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Aflatoxigenic potential of *Aspergillus* section *Flavi* isolated from maize seeds, in Burkina Faso

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The frequency of occurrence and four principal kinds of aflatoxin concentration in maize seeds grown in Burkina Faso was investigated. Ten (10) samples collected, were analyzed by high performance liquid chromatography (HPLC) with post-column derivatisation after immunoaffinity column cleanup. Eight strains of *Aspergillus* section *Flavi* were previously isolated from these samples and cultivated on "*Aspergillus flavus* and *parasiticus* agar (AFPA)" to ascertain if they belong to *A. flavus* or *A. parasiticus* species. The qualitative ability of aflatoxin production was also previously performed by fluorescence emission under ultra violet light at 365 nm after four (4) days of incubation at 30 °C on Coconut Agar Medium (CAM). Results showed that 70% of samples were contaminated by aflatoxins. The levels ranged from 0.93 to 58.94 µg/kg. Samples M1 and M10 had high concentrations, 58.94 µg/kg and 70.73 µg/kg; whereas M4 and M5 had low concentrations from 1.68 to 0.93 µg/kg, respectively. In these samples, four were contaminated with aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1), two with AFB1 and aflatoxin B2 (AFB2) and one (01) with AFB1 only. We notice that AFB1 was the most prevalent member of aflatoxins, and AFG2 was absent in all samples.

Key words: Maize, *Aspergillus*, aflatoxins, HPLC, Burkina Faso.

INTRODUCTION

Cereals are a staple food for humans and animals. In Burkina Faso, their annual consumption is estimated at 62% of the food consumed by households (Waongo et al., 2013). Among food crops, maize is the most used product by over 98% of rural households (Bambara, 2021). In Burkina Faso, maize ranks second among

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cultivated cereals, both in area, production and consumption (Somda, 2016). It also makes a significant contribution to the country's economies. The area devoted to maize cultivation in the country increased from 790,321 ha in 2010 to 1,019,181 ha in 2018 (GDSSS/MAHAD, 2020). National maize production reached 1,700,127 T in 2018 against 1,133,480 T in 2010 (GDSSS/MAHAD, 2020). Maize is grown widely around the tropical world owing to its good adaptation to climate and its popularity. Besides being distributed widely, maize can be used for many purposes, such as animal feed, industrial uses, and is even the staple food in many developing countries. It also makes a large contribution to the economies of developed and developing countries. Nevertheless, the aflatoxin phenomenon is undermining the sector in Africa (Chauhan et al., 2016). Thus, maize is food crop that is easily contaminated with mycotoxins such as aflatoxins that are cancer-causing, immuno-suppressive mycotoxins (Makhlouf, 2019). According to Bambara (2021), 40% of maize production is affected by aflatoxins in developing countries. Contamination of crops with aflatoxin is a global food safety issue (Compaoré et al., 2021). The most important members of AFs are AFB1, AFB2, AFG1 and AFG2. The International Agency for Research on Cancer (IARC) has classified AFB1, AFB2, AFG1 and AFG2 as Group I human carcinogens (IARC, 2002). Among the many known toxins in the world, aflatoxins are highly toxic and carcinogenic compounds that can cause diseases in livestock and humans (Ouattara-Sourabié, 2018). AFs are a group of mycotoxins produced as secondary metabolites by species in *Aspergillus* section *Flavi*. The species most notorious for aflatoxin production are *Aspergillus flavus* (produces only aflatoxins B) and *Aspergillus parasiticus* (produces both B and G aflatoxins) (Kachapulula et al., 2017).

