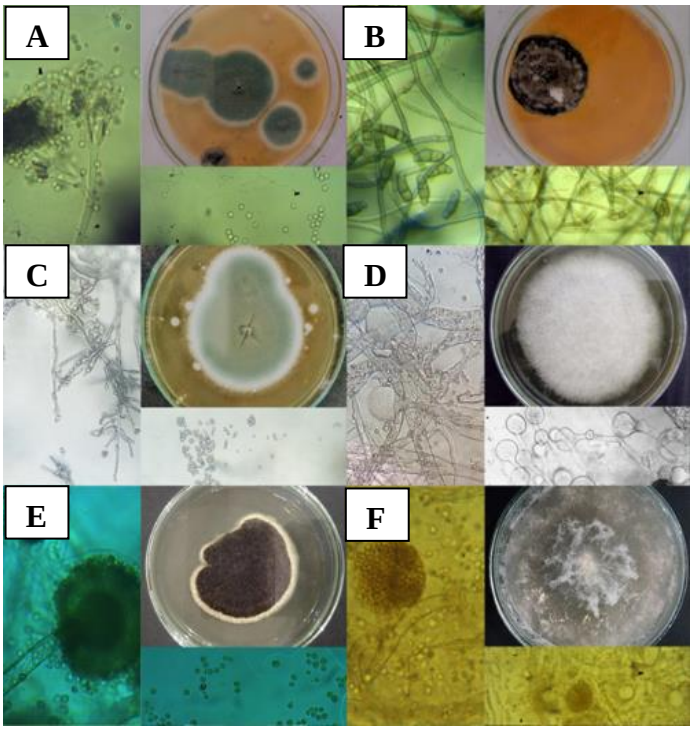
The different codes assigned to the purified mold isolates based on different district markets, different warehouses, are represented in the table below.

Table 2 : Code for mold isolates

Source	Number	Warehouse	Code
Total market	35	W1	G1, G2, G3, G4, G5, G6, G7, G8, G9, G10, G11
		W2	A1, A2, A3, A4, A5, A6, A7, A8, A9, A10
		W3	C1, C2, C3, C4, C5, C6, C7, C8, C9
		W4	D1, D2, D3, D4, D5
Texaco market	26	W1	B1, B2, B3, B4, B5
		W2	V1, V2, V3, V4, V5, V6, V7, V8, V9
		W3	J1, J2, J3, J4, J5, J6, J7, J8
		W4	L1, L2, L3, L4
PK market	21	W1	M1, M2, M3
		W2	P1, P2, P3, P4, P5
		W3	H1, H2, H3, H4, H5, H6, H7
		W4	N1, N2, N3, N4, N5, N6

3.2. Morphological identification of isolated molds

Phenotypic typing of the 82 mold isolates revealed thallus of different aspects, cottony, powdery, yellow, white, blue, black, green and slow and fast growing. Microscopic characterization revealed septate and non-septate filaments, circular, ovoid, macro and micro curved spores as well as conidiophores and sporangiospores.



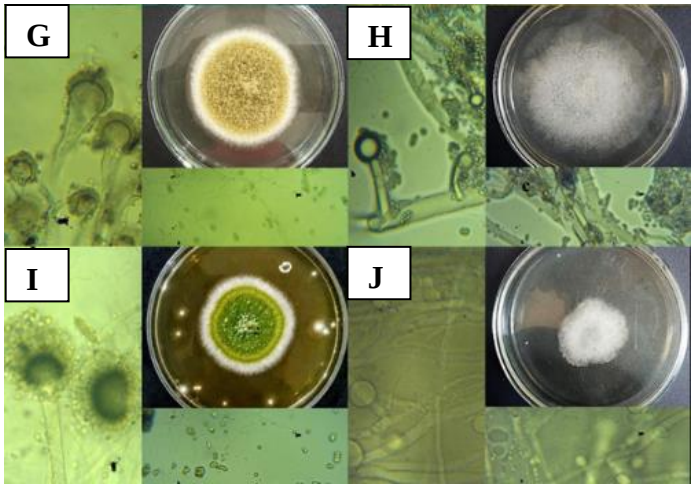


Figure 3: Macroscopic and microscopic images at 40× magnification of different mold isolates
[A] **V3**, [B] **P2**, [C] **V1**, [D] **J1**, [E] **A9**, [F] **G2**, [G] **D5**, [H] **G4**, [I] **M1**, [J] **H6**

The identification results following the macroscopic and microscopic characteristics revealed the fungal genera, illustrated in the table below.

