Analysis of the contrast between natural occurrence of toxigenic Aspergilli of the Flavi section and aflatoxin B1 in cassava

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Key words

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Abstract

Aflatoxin B1 (AFB1) is a carcinogenic mycotoxin produced by *Aspergillii* of the section *Flavi* that may contaminate food, in the field or during storage. Cassava represents an important staple food in sub-saharian Africa. The analysis of aflatoxigenic fungi in 36 cassava samples obtained from producers in Benin indicated that 40% were contaminated by *Aspergillii* of the section *Flavi*. Upon morphological and molecular characterization of the 20 isolates, 16 belonged to *A. flavus*, 2 to *A. parvisclerotigenus* and 2 to *A. novoparasiticus*. This is the first time that this latter species is isolated from food. Although most of these isolates were toxigenic on synthetic media, no AFB1 contamination was observed in these cassava samples. In order to determine the action of cassava on AFB1 synthesis, a highly toxigenic strain of *A. flavus*, was inoculated onto fresh cassava and despite a rapid development, no AFB1 was produced. The anti-aflatoxin property was observed with cassava from different geographical origins and on other aflatoxigenic strains of the section *Flavi*, but it was lost after heating, sun drying and freezing.

Our data suggest that fresh cassava is safe regarding AFB1 contamination, however, processing may alter its ability to block toxinogenesis leading to secondary contamination.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by fungi and are common contaminants of food and feed commodities worldwide. Over 400 mycotoxins are known today; due to their occurrence and toxicity, aflatoxins, fumonisins, ochratoxin, zearalenone and trichotecenes are usually considered as the most important for human health (Leslie et al., 2008).

The most toxic and dangerous mycotoxins are aflatoxins. Indeed, aflatoxin B1 (AFB1) is the most potent hepatic carcinogen known in mammals and has been classified by the International Agency for Cancer Research in the group I of molecules that are carcinogenic for both humans and animals (IARC, 1993). AFB1 also possesses immunosuppressive properties (Meissonnier et al., 2008) and is involved in growth impairment observed in children (Gong et al., 2004; Khlangwiset et al., 2011). Exposure to aflatoxins in sub-Saharan Africa is very frequent. In some areas, 99% of children tested have aflatoxins in their blood (Gong et al., 2002) and this high exposure contributes to appearance of chronic hepatomegaly in children (Gong et al. 2012). Contamination of food by very high levels of aflatoxins can lead to fatal consequences such as the reported death of 125 people in Kenya (Lewis et al., 2005; Probst et al., 2007). The contamination of food by AFB1 may therefore have important consequences for human health and more than 100 countries have established maximal tolerable limits for this molecule (FAO, 2003; EU, 2006).

In the tropical countries highly exposed to aflatoxin, cassava (*Manihot esculenta Crantz*) belongs to the short list of most consumed foodstuffs. It is ranked as the third most important food crop in tropical regions after rice and maize. Due to its high energetic value, it represents an important part of the diet of almost one billion people, mostly in sub-Saharan Africa. In Africa, cassava is mostly devoted to human food, livestock feedstuff, but it is also used as a raw material in various industries that produce flour, starch, adhesives, etc. As human food, cassava may be consumed as fresh roots usually as a snack, but also after being peeled, grated or soak in water to ferment and then processed into a wet mash (fufu) or varied dry products (gari, chips/crumbs/chunks, or milled into flour) (Akoroda, 2007). The conditions of production and storage of cassava and its traditional derivatives may be favorable to the contamination and development of molds (Westby and Twiddy, 1991). Generally such uncontrolled mold developments

may not only result in alterations in the organoleptic properties of the foodstuffs but may also lead to the accumulation of toxic secondary metabolites.

Due to its nutritional and economic importance in many tropical countries, it is necessary to assess the contamination of these foods by mycotoxins. Previous surveys performed in order to evaluate fungal and mycotoxin contaminations showed that some mycotoxins such as patulin, cyclopiazonic, ochratoxin A, zearalenone could be found in cassava (Wareing et al., 2001). However, no trace of aflatoxins has been mentioned, although the warm and humid climate that prevails in tropical areas can promote the development of aflatoxin-producing *Aspergillus* species (Muzanila et al, 2000; Jimoh and Kolapo, 2008). Recently, we did a preliminary survey on cassava chips sold in Beninese markets and found no aflatoxin contamination of cassava chips, despite a high presence of *A. flavus* spores (Gnonlonfin et al., 2012).

Within this context, the objective of this current study was to understand the absence of AFB1 in cassava products. Consequently, we firstly did a small survey to analyze fungal flora and aflatoxins in cassava chip samples taken directly from producers in order to evaluate the fungal contamination at this critical point in the food chain. We then characterized the isolates of *Aspergillus* of the section *Flavi*, isolated during this survey and the previous one, regarding their toxigenic potential. Finally, we investigated the action of cassava on AFB1 synthesis and demonstrated that fresh cassava was able to block AFB1 synthesis by toxigenic strains of *A. flavus*.

2. Material and methods

2.1 Chemicals and reagents

All reagents including solvents were purchased from Fisher Scientific (Fontenay sous Bois, France) and were of analytical grade. Aflatoxin B1 (AFB1), aflatoxin G1 (AFG1), scopoletin and ergosterol standards were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). AFB1 and scopoletin were dissolved in methanol and ergosterol in ethanol. Taq DNA polymerase used for molecular identification was purchased from Invitrogen (Carlsbad, CA, USA). Agarose gel and primers used for gene determination were purchased from Eurobio (Courtaboeuf, France).

2.2 Cassava samples

For the survey, thirty six samples of cassava of about 1kg each were purchased from local producers in the north of Benin during the dry season (December 2010) (Figure 1). Samples were randomly chosen and checked for the absence of macroscopically visible alteration or dryness.

For the experiments on *Aspergillus* development and toxinogenesis, two varieties of cassava were used. The first one, named *Logoguesse Kotorou* in Bariba dialect was grown in the north of Benin. The second variety, grown in Costa Rica, was purchased on the market in Toulouse, France. These roots were protected from contamination and alteration by a thin pellicle of wax.