Previous studies in Africa have found that the occurrence of aflatoxins in food products is mainly influenced by favorable conditions such as high moisture content and temperature (Waré et al., 2017). In fact, in the Sahelian zone dry, post-harvest conservation is the only means of ensuring the link between the harvest occurring once in the year and consumption that is permanent and obligatory. The harvests, kept in general under inappropriate conditions, are attacked by insects, rodents and molds (Waongo et al., 2013). Also, in Burkina Faso the improper storage or preservation methods used such as maize bad drying would also lead to attacks by micro-organisms including molds of the genus *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* (Sanou, 2000). Aflatoxins contamination of maize has always remained a topic of debate in terms of international market as well as economic development of country which are part of trade market (Chauhan et al., 2016). In view of the huge economic losses and health problems caused by mycotoxins; a great deal of interest is currently accorded to them throughout the world. Thus to guarantee the health of consumers, each country is obliged to adopt specific legislation for the main mycotoxins in foods liable to harbor toxigenic molds (Waré et al., 2017). Several countries in the world have established or proposed regulatory limits for mycotoxins in foods. European Union countries edited regulations that have been revised periodically to limit their presence in the foods in Europe (EU Regulation 1881, 2006). In Africa certain countries also have regulations on mycotoxins and produce significant research, especially on aflatoxins and fumonisins that affect health (Ezekiel et al., 2014). Unfortunately, Burkina Faso, a country of West Africa has not set a mycotoxin regulation and uses those of *Codex alimentarius* which fixes the maximum levels of AFB1 and total aflatoxins respectively at 2 µg/Kg and 5 µg/Kg (EU Regulation 1881, 2006). Indeed, there are few studies and surveys about the contamination of mycotoxins in maize in Burkina Faso and this does not really reflect the problem in our country (Sanou, 2000). Our farmers therefore often find it difficult to export their products to countries whose mycotoxin regulations are in force. Indeed, because crops in tropical and subtropical regions are more susceptible to contamination due to favorable climatic and their inadequate storage conditions which facilitate the proliferation of molds and their secondary metabolites (Waré et al., 2017). Therefore, the aim of the proposed work is to determine the fungal load of maize samples from Ouagadougou City in Burkina Faso and to quantify the concentration of all kinds of aflatoxins using high performance liquid chromatography. This will confirm the results of the qualitative demonstration of aflatoxins production capacity of molds previously isolated from these maize samples, such as fluorescence emission under ultra violet light at 365 nm and those in "*A. flavus* and *parasiticus* agar (AFPA)".

MATERIALS AND METHODS

Physicochemical analyses

The maize samples were first subjected to physicochemical analyses such as humidity and pH using standards methods. The tests were repeated three times.

Sampling and fungi isolation from maize seeds

A total of ten (10) maize samples in commercially available were collected during the period from December to February 2021 at Ouagadougou markets. Fungi were isolated and purified on Potato Dextrose Agar (PDA) and subculture in "*Aspergillus flavus* and *parasiticus* agar (AFPA)" to identify *A. flavus* and *A. parasiticus* according to Pitt et al. (1983) and Cotty (1994) protocols. Systematic determination and the strains identification were made on Potato Dextrose Agar (PDA) at 25 and 37 °C depending on the methods used by Christensen (1981), Hocking (1982) and Cooney and Emerson (1964). Inoculation was done in three points equidistant.

Reference strains

For comparison of cultural and microscopic characters between strains isolated from maize seeds and those of reference strains, three references belonging to *Aspergillus* section *Flavi* were used. They were UBOCC-A-106031 (*A. flavus* aflatoxinogenic) of French origin, UBOCC-A-111042 (*A. parasiticus var. globosus* aflatoxinogenic) of Japanese origin and S_2 (*A. flavus* aflatoxinogenic) previously isolated from groundnuts and identified in Burkina Faso using molecular method by two (2) PCR based on 28S ribosomal sub unit (D1-D2 region) and the hyper variable ITS1-5.8S-ITS2 region, (Compaoré et al., 2016). This comparison was performed on Potato Dextrose Agar (PDA) medium at 30 °C for seven days.

Aflatoxins quantification

Sample extraction (AOAC, 2005)

Aflatoxins production is confirmed by HPLC using aflatoxins B and G Standard, Romer Biopure, and a blank consisting of the extraction solution. HPLC analysis was performed at the Toxicological Department of the National Public Health Laboratory (LNSP) in Ouagadougou. Ten (10) maize samples were subjected to this analysis.

The principle was used to extract aflatoxin from the samples using suitable organic solvents, to purify this aflatoxin on an immunoaffinity column and then to identify and quantify it. To do this, 25 ± 0.2 g of mix ground maize was weighed and added approximately 3 g of sodium chloride and placed in blender cup. 125 ml of extraction solution methanol-distilled water (70:30; v/v) was added to the sample and the whole was stirred for 20 min. The solution was then filtered through Whatman No.4 filter paper and 15 ml of the filtrate was transferred into a beaker and diluted with 30 ml of distilled water. The diluted homogenized sample solution was filtered through glass microfiber membrane.

