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Full Length Research Paper

Impact of agro-ecological areas on the distribution of aspergillus section flavi in maize in Benin

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Maize is the first cereal grown in the world and adapted as a food crop in sub-Saharan Africa. In Benin, it is the leading cereal crop with nearly 70% of the cereal area sown. But the post-harvest storage conditions of these cereals favour the development of moulds and mycotoxins. Previous work has revealed *Aspergillus flavus* and aflatoxins in stored maize, naturally occurring substances that are carcinogenic to humans. The objective of this work is to evaluate the contamination of maize in storage by agro-ecological area. To do this, the study had collected 50 samples from différents agro-ecological zones in Benin. On them, evaluated aflatoxin contamination by HPLC-PDA, determined total fungal flora by direct culture and aflatoxinogenic nature of strains of *Aspergillus Flavi* by culture in différents media and thin layer chromatography. At the end of this work, 30.43% of the samples collected are contaminated by aflatoxins with an average of 6,41µg/kg. Contaminated samples were taken from agro-ecological zones 1, 3, 5 and 6 with mean aflatoxins levels of 1.88 µg/Kg; 5.81 µg/Kg; 8.38 µg/Kg and 9.59 µg/Kg respectively. Mycological analysis revealed five fungal genre : *Aspergillus* (53.38%), *Fusarium* (23.31%), *Penicillium* (17.29%), *Rhizopus* (2.26%) and *Mucor* (1.50%). By agro-ecological zone, a similarity is noted with regard to the total fungal flora. Of the 67 strains of *Aspergillus Flavi* isolated, 65 are aflatoxin-producing and the highest incidence found in agro-ecological zone 6 followed by zone 5. The contamination of maize stored in Benin by aflatoxins and the various subspecies of the *Flavi* section follows a distribution by agro-ecological zone due to different climatic conditions. The production of a distribution map of pathogenic strains by speculation could help to monitor the evolution of the quality of agricultural products.

Keywords: Map, aflatoxins, fungi, climate

INTRODUCTION

Aflatoxins are mycotoxins produced mainly by fungi, whose main producers are the species *Aspergillus flavus* and *Aspergillus parasiticus*, widespread throughout the world and especially in tropical countries but which have

appeared in recent years in temperate countries (Battilani et al., 2016). This raises the problem of redefining the geographical distribution map of fungal strains. Some countries, such as Benin, which is relatively hot due to its geographical location, have different characteristics from one region to another. These temperatures are stable all year round in the south (28 to 32°C) and oscillate between 30 and 38°C in the north of the country and rainfall is abundant during the rainy seasons. All these climatic conditions are adapted to the development of fungi and the production of aflatoxins. On the basis of soil and climate factors and the different crops grown, eight (08) agro-ecological zones are distinguished, which offer a variability of conditions for the development of *Aspergillus* aflatoxin producers. This work focused on the geographical distribution of *Aspergillus* strains from the *Flavi* section found in maize, the most widely produced and consumed cereal in Benin (PSRSA, 2016). and which is highly susceptible to this contamination. (Hell et al., 1995 and 2003; Bandyopadhyay et al., 2007).

1. MATERIAL AND METHOD

1.1 Sampling

The collection of maize samples (*Zea mays* L.) was carried out during the month of August 2018 in the main markets of the localities where agricultural products converge in each of Benin's eight (8) agro-ecological zones. These are the cities of : Malanville, Kandi, N'dali, Nikki, Natitingou, Djougou, Bassila, Parakou, Tchaourou, Glazoué, Bohicon, Abomey-Calavi, Cotonou, Kétou and Porto-Novo. To ensure the representativeness of the samples, four (04) 250g samples were collected per site. Before collection, resellers were asked to complete a questionnaire that took into account the following parameters: harvesting season, storage location and storage time.

1.2. Fungal contamination

The direct seeding technique is used. Three maize grains treated with the Usler method are grown on dextrose agar potatoes (PDA). The Petri dishes were incubated at 25 ± 2°C for 5 to 7 days. And the different colonies transplanted onto agar extract malt medium for identification. The morphological identification of the species was done according to the descriptions made by Pitt and Hocking, 2009. All the culture media used are Oxoids industry branded.

1.3. Molecular characterization

Fungal strains identified as belonging to the *A. Flavi* section are molecularly characterized.

- *DNA extraction*

To extract genomic DNA, each isolate was cultured on PDA. After culture at 25°C for 5 days in the dark, mycelium is collected and DNA prepared by extraction of phenol-chloroform according to the method of Aamir et al., 2015. Total DNA was quantified and visualized by gel electrophoresis to ensure quality. In addition, PCR amplifications of the B-tubulin region were used to verify the quality of genomic DNA extraction.