These two varieties were intended for human consumption and corresponded to sweet varieties with concentrations of cyanogenic compounds below 50 mg/Kg as evaluated according to Essers et al. (1993).

2.3 Isolation and Identification of fungi

2.3.1 Origin

Fungal analysis of cassava samples was done according to Gnolongin et al. (2012). In brief, 1 ml of decimal dilutions were plated on Petri dishes containing dichloran glycerol agar (DG18). Petri dishes were incubated at 25°C in alternating 12h periods of

fluorescence light and dark for 5 days. Colony were isolated by several plating out and further identified as described below.

Twelve isolates coming from a survey done in 2009-2010 on cassava chips collected in markets in Benin were added to the study (Gnonlonfin et al., 2012). Other strains of *Aspergillus* of the section *Flavi* used in this study were obtained from the Agricultural Reseach Service collection (NRRL) and CBS-KNAW Fungal Biodiversity Centre collection (CBS): *A. flavus* NRRL 1957, *A. parasiticus* NRRL 5862, *A. parasiticus* NRRL 4123, *A. tamarii* NRRL 20818, *A. bombycis* NRRL 26010, *A. pseudatomarii* NRRL 25517, *Aspergillus parvisclerotigenus* CBS 121.62, *Aspergillus minisclerotigenes* NRRL 29000, *Aspergillus minisclerotigenes* CBS117635.

2.3.2 Morphological and molecular identification

The 32 strains isolated from cassava were cultivated on Potato Dextrose Agar (PDA) (24 g/l potato-dextrose broth, 15% (w/v) technical agar (Becton, Dickinson and Co., Franklin Lakes, NJ, USA)) at 25°C for seven days. They were identified as *Aspergillus* section *Flavi* isolates by both macroscopic and microscopic examination according to Pitt and Hocking (2009) and Varga et al. (2011).

Identification of *Aspergillus flavus* isolates was also performed by PCR amplification of the *cypA*/*norB* region as described in Probst et al. (2012). The culture conditions and genomic DNA isolation were described by El Mahgubi et al. (2013). PCR reactions were carried out in a GenAmp PCR 2700 thermocycler (Applied Biosystems, Forster City, CA, USA). This identification was completed for 10 randomly selected isolates which showed 0.3 or 0.9 kb fragments by internal transcribed spacers (ITS) rRNA and 5.8S rRNA gene amplification and sequencing. For the other isolates displaying no gap, added to ITS, the molecular identification was performed by calmodulin, beta-tubulin or acetamidase (*Amds12*) and o-methyltransferase (*Omt12*) genes amplification and sequencing. Primers used in this study are listed in Table 1. For ITS amplification, PCR conditions were those described by White et al. (1990). Beta-tubulin, calmodulin were amplified under conditions previously described by Peterson (2008) whereas (*Amds12*) and o-methyltransferase (*Omt12*) genes were amplified according to Geiser et al. (1998).

PCR products were purified with GenElute PCR clean–up kit (Sigma-Aldrich) and sequenced by the dye terminator technology on an ABI3130XL sequencer (Applied Biosystems). PCR products were sequenced in both directions.

Nucleotide sequence accession numbers for internal transcribed spacers (ITS) rRNA and 5.8S rRNA, beta-tubulin, calmodulin, acetamidase and O-methyltransferase of tested isolates are reported to Table 2.

2.3.3 Analysis of toxin pathway genes

The toxigenic status of the *A. flavus* isolates was determined by analyzing the presence of six genes involved in the aflatoxin biosynthesis pathway: *AflD* (*Nor-1*), *AflO* (*OmtB*), *AflP* (*OmtA*), *AflQ* (*OrdA*), *AflR*, and *AflS*. The primers used were designed using the sequences of the *A*. *flavus* strain NRRL 3357 deposited in GenBank and are reported in Table 1. The PCR amplification was carried out in 50 µL reaction mixture (5 µl of Taq polymerase buffer 10X, 1.5 µL of 50 mM $MgCl_2$, 1 µL of 10 mM dNTP (Promega, Madison, WI, USA), 1.5 µM of each primer, 1.5 units of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5µl of genomic DNA (100 ng/µl), and 31 µl of pure water (Laboratoire Aguettant, Lyon, France)). Reaction conditions were: 94◦C for 5 min, 40 cycles (1 min at 94◦C, 1 min at 58◦C, and 1 min at 72◦C) followed by 10 min at 72◦C. The amplified products were examined by 1.2% w/v agarose gel electrophoresis.

2.4 Aflatoxin B1 quantification

2.4.1 In cassava chip samples

AFB1 quantification in cassava chip samples was done on site according to the method developed and validated by Gnonlonfin et al. (2010). Briefly, each sample (10 g) was extracted with 1 g of sodium chloride and 25 mL of extraction solution [methanol/water (80/20, v/v)] and purified on an Aflatest®immunoaffinity column (VICAM, Watertown, MA, USA). The extract was dried under nitrogen gas at 60°C, dissolved in 200 µL of methanol and analyzed by HPLC with a post-column derivatization using a photochemical reactor for enhanced detection (PHRED). The limit of detection (LOD) of the method was 0.1 µg/kg.

2.4.2 In culture medium

AFB1 production by *A. flavus* strains was determined after culture on PDA for 7 days at 25°C. Toxins were extracted from culture medium by mechanical agitation with 70 ml of chloroform. The organic phase was evaporated in a rotary evaporator at 50°C. The

residue was taken up in 400 µl of methanol, and this suspension was filtered-through a 0.45 mm pore-size filter before analysis.

