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Contamination of rice marketed in Benin by aflatoxin and aflatoxinogenic moulds

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Abstract

Contamination of food with aflatoxins, a carcinogenic substance secreted by moulds of the genus *Aspergillus* section *Flavi*, is a major public health concern, particularly in tropical countries. In Benin, rice (*Oryza* spp. L.) is one of the most widely consumed food crops. However, poor storage conditions predispose it to fungal invasion. *Aspergillus* spp. are common contaminants in rice stored in tropical regions. Their presence could, under certain conditions, lead to the production of aflatoxins. This study focuses on the occurrence of moulds and aflatoxins in local and imported rice in Benin. According to mycological analyses, carried out by morphological characterization, all local rice samples and 47.27% of imported rice samples are contaminated by moulds with a low level of contamination ranging from 9 cfu/g to 2.52 10^2ufc/g. The species of the genus Aspergillus spp. are mainly represented with 23.63% of Flavi section and 12.27% of nigri section. The presence of aflatoxins was evaluated in rice after extraction and purification on an immuno-affinity column by HPLC coupled with a fluorescent detector (detection limit: 0.15µg/kg (AFB1 and AFG1) and 0.13µg/kg (AFB2 and AFG2)). After analysis, only one sample of rice contaminated with aflatoxins. This work shows that rice marketed in Benin is highly contaminated with fungal spores, mainly those of *Aspergillus* section *Flavi*, which produce aflatoxins, but is not very susceptible to aflatoxins.

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Introduction

Ranked third in the world for cereals after wheat and maize, rice (*Oryzae spp.*) is the main food for nearly 50% of the world's population and contributes more than 20% to the global supply of calories consumed (WARDA, 2006).

In Benin, rice ranks third in terms of cereal production after maize and sorghum (Abel, 2009) and second in terms of consumption after maize (Konnon, Sotondji, and Adidehou, 2014) with an average consumption of 25 to 30 kg per capita per year, or between 175 000 and 210 000 tonnes nationally, of which more than 80% is covered by imports (MAEP, 2010). After harvesting, rice by its nature is one of the most contaminant-friendly cereals and under poor storage conditions an excellent substrate for moulds and most often those of the genus *Aspergillus*. The development of these moulds on cereals results not only in a deterioration in the marketability of the cereal with significant economic losses but also in contamination of cereal grains by mycotoxins and most often aflatoxins which are hepato-carcinogenic substances. Although several studies described aflatoxins as frequent contaminants in rice (Sales and yochizawa, 2005; Nguyen *et al.*, 2007; Reddy *et al.*, 2009; Makun *et al.*, 2011; Bansal *et al.*, 2011; Almeida *et al.*, 2012;, Amanloo *et al.*, 2013; Ruadrew *et al.*, 2013), no studies in Benin have yet evaluated fungal mycoflora and aflatoxins in Benin rice. Faced with this lack of data, and given the importance of this agricultural product in Benin's diet, we have set ourselves the objective of assessing the contamination of rice marketed by aflatoxins and identifying its fungal flora.

Materials and methods

Rice samples collections

Two types of rice are marketed in Benin: local rice produced and imported rice. Rice sampling was carried out during the month of August 2018.

55 samples of imported rice were collected at the largest market in Benin's economic capital Market (Dantokpa), which is the distribution hub for imported rice in the country.

09 samples of locally produced rice were collected in Cotonou (Dantokpa market, Label Bénin and Mont Sinaï supermarkets), Bohicon (Bohicon market) and Tanguiéta from distributors of agricultural products manufactured in Benin.

Moisture content

The water contents of these samples were determined according to AOAC 991.43 (Association of Official Analytical Chemists).

Fungal flora count

The list of moulds was compiled using the dilution method (Microbiology of food, 2008). From each sample, a stock solution was made with 0.1% sterile peptone water. 100µl of successive dilutions are inoculated on Potatoes Dextrose Agar (PDA) medium and incubated in the oven for 7 days at 30°C.

Identification of moulds

Morphological characterization

Mould identification is performed from cultures on Malt Extract Agar (MEA) media using the morphological characteristics described by (Frisvad *et al.*, 2019; Pitt & Hocking, 2009). Aspergillus flavus et parasiticus Agar (AFPA) media is used for the isolation of Aspergilli section Flavi.