- *Polymerase chain Reaction*

PCR is performed according to Adjovi et al, 2014. Four genes belonging to the aflatoxin biosynthesis pathway (AflO, AflD, AflP and AflR) have been amplified. The primers used were at a concentration of 100 pmol/µl, the amount of DNA 50 ng, Taq Ozyme Purple mix 2, 25µl for a total volume of 50 µl.

PCR conditions were as follows: denaturation at 95°C for 5 minutes; 40 cycles of 94°C for 45 seconds, specific priming temperature (at 60°C for b-tubulin and 58°C for all others) for 1 minute, and extension to 72°C for 1 minute; and a final extension at 72°C for 10 minutes. The reactions were carried out in the Thermal Cycler in Eppendorf. The PCR primers used are described by Adjovi et al., 2014 and presented in Table 1.

- *Restriction fragment length Polymorphism (RFLP)*

The RFLP of a B-tubulin segment was performed to identify the clade of *Aspergillus* species isolated from the *Flavi* section. After PCR of the partial sequence of b-tubulin B, 20µL of product is digested by the enzyme BstYI. The profiles to be obtained for species close to the subgroups of the *Flavi* section are : *A. flavus* (336, 438, 581bp), *A. parasiticus* (176, 263, 334, 576bp).

- Metabolic profile

Extraction

Aflatoxins were extracted from 7-day cultures of morphologically identified *Aspergillus* Section *Flavi* strains on malt extract agar (MEA). Each crop is extracted with methanol / water (80/20, V / V). The extracts are evaporated to dry at 60°C. The dry extract taken up is absorbed in methanol 400µl.

Aflatoxins detection

Aflatoxins were detected by thin layer chromatography. 10 µL extracts and aflatoxin standards (Sigma Aldrich) were

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Table 1: Primers

Genes	Nucleotides
b-tubulin control	Btub1F: GTTCACCTTCAGACCGGCCAGTG
	Btub1R: CTCCTTCATGGAGACCTTTCCGCG
b-tubulin 2 (RFLP)	Bt2a : GGTAACCAAATCGGTGCTGCTTTC
	Bt2b : ACCCTCAGTGTAGTGACCCTTGGC
AflD	AflDF : CGGTGTATTTGGTCACCGGGGC
	AflDR : CGGCTGCCTGGGCATCAGTTTC
AflQ	AflQF : CGTTATGGGAGGATCGGACACG
	AflQR : CCCAGATCTGATCCTCCTGCG
AflP	AflPF : GGGCATTCATGCCTTGGTTG
	AflPR : CCCATACCTAGATCAAAGCGG
AflR	AflRF : GTCGATTTCTTGGCCGAGTC
	AflRR : CTCAGCAAGTAGCCATCCTG

deposited on a fluorescent chromatographic plate (1.05553. DC Alufolien Kieselgel 60). The migration solvent is a mixture of ether / methanol / water (96 : 3 : 1, v / v / v / v / v / v). After migration the plates are dried and observed with UV 365nm.

1.3. Aflatoxins detection in maize

- Extraction and purification

Mycotoxins are extracted, in 50g corn with methanol/water, followed by a passage on immuno-affinity column and an elution of aflatoxins to methanol.

- HPLC analysis condition

The determination of aflatoxins B1, B2, G1 and G2 is carried out by separating the components of the injected solution in a chromatograph equipped with a C18 silica gel column (Spherisorb ODS1-Excel 25 cm x 4.6 mm, 5 µm) in the reverse phase, followed by fluorescence detection (λexc = 360 nm and λem= 440nm). The system is executed in isocratic mode. The mobile phase consists of water/acetonitrile/methanol (6+2+3) (v+v+v) containing 120 mg potassium bromide and 350 µl nitric acid at 4 mol/L. It is injected with a flow rate of 1 ml/min. The injection volume is 10 µl and the retention times of the AFB1, AFB2, AFG1 and AFG2 are 3min, 4min, 5min and 6min respectively.

- Solvents and standards

All reagents used (sodium chloride, nitric acid, potassium bromide) are of Merck Industry analytical quality. All solvents used (methanol, acetonitrile, toluene) are of HPLC analysis quality. All aqueous solutions and the mobile phase used in HPLC were prepared with deionized (or demineralized) water. The standard solutions for each aflatoxin (AFB1, AFB2, AFG1 and AFG2) used are from Sigma Aldrich. Aflatoxin solutions B1 and G1 at 1000ng/ml and aflatoxin solutions B2 and G2 at 200ng/ml are the stock solutions used. The concentration ranges of the standard solutions of aflatoxins B1 and G1 range from 5ng/ml to 20ng/ml and the concentration ranges of aflatoxins B2 and G2 range from 1ng/ml to 4ng/ml.