The mycotoxin was quantified by high performance liquid chromatography with photodiode array detector (Thermo-Finnigan Suveyor HPLC-PDA system, ThermoElectron Corporation, Whaltham, MA, USA). The liquid chromatography separation was performed using a 150 mm x 2.00 mm Luna 5 µm C18 column (Phenomenex, Torrance, CA, USA). HPLC was done with a linear gradient elution using 33 mM acetic acid (solvent A) and acetonitrile (solvent B) with a flow rate of 200 µl/min. The compounds were eluted starting from 80% of solvent A for 5 min, followed by a 50% solvent B step, then a linear gradient up to 90% solvent B within 5 min. After isocratic elution for 5 min, the gradient was decreased to its initial value within 2 min, and maintained at it for the last 5 min.

Calibration curves for AFB1 were constructed using standard solutions in methanol (from 0.1 to 100 µg/ml) at 365nm. The LOD was 2 ng/ml.

2.5 *A. flavus* growth and toxinogenesis on cassava

2.5.1 Culture conditions

Inoculum preparation

Strains were cultured on PDA at 25°C in the dark for 7 days. After this period, the plates were washed with 10 ml of 0.05% Tween and a spore suspension was harvested and adjusted to 10^5 spores/ml after counting in a Malassez cell.

Culture on cassava

Before inoculation, the water activity of cassava was measured with an HC2-AW device (Rotronik AG, Basserdorf, Switzerland) and adjusted to 0.98 by addition of the required quantity of sterile water.

After peeling, cassava was cut into thin slices and inoculated immediately (fresh) or after heat treatment (120°C for 20 min), freezing (-20°C for 2 months) or drying. For this later treatment, cassava chips were processed as previously described (Gnonlonfin et al., 2010). Everyday, the evolution of the water content was followed according to AOAC 934.01 (AOAC, 2000) and 200 g of cassava were used and inoculated as follow.

Fifteen grams of fresh or treated cassava were inoculated with 100 µl of a spore suspension (10^4 spores) and incubated at 25°C for 3 and 6 days in dark and high humidity conditions. Each experiment was done in triplicate.

2.5.2 Fungal development measurement

Conidia formation:

Spore numbers were determined after grinding cultures in phosphate buffer [(0.1M, pH7.4); 50 ml for 15 g cassava]. Ten-fold dilutions were prepared with tween 0.05% and the numeration of spores was done both by counting in a Malassez cell and by plating dilutions on malt agar and determining Colony Forming Units per gram (CFU/g) after 5 days at 25°C.

Ergosterol concentration

Ergosterol measurement was done according to Bailly et al. (1999). In brief, after saponification in methanol, ethanol, KOH and pyrogallol and extraction with petroleum ether, ergosterol was quantified by fluorodensitometry with a Shimadzu CS930 densitometer (Shimadzu, Kyoto, Japan) after separation of the extracts by thin layer chromatography on silica plates (Macherey-Nagel, Düren, Germany) in toluene-acetonitrile (70:30). Quantification was done by comparison with known amounts of standard spotted on the same plate.

2.5.3 Aflatoxin B1 measurement in cassava

The aflatoxin B1 was extracted from cassava with chloroform (50 mg/ml) and filtered-through a phase separator PS Whatman filter (GE Healthcare Biosciences, Pittsburgh, PA, USA). The extract was evaporated to dryness in a TurboVap Lv Evaporator (Zymark, Hopkinton, MA, USA) under N_2 atmosphere. Dry extracts were re-suspended and analyzed by HPLC-PDA as described above.

For spiking experiments, pure AFB1 was added on fresh or autoclaved cassava (5 ng/g cassava). Extraction and quantification of AFB1 was then done as described above.

2.6 Statistical analysis

Differences for ergosterol production and sporulation were evaluated for statistical significance using an analysis of variance (two-way ANOVA). P≤0.05 was required for significance.

3. Results

3.1 Contamination of cassava chips with *Aspergillus* section *Flavi*

A survey was done to evaluate contamination with *Aspergilli* and the presence of AFB1 on 36 samples randomly collected from producers located in the North of Benin (Figure 1).

3.1.1 Morphological and molecular characterization of fungal isolates

Identification of fungal isolates from cassava samples was firstly done by morphologic examination after growth on PDA medium. Nearly forty percent of samples collected (38.9%) were found to be contaminated with strains that were morphologically identified as *Aspergillus* of the section *Flavi* and a total of 20 different strains were isolated, two samples being contaminated with 2 different strains. The presence of sclerotes was observed in half of the strains.

Identification of these isolates to the species level was performed using molecular methodologies. Twelve additional isolates previously isolated form marketed cassava cheaps (Gnolonfin et al., 2012) were also submitted to such identification. As a first step, identification of these isolates to the species level was performed by the determination of the presence of a gap in *cypA*/*norB* region. The amplification of *cypA*/*norB* of 28 isolates revealed 0.9 or 1.5 kb deletion. Among the 32 isolates , 18 displayed 0.9 kb gap and 10 displayed a 1.5 kb deletion (Table 3). The presence of these deletions strongly suggested that the isolates belonged to *Aspergillus flavus* species. Indeed, the cytochrome P450 monooxygenase encoded by *cypA* is required for aflatoxins G_1 and G_2 production and the partial deletions of *cypA* explain that *A. flavus* lacks capacity to synthetize aflatoxins G (Ehrlich et al., 2004). Molecular identification based on ITS

sequences was also performed on 10 strains randomly selected from these isolates. This confirmed that all belonged to *A. flavus* species (Table 2).

The amplification of *cypA*/*norB* region (1.8 kb) of the 4 remaining isolates (AFc31, AFc32, AFc35 and AFc36) revealed an absence of gap. For these isolates, the alignment of *cypA*/*norB* region sequences (KC990464-KC990468) to the full length sequence of *Aspergillus parasiticus* NRRL 5862 (SRRC143) strain suggested that they could be able to produce aflatoxins G.

For AFc31 and AFc32 isolates, the sequences obtained after ITS sequencing matched 100% with those of *A. novoparasiticus* species. In order to confirm that these isolates belong to *A. novoparasiticus*, fragments of *AmdS12* and *Omt12* genes were amplified and sequenced. The obtained sequences (Table 2) matched 100% with the *A. novoparasiticus* sequences previously deposited in GenBank (Gonçalves et al., 2011).