Molecular characterization

- DNA extraction

To extract genomic DNA, each isolate was cultured on PDA. After culture at 25°C for 5 days in the dark, mycelium was collected and DNA prepared by phenol-chloroform extraction using the method of Aamir *et al*, 2015.Total DNA was quantified and visualized by gel electrophoresis to ensure quality. PCRs of the gene common to *Aspergillus*, β-tubulin, were used as a control.

Restriction fragment length polymorphism

The RFLP of a segment of β-tubulin was performed to identify subgroups of *Aspergillus* species isolated from the *Flavi* section. After PCR of the partial

sequence of β-tubulin B, 20µL of product is digested by the enzyme BstYI. The profiles obtained are for the clade of *A. flavus* (336, 438, 581bp) and for *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. Each crop is extracted with methanol / water (80/20, V / V). The extracts are evaporated to dry at 60°C. The dry extract is included in 400µl of methanol.

Aflatoxin detection

Aflatoxins were detected by thin layer chromatography. 10 µL extracts and aflatoxin standards (Sigma Aldrich) are deposited on a fluorescent chromatographic plate (1.05553. DCAlufolien 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 at UV 365nm.

Determination of aflatoxins in rice samples

The determination of aflatoxins in rice was performed by HPLC using the method described by Jorg Stroka *et al.* in 2011.

Extraction and purification

The mycotoxins are extracted in 50 g of rice with a methanol/water mixture, followed by a passage on an immuno-affinity column and an elution with methanol.

HPLC analysis condition

The determination of aflatoxins B1, B2, G1 and G2 is done by HPLC on a C18 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.

Results

Moisture content

The average water content of imported rice samples is 2.88% ± 1.68 while that of local rice samples is 5.49% ± 0.43. It was noted that among the imported rice, samples of Thai origin are the wettest, with an average content of 3.49 ± 1.44.

Table 1. Fungal load and incidence of contamination (I.C.) of imported and local rice samples Description: this table shows the average fungal load of imported and local rice samples with details on the most contaminated samples.

Rice	Parameters	Means
Imported	Fungal charge (ufc.g^{-1})	2.63x10^1 ± 1.60 x 10^1
	I.C. (%)	47.27
Thaïlande	Charge fongique (ufc.g^{-1})	3.24 x 10^1 ± 1.33 x 10^1
	I.C (%)	45
Inde	Charge fongique (ufc.g^{-1})	2.58 x 10^1 ± 2.37 x 10^1
	I.C. (%)	43.75
Autres	Charge fongique (ufc.g^{-1})	2.37x 10^1 ± 1.98 x 10^1
	I.C. (%)	42.10
Local	Fungal charge (ufc.g^{-1})	1.76 x 10^2 ± 3.11 x 10^1
	I.C. (%)	100

Fungal flora count

After analysis of the collected samples, fungal spore contamination was observed in all locally produced rice samples and in 47.27% of imported rice samples. This fungal load was lower in imported rice samples (2.63. 101 cfu.g-1 on average) than in local rice

samples (1.76 x 102 cfu.g-1 on average) (Table 1). A significant difference between the two sample batches (imported and local) was noted on this point. The differences in fungal loads observed between the countries of origin of imported rice samples are not significant. However, among the imported rice samples, samples of Thai origin are the most contaminated with fungal spores (3.24 x 101 ± 1.33 x 101 cfu.g-1).

Identification of moulds

Morphological characterization

Moulds of the type *Aspergillus* section *Flavi* are the most isolated in commercial rice (Table 2). 44 strains of *Aspergillus Flavi* section were isolated from imported rice and 12 strains of local rice.

They were isolated in 23.63% of imported rice samples (13 out of 55 in total) and 88.88% of local rice samples (8 out of 9 in total). The least frequent species are: those belonging to the genus *Aspergillus nigri* section which contaminated 9.09% of imported rice and 44.44% of local rice; followed by Spergillus candidus found in only 3.63% of imported rice. Moulds belonging to other genera, namely *Penicillium* spp. and *Mucor* spp., were also detected.