1.4. Statistical analysis

The statistical analysis was done with the SPSS software. The ANOVA univariate tests, with a significance of 0.05 and a mean comparison test were performed.

2. RESULT AND DISCUSSION

2.1. Moisture content

Table 2 presents the average moisture content of maize samples according to agro-ecological zones and their characteristics. From this table, it can be seen that the water content of the different maize samples is relatively low and ranges from 3.24 to 4.69%.

2.2. Fungal analysis

The maize samples collected were analyzed for the determination of the total fungal flora. Table 3 presents the frequencies of occurrence and relative density of isolated fungal genera according to agro-ecological zones. From this table it can be seen that isolated strains mainly belong to the genres: *Aspergillus*, *Mucor* and *Penicillium*. The genus *Aspergillus* is mainly found with a frequency of appearance that varies from 63.64% to 100% and a relative density that varies from 88.89% to 73.53%.

Table 2: Moisture content

Agro-ecological zones	Characteristics	Moisture
Zone 1 : Extreme North	Sudano-Sahelian zone with a rainy season of less than 900 mm per year. Temperature varies between 18° and 38°C	4.35
Zone :2 North Cotton growing area	in northern Sudan with a rainy season of 1000 to 1300 mm per year	3.24
Zone 3 : South Borgou food bank	The main feature of this area is the very high availability of agricultural land, which is a major asset for food security. This is the domain of the Sudanese wet climate marked by a rainy season from April to September and a dry season that lasts nearly five months.	3.36
Zone 4 : Atacora West	Sudanese climate with a wide disparity in average rainfall, ranging from 800 to 1500 mm	4.69
Zone 5 : Center Cotton growing	sub-equatorial to two rainy seasons in the south and one rainy season in the north, rainfall is between 1000 and 1200 mm per year. Temperature is on average 27°C	3.60
Zone 6 : Sub-equatorial bar lands	with two rainy seasons with 900 to 1200 mm of water per year in the West and 1100 to 1400 mm of water per year in the East. Average annual temperature is 26.5°C	3.48
Zone 7 : Depression zone	It is the smallest of the eight agro-ecological zones in terms of area. In terms of climate, it is quite comparable to the barren land area, but with a high relative humidity (about 85%)	
Zone 8 : fisheries zone	sub-equatorial climate with 2 rainy seasons; rainfall of 1,000 to 1,400 mm over 100 days; duration of the period favourable to vegetative growth: 240 days.	4.62

Variance analysis (ANOVA) showed that there is no significant difference in total mycoflora depending on the agro-ecological zones.

2.3. *Aspergillus* section *Flavi* isolated

- *Asperillus* section *Flavi* strains repartion by agro-ecological area

Table 3 provides details of the profiles of the presumed strains in the *Flavi* section by morphological and biochemical characterization. From this table, it can be seen that aflatoxin-producing strains are present in all agro-ecological zones. Figure 1 shows the geographical distribution of strains belonging to the *Aspergillus flavus* and *Aspergillus parasiticus* clades according to agro-ecological zones.

From Table 4 and Figure 1, it appears that in the ZEN, ZCN, ZVSB, and ZOA areas, there is a predominance of strains close *to Aspergillus parasiticus* producing aflatoxin B and G; while the ZCC, ZTB, ZP areas, are marked by the predominance of strains close to *Aspergillus flavus* producing aflatoxins B.

2.4. Aflatoxins from maize

After the identification of *Aspergillus* strains in the *Flavi* section contaminating maize, the aflatoxin content of the samples was evaluated.

Table 5 gives the average levels of aflatoxins B1, B2, G1, G1, G2, in maize samples according to each agro-ecological zone. From this table, it appears that the samples in zone 6 have the highest aflatoxin B1 content, with an average content of 10.82µg/Kg. Samples from

