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International Journal of Current Research
Vol. 7, Issue, 06, pp. 16644-16649, June 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

FUNGAL CONTAMINATION AND OCCURANCE OF OCHRATOXIN A IN COCOA BEANS PRODUCED IN TWO REGIONS OF CÔTE D' IVOIRE

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ARTICLE INFO

Article History:
Received 12th March, 2015
Received in revised form
29th April, 2015
Accepted 19th May, 2015
Published online 27th June, 2015

Key words:

Aspergillus ochraceus,
Cocoa bean,
Mycoflora,
OTA.

ABSTRACT

The presence of mycotoxins in cocoa beans and chocolate products is emerging as an important public health. There is a réal need for more information about the occurrence of mycotoxigenic fungi in cocoa beans. The mycoflora of cocoa beans in two producing regions was assessed and its potential for ochratoxin A (OTA) production was evaluated. A total of 37 fungal strains were isolated by plating method. Six species of moulds belonging to four genera were isolated from all cocoa samples tested: *Absidia, Rhizopus, Aspergillus niger, A. carbonarius, A. ochraceus, A. flavus, A. versicolor, A. clavatus, Penicillium chrysogenum*. The OTA content producing for ochratoxigenics species was also determined. Three OTA-producing strains were isolated, belonging to the species *Aspergillus carbonarius*, *Aspergillus niger* and *Aspergillus ochraceus* OTA was detected in medium, up to 54.04 µg/kg. The highest content of ochratoxin A was observed with the species *A. ochraceus*. The results of this study highlight the risk associated with the presence of ochratoxinogenic in cocoa from Côte d'Ivoire.

***Citation*:** Agnès Kouakoubla Ama KOUADIO, Pamphile Bony Kouadio KOFFI, Aw Sadat, Soumaila DABONNE, Nogbou Emmanuel ASSIDJO and Lucien Patrice KOUAME, 2015. "Fungal contamination and occurance of ochratoxin a in cocoa beans produced in two regions of côte d' Ivoire", *International Journal of Current Research,* 7, (6), 16644-16649.

INTRODUCTION

Cocoa is widely consumed as chocolate. It is also used in processing of beverages, cosmetics, pharmaceutical products. World production of cocoa beans is estimated to have decreased by 3.7% to 3.931 million tonnes in 2012/2013, compared to the previous season. The combined production for Côte d'Ivoire and Ghana dropped by 85,000 tonnes, to 2.280 million tonnes in 2012/2013, but still represented 58% of total world cocoa output (ICCO, 2014). Côte d'Ivoire is the world's leading exporter of cocoa beans. Nowadays, one of the most widespread problems in advanced technological countries is food quality and safety. The raw cocoa bean and cocoa products quality is more and more in the heart of the future standards of quality of UE countries dealing with the presence of mycotoxins such ochratoxin A (EEC, 2005). In the same time, the economy of the most developing countries and particularly Côte d'Ivoire based primarily on their agricultural resources is strongly dependent on the standards and the often rigorous and rigid quality standards fixed by the developed countries. The quality of cocoa beans is highly dependent on processing technologies and storage conditions. In all warm and wet countries, weather and agronomic conditions are favourable for fungi growth and consequently food quality spoiling. Mycotoxinogenic fungi contamination may be possible at many critical points of the producing chain. It is difficult to predict mould contamination of food because it depends on a complex interaction of factors, such a temperature, moisture, endogenous fungal species, storage history and storage time (Chelack *et al.,* 1991). Mycotoxin refers to a group of secondary metabolites produced by some fungal species and they can cause diseases or death when ingested. Mycotoxins biosynthesis is related to environmental conditions such as temperature, humidity and, harvesting, post-harvesting and storage periods of the agricultural products. Fungal contamination is not always synonymous with the presence of mycotoxin (Hussein and Brasel, 2001). Generally, poor post-harvest management can lead to rapid deterioration of quality, decreasing of commercial and nutritional values of cocoa beans (Magnoli *et al.,* 2006).

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Unfortunately contamination by certain species of fungi such as *Aspergillus* and *Penicillium* responsible for the production of ochratoxin A (OTA) can produce mycotoxins in cocoa beans (Naresh *et al.,* 2003). In Côte d'Ivoire there is no available information on the natural occurrence of moulds in raw cocoa beans. The objective of this study is to isolate and identify fungi strains naturally contaminating beans cocoa and to their toxin producing potential.