Table 2. Fungal species isolated from imported rice samples Description: This table presents the incidence of the main fungal species found in the samples collected according to their origin (local or imported production).

Rice	Eungal species	Incidence (%)	Charge (ufc.g^{-1})
Imported	*A.* section *Flavi*	23.60	9 – 2.52 x 10^{2}
	A. section *Nigri*	9.09	9 – 5.40 x 10^{1}
	Aspergillus candidus	3.63	9
	Penicillum spp.	5.45	9
	Mucor spp.	7.27	9
Local	*A.* section *Flavi*	88.88	9 - 9 x 10^{2},
	Aspergillus niger	44.44	9 – 5.40 x 10^{1},
	Mucor spp.	33.33	Low

Molecular characterization

A total of fifty-six strains of *Aspergillus Flavi* section were isolated from the collected samples, including forty-four from imported rice and twelve from local rice.

The amplification of a portion of the b-tubulin gene confirmed the genus of forty-seven (47) of the isolated strains.Digestion of the amplification products by the restriction enzyme BstYI produced two types of profiles, one with three fragments and the other with four fragments. The three-fragment RFLP profiles are characteristic of *A. flavus* clade (336, 438, 581bp) and the four-fraction RFLP profiles are characteristic of *A. parasiticus* clade (176, 263, 334, 576bp) (Figure 1). After viewing the RFLP profiles, the most represented species is *A. parasiticus*, constituting 65.78% (25 out of 38 strains) of *Aspergillus* strains section *Flavi* isolated from imported rice samples and 77.77% (7 out of 9 strains) of those isolated from local rice.

Metabolic profile

The metabolic profile assessment showed that all strains of *Aspergillus* section Flavi isolated from imported rice produce aflatoxins while 8/9 of those from local rice produce aflatoxins. The majority of strains isolated from the two sample batches produce aflatoxins B and G (Table 3).

Occurrence of aflatoxins in rice marketed in Benin

Of the fifty-five (55) imported rice samples analyzed, only one, SUPER SARI rice brand, is positive for at least one of the four types of aflatoxins tested in the samples. Aflatoxin B1 is the only one detected in this sample with a content of 0.48µg/kg. All other samples are negative, i.e. below the detection limit (0.15 µg/kg for AFB1 and AFB2: 0, 13 µg/kg for AFG1 and AFG2).For local rice samples, none of the nine

samples tested were positive for aflatoxins (contamination rate below the detection limit).

Discussion

According to the International Commission on Microbiological Specifications of Foods (1974) cited by Feroz *et al* (2016), any food is unfit for consumption if its total fungal load exceeds 104 cfu.g-1. In addition, a water content of less than 14% generally protects rice against fungal attack (Codex Alimentarius, 2016).

Table 3. Differents types of Aflatoxins produced by isolated *Aspergillus* strains of *Flavi* section Description: Different types of aflatoxins that isolated strains can produce are evaluated in order to estimate the contamination capacity of rice by aflatoxins B and G.

Aflatoxins	Numbers of productives strains	
	Imported rice	Local rice
AFB	9	2
AFB et AFG	29	6
None	0	1

The fungal loads of imported rice samples (2.63.101 cfu.g-1) and local rice samples (1.76 x 102 cfu.g-1) found in this study are below the above standards for tolerated fungal load in food. This is probably due to the low water content of the samples (2.88% for imported rice and 5.49% for local rice). These results are in agreement with those of wallace (1973) cited by El-Said (2014) who stipulate that a water content of less than 13.5% reduces the colonization of cereal stocks by storage moulds (*Aspergillus* spp. and *Penicillium* spp.).