AFc35 and AFc36 share identical ITS sequences with *A. flavus.* Since ITS sequence is not discriminatory to differentiate *A. flavus* from *A. parvisclerotigenus*, the sequences of parts of beta-tubulin and calmodulin gene were determined and displayed 100% identity with those of *A. parvisclerotigenus* (Table 2).

3.1.2 Toxin pathway gene profile of fungal strains isolated from cassava

To evaluate the toxigenic potential of *A. flavus* isolates, the presence of 6 different genes (*AflD, AflO, AflP, AflQ, AflR* and *AflS*) involved in the aflatoxin biosynthetic pathway was investigated by PCR. All the strains possessed these six genes in their genome (Table 3).

The ability of the strains to produce mycotoxins was then confirmed by AFB1 and AFG1 analysis after culturing in conditions known to favor mycotoxin synthesis. Table 3 shows that most of the isolates were able to produce AFB1. Although the biosynthetic pathway genes detection was positive, the culture extracts from two strains do not showed any AFB1 trace. Four isolates identified as *A. novoparasiticus* (AFc31 and AFc32) and *A. parvisclerotigenus* (AFc35 and AFc36) were also found capable to produce AFG1. These results are in agreement with results of sequences analysis of *cypA*/*norB* region reported above.

3.1.3 Detection of aflatoxin B1 in cassava chips

As shown in Table 2, despite the aflatoxigenic character of the *A. flavus* strains isolated from cassava chips collected in Benin, no trace of AFB1 was detected neither in the chips collected in the present survey nor in the samples obtained from the previous survey.

3.2 Effect of cassava on *A. flavus* growth and toxinogenesis

In order to understand the absence of contamination of cassava by AFB1 despite the presence of toxigenic strains of *A. flavus*, the ability of cassava to inhibit AFB1 synthesis was investigated.

3.2.1 Effect of fresh cassava on aflatoxinogenesis

One highly toxigenic strain of *A. flavus* isolated from cassava chips (strain AFc5) was used to inoculate fresh cassava pieces of two varieties from different geographic origins. As shown on Figure 2, fungal development was macroscopically visible after 3 and 6 days of incubation, whatever the variety of cassava was used. The measurement of the ergosterol content in the cultures and spore formation confirmed that the *A. flavus* strain AFc5 was able to develop on both cassava varieties (Table 4).

As expected from the survey data, the fungal development and sporulation was not associated with AFB1 synthesis, regardless of the time and the cassava variety tested (Table 4). A similar inhibition of AFB1 production was observed when toxigenic strains belonging to three other *Aspergillus* section *Flavi* species (*A. parasiticus*, *A. bombycis* and *A. pseudotamarii*) were grown on cassava (Table 5), although these strains produced AFB1 when cultured on PDA medium.

The spiking of both fresh and autoclaved cassava with known amount of pure AFB1 demonstrated that the lack of AFB1 after *A. flavus* development on cassava was not linked to a matrix interference. Indeed, 71 and 74% of the added toxin could be recovered from fresh and autoclaved spiked samples respectively.

3.2.2. Effect of cassava processing on AFB1 synthesis

The impact of different processing used during the production or the storage of cassava products were then tested for their effect on AFB1 synthesis.

Cassava pieces were submitted to different processing before inoculation with toxigenic *A. flavus* strain AFc5. Figure 3 shows the effect of heat treatment on both mold

development and AFB1 synthesis. A small but significant increase in spore numbers after development on heat-treated cassava was noted (Figure 3a). By contrast, no difference was observed for ergosterol content with the exception of an increased value after development of AFc5 strain on fresh cassava from Costa Rica for 6 days (Figure 3b). Regarding toxinogenesis, when the toxigenic strain was inoculated on heat-treated cassava, AFB1 was detected after 3 (data not shown) and 6 days of incubation (Figure 3c) whereas no toxin was detectable after mold development on fresh cassava.

The effect of other process methods was also tested. Table 6 shows that sun drying of cassava pieces may also interfere with the ability of this raw material to prevent AFB1 synthesis by a toxigenic strain of *A. flavus*. Indeed, after 7 days of drying, a re-moistening of cassava chips allowed AFB1 production (5.5 ± 0.5 ng/g).

Similarly, we observed that a 2-month freezing period at -20°C inhibited the anti-AFB1 ability of cassava, the mycotoxin becoming detectable after inoculation with the toxigenic strain Afc5 and incubation for 7 days at 25°C (42 ± 18 ng/g).

3.2.3. Role of scopoletin in the inhibition of AFB1 synthesis

Among constituents of cassava, scopoletin is known to have anti-fungal properties. Even if no antifungal activity was noted, we nevertheless investigated if this compound could explain the inhibition of AFB1 biosynthesis in fresh cassava.

Table 7 shows that there were no significant differences in the scopoletin concentration in heat treated cassava compared to fresh one (p=0.159) whereas in the former condition, AFB1 was detected after *A. flavus* development. The same results were obtained using the two varieties of cassava that contained comparable concentrations of scopoletin (Table 7).

4. Discussion

Cassava is a major crop in many regions of the world where it is used as human food and animal feed. The aim of this study was to assess the fungal and mycotoxin contamination that could occur after harvest and during processing of cassava.

In order to characterize fungal contamination of cassava at the first step of the processing chain, cassava chips were collected from several local producers in the north of Benin. Almost 40% of the samples were contaminated with *Aspergillus* of the section *Flavi* demonstrating that these fungal species may contaminate cassava before processing. Among the 20 isolates from these samples, sixteen were identified as *Aspergillus flavus*. All isolates previously collected from cassava on Benin markets were also identified as *A. flavus*. This confirmed the data published by Gnonlonfin et al. (2008; 2012) which have identified *A. flavus* as a frequent contaminant of cassava chips marketed in Benin as well as other studies done elsewhere in Africa (Manjula et al., 2009; Westby et al., 1995; Essono et al., 2007). This is also in agreement with the observation that *A. flavus* is more prevalent in foods and feeds than *A. parasiticus* (Essono et al., 2009).