Sample cleanup

The immunoaffinity columns were previously conditioned by passing 10 ml phosphate buffered saline (PBS) through the column by gravity and placed on the variant cuvette; the silica gel was allowed to flow. 15 ml of the diluted filtrate (1g sample equivalent) was taken and poured into the immunoaffinity column where it retained the desired molecules. The molecules were then washed with 10 ml of distilled water which was poured twice into the immunoaffinity column. Under gravity, the bound aflatoxins were eluted with 1 ml of pure methanol and air was pushed through the column to collect the last drops of eluate. 1 ml of distilled water was added to the eluate. The eluate was filtered through 0.45 µm Methanol-compatible membrane filter (PTFE or Nylon) and collected into a micro sample vial. Then this solution containing the aflatoxin molecules was sent to the HPLC for the detection and quantification of these aflatoxin molecules. Each experiment was conducted in triplicate and aflatoxins contents were determined according to their corresponding standard curves.

HPLC analyses

Spiked samples were prepared on relevant matrix for each batch of sample to obtain the % recovery. 0.5 ml of the working solution was added (100 µg/kg B1, G1 and 25 µg/kg B2, G2) per 25 g of matrix, mixed well and proceeded to sample extraction. The chromatographic system consisted of an automatic Agilent 1200, with Immunoaffinity column cleanup and post-column derivatisation manufactured by Shim-pack VP-ODS, 4.6 mm (ID) x 150 mm (L). The post-column derivatisation was achieved using a Kobra Cell to obtain electrochemically generated bromine (ISO 16050 CEN/TC-34, 2006); Romer Biopure). It is equipped with an auto-sampler (10 µl, injector vol), a Shim-pack VP-ODS column with a Reverse-Phase C18 (4.6 (ID) x 150 mm (L)) and a fluorescence detector. The detector was set at EX= 350 nm, EM = 450 nm. The mobile phase was isocratic and composed of methanol: water (45:55)-KBr-HNO_3 mixture with 450 ml of methanol, 119 mg of potassium bromide and 87.5 µl of 16 M nitric acid per liter of mobile phase. The flow rate was set at 1 ml/min.

The quality control standard (40 µg/kg calibration solution) was injected 3 times. The % CV of the peak area corresponding to the consecutive injections shall be within ± 10%. 10 µl of each of the Calibration Standards Solutions (2.5 µg/kg, 5 µg/kg, 10 µg/kg, 20 µg/kg, 40 µg/kg and 80 µg/kg) was injected into the HPLC system, followed by sample.

The concentration of Aflatoxins in the sample is calculated using the following formula:

$$\text{C spl } (\mu g/kg) = \frac{\text{A spl}}{\text{Gradient of calibration curve}} \times \frac{\text{Inj Vol std}}{\text{Inj Vol spl}} \times F$$

Where

A spl : Peak area of sample

$$\text{Gradient of calibration curve}: \frac{\text{Peak area of standard}}{\text{Concentration of standard}}$$

F : Dilution Factor per gram (× 200)

$$F: \frac{\text{Total sample extract (ml)} \times \text{Vol of final sample extract } (\mu l)}{\text{Volume for column (ml)} \times \text{Vol for injection into HPLC } (\mu l) \times \text{Weight of sample(g)}}$$

Statistical analysis (XLSTAT. 2016)

The differences in aflatoxins concentration in maize samples between the Ouagadougou zones Burkina Faso and those of physicochemical analyzes were compared by Analysis of variance (ANOVA) using XLSTAT-Pro 7.5.2 software. Interpretation of values was performed using Newman-Keuls test at probability level p = 5%. The results were expressed as mean ± SD and the measures were repeated three times (n=3).

RESULTS AND DISCUSSION

Physicochemical analyses of maize sample

Humidity rate analysis carried out on the ten (10) samples of maize seeds revealed that our samples are not very humid with an average value between 3.91 and 4.66% (Table 1).