MATERIALS AND METHODS

Sampling

cocoa producing regions in Côte d'Ivoire characterized by differing climatic conditions during harvesting were selected for study : (1) Yamoussoukro in the center of the country, a moderate hot rainy region with an average of 21°C-31°C during the harvest season, (2) Soubré in South-western area, a relatively cold and high rainy region with an average of 23-24°C and 70 mm/month rainfall, (3) Soubré in the western center region, a relatively high rainy region with an average of 20.6-34.1°C.

Collection of Samples

Sample preparation, including cocoa harvesting, pod-breaking, fermentation, drying, and storage, was repeated 2 times between 2009 - 2010. 528 samples were collected from the study to evaluate the effects of pod-opening (27 samples), fermentation (158 samples), drying (241 samples), and storage (102 samples).

Determination of pH and water content

A sample of 20 g of cocoa beans was immersed in 100 mL of distilled water and the pH was measured in the supernatant (Lopez *et al*., 1989). Water content was determined in compliance with international standard ISO 2291–1972 (drying at 103 °C for 16 h) on cocoa beans at different stages of post harvest.

Isolation and identification of fungi

Cocoa beans samples were collected aseptically. These samples were immediately plated for yeast and mould counts using potato dextrose agar (PDA). The plates were incubated at 25 ºC for seven days and moulds grown on the plates were maintained using czapek dox agar to observe the morphological and microscopical characters of the isolates.
The characters such as growth rate colour of the colony, colour changes on mycelial growth, colour on the reverse of the plate, texture of the culture on agar surface and micrometry of the reproductive structures were studied for the identification of the moulds. Reference was made to Jamaluddin *et al*. (2004), Pitt and Hocking (2009). The relative percentage frequency (%) and relative density (%) of the fungal isolates was calculated using the formula described by Ghiasian *et al*. (2004).

$$\text{Relative percentage frequency (\%)} = \frac{Number\ of\ samples\ with\ a\ species\ or\ genus}{Total\ number\ of\ samples} X100$$

$$\text{Relative percentage density (\%)} = \frac{No.\ of\ isolats\ of\ fungus}{Total\ no.\ of\ fungus\ isolated} X\ 100$$

Mycotoxins analysis

Isolated strains of *Aspergillus* was inoculated on yeast extract sucrose agar medium (YES) and Czapek Yeast Extract Agar (CYA) and incubated at 27°C for seven days in dark. Fungal cultures were extracted by micro-scale extraction (Samson *et al.*, 2002). The plugs were transferred to a 10 ml glass screw-capped vial containing 3 ml solvent mixture of methanol-dichloromethane-ethyl acetate (1:2:3), 1% (v/v). Extraction was made formic was mad ultrasonically for 60 minutes. A 0.5 ml of the extract was shifted to a glass vial and evaporated to dryness under a gentle stream of nitrogen. The evaporated residues of 0.5 ml extract were re-dissolved ultrasonically for 10 minutes in 400 µl methanol containing 0.6% (v/v) formic acid, 0.02% v/v) hydrochloric acid and 2.5% (v/v) water. Analysis of OTA was performed on the high pressure liquid chromatography system (Prominence TM, Shimadzu®) equipped with florescent detector RF-10AXL® (Shimadzu) by using C-18 column**,** Mediterranean Sea18® 5µm 25cm x 0.46 (Teknokroma, Spain). OTA Analysis was performed using a mixture of acetonitrile: water: acetic acid (57: 41: 2) as mobile phase with a flow rate of 1.0 ml/min at 40°C. The emission and excitation wavelengths were 333 nm and 477 nm, respectively. OTA was confirmed by methyl ester formation (Zimmerli and Dick, 1995).

Statistical analysis

The statistical analysis of the results was carried out using the standard software STATISTICA 7.1. The significance of the differences between the results was calculated with the test of student, $P < 0, 05$.