Most of the isolates produced aflatoxin B1 when cultured on PDA. This proportion of toxigenic isolates appeared quite high compared to what is usually reported. Indeed, the proportion of toxigenic *A. flavus* often ranges between 40% and 65% in raw materials such as maize (Saleemi et al., 2012; Giorni et al., 2007), cocoa beans (Sanchez-Hervas et al., 2008), groundnut (Bankole et al., 2004) or spices (El Mahgubi et al., 2013; Elshafie et al., 2002). Four isolates were found able to produce aflatoxin G_1. These strains were further identified as *A. parvisclerotigenus* and *A. novoparasiticus*.

This last species was recently discovered and characterized (Gonçalves et al., 2012). With one exception, all strains have been isolated from patients and hospital environment samples. So far, its geographical distribution was limited to South America (Brazil and Colombia). This is the first report which mentions the presence of *Aspergillus novoparasiticus* in another continent other South America. It is also the first time that this species is isolated from food products.

Unlike *A. flavus*, *A. parvisclerotigenus* is a poorly documented species. Although the first mention regards atypical isolates from tropical soils in Thailand (Saito and Tsurata, 1993) all isolates which were subjected to molecular characterization came from Guinea

Gulf area (Nigeria, Benin). Recently, five isolates were identified from dried edible mushrooms in Nigeria (Ezekiel et al., 2013) while the Type strain (CBS 121.62) was isolated from peanut, also in Nigeria (Frisvad et al., 2005). Blast analysis of *cypA*/*norB* region sequences of AFc35 and Afc36 isolates displayed a 100% identity with those of the type strain but also with those of BN008R (ATCC MYA-379) strain, an atypical S_{BG} strain collected from agricultural soil in Benin (Cotty and Cardwell, 1999). *A. parvisclerotigenus* occurrence observed in our study is very low (<6%) as already described (Ezekiel et al., 2013).

Despite the high prevalence of contamination with toxigenic isolates, the analysis of AFB1 contamination of cassava samples revealed no trace of this toxin in the chip samples. Similar results were obtained on cassava chips sampled at the market stage in Benin (Gnonlonfin et al*.,* 2012). This confirms that, in spite of a high occurrence of *A. flavus* spores, cassava chips are not contaminated with aflatoxins.

This observation suggests that cassava may interfere with AFB1 production. To characterize the interaction between cassava and aflatoxinogenesis, fresh cassava chips were inoculated with spores of a highly toxigenic *A. flavus* strain. The fungus was able to rapidly grow on cassava, and was macroscopically visible after 3 days of incubation. This growth was confirmed by both ergosterol measurement and spore numeration. Nevertheless, the fungal development on fresh cassava was not associated with AFB1 production. So it demonstrates that fresh cassava is able to block AFB1 production when colonized by a toxinogenic strain of *A. flavus*. This inhibition of AFB1 production was observed using 2 different varieties of cassava and with several AFB1 producing species of the *Flavi* section. To the best of our knowledge, this is the first report indicating that a raw material, which allows fungal development, may interfere with aflatoxin B1 synthesis. Cassava can therefore be considered as resistant to the contamination with this mycotoxin. Plant extract/products have been described that inhibit AFB1 production. For instance, Yoshinari et al. demonstrated that German Chamomille blocks aflatoxin G production (Yoshinari et al., 2008). Razzaghi-Abyaneh et al. (2009) also showed that *Carum carvi L*. inhibits AF production without any obvious effect on fungal growth. More recently, Gorran (2013) showed that an aqueous extract of *Thymus daenensis* was able, at a concentration of 2000 mg/l, to reduce the production of AFB1 by 97%.

For cassava, our data suggest that cassava is a substrate non-permissive for secondary metabolism. Indeed, HPLC analysis of extracts revealed that no peak corresponding to secondary metabolites was observed on a chromatogram when the strain AFc5 was grown on cassava in contrast to what was observed when the *A. flavus* strain was grown on the usual media (PDA, MEA or YES) (data not shown). The absence of *A. flavus* secondary metabolites despite normal growth suggests that cassava acts on the global regulation of secondary metabolism. In the last decade, several transcription factors have been found to regulate secondary metabolism in *A. flavus*. The alteration of transcription factors is often linked to fungal development anomalies. An inhibition of the recently described transcription factors nsdC and nsdD is very unlikely. Indeed, the Δ*nsdC* and Δ*nsdD* mutants exhibit columnar arrangement of the conidial chains on the conidiophores and shorter conidiophore stipes (Cary et al., 2012). In the present study, no macroscopic or microscopic morphological alteration was observed when *A. flavus* grew on fresh cassava. A heterotrimeric nuclear complex composed of three proteins LaeA, VeA and VelB regulates the secondary metabolism (Bayram et al., 2008). LaeA has a major effect on aflatoxin B1 synthesis in *A. flavus* as well as on the whole secondary metabolism in other *Aspergillus* species (Kale et al., 2008). The disruption of *VeA* leads to decreased activation of the aflatoxin biosynthesis pathway in *A. parasiticus* and *A. flavus* (Calvo et al., 2004; Amaike and Keller, 2009). In *A. flavus*, VeA is also necessary for the synthesis of cyclopiazonic acid and aflatrem (Duran et al., 2007). VelB regulates the same biosynthetic pathway (aflatoxin/sterigmatocystin) in *A. nidulans* (Bayram et al., 2008). In contrast to *nsdC* or *nsdD* genes, the deletion of *LaeA, VeA* or *VelB* only marginally modifies the morphological aspect of the fungi. For instance, disruption of *VelB* leads to a considerable decrease in conidia production whereas deletion of *VeA* or *LaeA* only induces a slight decrease in spore numbers (Park et al., 2012). This reduction of conidiogenesis is accompanied by a decrease in the growth diameter when the Δ*laeA* or Δ*veA* mutants grow on a media plate. The deletion of both *LaeA* and *VeA* genes is characterized by an inability to produce sclerotia. In the present study, when the *A. flavus* strain AFc5 was grown on fresh cassava, we observed a small decrease in sporulation and the absence of sclerote formation as well as an inhibition of whole secondary metabolism. Despite the absence of an effect on fungal growth, these observations suggest that fresh cassava may act on the velB/VeA/LaeA complex.