Table 3: Fungal genus by agro-ecological area

Agro-ecological area	Sites	Main isolated fungal genre
1- Extreme north	Malanville	**Aspergillus :** Fq= 100% et Ri= 76,92%; **Mucor** : Fq=25% et Ri = 7,69%; **Penicillium** : Fq= 25% et Ri= 15,38%
2- North Cotton growing Zone	Kandi	**Aspergillus :** Fq= 100% et Ri= 75%; **Mucor** : Fq = 25% et Ri = 12,5%; **Penicillium** : Fq= 25% et Ri= 12,5%
3- South Borgou food bank Zone	Nikki, N'dali	**Aspergillus :** Fq= 75% et Ri= 80%; **Mucor** : Fq= 25% et Ri = 20%; **Penicillium** : Fq=0% et Ri= 0%
4- Zone west-Atacora	Djougou, Natitingou	**Aspergillus :** Fq= 100% et Ri= 88,89%; **Mucor** : Fq= 12,5% et Ri = 11,11%; **Penicillium** : Fq=0%et Ri= 0%
5- Center cotton growing Zone	Parakou, Tchaourou, Bassila, Glazoué, Kétou	**Aspergillus :** Fq = 92,86% et Ri= 73,53%; **Mucor** : Fq = 64,29% et Ri = 20,59%; **Penicillium** : Fq= 13,33% et Ri= 5,88%
6- Sub-equatorial bar lands Zone	Bohicon, Abomey-Calavi, Porto-Novo	**Aspergillus :** Fq= 63,64% et Ri= 74,07%; **Mucor** : Fq = 45,45% et Ri = 29,17%; **Penicillium** : Fq= 0% et Ri= 0%
8- Fisheries Zone	Cotonou	**Aspergillus :** Fq= 100% et Ri= 84,62%; **Mucor** : Fq= 50% et Ri = 15,38%; **Penicillium** : Fq=0% et Ri= 0%

Fq (Frequency of occurrence) **=** Number of samples contaminated by a genre x 100 / Total number of samples
Ri (Relative density) **=** Number of strain isolates (ni) x 100 / Total number of moulds (Ni)

Table 4: Number of strains of *Aspergillus* section *Flavi* clade by agro-ecological zones

Agro-ecological area	Number of strains of *Aspergillus flavus* clade	Number of strains of *Aspergillus parasiticus* clade
1- Extreme north	2	4
2- North Cotton growing Zone	4	7
3- South Borgou food bank Zone	1	5
4- Zone west-Atacora	3	6
5- Center cotton growing Zone	17	2
6- Sub-equatorial bar lands Zone	8	0
8- Fisheries Zone	3	0

Table 5: Aflatoxins in maize according to agro-ecological zones

Agro-ecological area	Sites	AFB1	AFB2	AFG1	AFG2	H2O
1- Extreme north	Malanville	1,57	0,30	<0,13	<0,13	0,96
2- North Cotton growing Zone	Kandi	<0,15	<0,15	<0,13	<0,13	0,97
3- South Borgou food bank Zone	Nikki, N'dali	5,40	<0,15	0,83	<0,13	0,97
4- Zone west-Atacora	Djougou, Natitingou	<0,15	<0,15	<0,13	<0,13	0,96
5- Center cotton growing Zone	Parakou, Tchaourou, Bassila, Glazoué,	6,47	1,76	0,23	<0,13	0,97
6- Sub-equatorial bar lands Zone	Bohicon, Abomey-Calavi, Porto-Novo	10,82	0,87	0,01	<0,13	0,96
7- Depression zone	Kétou	<0,15	<0,15	<0,13	<0,13	
8- Fisheries Zone	Cotonou	<0,15	<0,15	<0,13	<0,13	0,96