RESULTATS AND DISCUSSION

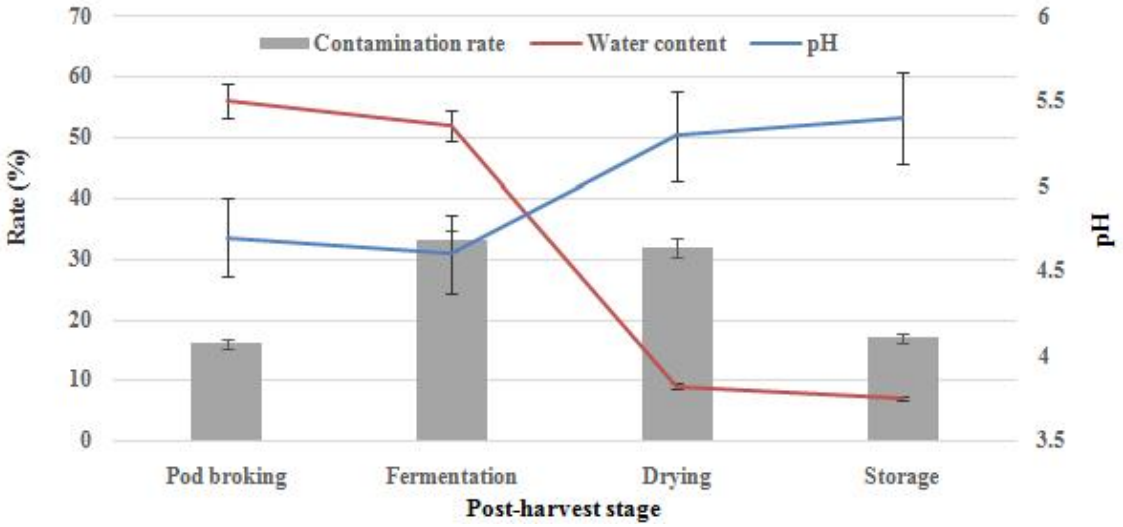
Results in Table 1 show that water rate in cocoa beans decreases during post-harvest treatments. Water content in the beans varies from 51% to 67% at the opening of the pods and from 4% to 13% during storage. Value of pH is comprised between 4.7 and 5.4. pH increases from beginning to end stages of operations although it drops during fermentation. The minimum and the maximum amount of pH in the beans at the opening of the pods are 4.3 and 5.4 respectively while in storage they are 4.7 and 6.5.

Table 1. Physico-chemical characteristics of cacao beans

Phases	Parameter	Average	Minimum	Maximum	Standart deviation
Broking up	pH	4.7	4.3	5.4	0.27
	%Water	56	51	67	3.48
Fermentation	pH	4.6	3.9	5.9	0.47
	%Water	52	42	57	2.91
Drying	pH	5.3	4.5	6.7	0.51
	%Water	9	5	15	2.37
Storage	pH	5.4	4.7	6.5	0.43
	%Water	7	4	13	1.69

%: percentage

The correlation between physico-chemical parameters and the frequency of contamination during the different stage of post-harvest treatment is shown by figure 1. Contamination rate of cocoa beans increases during fermentation.



pH: potential of hydrogen

Figure 1. Evolution of the physico-chemical parameters and the rate of fungi

It then decreases gradually in cocoa beans while losing water and pH rising during the post-harvest processing. Fungi have proliferation during fermentation before decrease in drying and storage. Motoring of pH values show low values that increase during fermentation from 4.6 to 5.4. In food products such as cocoa, the availability of water has an influence on the development of the fungi (Gwaldy and Julien, 2004). The observed pH values (4.7 to 5.4) are a favorable to optimum groth of fungi as established by Duron (1999) which validated the interval of pH from 3.0 to 8.0. Moisture which is the amount of free water available in the sample is responsible for several biological alterations of food including mycological phenomena Moreau (1996). Drying is process removed initial humidity from 56% to 9%. This step is important to stop acidification and stabilize beans. The particular aroma, the color and the taste of chocolate is due to the chemical processes that take place inside the bean during fermentation (Lopes *et al*., 2003).