However, a further molecular study should be carried out in order to confirm this hypothesis.

In order to better understand the mechanisms underlying the inhibition of AFB1 synthesis by cassava, the impact of several processes was studied. We observed that the ability of cassava to block AFB1 production by a toxigenic strain of *A. flavus* was inhibited by heat treatment, sun drying or freezing of cassava samples. When each of these processes was applied, the growth of a toxinogenic strain of *A. flavus* on treated cassava was associated with the production of AFB1. These assays demonstrated that the molecule responsible for the inhibition of toxin production is quite sensitive and could correspond to a peptide or small protein.

Among candidate compounds that may participate to AFB1 inhibition by cassava, the role of scopoletin was investigated. This molecule is a thermostable coumarin phytoalexin with medical properties. It is accumulated in some plants such as carrot (Coxon et al., 1973), cotton (Zeringue, 1984), sunflower (Gutierrez et al, 1995), noni or *Morinda Citrifolia* (Deng et al., 2010) and cassava (Buschmann et al., 2000) in response to both biotic and abiotic stress (Edwards et al., 1997). It can also accumulate in roots and tubers as a result of post-harvest physiological deterioration. This compound has been reported to be a potent anti-microbial molecule with fungicidal properties (Rodriguez et al., 2000; Gomez-Vasquez et al., 2004). In the present study, scopoletin was not responsible for AFB1 inhibition. Indeed, thermal treatment (120°C, 20 min) did not modify the scopoletin content of cassava chips whereas it restored AFB1 production by toxinogenic *A. flavus* strain.

Similarly, cyanogenic compounds present in fresh cassava may not be involved in the anti-aflatoxigenic ability of the product since the varieties used in our study were sweet ones characterized by a low content of cyanogenic compounds. Moreover, many fungi display natural linamarase activity and are therefore able to break down cyanogenic glucosides present in cassava (Birk et al., 1996; Amoa-Awua et al., 1997).

In conclusion, this study demonstrated for the first time that fresh cassava can be considered as a crop that is resistant to aflatoxin B1 contamination. This report is very important as a food safety issue due to the importance of cassava in the diet of millions of people. Indeed, it appears that cassava is naturally protected from AFB1 contamination during the pre-harvest period. This is usually a critical step regarding

503 mycotoxin synthesis due to the high water activity of plants. After that, since this ability
504 will be lost by cassava during drying, it is necessary to ensure good storage procedure
505 and avoid remoistening of chips. It is now important to identify the active compound(s)
506 and the precise mechanism of action in order to be able to inhibit toxin production
507 during processing and storage and to possibly use it in other applications.
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509
510
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Figure legends

Figure 1: Benin map with sampling sites. ■ producer sites. ⋆ market sites.
For the strains isolated at the cassava producers, the survey was performed during the dry season in 2010. One sample was collected from 6 different producers in 6 villages (total number of 36 samples).

Figure 2: Visual aspect of cassava after inoculation with a toxigenic *A. flavus.*
Sterile cassava from Benin and from Costa Rica were contaminated with 10^4 spores of *A. flavus* strain AFc 5, and incubated at 25°C for 3 or 6 days.

Figure 3: *A. flavus* development and toxinogenesis on non-autoclaved (NA) and autoclaved (A) cassava from Benin and Costa Rica after 6 days of incubation.
Autoclaved and non-autoclaved cassava were contaminated with 10^4 spores of *A. flavus* strain AFc5 and incubated at 25°C for 6 days.
a) Spore formation, b) Quantification of ergosterol, c) AFB1 production.
Two-way ANOVA indicated a significant effect of heat treatment on spore numbers and on ergosterol concentration on cassava from Costa Rica after 6 days of culture ($p < 0.05$)

Table 1: Primers used for molecular characterization of fungal strains isolated from cassava samples

Genes	Primers	Nucleotides 5'➔3'	References
ITS	ITS5	GGAAGTAAAAGTCGTAACAAGG	White et al, 1990
	ITS4	TCCTCCGCTTATTGATATGC	
β-tubulin	Bt2a	GGTAACCAAATCGGTGCTGCTTTC	Glass et al, 1995
	Bt2b	ACCCTCAGTGTAGTGACCCTTGGC	
Calmodulin	cmd5	CCGAGTACAAGGAGGCCTTC	Hong et al, 2006
	cmd6	CCGATAGAGGTCATAACGTGG	
Acetamidase AmdS12	amdS1	CCATCGGTATAGGAACTGA	Geiser et al, 1999
	amdS2	AGGGTGCCACGGTATGTC	
O-methyltransferase Omt12	omt1	GGAGTATCAGAGGATTTA	Geiser et al, 1999
	omt2	AGTGCTGTAATAGTCAAA	
NorB-CypA region	AP1729	GTGCCCAGCATCTTGGTCCACC	Ehrlich et al, 2004
	AP3551	AAGGACTTGATGATTCCTC	
AflD	AflDF	CGGTGTATTTGGTCACCGGGGC	This study
	AflDR	CGGCTGCCTGGGCATCAGTTTC	
AflQ	AflQF	CGTTATGGGAGGATCGGACACG	This study
	AflQR	CCCAGATCTGATCCTCCTGCG	
AflP	AflPF	GGGCATTCATGCCTTGGTTG	This study
	AflPR	CCCATACCTAGATCAAAGCGG	
AflO	AflOF	CTCTGGCGAAGGTCGGCATTG	This study
	AflOR	CTCTCGGCCAGGAAGTCAGG	
AflR	AflRF	GTCGATTTCTTGGCCGAGTC	This study
	AflRR	CTCAGCAAGTAGCCATCCTG	
AflS	AflSF	CAATTGATGCCGGCGTGGAG	This study
	AflSR	CAAGTGATGCGTGCGCGTAG	