These values are lower than the average moisture content of mold growth. In fact, the minimum humidity for certain molds to start growing is 10% (Compaoré, 2017). Our data could be explained by contamination of samples in the field or during storage. Mold strains are able to survive unfavorable conditions for their growth by producing large numbers of spores (Tabuc, 2007). Thanks to its great adaptability to environmental conditions,

Table 1. Humidity rate of maize samples

Sample	Humidity (%)
M1	4.10 ± 0.07[cd]
M2	4.45 ± 0.07[ab]
M3	4.07 ± 0.07[cd]
M4	4.71 ± 0.07[a]
M5	3.99 ± 0.07[cd]
M6	4.22 ± 0.07[bc]
M7	4.48 ± 0.07[ab]
M8	3.91 ± 0.07[d]
M9	4.66 ± 0.07[a]
M10	4.26 ± 0.07[bc]
Significance level	< 0.0001

For each column and for each maize sample, the means which are in common the same letter are not significantly different according to the Newman-keuls test at the 5% probability level.

Table 2. pH values of maize samples.

Sample	pH
M1	5.93 ± 0.04[a]
M2	5.83 ± 0.04[a]
M3	5.89 ± 0.04[a]
M4	5.95 ± 0.04[a]
M5	5.98 ± 0.04[a]
M6	5.94 ± 0.04[a]
M7	5.96 ± 0.04[a]
M8	5.99 ± 0.04[a]
M9	5.93 ± 0.04[a]
M10	5.96 ± 0.04[a]
Signifiance level	< 0.0001

For each column and for each maize sample, the means which are in common the same letter are not significantly different according to the Newman-keuls test at the 5% probability level.

A. flavus can grow both on crops in the field, during harvest as well as later, during storage (Makhlouf, 2019). For the different samples, statistical analyses showed significant differences between the different humidity values. From this analysis it appears that the humidity is very variable from one sample to another. The pH results of the various maize samples analyzed indicate that all of the samples are slightly acidic, with pH values ranging from 5.83 to 5.99 (Table 2).

The pH values are very favorable to the growth of molds because according to Gauthier (2016), molds can grow in a pH range from 3 to 8, with optimal growth being rather between 5 and 6. The production of mycotoxins takes place for pH close to optimal growth pH (Makhlouf, 2019). Due to their acidity, many foods are much more prone to fungal than bacterial spoilage (Tabuc, 2007). Statistical analyses showed no significant difference between the different pH values.

Fungi isolation from maize seeds

In our study, ten (10) maize samples were used as a matrix for the collection of strains of *Aspergillus* section *Flavi*. From the consortium of fungi grown on maize, twenty-three (23) isolates were collected. Macroscopic observation of the Petri dishes made it possible to retain only the isolates forming colonies of yellowish and greenish color and have powder aspect. In optic microscopy, we were interested in those with non-septate and hyaline conidiophores. Eight (08) local *Aspergillus* section *Flavi* strains were therefore isolated from the

Table 3. Number of *Aspergillus* section *Flavi* isolates from collected maize samples.

Sample	Number of *Aspergillus* section *Flavi* isolates	Isolates code
M1	1	A_2
M2	1	A_5
M3	0	-
M4	0	-
M5	3	A_4, A_6 et A_8
M6	0	-
M7	0	-
M8	0	-
M9	1	A_3
M10	2	A_1 and A_7
Total	8	-

consortium of fungi grown on maize seeds (Table 3).

Aflatoxins level in maize samples

Results of the quantitative aflatoxin analysis are reported in Table 4. 70% of the samples were found to be contaminated with total aflatoxins. These were M1, M2, M4, M5, M6, M9 and M10. The total aflatoxin contents in the maize samples ranged from 0.93 to 70.78 µg/kg. M1 and M10 had high concentrations (58.86 and 70.78 µg/kg respectively) and M4 and M5 had low concentrations with respective values of 1.67 and 0.93 µg/kg. In all the contaminated samples, aflatoxin B1 was present and the most concentrated with however the absence of aflatoxin G2 as it can be seen from the chromatograms in Figure 1. Out of seven (7) samples, four (04) were contaminated with aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1), two (02) with AFB1 and aflatoxin B2 (AFB2) and one (01) with AFB1 only.