Low moisture content confers higher shelf-life to the cocoa powder and preserves the nutritional value. Fungal analysis in this study showed a prevalence of the genera *Aspergillus, Penicillium* and *Fusarium* in all samples of cocoa beans analysed. These fungal species are common mycotoxigenic fungi generally found in human food such as in grains, nuts, seeds, fruits, tubers, grain-based products (Kumar and Rajendran, 2008; Sánchez-Hervás and Martínez-Culebras, 2008). Occurrences of some of these species of fungi have been reported in other agricultural products in Côte d'Ivoire (Boli *et al*., 2014; Assiri *et al*, 2015). Mycological analysis in this study showed a wide range of filamentous fungal species belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* contaminating food commodities indicated in Table 2. 98.10 of the samples were infected by filamentous fungi. Cocoa samples were contaminated mainly by fungal species belonging to the genera *Rhizopus* (78.91%) and less by *Aspergillus* (54.05%), *Penicillium* (30.4%) and *Fusarium* (21.81%).

Table 2. Isolation frequency (Fr) and relative densities (RD) of different genera of fungi isolated from cocoa beans

Fungi isolated	*Aspergillus*	*Mucors*	*Fusarium*	*Penicillium*
Fr (%)[1]	54.05	78.91	21.81	30.4
RD (%)[2]	54.04	18.81	10.81	5.4

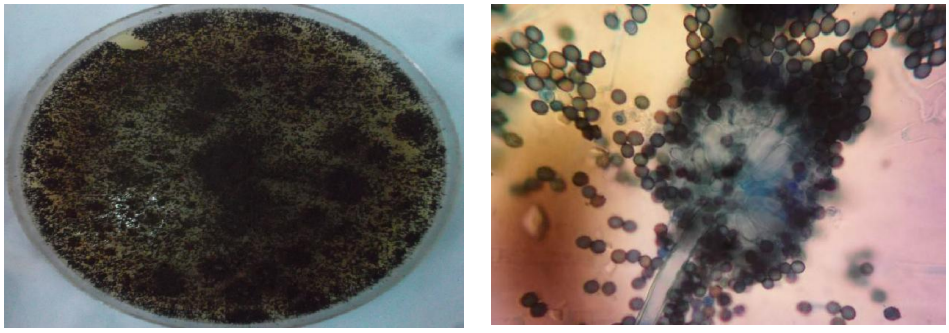
[2]Fr = Isolation frequency; Cocoa beans (n= 528), [1]RD = Relative density

The distribution of fungi gives 54.04% for the genera *Aspergillus*, 18.81% (*Rhizopus* and *Absidia*, 10.81% for *Fusarium* and 5.4% for *Penicillium*. There is a dominance of the genera *Aspergillus*. The genera *Aspergillus* presents the greatest number of species isolated from cocoa beans with *A. niger* and aggregates (36.54%), followed by *A. flavus* (22.64%), *A. fumigatus* (16.98%), *A. versicolor* (13.21%), *A. carbonarius* (4.97%), *A. ochraceus* (3.77%) and *A. clavatus* (1.89) (Table 2). Presence of *Aspergillus* in foods such as cereals has been showed through several works (Riba *et al*., 2005) but also in cocoa (Egbuta, 2015). *Aspergilli are* considered as storage fungi (Withlow and Hagler, 2001). The other genres like the mucorales and *Fusarium* isolated are naturally occurring in crops coming from the fields in the soil (Christensen *et al*., 1977). All Species isolated in the beans during post-harvest treatment are listed in tale Table 3.

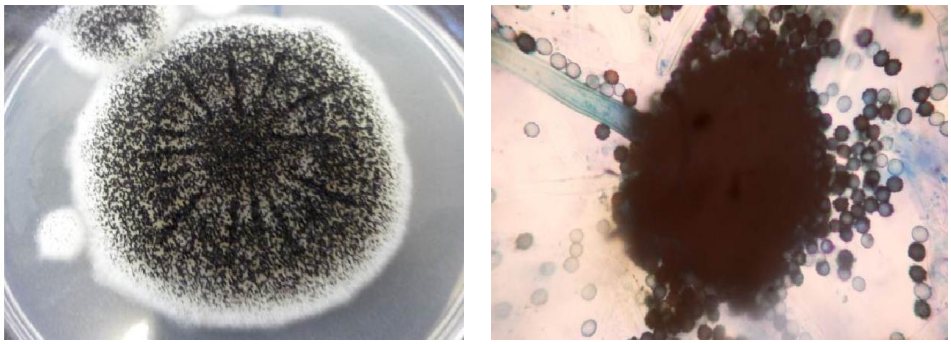
Filamentous fungi isolated during this study are *A. fumigatus, A. flavus, A. versicolor, A.clavatus, A. ochraceus, A. carbonarius, A. niger, P.chrysogenum, Rhizopus sp., Absidia sp., and Fusarium sp*. In previous studies, *Mucor, Penicillium, Rhyzopus, Absidia* and particularly *Aspergillus* have been frequently isolated in cacao beans (Schawn and Wheals, 2004, Oyetungi, 2006). According to these authors, fungi appear to occupy an important place in the microbial contamination of cocoa beans. Differents results were found by some authors who have isolated *A. tamarii, P. paneum, P. crustosum, Aspergillus glaucus*, and *Penicillium sp*. fermented beans (Moujouenpou *et al*., 2008) or dried beans (Sánchez-Hervas *et al*., 2008).