Table 3: Different types of mold genera identified and related to each coding

Code	Genus	Code	Genus
M1	*Aspergillus sp1*	**P1**	*Alternaria sp1*
A4	*Aspergillus sp2*	**P2**	*Alternaria sp2*
G6	*Aspergillus sp3*	**V1**	*Cladosporium sp 1*
G7	*Aspergillus sp4*	**V2**	*Cladosporium sp 2*
A5	*Aspergillus sp5*	**H1**	*Cladosporium sp 3*
G10	*Aspergillus sp6*	**H2**	*Cladosporium sp 4*
D4	*Aspergillus sp7*	**V7**	*Fusarium sp1*
D5	*Aspergillus sp8*	**V8**	*Fusarium sp2*
J6	*Aspergillus sp9*	**V9**	*Fusarium sp3*
B4	*Aspergillus sp10*	**A1**	*Mucor sp1*
B5	*Aspergillus sp11*	**A2**	*Mucor sp2*
L4	*Aspergillus sp12*	**A7**	*Mucor sp3*
J4	*Aspergillus sp13*	**C5**	*Mucor sp4*
L1	*Aspergillus sp14*	**C6**	*Mucor sp5*
L2	*Aspergillus sp15*	**G4**	*Mucor sp6*
P3	*Aspergillus sp16*	**G5**	*Mucor sp7*
P4	*Aspergillus sp17*	**V3**	*Penicillium sp1*
P5	*Aspergillus sp18*	**V4**	*Penicillium sp2*
H3	*Aspergillus sp19*	**C1**	*Penicillium sp3*
H4	*Aspergillus sp20*	**C2**	*Penicillium sp4*
G1	*Aspergillus sp21*	**C7**	*Penicillium sp5*
G3	*Aspergillus sp22*	**C8**	*Penicillium sp6*
G8	*Aspergillus sp23*	**C9**	*Penicillium sp7*
G9	*Aspergillus sp24*	**G2**	*Phoma sp1*
A8	*Aspergillus sp25*	**D1**	*Rhizopus sp1*
A9	*Aspergillus sp26*	**D2**	*Rhizopus sp2*
D3	*Aspergillus sp27*	**J1**	*Rhizopus sp3*
B1	*Aspergillus sp28*	**J2**	*Rhizopus sp4*
B2	*Aspergillus sp29*	**J7**	*Rhizopus sp5*
V5	*Aspergillus sp30*	**J8**	*Rhizopus sp6*
V6	*Aspergillus sp31*	**N1**	*Scedosporium sp1*
J5	*Aspergillus sp32*	**N2**	*Scedosporium sp2*
B3	*Aspergillus sp33*	**H5**	*Scedosporium sp3*
L3	*Aspergillus sp34*	**H6**	*Scedosporium sp4*
J3	*Aspergillus sp35*	**H7**	*Scedosporium sp5*
N5	*Aspergillus sp36*		
N6	*Aspergillus sp37*		
M3	*Aspergillus sp38*		
N3	*Aspergillus sp39*		
N4	*Aspergillus sp40*		
A6	*Aspergillus sp41*		
G11	*Aspergillus sp42*		
A3	*Aspergillus sp43*		
A10	*Aspergillus sp44*		
C3	*Aspergillus sp45*		
C4	*Aspergillus sp46*		
M2	*Aspergillus sp47*		

The overall identification of molds revealed a great diversity of molds at the level of stored peanut grains, among which several genera, namely nine (9) genera of molds in total, including *Aspergillus* (57%), *Alternaria* (2%), *Cladosporium* (5%), *Fusarium* (4%), *Mucor* (9%), *Penicillium* (9%), *Phoma* (1%), *Rhizopus* (7%), *Scedosporium* (6%), as indicated below.

