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Cyanide influence on the growth of mycotoxigenic fungi from cassava flour *in vitro*

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The hypothesis that residual cyanide present in cassava flour influences the growth of mycotoxigenic *Aspergillus* was established. Therefore, cyanide concentrations were measured in three types of flour: mixed, dry and watery (*n=30*), as the basis for the establishment of doses to be used. Fungi sowing were performed in solid Saboraud medium with a central well (500 µL), in which 5, 10, 15 and 20 mg CN kg^{-1} concentrations were applied, and control group was established using distilled water (0 mg CN kg^{-1}). Plates were incubated at 25°C for 24, 48 and 120 h and evaluated on direct observation of fungal growth. Results showed that fungi grown unevenly according to the applied concentration. The initial development at all concentrations, in both genera, was by the edge of plates, at a slower rate in larger doses. After 120 h only the 20 mg kg^{-1} concentration did not grow near the center circle. Thus, results show that cyanide slows fungi growth of mycotoxigenic *Aspergillus flavus* assessed, with dose-dependent effect. It is important to apply the good practices in cassava flour production in order to control cyanide levels, as well as the reduction of toxigenic fungi, to promote the food safety.

Key words: Mycotoxin, linamarin, *Manihot*, *Aspergillus.*

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is part of the food base and cuisine of Latin America and Africa. In Brazil, where the plant is domesticated, its importance is not only economic but also social and cultural. Among its main derivatives, the flour is of great prominence due to the heterogeneity of manufacturing processes, resulting in typical products of each region. In Brazil, a classify-cation criterion was established dividing the flour into

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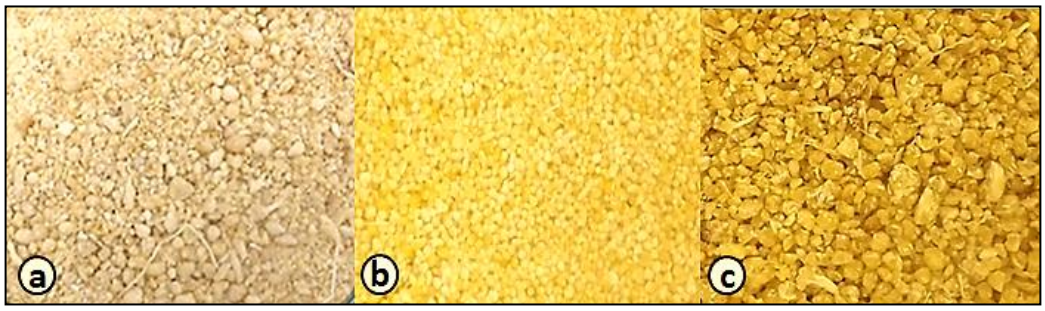

Figure 1. Cassava flour. a) Mixed; b) dry and c) watery.

groups (dry, watery and "Bijusada"), classes (fine, medium or semi-coarse and coarse) and types (1, 2 and 3) (Brazil, 2011). In the Amazon region (northern Brazil), the main type of flour used is the watery. This type of flour is produced through an empirical process of roots fermentation, followed by pressing, grating and roasting (Cereda and Vilpoux, 2010; Chisté and Cohen, 2011). This product is available heterogeneously in the market due to a large physicochemical and microbiological variation.

Microbiological evaluations of cassava flours show the incidence of mycotoxigenic fungi, especially from genera *Aspergillus* and *Penicillium* (Gomes et al., 2007; Santos et al., 2012), which mainly produce Aflatoxins (AFLs), Ochratoxin A (OTA), Citrinin and Patulin (Santos et al., 2012; Morales et al., 2007; Singh et al., 2007; EFSA, 2006). Due to a high toxicity (hepatotoxicity, nephrotoxicity, neurotoxicity, blood toxicity and even death), human exposure to food consumption is a matter of public health concern in the world (Caldas et al., 2002). The culinary use of cassava could be more valued, even for export, if not for the concern for indigenous cyanide content and its possible toxicity. However, during the flour processing steps, cyanogenic compounds (linamarin and lotaustralin) present in the roots decrease substantially (Unung et al., 2006; Chisté and Cohen, 2008), decreasing the possibility of human poisoning (Cereda, 2003; Brito et al., 2013). For example, during processing stages, the cyanide levels decrease in flours (Cardoso et al., 2005) and fermentation of tubers (Chikezie and Ojiako, 2013).