The contamination of our samples with aflatoxins could be explained by the presence of toxigenic strains *Aspergillus* section *Flavi*. Indeed, the latter are the fungal contaminants most frequently encountered in cereals and which secrete aflatoxins there when environmental conditions (temperature, humidity, pH, etc.) are met, as is the case with the pH of our maize samples which are slightly acidic with a pH around 5.83 to 5.99 (Table 2). Maize is considered among the fragile crops at high risk of contamination by toxigenic molds unlike other cereals, particularly barley and wheat (Brahmi and Zahi, 2016).

According to the *Codex alimentarius*, the maximum levels of AFB1 and total aflatoxins (B1, B2, G1 and G2) are respectively 2 and 5 µg/Kg (EU Regulation 1881, 2006). Our results revealed that the contamination rate of the different samples varied from sample to sample. Four (04) samples had total aflatoxin levels between 13 and 71 µg/Kg and therefore well above the *Codex alimentarius* standard. Only the M2, M3, M4, M5, M7 and M8 samples complied with the standard. The high concentration of aflatoxins in our samples could be explained by climatic conditions in Africa, poor agricultural practices and poor crop storage conditions. According to Zinedine (2004), crops grown in hot, humid climates and exposed to toxigenic molds provide optimal conditions for mold growth and can be contaminated with mycotoxins. According to Makhlouf (2019), the infestation of maize, by *Aspergillus flavus* (producer of AFB) before harvest, is often linked to the aggression of the plant by insects and rodents in the field. Cereals can be contaminated either by the spores that are initially found in the cereals or later during storage mainly if it is bad (Waré et al., 2017). Indeed, overripe crops and grain damaged during threshing are postharvest conditions that promote fungal growth in crops (Bhat et al., 2015). In addition, crops, usually stored in inadequate conditions, are attacked by insects, rodents and molds (Waongo et al., 2013). In Burkina Faso, the ears of maize are kept either in bundles or sheaves and hung from branches or above the hearths, or in straw or mud granaries. These storage systems do not protect the food from direct attack by insects and many other pests such as birds and rodents which carry microorganisms (Somda, 2016).

Aflatoxin B1 (AFB1) was predominantly present in all seven (7) contaminated samples with concentrations ranging from 0.54 ± 0.08 to 63.24 ± 0.07 µg/Kg. The maximum values of AFB1 that we obtained are much lower than the results obtained by Vargas et al. (2001) in Brazil, where the level of contamination of maize by aflatoxins reached 129 µg/kg. Nevertheless, our results are superior to those obtained in Ethiopia by Chauhan et al. (2016) which was 53 µg/Kg.

Aflatoxins are produced mainly by *Aspergillus* section *Flavi* species, mainly *A. flavus* and *A. parasiticus*. These two species are both producers of type B aflatoxins, but *A. parasiticus* also produces type G aflatoxins (Vargas et al., 2001). Out of seven (07) samples, three (03) were only contaminated with AFB. *Aspergillus spp.* isolated from these

Table 4. Aflatoxin level in maize samples.

Aflatoxin (µg/kg)	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	*Codex* limit
B1	55.70± 0.21[b]	1.81± 0.04[e]	nd	1.67± 0.12[e]	0.54± 0.08[f]	9.58± 0.05[c]	nd	nd	6.73± 0.06[d]	63.24± 0.07[a]	2
B2	3.16± 0.07[b]	nd	nd	nd	nd	nd	nd	nd	nd	7.54± 0.11[a]	-
G1	nd	0.62± 0.04[c]	nd	nd	0.39± 0.01[d]	5.73± 0.06[b]	nd	nd	6.39± 0.03[a]	nd	-
G2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	-
Significance level					< 0.0001						
AFs	58.86	2.43	nd	1.67	0.93	15.31	nd	nd	13.12	70.78	5

nd= not detected. For each line and for each maize sample, the means which are in common the same letter are not significantly different according to the Newman-keuls test at the 5% probability level.

maize samples would therefore belong either to *A. flavus* or to *A. parasiticus*. Four (04) on the other hand were contaminated with AFB and AFG, indicating the presence in these samples of strains of mold belonging to the species *A. parasiticus*. Samples M1, M4 and M10 would therefore be contaminated by *Aspergillus flavus* strains while M2, M5, M6 and M9 would be contaminated by *A. parasiticus*.