Table 3. Microflora during post harvest treatments

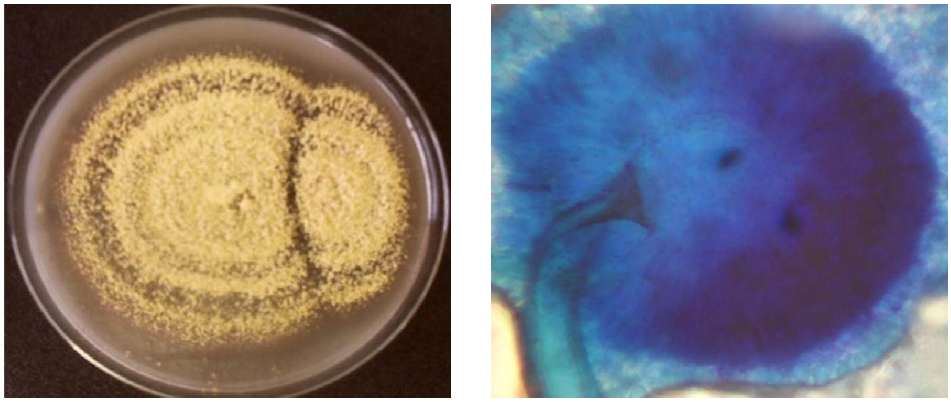
Post harvest stage	Species
Pod broking	*Aspergillus flavus, Aspergillus niger, Aspergillus carbonarius, Aspergillus fumigatus, Penicillium chrysogenum, Penicillium sp, Absidia, Rhizopus, Fusarium sp*
Fermentation	*Aspergillus flavus, Aspergillus versicolor, Aspergillus ochraceus, Aspergillus niger, Aspergillus carbonarius, Aspergillus fumigatus, Aspergillus clavatus, Penicillium chrysogenum, Penicillium sp, Absidia, Rhizopus, Fusarium sp.*
Drying	*Aspergillus flavus, Aspergillus clavatus, Aspergillus versicolor, Aspergillus ochraceus, Aspergillus niger, Aspergillus carbonarius, Aspergillus fumigatus, Penicillium chrysogenum, Absidia, Rhizopus, Fusarium sp.*
Storage	*Aspergillus niger, Aspergillus carbonarius, Aspergillus ochraceus, Aspergillus flavus, Aspergillus versicolor, Aspergillus fumigatus, Absidia, Rhizopus, Fusarium sp, Penicillium chrysogenum*



Aspergillus niger



Aspergillus carbonarius



Aspergillus ochraceous

Figure 3. Species ochratoxigenics in cocoa beans

It therefore appears that the biodiversity of fungal strains present in the processing of cocoa is very strongly related to the geographic, climatic, ecological and human context (local traditional practices and unevenly respected hygiene conditions). Regardless of the condition of pod and the treatment type, a proliferation of filamentous fungi has been observed during fermentation. This can be explained by the existence of the sweet mucilage beans, which strongly favours the development of fungi. However, drying helps to reduce the mycoflora. The fungi coming from the fields (*Mucor, Fusarium*) disappears at the advantage of the storage fungi (*Aspergillus, Penicillium*) (Christensen *et al*., 1977).