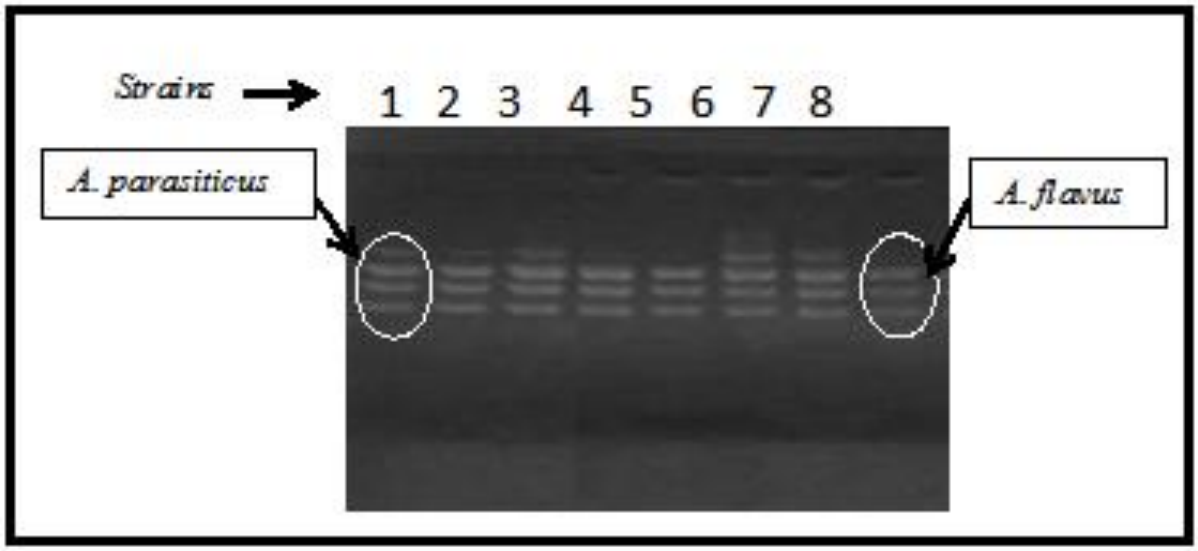

Fig. 1. BstYI restriction scheme after digestion of the amplification products of a discriminating portion of b-tubulin.

On the other hand, this low contamination of the samples may also be due to the structure of the rice found on the markets. In general, it is milled rice, from which the pericarp and germ are removed. However, for most of the time, the fungal attack of cereals occurs at the level of the germ (Lahouar, 2016). Compared to local rice samples, imported rice samples are less infected with mould. This is probably related to their low water content but probably also to the preservatives (benzoic acid, propionic acid or

their esters) used by exporters (Pitt and Hocking, 2009).

Moulds belonging to the genus *Aspergillus* spp. more precisely *Aspergilli* section *Flavi* and *Aspergilli* section *Nigri* are the most predominant in the fungal flora of all rice samples collected.

These results confirm previous investigation results that have also reported that *Aspergillus* spp. is one of the most predominant fungi in rice grains (Fredlund *et al.*, 2009; Pitt and Hocking, 2009; Reddy *et al.*, 2009; Ruadrew *et al.*, 2013; Amanloo *et al.* 2014).

Of the strains of *Aspergillus* section *Flavi* isolated in our study, the species *A. parasiticus* is the most represented. These results are consistent with those of Sales and Yoshizawa (2005) who describe *A. parasiticus* and *A. flavus* as frequent rice contaminants. In 2007, Makun *et al.* reported similar results from rice samples collected in Niger State, Nigeria. Although rice grains marketed in Benin are contaminated with *Aspergilli* spores from the *Flavi* section, they are practically not contaminated with aflatoxins. Only one of the 64 samples collected was contaminated with a low level of aflatoxin B1 (0.49µg.g-1).

This contamination level is below the aflatoxin standards (4µg.g-1) in foods according to the codex alimentarius (2003). Similar results have been found by Palaniswami *et al* (1989) and Amanloo *et al.*, (2014).

This shows that rice is not a substrate very favourable to aflatoxin contamination, unlike other cereals such as maize. However, the presence of *Aspergillus* spores from the aflatoxin-producing Flavi section could promote aflatoxin contamination of rice when storage conditions favouring an increase in water content occur.

Conclusion

Local rice and rice imported into Benin are slightly contaminated with aflatoxin although they are contaminated with moulds of the genus Apergillus section Flavi aflatoxinogens. Among the rice marketed in Benin, imported rice is less likely to be contaminated by moulds, probably because of the conservation products used by exporters. It would be important to continue this work by researching the different conservation products used in rice imported into Benin.

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