Control of aflatoxin production in cassava produced by dry fermentation in North Kivu, Democratic Republic of Congo

Original research papers

ABSTRACT

Traditionally, cassava (Manihot esculenta Crantz) is transformed by fermentation in water (retting) or in the open air (dry fermentation) in the DRC. In the east of the country (North Kivu), dry fermentation is the main technique for processing cassava for its detoxification and conservation. The Congolese farmers ferment the cassava to the open air using a preselected microferment contained in the scrapings of the fermented cassava previously called "MUSIYIRO". These fermentations are spontaneously directed by the microorganisms of the uncontrolled autochthonous flora. Unfortunately, toxinogenic molds are often more active in the fermentation process during which they also produce aflatoxins. This study was undertaken to help prevent the production of aflatoxins in cassava during this process. To do this, we substituted the traditional ferment with a strain of *Rhizopus oryzae* used as starter (microferment). Six successive replications, in controlled fermentation and uncontrolled fermentation, in a peasant environment (Beni, North Kivu) and fermentation directed by the strain of *R. oryzae* were carried out. Aflatoxins were then dosed in cassava flour. The results of the assay revealed an absence of aflatoxins in cassava fermented by scrapings from fermentation led by *R. oryzea*, while the non-directed fermentation controls were all contaminated with aflatoxins. These results show that it is possible to prevent the production of aflatoxins in cassava during fermentation when an aflatoxin-inhibiting microbial biomass is used which can progressively invade and colonize the fermentation site and thereby control the fermentation activities of cassava.

Keywords: Aflatoxins inhibition; Rhizopus oryzae; Dry fermentation; Cassava.

1. INTRODUCTION

Cassava (*Manihot esculanta* Crantz) is the staple food and source of nourishment for more than one billion people worldwide [1] especially in Africa, Asia and South America. Because of its high water content, cassava must be transformed into various derivatives to ensure its availability outside harvest periods and to reduce post-harvest losses. The cassava products can be fermented, dried or roasted, and the most common form is chips. This product serves as food for both human and animals and can be maintained up to one year [2].

However, tropical climate of some geographical areas of cassava production may contribute to fungal development of many species and subsequent toxinogenesis on such raw material [3]. Moreover, the processing conditions and storage premises are not always well adapted to protect cassava products from secondary contamination and/or fungal development.

The FAO estimates that 25% of the world food crops are contaminated by mycotoxin, of which the most notorious are the aflatoxins (AFTs) [4-6]. AFTs are metabolites produced primarily by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. There are four major naturally produced AFTs, referred to as B1, B2, G1 and G2 [7, 8]. The B1 is the most toxic of the AFTs and potent naturally occurring liver carcinogen [10]. Reports estimated that more than 5 billion people in developing countries world-wide are at risk of