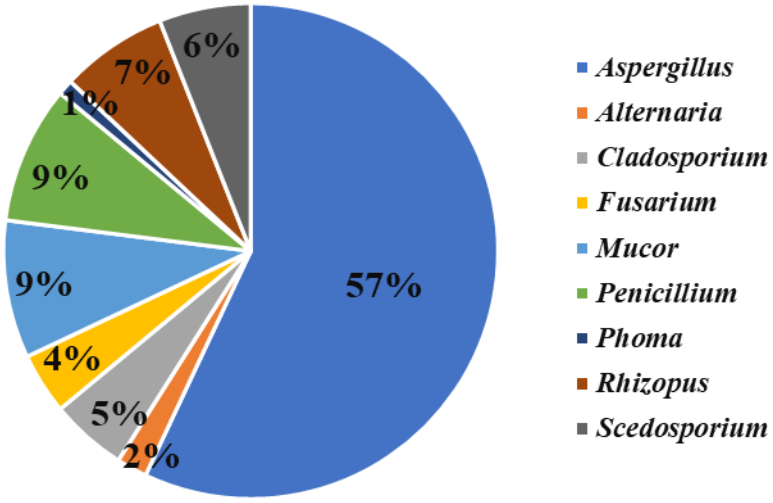


Figure 4: Global distribution of identified molds

The distribution of molds isolated and identified according to sampling points mainly in the city's markets is presented in the following the figure below.

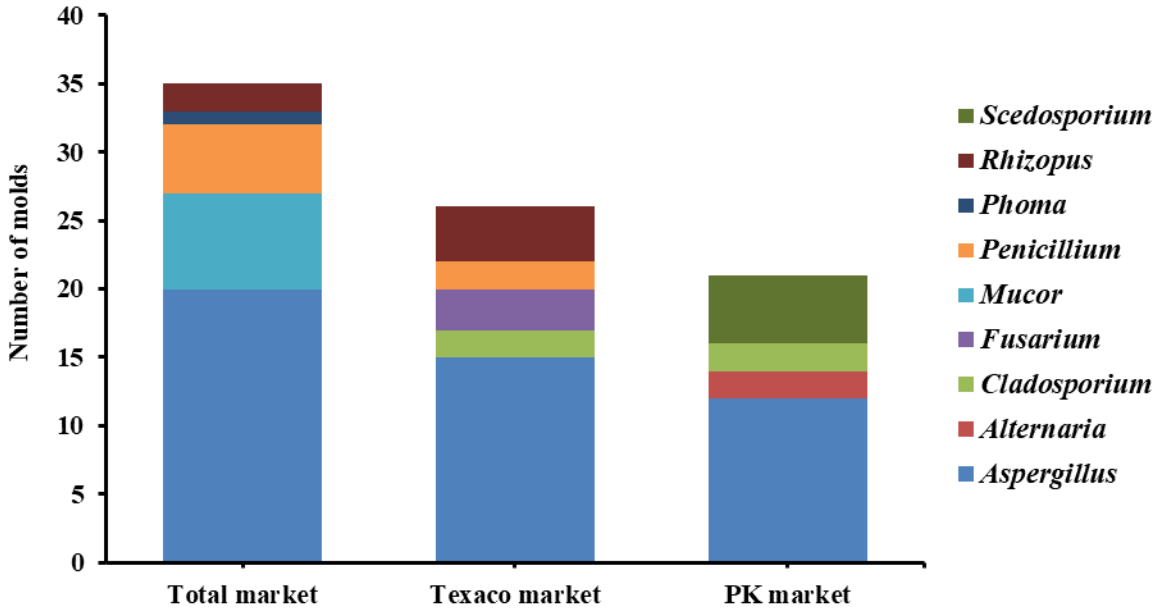


Figure 5: Mold identified according to markets

3.3. Screening of mycotoxins production

Mycotoxin production capacity was evaluated in potentially toxigenic molds of the genera *Aspergillus*, *Penicillium* and *Fusarium*, largely being incriminated in mycotoxin production. The different isolates selected have been represented in the table below.

Table 4: Molds tests

Genus	Code
Aspergillus	M1, G10, B4, H4, A8, D3, J6, L4
Fusarium	V7, V8, V9
Penicillium	C1, C2, V3, V4

The test of mycotoxin production capacity of the colonies found in the Petri dishes was performed on CAM agar. The presence of a fluorescent ring around the colonies found in the Petri dishes under UV light was characteristic of mycotoxin production. Below are the different observations.