The presence of the different types of aflatoxins in our maize samples would confirm the morphological characterization of our different isolates carried out previously. Thus, the A_1, A_2 and A_7 isolates from sample M10 would be strains of *A. flavus*. A_5 isolated from M2; A_4, A_6, A_8 isolated from M5 and A_3 isolated from M9, would all be strains of *A. parasiticus*. Indeed, in a previous study by Compaoré et al. (2021), we determined by qualitative methods the ability of A_2 and A_3 isolates to produce aflatoxins through blue fluorescence emission when cultured on Coconut Agar Medium (CAM). Both isolates were also subcultured on "*Aspergillus flavus* and *parasiticus* Agar" (AFPA) medium a four days incubation at 30 °C to study their ability to produce aspergillic acid. The results showed that both isolates are capable of producing aflatoxin. Reference strains UBOCC-A-111042 and S_2 which are aflatoxinogenic were also tested in order to compare the obtained results with those of our two maize isolates. The results showed that both isolates are capable of producing aflatoxin. In the present study, the quantitative analysis of the different types of aflatoxins came to confirm the morphological identification carried out previously.

Aflatoxins production and their determination by fluorescence HPLC

Results of the qualitative aflatoxin analysis are reported in Figure 2. Aflatoxin production abilities tested previously by fluorescence under UV light of strains by cultivating them on Coconut Agar Medium (CAM) and AFPA medium were in concordance with those obtained by HPLC determination (Table 5). We have found that both A_2 and A_3 isolates showing fluorescence under UV light produced aflatoxins in CAM.

Conclusion

The present study included ten (10) samples of maize seeds grown in Burkina Faso. The results of the aflatoxin analysis showed that the majority of these are of unsatisfactory sanitary quality and have a fairly high average mold load predominantly dominated by genus *Aspergillus*. A total of eight (8) fungal strains belonging to *Aspergillus* section *Flavi* were isolated and characterized. The quantitative aflatoxin analysis method such as HPLC performed in the present study attests to the results of aflatoxin-producing ability previously performed and confirms that isolates A_2 and A_3 belong respectively to *A. flavus* and *A. parasiticus*. Nevertheless, this identification should be confirmed by Biology molecular methods. In view of the high levels of aflatoxins in cereals in Burkina Faso and the danger represented by the ingestion of contaminated food. The players in the maize sector must observe the Good Practice pre and post-harvest as well as decontamination methods such as biopreservation by lactic acid bacteria and *Bacillus* in order to preserve the health of consumers.