On the other hand, as the exposure to cyanide is inevitable due to natural cianoglycosides, many organisms have developed mechanisms to perform the detoxification of this compound. Only when the amount of cyanide is higher than the amount that natural detoxification mechanisms are able to eliminate, the possibility of cyanide poisoning is considered (Ramalho et al., 2010). In this sense, the presence of residual cyanide in cassava flour can potentially provide an inhibitory effect on the development of mycotoxigenic fungi. The aim of this study was to evaluate the influence of cyanide on the growth of mycotoxigenic *Aspergillus flavus in vitro*.

MATERIALS AND METHODS

Samples of cassava flour were selected, according to the manufacturing process, corresponding to groups mixed (*n=16*), dry (*n=3*) and watery (*n=11*) (Figure 1).

Samples were collected in the local market of Coari-Amazonas (Brazil), in different batches sampled randomly. They were placed in sterile plastic bags (300 g), identified and stored under controlled temperature and light conditions until analysis. The flowchart of cassava flours production steps is in Figure 2.

Methods

Cyanide content

Cyanide analysis was performed according to that described by Brito et al. (2009) using potassium cyanide (KCN Vetec®, 96% purity). Standard curve was established from free cyanide increasing doses, ranging from 0.00104 to 0.0520 mg. The color reaction with alkaline picrate developed a color gradient according to CN concentration variation, measured through a spectrocolorimeter (Bel Photonics® spectrophotometer SP 1105) at 535 nm. Absorbance values obtained in analyzes were computed, obtaining the equation $Y = 0.6635.X - 0.0215$ with $r^2 = 0.996$. Samples were suspended in 0.01 M sulfuric acid solution, homogenized for 10 min, followed by filtering and collection of the supernatant. Colorimetric reaction with alkaline picrate was subsequently performed (15 myths at 37°C), followed by spectrophotometry (triplicate). Absorbance values were calculated according to the calibration curve. Results were expressed as milligrams of total cyanide per kilogram of dry weight ($mgHCNkg^{-1}$ DW).

Fungi

The isolation was obtained by using 10 g of each sample, diluted in 90 ml of 0.1% peptone water and submitted to consecutive decimal dilutions up to 10^{-4}. Sowing was performed in triplicate, using 0.1 mL from the extract on the surface in 18% Dichloran Glycerol Agar (DG18), at 28°C for 07 days (Pitt and Hocking, 2009). Then, colonies were purified in test tubes containing Malt Extract Agar (MEA). Purified fungi were identified to genus level by microculture technique: fungi were subcultured on plates containing MEA at three equidistant points. From the same plate, a cube of approximately 1 cm^3 was cut and the sample was seeded in its surface. The cube was then covered with a coverslip and the plate was incubated at 28°C/07 days. After, the coverslip was removed to prepare a slide, to identify the fungus genus under a microscope (Kern and Blevins, 1999).