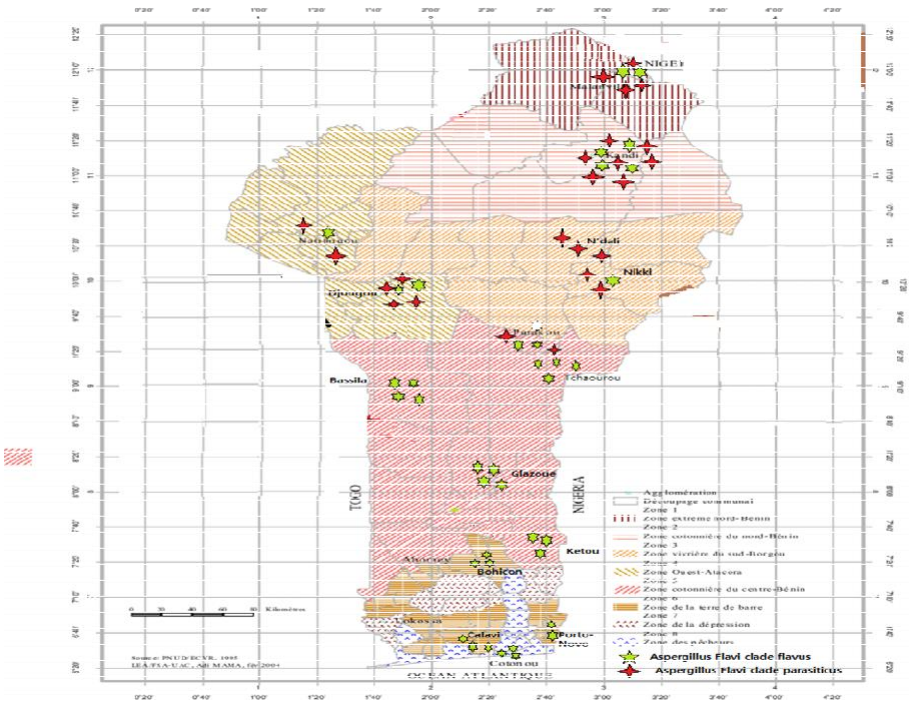

Figure 1: Geographic répartition of *Aspergillus* section *Flavi* clade

zones 2, 4, 8 (ZCN, ZOA, ZP) have an average content below the detection limits for the four types of aflatoxins which are 0.15µg/Kg for aflatoxins B1 and B2 and 0.13µg/Kg for aflatoxins G1 and G2.

3. DISCUSSION

In Benin, maize is the staple food with maize-based meals consumed up to three times a day (Lutz, 1994). It is widely cultivated in all regions of the country, due to its adaptability to the climatic conditions of tropical countries (Asiedu, 1989). On the basis of these climatic conditions, Benin is subdivided into three main zones: a rather dry northern zone, characterized by a Sudanese climate with temperatures ranging from 25 to 38°C; a much more humid central and southern zone, characterized by a sub-equatorial climate with an average temperature of 27°C (Dayou et al., 2017). The objective of this study is to assess the impact of climatic conditions in agro-ecological zones on the development of *Aspergillus* strains in the *Flavi* section and the synthesis of aflatoxins in maize in Benin. Indeed, the southern areas: Fisheries Zone Depression Zone Barrier Lands Zone and Central Cotton Zone, are the areas most exposed to the development of *Aspergillus flavus* strains and aflatoxin production compared to other areas, due to the binomial distribution of rainfall that encourages farmers to harvest part of the maize crop during the rainy season.

Maize is being a favorable substrate for the development of microorganisms, in particular *Aspergillus flavus* (Bandyopadhyay et al., 2007), if the moisture content of the grain is not below 17% (Kawasugi et al., 1988). In this work, water content reveals that all maize samples collected are below the threshold necessary for fungal growth and aflatoxin production. As far as the total fungal flora is concerned, the majority of genres found are: *Aspergillus, Penicillium* and *Mucor*. The genus *Aspergillus* is the most predominant with a frequency of occurrence per agro-ecological zone ranging from 63.64% to 100%. The predominance of the genus *Aspergillus* and low moisture content of the samples confirmed the hypothesis that a high infection with the genus *Aspergillus* observed in maize in storage could be due to a significant infection before and during harvest.

Among these strains, the genus *Aspergillus* is the most dangerous, especially the *Aspergillus* of the *Flavi* section, which are the producers of aflatoxins. The Aspergillus strains of the Flavi section found in the samples were isolated and characterized according to their toxigenic nature. These results reveal that 66.66% of the *Aspergillus* strains in the *Flavi* section are aflatoxin B producers, 25.76% are aflatoxin B and G producers. The EN, CN, VSB and OA zones are marked by the predominance of aflatoxin B and G producing strains confirmed by RFLP as strains of the rock of the *A. parasiticus* species while the central and southern zones are marked by the predominance of aflatoxin B clade *A. flavus* producing strains only. This distribution is shown in Figure 1. The preferred areas for *Aspergilli* clade *parasiticus* have a Sudanese (ZOA, ACT, ACT,) and Atacorian (Z) climate composed of two seasons: a dry and rainy season with rainfall ranging from 900 to 1200 mm in Sudanese climate and 1300mm in Atacorian (Adam and Boko, 1983). As for the region dominated by *Aspergilli* of the *flavus* clade, these areas have a sub-equatorial or Beninese climate with rainfall of 1200 mm per year (Adam and Boko, 2003). However, despite this distribution, the search for aflatoxins in maize samples showed rather high contamination in samples from southern wetland areas, especially the ZTB barren zone (Table 5).

CONCLUSION

This work shows that the *Aspergillus* strains in the *Flavi* section follow a geographical distribution according to the subgroups close to *A. flavus* and *A. parasiticus*. Those close to *A. parasiticus* identified by RFLP are found in the northern part of the country which is warmer and drier while those close to *A. flavus* in the central and southern coastal areas are more humid and less hot. Taking all the above into account, it can be said that environmental conditions have an influence on the *Aspergillus* species in the *Flavi* section that contaminate food.

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