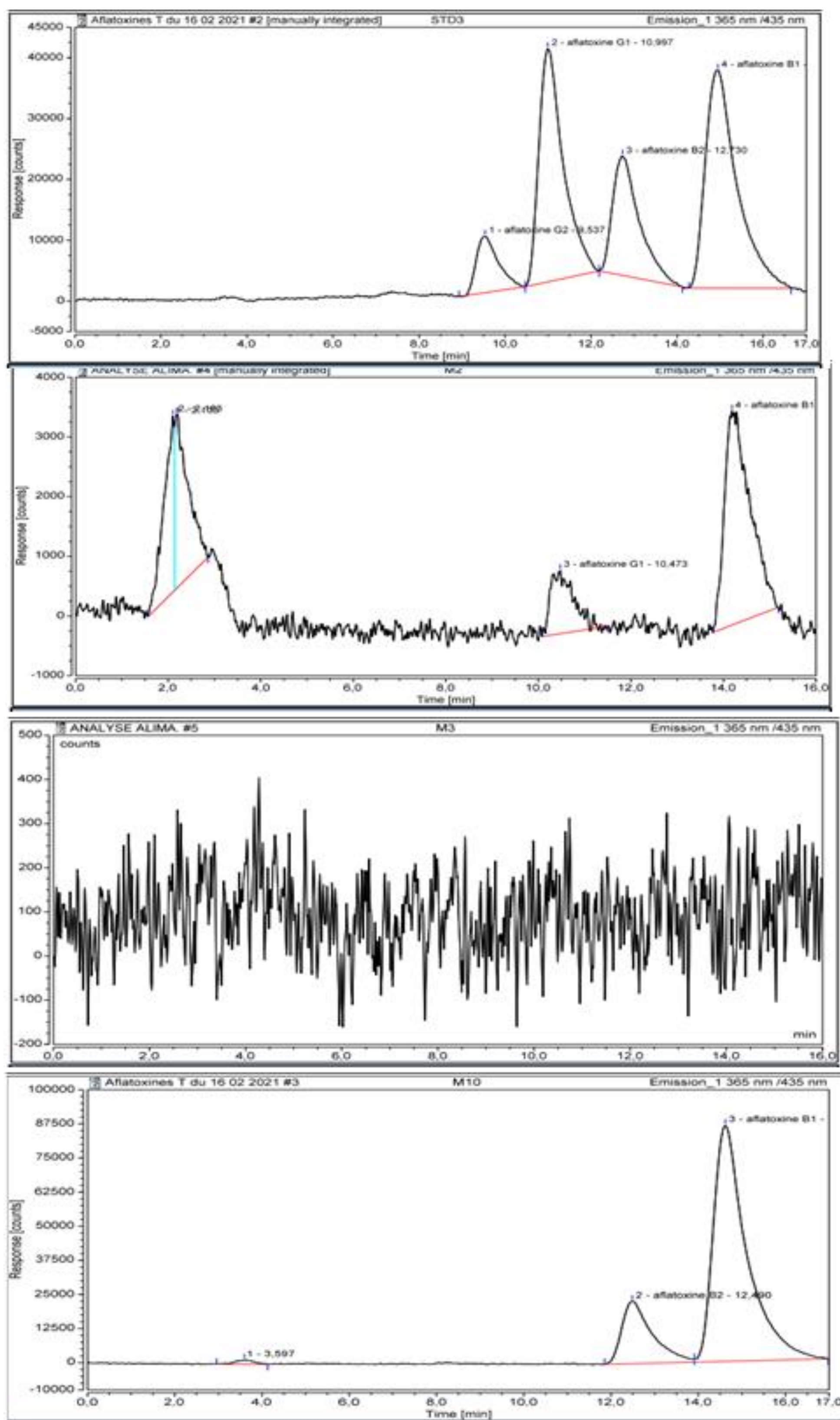

Figure 1. HPLC chromatograms of aflatoxins standards (AFB1, AFB2, AFG1 and AFG2); AFB1 and AFG1 in sample M2, lake of Aflatoxin in sample M3, AFB1 and AFB2 in sample M10.

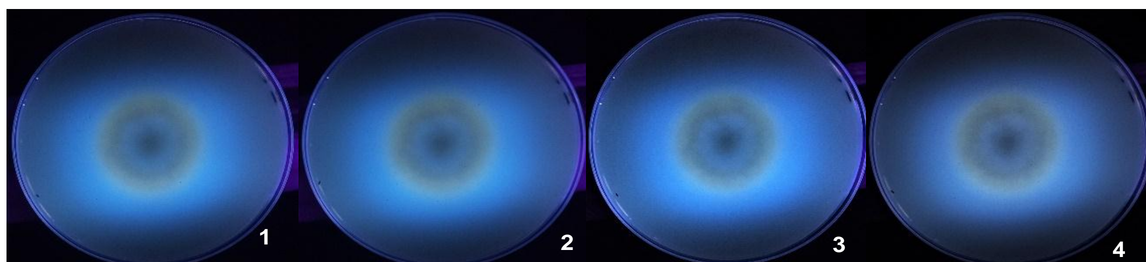

Figure 2. Showed the detection of aflatoxigenic strains by Coconut Agar Medium under UV light at 365 nm on the fourth (4th) day of incubation at 30 °C. (1) control of aflatoxigenic strain UBOCC-A-111042, (2) S_2 aflatoxigenic, (3) A_2 isolate and (4) A_3 isolate showing a blue-green fluorescent ring around the colony.

Table 5. Comparison of aflatoxigenic strains responses for three methods of identification (response on AFPA, fluorescence under UV light on CAM and HPLC response of maize samples source of A_2 and A_3 *Aspergillus spp.* isolates).

Isolate	Response on AFPA	Fluorescence under UV light on CAM	Aflatoxins production in maize sample
A. flavus UBOCC-A-106031 France	+	+	na
A. parasiticus var. globosus UBOCC-A-111042 Japan	+	+	na
A_2	+	+	+
A_3	+	+	+
A_5	+	+	+
A_4, A_6 and A_8	+	+	+
A_1 and A_7	+	+	+

(-) negative; (+) positive; (na) not analyzed

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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