840 **Table 2**. Accession number of the sequences deposited at Genbank

	Gene sequences				
Aspergillus species	ITS	Acetamidase	O-methyltransferase	Beta-tubulin	Calmodulin
Isolate number		*Amds12*	*Omt12*		
Aspergillus flavus					
AFc4	JX456207	---	---	---	---
AFc5	KC153995	---	---	---	---
AFc6	KC153996	---	---	---	---
AFc21	KC990469	---	---	---	---
AFc22	JX456209	---	---	---	---
AFc25	KC994648	---	---	---	---
AFc33	KC990470	---	---	---	---
AFc34	KC990471	---	---	---	---
AFc37	JX456208	---	---	---	---
AFc40	KC994649	---	---	---	---
Aspergillus novoparasiticus					
AFc31	KC964099	KC921994	KC964097	---	---
AFc32	KC964100	KC921995	KC964098	---	---
Aspergillus parvisclerotigenus					
AFc35	KC964101	---	---	KC954603	KC954605
AFc36	KC964102	---	---	KC954604	KC954606

841 --- : Not tested

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843

844 **Table 3**: Aflatoxin contamination of cassava samples and toxigenic profile of *Aspergillus*
845 section *Flavi* isolated from cassava chips

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Origin of chips	Chip sample (city/N°sample)	AFB1 concentration in chips	Characterization of *Aspergillus* section *Flavi* isolates			
			Isolates number	Presence of AF biosynthetic pathway genes	*NorB-CypA* gap (bp)	Aflatoxins production
PRODUCER	**Gobé 1**	ND	AFc 21	yes	900	B
	Gobé 2	ND	AFc22	yes	1500	B
	Gobé 6	ND	AFc 23	yes	900	B
	Savè 2	ND	AFc 24	yes	1500	B
	Tchaourou 1	ND	AFc 25	yes	900	B
	Tchaourou 1	ND	AFc 26	yes	900	B
	Tchaourou 2	ND	AFc 27	yes	1500	B
	Tchaourou 3	ND	AFc 28	yes	900	B
	Tchaourou 5	ND	AFc 29	yes	900	B
	Tchaourou 6	ND	AFc 30	yes	900	B
	Goro 1	ND	AFc31	yes	-	B+G
	Goro 2	ND	AFc32	yes	-	B+G
	Ina 1	ND	AFc 33	yes	900	B
	Ina4	ND	AFc 34	yes	1500	B
	Sinissou 1	ND	AFc 35	yes	-	B+G
	Sinissou 2	ND	AFc36	yes	-	B+G
	Sinissou 8	ND	AFc 37	yes	1500	B
	Ina 3	ND	AFc 38	yes	900	B
	Ina 2	ND	AFc 39	yes	900	B
	Ina 2	ND	AFc 40	yes	1500	B
MARKET	**Parakou/Depot 1**	ND	AFc 1	yes	900	B
	Save 5 S1	ND	AFc 2	yes	900	B
	Save 5 S2	ND	AFc 3	yes	900	-
	Cocotomey	ND	AFc 4	yes	1500	B
	Gobe 2	ND	AFc 5	yes	900	B
	Bohicon 3	ND	AFc 6	yes	1500	-
	Gobe 3	ND	AFc 7	yes	900	B
	Parakou/Arzeke 2	ND	AFc 10	yes	900	B
	Cocotomey 4	ND	AFc 11	yes	900	B
	Save 3	ND	AFc 12	yes	900	B
	Bougou 5	ND	AFc 13	yes	1500	B
	Kandi	ND	AFc 14	yes	1500	B

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848 ND: not detected (< 0.1 µg/kg)

849 yes: presence of the six biosynthesis genes tested (*AflD*, *AflO*, *AflP*, *AflQ*, *AflR*, *AflS*);

850 All isolates were identified as *Aspergillus flavus* except AFc31, Afc32 (*A. novoparasiticus*)
851 and AFc35, Afc36 (*A. parvisclerotigenus*).

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Table 4: Effect of fresh cassava on *A. flavus* growth and toxinogenesis

	Origin of cassava			
	Benin		Costa Rica	
Culture duration (days)	3	6	3	6
Ergosterol (µg/g)	13.82± 0.63	26.47 ± 4.37	18.09 ± 1.49	50.67 ± 2.77
Spores (x 10^7)	2.33 ± 0.38	133.00 ± 11.50	7.43 ± 2.24	310.00 ± 26.50
AFB1 (ng/g)	ND	ND	ND	ND

ND: Not detected

Results are expressed as mean ± SD of three independent experiments

878

879 **Table 5**: Effect of fresh cassava on aflatoxin B1 production by different *Aspergillus*
880 species of the section *Flavi*

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	Fungal species		
	A. parasiticus NRRL 4123	*A. bombycis* NRRL 26010	*A. pseudotamarii* NRRL 25517
AFB1 production on MEA (ng/g)	477.14 ± 56.88	9.43 ± 3.10	190.53 ± 77.26
AFB1 production on fresh cassava (ng/g)	ND	ND	ND
Ergosterol on cassava (µg/g)	1.18 ± 0.05	1.301 ± 0.05	1.456 ± 0.15

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883 AFB1 and ergosterol measurement were done after 6 days of incubation at 25°C;
884 ND: Not detected.
885 Results are expressed as mean ± SD of three independant experiments

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Table 6: Impact of drying on *A. flavus* development and toxinogenesis on cassava chips

	Drying time (days)			
	2	3	4	7
Ergosterol (µg/g)	36.63 ± 4.23	40.81 ± 7.03	56.51 ± 2.97	43.62 ± 2.0
Spores (x 10^7/g)	6.18 ± 0.44	9.03 ± 2.68	14.9 ± 0.95	35.1 ± 9.95
AFB1 (ng/g)	ND	ND	ND	5.5 ± 0.5

ND: Not detected

Results are expressed as mean ± SD of three independent experiments.