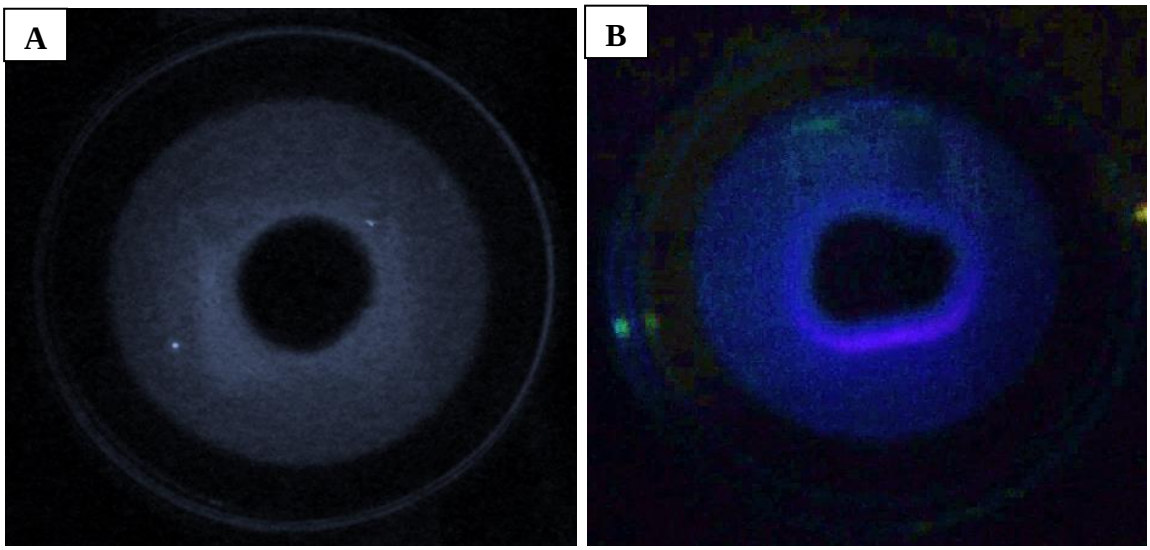


Figure 6: Visualisation of mycotoxins on agar medium (CAM) under UV light
[A] Petri dish containing CAM medium with a colony of mold A8 negative to mycotoxin production photographed under UV light (365nm) in a transilluminator, [B] Petri dish containing CAM medium with a colony of mold M1 positive to mycotoxin production photographed under UV light (365nm) in a transilluminator.

Below the TLC Plates demonstrated the fluorescence of mycotoxins under long wave UV illumination (365nm). The mycotoxin bands are noted on the right side of the image. The YES standard, as negative control which has no band, is noted on the lower left line.

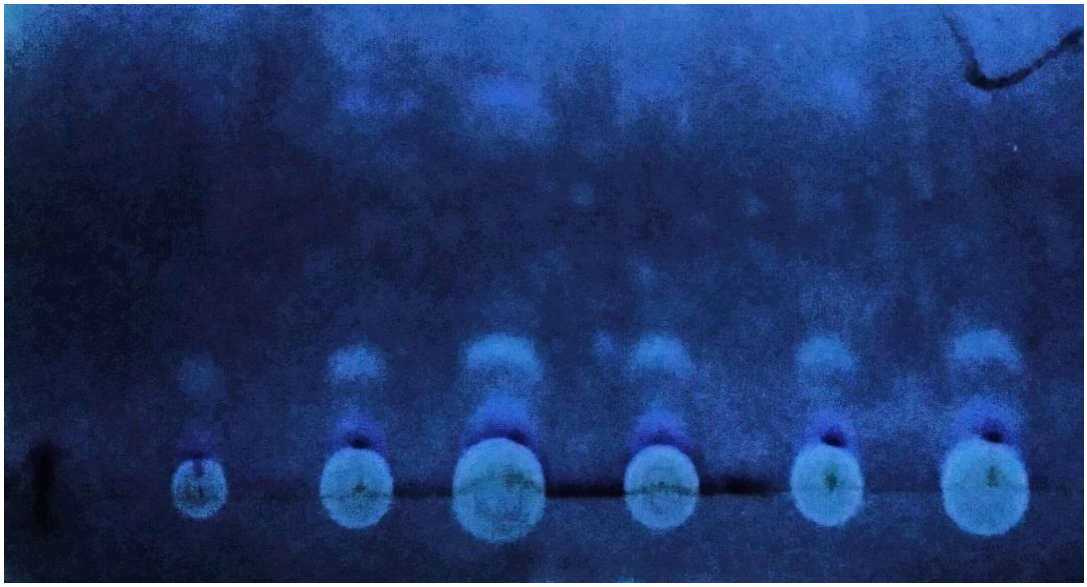


Figure 7: Visualisation of mycotoxins spots on thin layer chromatography (TLC) under UV light