chronic exposure to AFTs through contaminated foods [9, 10]. AFTs affect livestock and poultry causing reduced feed efficiency, subtle immunosuppression, growth rate and death of animals [6, 11]. Other economic adverse effects of AFTs include low yields of food and fiber crops [12].
Considering the importance of this crop in Democratic Republic of Congo (DRC) and subsequent possible fungal toxin production, various studies have attempted to evaluate cassava products contamination with moulds and mycotoxins. A number of potentially mycotoxigenic fungi have been isolated from cassava products and mycotoxins contamination of cassava has been documented but the potential sanitary risk of such contamination was not fully assessed [13, 14].
The populations of Ituri, North and South Kivu in the north-east of the DRC, mainly consume a dry fermentation product of cassava tubers (*Manihot esculenta* Crantz) called "Kithabiro". Unfortunately, to date, these cassava transformations are still artisanal, rudimentary and unhygienic; fermentations are spontaneously directed by the microorganisms of the uncontrolled autochthonous flora.
Among the microorganisms involved in these cassava fermentations, the following strains were isolated: *A. flavus* (LINK), *A. flavus oryzae*, *A. niger*, *A. fumigatus* and *A. glaucus*, *Rhizopus oryzae* and *Mucor mucedo* [14].
The toxigenic molds of which *A. flavus*, *A. fumigatus* and *A. niger* responsible for the production of aflatoxins are almost permanent and actively participate in the softening of cassava and the development of characteristic aromas of traditional fermented cassava [15].
In fact, farmers conduct cassava fermentation in the open air using a preselected microferment contained in the fermented cassava scrapings previously called "MUSIYIRO". This "Musiyiro" is transplanted each time into the cassava batch to be fermented, basted and then covered with banana leaves and cotton bags to create the moisture needed for microbial growth and cassava fermentation without water.
After several successive subcultures, the various microorganisms of the site stabilize and constitute the STARTERS (Microferment) for the fermentation and the softening of cassava without water.
The studies carried out by Yandju [16] and Djoulde [17] in the raking of cassava called wet fermentation, have shown that the use of a non-toxinogenic efficient microferment has made it possible to obtain stable fermented cassava products and to guarantee the sanitary quality of these products.
Considering the high dependence of populations on cassava Fufu from dry fermentation, it is essential to consider improving cassava processing techniques in the farmer sector. Within this context, the quality and safety of agricultural products and food are surveyed to limit consumer exposure to aflatoxins.
The main objective of this study is to inhibit the production of aflatoxins during the dry fermentation of cassava called "fufu kithabiro" of North Kivu by the substitution of the traditional "Musiyiro" by a pure culture biomass of *R. oryzae*, molds selected for this purpose.

2. MATERIALS AND METHODS

2.1. Collection of samples

Fifty seven (57) samples (fermented cassava Fufu) were collected from the eighteen (18) sites in North Kivu used for the aflatoxin assay and thirty six (36) samples obtained from the experimental fermentation with the *R. oryzae* strain.

2.2. Assessment of the presence of aflatoxins in the Fufu of North Kivu markets

Approximately two hundred and fifty grams of dry ferment cassava chips were aseptically collected in markets in twenty one locations in North Kivu due to three samples per site for aflatoxin and isolation of non-toxinogenic and potent *R. oryzae* in the directed fermentation of cassava [17].
During the sampling, a macroscopic analysis of the organoleptic properties of the dry fermenting cassava Fufu was carried out on site before being sent to the laboratory for the isolation of the seeds and the aflatoxin determination. The analysis concerned the surface color of cassava chips and their aroma [18, 19].

2.3. Determination of aflatoxins in fermented cassava samples

Aflatoxins were evaluated by biological detection and spectrophotometric assay. For biological detection, aflatoxins were detected by inhibiting the growth of sensitive *E. coli* C600 on nutrient agar following their diffusion.
The aflatoxin assay was done by the rapid multitoxin assay method using the Acquity Spectrophotometer HPTLC and the Quattro Preparier XE mass spectrophotometric at South Africa's Perishable Products Export Control Board (PPECB) (RSA).

2.4. Directed fermentation of cassava by non-toxigenic microferment

A culture of *R. oryzae* has been selected to serve as a microferment to replace the scrapings of the traditional fermentation called “Musiyiro” in Nande dialect. After massive seeding on a first batch of cassava under aseptic conditions, scrapings consisting of *R. oryzae* mycelium were subcultured successively six times on six batches of cassava in the presence of two controls, the first taking up the traditional fermentation and the second one seeded with *R. oryzae* strain and incubated under aseptic conditions.
The incubation was done under the traditional fermentation conditions until the softening of cassava in order to put in the peasant conditions. After 72 hours of incubation, the spores were collected by immersion and introduced into sterile tubes; an enumeration was then made by counting in the Malassez cell. Concentrates of 10^3 spores / g of cassava were made.
The fermented, dried cassava was removed from the site for analysis and sent to the laboratory for aflatoxin determination [20].

2.5. Statistical data analysis

Data were analyzed by descriptive statistics and analysis of variance (ANOVA) using Statistix Ver.8 software. Difference in the levels of aflatoxin contamination was determined by the comparison of mean using least significant difference (LSD) at 5% level of significance. The mean contents of aflatoxins were transformed into log (x+1) to normalize data prior to analysis.