929

930 **Table 7:** Scopoletin and aflatoxin concentrations in non-autoclaved and autoclaved
931 cassava samples from Benin and Costa Rica after 3 days of incubation

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	Origin of cassava			
	Benin Cassava		Costa Rica Cassava	
	Non autoclaved	Autoclaved	Non autoclaved	Autoclaved
Scopoletin (µg/g)	13.72 ± 0.30	12.62 ± 0.30	13.45 ± 4.40	10.53 ± 3.80
AFB1 (µg/g)	ND	1.20 ± 0.4	ND	1.69 ± 0.5

933

934 ND: Not detected

935 Results are expressed as mean± SD of three independant experiments

936

Figure 1

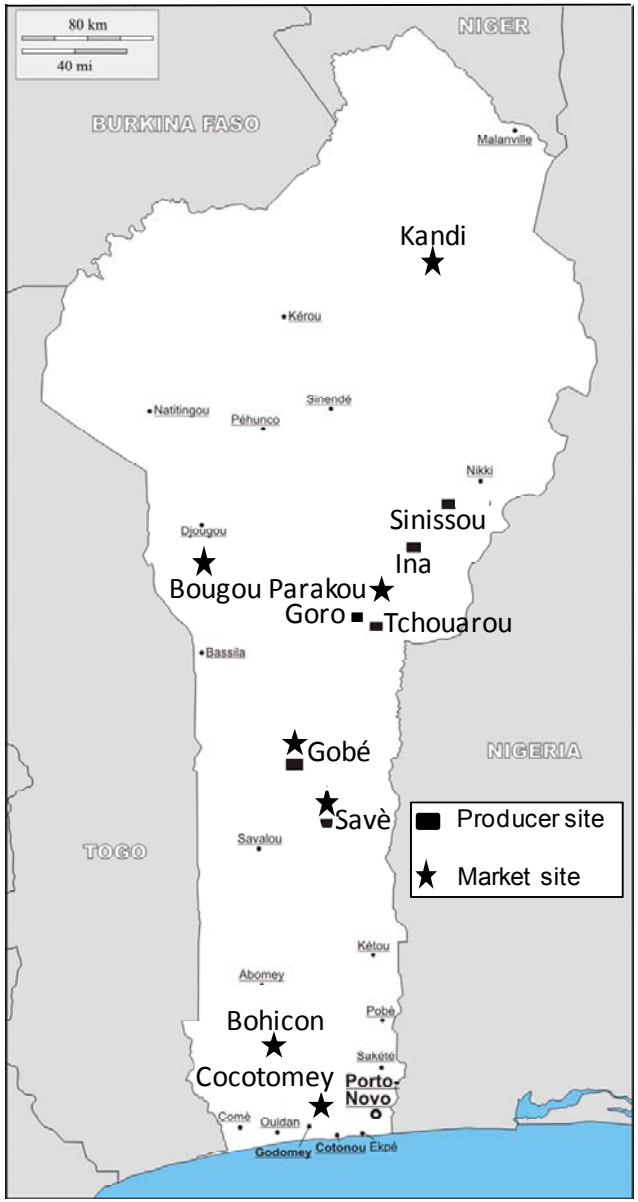

Figure 1 *Adjovi et al.*

Figure 2

Benin	Costa Rica	
		3 days of incubation
		6 days of incubation

Figure 2 *Adjovi et al.*

Figure 3

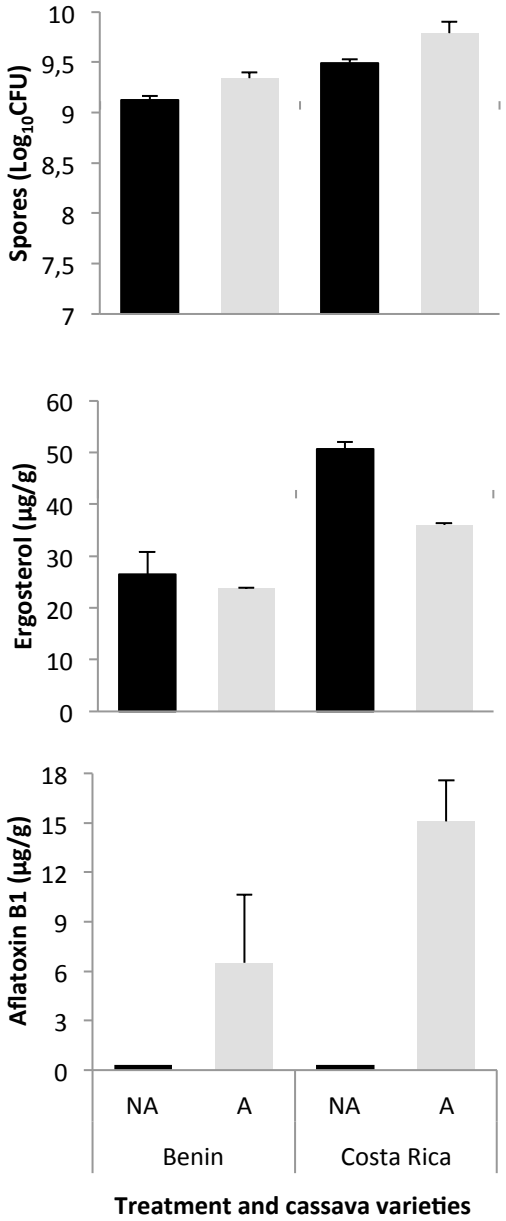

Figure 3 *Adjovi et al.*

Research Highlights

- Cassava is contaminated with toxigenic *Aspergillus flavus* but not by aflatoxin B1
- Cassava can be contaminated with *A. novoparasiticus*.
- Fresh cassava is able to block AFB1 synthesis by *Aspergillus flavus*
- But cassava does not modify *Aspergillus flavus* development
- Thermal treatment of cassava leads to a loss of the anti-aflatoxin ability