The comparison of mycotoxin production capacity by toxigenic molds isolates performed under UV at 365nm on CAM agar medium and on YES medium associated with thin layer chromatography has been represented in the table below.

Table 5: Distribution of molds according to the tests carried out

Genus	*Aspergillus*								*Penicillium*				*Fusarium*		
	M1	G10	B4	H4	A8	D3	J6	LA	C1	C2	V3	V4	V7	V8	V9
CAM	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-
TLC	+	+	+	+	-	-	-	-	+	+	+	+	-	+	-

IV. DISCUSSIONS

Molds are unpredictable and unavoidable contaminants in food and feed throughout the world. Their presence in food grains poses a serious risk to human and animal health, while contributing to massive economic losses for the agricultural industry. Microbial analysis of a food provides information on the number and types of microorganisms present, which gives an indication of the quality of the sample and the severity of the potential risk to sensitive consumers. Fungal analysis of peanut grains showed that all samples collected from Total, Texaco, PK markets were contaminated with mold, such results have already been recorded in peanut grains stored in Cote d'ivoire, Benin, Nigeria, Kenya [20][21], which also showed a high prevalence of molds on the grains. This justifies that peanut grains are an excellent substrate for molds.

The identification of the fungal flora reveals field molds such as *Mucor*, *Alternaria*, *Rhizopus* and storage molds such as *Aspergillus*, *Penicillium* and *Fusarium*. These molds are often commonly associated among the constant mycoflora of stored grains and responsible for the deterioration of the grains via their enzymatic potentials, as these results are identical to those found by Abdulla and others [22][23], regarding the types of fungal flora present in stored grains.

It was found the low prevalence of field molds of the genus *Mucor* (9%), *Fusarium* (3%), *Rhizopus* (7%) etc. These results are similar to the one found by Laouid [24], in terms of a low occurrence. This is normal because the persistence of field molds in storage may be an indication of the short storage time of the grain bags, of the inefficiency of physical preventive methods, but also of the ability of their spores to resist the storage conditions. The presence of molds of the Mucorales group can also be dangerous because they are also incriminated in most rare infections of the Mucormycosis type that affects the sinuses or lungs after inhalation of airborne fungal spores whose clinical complications are vascular invasion causing necrosis of infected tissues and perineural invasion followed by death [25].

Also the daily inhalation of fungal spores by traders in warehouses could lead to fungal infection.

Considering that molds of the genus *Aspergillus* were dominant in the fungal flora, the strong presence of this genus was also demonstrated by Temesgen and others [23], in their work. Molds of the genus *Aspergillus*, *Penicillium* and *Fusarium* are qualified as potential toxigenic. The high prevalence of these genera could therefore be a source of mycotoxins mainly aflatoxin and ochratoxin as already demonstrated in the work of El-Khoury and others [26][27].

V. CONCLUSION

This study showed that all peanut grains collected in the three markets of the city of Brazzaville were heavily contaminated with a number of toxigenic molds. The genus *Aspergillus* was distributed in all collected grain samples. The high contamination of stored peanut grains by *Aspergillus* molds in the samples is alarming because *Aspergillus* and some isolates of *Penicillium* and *Fusarium* are toxigenic, and some have been shown to produce mycotoxins. The high presence of molds of the mucoral group such as *Mucor*, *Rhizopus* etc, which are opportunistic pathogens, would also be a danger factor. Such results invite us to identify the factors failing in the preservation of the grains at the level of the warehouses to improve the conditions of storage, but also it becomes important to evaluate the presence of mycotoxins and to quantify it at the level of the stored peanut grains in order to prevent any sanitary risk related to their consumption.

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