3. RESULTS AND DISCUSSION

Table 1. Overall aflatoxins contamination incidence in cassava samples

Classes of aflatoxins	Cassava samples			
	Sample size	Positive sample (%)	Mean	Range
Aflatoxin B1	54	29	0.96[a]	0.34-1.95
Aflatoxin B2	54	29	0.36[b]	0.12-0.74
Aflatoxin G1	54	nd	nd	nd
Aflatoxin G2	54	nd	nd	nd

The mean aflatoxin levels with the different superscript letters in the same column are significantly different (p<0.05); mean aflatoxin levels were transformed into log (x+1) prior to analysis
nd= the levels of the aflatoxin analyzed were lower than the limit of detection (0.15 ppb)

Cassava fufu samples analyzed did contain two types of aflatoxins. Aflatoxins B1 and B2 were present in fermented cassava and in 4 of our 21 fermented cassava samples from North Kivu province while aflatoxins G1 and G2 were absent in all fermented cassava samples analyzed. Aflatoxins B1 are significantly higher ($p<0.05$) than B2. A plausible reason could be that aflatoxins levels in samples of cassava analyzed were below the limit of detection (LOD): 0.2 ppb and 0.5 ppb for aflatoxin G1 and G2; respectively. Also, previous studies suggest that cassava is unlikely to be a source of aflatoxin [21, 22]. Another study investigated the fungal and aflatoxins contamination of cassava products and found that aflatoxins B1, B2, G1 and G2 were lower than the limit of detection (2ppb) of analytical method (VICAM Afla Test immunoaffinity fluorometric method) that they used [23].

115 However, other studies have shown the presence of aflatoxins in cassava products [5, 24, 25]. The study
116 conducted by Jonathan *et al* [25] showed that major spoilage (biodeteriorating) fungi of Attiéké from
117 Ejigbo, Iwo and Adjame in West Africa were mostly molds with *A. niger* and *A. flavus* having highest
118 occurrence and *Candida albicans* and their percentage occurrence has direct effect on its food values.
119
120 **Table 2. Aflatoxin B1 contamination (ppb) in cassava samples**

Source of samples	Cassava samples		
	Sample size	Positive sample (%)	Mean
Alungupa	3	40	0.89[a]
Beni ville	3	20	1.2[a]
Butembo	3	nd	nd
Goma	3	nd	nd
Itendi	3	nd	nd
Kabasha	3	nd	nd
Kalunguta	3	nd	nd
Kanyabayonga	3	nd	nd
Kayina	3	20	1.05[a]
Kiantshaba	3	nd	nd
Kiwandja	3	nd	nd
Lubero	3	nd	nd
Mabaya	3	nd	nd
Mbau	3	nd	nd
Mukuliya	3	nd	nd
Musienene	3	nd	nd
Oicha	3	20	0.71[a]
Rutshuru	3	nd	nd

121 ***The mean aflatoxin B1 contents with the same superscript letters in the same column are not significantly different***
122 *(p>0.05); mean aflatoxin levels were transformed into log (x+1) prior to analysis*
123 *nd= the levels of the aflatoxin analyzed were lower than the limit of detection (0.15 ppb)*

124 Table 2 shows that of the 21 markets sampled in North Kivu, only four markets whose cassava products
125 were contaminated by aflatoxin; it's about Oicha, Alungupa, Beni city and Kayina.The highest aflatoxin
126 contamination was noted in samples that came from Beni city market, with the mean of aflatoxins was 1.2
127 ppb for aflatoxin B1 followed by Kayina (1.05 ppb) and Alupunga market (0.89ppb). The least
128 contamination was observed to Oicha market. The same observation was made to aflatoxin B2 contents
129 as reported in Table 3. This presence is noticed in the samples from the 4 sites including Kayina, Oicha,
130 Alungupa and Beni city. The almost permanent presence of aflatoxins in the samples coming from the
131 same axes can be justified by the fact of permanent use of the previous fermentation scrapings by site
132 and by axis.