Among 20 isolates of *Aspergillus*, we observed that three species (15 %) have the capacity to produce OTA. The presence of *Penicillium* ochratoxigenic was not detected. Ochratoxigenic fungi such as *A. niger, A carbanarius* and *A ochraceus* was found during all the post-harvests operations, in the fresh, fermented and dried beans. *Aspergilus* species involved in the production of OTA in cocoa beans are *A. carbonarius* and *A. niger aggregate* with species *A. westerdijkiae*, *A. ochraceus*, and *A. melleus* (Coppetti *et al*., 2010; Abrokwa and Sackey, 2010). This high contamination may be due to the fact of direct contact with the surface of beans when pods are semi-open (Moujouenpou *et al*., 2008). Among the species ochratoxigenics, 100% of *Aspergillus ochraceus*, *A. carbonarius* and 75% of *Aspergillus niger* have shown their ability to produce OTA. This is an interesting result considering that Taniwaki *et al*. (2003) found in their study that 75% *A. ochraceus* and 3% of *A.niger* isolated have the ability to produce OTA in coffee.

Our study shows that fungi *A. niger*, *A. carbonarius* and *A. ochraceus* showed their ability to produce OTA on agar. Isolates of *Aspergillus* ochratoxigenics (*A. niger*, *A. carbonarius* and *A. ochraceous*) produced OTA range from 27.78 to 42.4 µg/kg. Production of OTA by *Aspergillus ochraceus*, *A carbonarius* and *A. niger* depending on various substrates presented in Table 3. OTA production by *Aspergillus ochraceus* on YES and CYA environments is more important than production by *A. carbonarius* and *A. niger*. The amount of OTA produced by *A.ochraceus* on YES and CYA environments are 42.4 and 55.4 µg/kg respectively. *A. niger* produced the lowest rates of OTA in the two agars.

Table 4. Average production of OTA according to the media and the species

	Media	Average (µg/kg)
A.ochraceus	YES	55,4±0,15
	CYA	42,4±0,56
A.carbonarius	YES	38,02±0,08
	CYA	29,89±0,45
A.niger	YES	30,42±0,64
	CYA	17,78±1,46

YES : Yeast Extract Agar ; CYA : Czapect Yeast agar

The rate of OTA produced on YES is 38.02 µg/kg while on CYA, this rate is 29.89 µg/kg. OTA produced by *A.niger* showed the same trends as that of *A.carbonarius* and *A. ochraceus*. The rate of production of OTA by *A. niger* in on YES and CYA is 30.42 and 17.78 µg/kg afte 14 days. Although the three strains are producing OTA, their metabolic activity (toxin production) is strongly influenced by the substrate in which they are located. Rosa *et al*. (2006) found similar rates on agar plates. OTA production on the substrate of CYA is extended from 25 to120 µg/kg. Production of ochratoxin A (OTA) by *Aspergillus ochraceus*, *Aspergillus niger* and *Penicillium nordicum* has been tested on three substrates, based on yeast extract (YES), coffee and cocoa for a period of 24 days. OTA productions ranged from 0.31 to 6.60 µg/g by isolating species varies depending on the substrate. Our results are closed with those of other authors on the effect of substrate on the production of mycotoxin (L - Ban Koffi *et al*., 2009). II is known that infection by OTA in Côte d'Ivoire, cocoa beans begins in the fields and the main sources of contamination are soil, equipment and surfaces of drying (Bastide *et al*., 2006; Cocoqual, 2007). In addition, the maturity and defects on the beens (broken, damaged and infested seeds) are important sources of contamination by OTA, indicating that the use of good raw materials contributes to the reduction of the toxin (Bucheli *et al*., 2000). Thus, depending on environmental conditions, some areas need more attention on the use of best practices of processing food products than others. Therefore, hazard analysis and control of critical points (HACCP) during the production of the raw material and the steps such as drying, transport, development and storage are essential to prevent the high-risk content OTA (Chiodini *et al*., 2006).

Conclusion

This study showed the presence of the species ochatoxigenics such as *Aspergillus carbonarius*, *A. niger* and *A. ochraceus* in cocoa beans samples in two regions of Côte d'Ivoire. However, good hygiene, handling and appropriate treatment should be used to reduce the contamination of the seed of cocoa. Isolated ochatoxiongenics mycoflora can degrade the cocoa beans thus reducing its market value.

Acknowledgements

The authors would like to express their gratitude to the members of Food and nutrition laboratory of Polytechnic Institut Houphouet Boigny of Yamoussoukro.

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