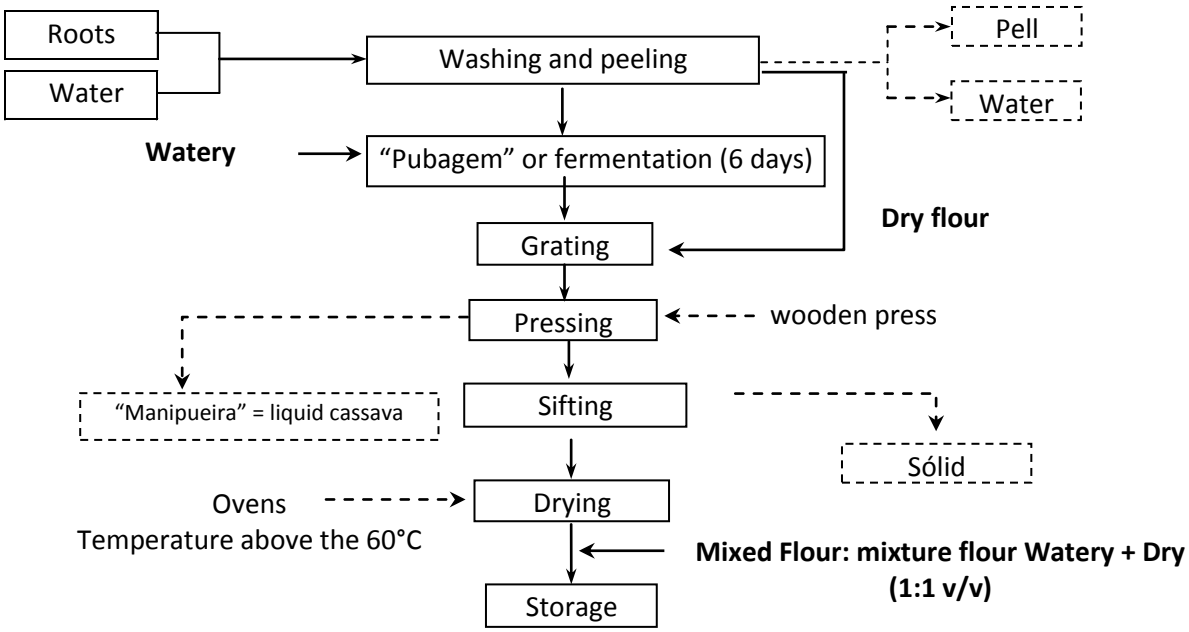

Figure 2. Flowchart of the basic steps of processing of watery, dry and mixed cassava flour. Adapted from Cereda and Vilpoux (2010) and Cardoso (2005).

Table 1. Cyanide concentrations in cassava flour from Coari-Amazonas (Brazil), according to the group (dry, mixed, watery).

Flour samples	Cyanide[a] (range) mgHCN.kg^{-1}
Dry	2.55±0.5 (0.50-4.20)
Mixed	2.91±0.8 (0.99-4.70)
Watery	5.46±1.2 (1.12-15.60)

[a]Results in DW±SD.

Cyanide versus fungi (in vitro tests)

Doses were established from the concentrations of cyanide determined in cassava flours. For growth tests, solid Saboraud medium was used into Petri dishes (Ø 10 cm), with wells established in the center of each plate (500 µL capacity) for the application of cyanide solution. A solution of potassium cyanide (KCN Vetec®, 96% purity) was applied in spaced concentrations of 5, 10, 15 and 20 mgHCN.kg^{-1}. Control group was established using 0 mgHCN.kg^{-1} of distilled water. The experiment was performed in triplicate. Plates were incubated at 25°C for 24, 48 and 120 h. The evaluations consisted of direct observation of fungal growth.

RESULTS AND DISCUSSION

Cyanide content

As expected (due to processing), mixed, dry and watery cassava flour evaluated had low concentrations of cyanide as compared to other authors (Table 1). Chisté et al. (2007) showed that the concentration of cyanide in cassava products could be different according to the cassava cultivar. Charles et al. (2005) found mean of 17.1 (8.3-28.8) mgHCN.kg^{-1} in different cassava genotypes. Cumbana et al. (2007) studied cassava flour samples from Mozambique, which the authors considered as typical for a year of average rainfall, and found an average of total cyanide of 4.3 (0.8-8.85) mgHCN.kg^{-1}. Chisté and Cohen (2008) studied cassava flour of another city from Northern Brazil (Belem), and their results ranged from 7.68-20.57 mgHCN.kg^{-1} (dry) and 3.57-12.36 mgHCN.kg^{-1} (watery). Comparing our findings with those authors' even in *watery* cassava flour with 5.46 (1.12-15.60) mgHCN.kg^{-1}, the results were lower.