133 **Table 3. Level (ppb) of aflatoxin B2 in cassava samples**

Source of samples	Cassava samples		
	Sample size	Positive sample (%)	Mean
Alungupa	3	40	0.34[a]
Beni ville	3	20	0.49[a]
Butembo	3	nd	nd
Goma	3	nd	nd
Itendi	3	nd	nd
Kabasha	3	nd	nd

Kalunguta	3	nd	nd
Kanyabayonga	3	nd	nd
Kayina	3	20	0.21[a]
Kiantshaba	3	nd	nd
Kiwandja	3	nd	nd
Lubero	3	nd	nd
Mabaya	3	nd	nd
Mbau	3	nd	nd
Mukuliya	3	nd	nd
Musienene	3	nd	nd
Oicha	3	20	0.41[a]
Rutshuru	3	nd	nd

***The mean aflatoxin B2 contents with the same superscript letters in the same column are not significantly different (p>0.05); mean aflatoxin levels were transformed into log (x+1) prior to analysis*

nd= the levels of the aflatoxin analyzed were lower than the limit of detection (0.2 ppb)

Table 4. Determination of aflatoxins in cassava by directed fermentation with *R.oryzae*

Fermentation number	Directed fermentation with *R.oryzae* Aflatoxin (µg/kg)	Control * Aflatoxin (µg/kg)
F1	0.0	0.5
F2	0.0	1.5
F3	0.0	1
F4	0.0	1.8
F5	0.0	2
F6	0.0	2.5

*with traditional fermentation, from Beni city

The survey established implies that cassava products are vital source of food and income for majority people in North Kivu. Generally, in Beni city, cassava products which contained fungal growth retained those till the time of preparation for milling when are scraped. This condition may contribute to high contamination of aflatoxins when compared to others districts.

The determination of aflatoxins in cassava samples of directed fermentation with *R.oryzae* showed that there was no aflatoxin; while the traditional fermentation cassava control incubated at the same site contained aflatoxins B1 and B2. After six subcultures of the scrapings constituting the microferment in the traditional fermentation, Table 4 shows disparities in the production of aflatoxins in control batches.

Their concentration is different with a tendency to increase which probably reflects the dominance and stability of aflatoxinogenic strains in the site after several subcultures. These results are consistent with the studies of Yandju, 1995. The results of aflatoxin determination in 6 batches of cassava inoculated with pure *R. oryzae* microferment showed an absence of aflatoxins in all batches, although incubation was done under the same conditions in a traditional setting.

Indeed, the inoculation of the selected germs during a natural fermentation, eventually impose a pure and quantitatively large initial biomass. This would gradually colonize cassava processing sites and destroy the effects of toxigenic microorganisms that play a negative role in cassava fermentation under peasant conditions [15, 17]. Most recently, R. Bandyopadhyay at al (2016) developed a technique using a non-aflatoxinogenic *Aspergillus* strain to provide biological control of maize in storage. To date, it is the only product called “Aflasale” consisting of a suspension of aflatoxinogenic mold spores to protect agricultural products against the production of aflatoxins by aflatoxinogenic molds.

The results obtained in this study suggest that *R. oryzae* strains used in controlled fermentation have been a potent inhibitor of aflatoxins during dry fermentation.

4. CONCLUSION

The results of this study indicates the presence of aflatoxins B1 and B2 in the cassava fufu samples from the Alungupa, Beni city, Kayina and Oicha sites while all the samples obtained from fermentations directed with *R.oryzae* did not have traces of aflatoxin.
Furthermore, the determination of aflatoxins in cassava samples fermented by scrapings from the biomass of *R. oryzae* non-aflatoxinogenes showed a complete absence of these even when the *Rhizopus* strains were sown without asepsis under peasant conditions.
It is therefore very advantageous to use non-aflatoxigenic strains for the dry fermentation of cassava. It will produce large quantities and popularize. This would undoubtedly reduce the risks associated with aflatoxins.

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