FAO/WHO (1991) establishes a LD_{50} value of 10 mg HCN kg^{-1} of body weight, a dose determined from HCN administered to experimental models via inhalation. The LD_{50} was established using extracted linamarin, orally applied, which is consistent with its usual absorption by the body, and the lethal dose was 35.35 mgHCN.kg^{-1} of body weight (Chisté at al., 2010). Thus, cyanide values found in these cassava flours from Brazil, suggest low risk to human health. It is important to emphasize the samples have passed through different processing steps, including heating, when some linamarin could be hydrolyzed to acetone cyanohydrin (catalyzed by endogenous linamarase) which decomposes to HCN gas, thus reducing the cyanogen content.

Cyanide versus fungi (*in vitro* tests)

Depending on the established concentration of cyanide in cassava flour, *in vitro* tests were performed to evaluate

Figure 3. Growth of *Aspergillus* after 24 h of incubation with cyanide (0.00; 5.00; 10.00; 15.00 and 20.00 mg kg^{-1}).

Figure 4. Growth of *Aspergillus* after 48 h of incubation with cyanide (0.00; 5.00; 10.00; 15.00 and 20.00 mg kg^{-1}).

Figure 5. Growth of *Aspergillus* after 120 h of incubation with cyanide (0.00; 5.00; 10.00; 15.00 and 20.00 mg kg^{-1}).

the effect of this compound on the growth of mycotoxigenic *A. flavus*. Qualitative assessments indicated that the initial development of fungi at all concentrations in both genera was by the edge of plates, at a slower rate in tests with higher doses, as shown in Figures 3 to 5. After 120 h of incubation, only the 20 mg kg^{-1} concentration had no growth of fungi near the center circle (Figure 5). However, it was observed that the total colonization of plates was only a matter of time. In the literature, there are some fungal pathogens of cyanogenic plants (*Stemphylium*, *Gloeocercospora* and *Fusarium* genera) that have detoxification capacity due to its colonization habits (Nazly et al., 1983).

Cyanide presented a delaying action on the growth of fungi genera studied, with dose-dependent effect, only though for a short period, indicating a possible inhibition of these types of fungi just after the flour preparation, thereby precluding the production of mycotoxins. In addition to the observations of fungi growth and cyanide influence, the possibility of AFL production by toxigenic strains, in cassava could be considered. On the other hand, previous work did not detect AFL in cassava flour samples (Muzanila et al., 2000). Adjovi et al. (2014) studied the ability of cassava to block AFB1 production by a toxigenic strain of *A. flavus*. The fungi was inhibited by heat treatment, sun drying or freezing of cassava samples. When each of these processes was applied, the growth of a toxigenic strain of *A. flavus* on treated cassava was associated with the production of AFB1. The assays demonstrated that the molecule responsible for the inhibition of toxin production is quite sensitive and could correspond to a peptide or small protein. Many fungi display natural linamarase activity and are therefore able to break down cyanogenic glucosides present in cassava. They conclude that cassava is a substrate non-permissive for secondary metabolism of fungi and aflatoxin production.

Despite the intrinsic mechanisms of cassava, it is important to promote the good practices in the cassava flour production to avoid the contamination of toxigenic fungi. Products intended for the most demanding markets must comply with strict standards of contamination control. Among several parameters that determine food quality, the most important are those that define their

microbiological characteristics and safety. Therefore, it is likely that in flours packed immediately after processing, there is a reduction in the risk of mycotoxigenic fungi growth. Nevertheless, hygienic conditions by manufacture, packaging and good storage practices remain as the main forms of product quality assurance and safety.

Conflict of interests

The authors did not declare any conflict of interest.

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