Rhizopus sp. as antagonist fungi against ochratoxin A production by *Aspergillus carbonarius* in raw cocoa beans

Abstract

Ochratoxin A (OTA) is produced generally by the mold of the genera *Aspergillus*in cocoa beans. This study evaluatedthe antifungal activity of a strain of *Rhizopus* sp. on an OTA-producing mold and the biocontrol of the toxin production by this species in cocoa. OTA contents were detected by HPLC in cocoa beans from different post-harvest treatments. Mold species were identified byconventional microbiology and PCR-DGGE methods according to OTA contents. Suspensions calibrated at 10^6, and 10^4conidia.mL^{-1} of *Rhizopus* sp. and *A. carbonarius* were co-inoculated in cocoa pods and incubated for 4 or 8 days in a climatic chamber.The results showedvarious fungal species of *Aspergillus*, *Aureobasidium*, *Chrysonilia*,*Mucor*, *Penicillium*, and *Rhizopus*. The number of fungalspecies obtained withPCR-DGGE was higher than that obtained on Petri dishes. *Aspergillus carbonarius* and *A. niger*were the main producers of OTA in the samples. The use of plastic crates as fermentation support in the presence of *A.Carbonarius* promoted a high production of OTA compared to wooden boxes and banana leaves. The co-inoculation of 10^4 spores.mL^{-1} of *A. carbonarius* and 10^6 spores.mL^{-1} of *Rhizopus* led to an OTA content in cocoa of 0.1 µg.kg^{-1} in 4 days of incubation while in 8 days, this concentration reached 5.6 µg.kg^{-1}. Finally, the co-inoculation of cocoa with 10^6 spores.mL^{-1} of *A. carbonarius* and 10^4 spores.mL^{-1} of *Rhizopus*sp. led in 4 and 8 days of incubation to respective OTA contents of 36.3 and 6.2 µg.kg^{-1} of cocoa.*Rhizopus* could inhibit OTA production in cocoa.

Keywords: Ochratoxin A, *Rhizopus* sp., *Aspergillus carbonarius*, Cocoa, PCR-DGGE

1. Introduction

Originating from Mexico, cocoa holds an important place in the world. It is cultivated on 8 to 10 million hectares by 3.5 million people in the world who produce 3.5 million tons per year. The cocoa beans are seeds from cocoa fruit pods. Cocoa consumption is estimated to be 3.63 million tons, corresponding to 5 billion dollars USA. Cocoa beans are the main raw material for the production of chocolate **(De Vuyst and Weckx, 2016)**. To obtain this raw material, the fresh cocoa beans undergo post-harvest treatments, particularly

fermentation and drying which significantly influence the final quality of raw cocoa. Fermentation is the main stage in cocoa post-harvest processing. Proper taste, color, and flavor associated with cocoa products are obtained during this step. Generally, fermentation is a spontaneous process carried out traditionally. Its methods, duration, and process vary according to the country, region, and farmers**(Kedjebo et al., 2015)**. The usual methods are fermentation in the basket, fermentation in banana leaves, and fermentation in perforate boxes. After opening the pods, the beans are extracted by hand. Whatever the method of fermentation, the beans are piled on a bed of banana leaves. The sweet mucilaginous pulp surrounding the beans promotes rapid yeast growth that initiates fermentation, followed by lactic and acetic bacteria**(Hamdouche et al., 2015)**. Cocoa beans are generally fermented for four to seven days with or without mixing (**Dano et al., 2013**). The fermented beans are immediately dried. There are two ways of drying in the world of cocoa. But the most used is solar drying which is generally carried out on a concrete floor, a plastic sheeting, or a sieve. Drying stopped the fermentation and reduced the water content of the beans to 7 to 8%.

Mold can contaminate cocoa from pods opening to storage. However, this contamination is increasing during drying. Molds population contaminating cocoa is dominated by species belonging to the genera *Fusarium, Mucor, Rhizopus, Penicillium,* and *Aspergillus***(Copetti et al., 2014)**.The identification of these molds is generally cured by conventional microbiology **(Guehi et al., 2017)**. To our knowledge so far, no study has used the molecular PCR-DGGE technique to identify molds in general and mycotoxigenic molds contaminating cocoa.However, PCR-DGGE was used to rapidly have the overview of microbial ecology **(Ercolini et al., 2001).** This method was successfully employed for investigating the fungal community of asparagus, yeasts, and bacteria community of coffee, and cocoa **(Hamdouche et al., 2015)**. Moreover, **El Sheikha (2019)**showed that PCR-DGGE can be used to identify mycotoxigenic fungi in food.

The presence of mold in cocoa should be avoided. Indeed,some species can produce enzymatic lipolysis and increase the content of free fatty acid (FFA) in cocoa beans **(Houphouët et al., 2023)**. Moreover, some species of the genera *Fusarium, Penicillium,* and *Aspergillus* are producers of mycotoxins such as ochratoxin A (OTA) (**Copetti et al., 2014**). OTA is a mycotoxin classified as carcinogenic, nephrotoxic, teratogenic, immunotoxic, and hepatotoxic for humans and animals **(Pfohl-Leszkowicz and Manderville, 2007)**. That toxin contaminates many foodstuffs from plant origin, including cocoa and cocoa products **(Turcotte et al., 2013)**. Chemical, physical, and biological methods have been proposed for

the detoxification of mycotoxins. However biological methods are the more promising ones. Indeed, biological methods use microorganisms such as yeast, bacteria, and mold, which can decompose, transform, or adsorb OTA to detoxify contaminated products or to avoid the toxic effects when mycotoxins are ingested**(Abrunhosa et al., 2010)**. Concerning molds, *Rhizopus oryzae* has been used to reduce aflatoxins B1, B2, G1, and G2 contents **(Hackbart et al., 2013)**. Preview studies showed that species of *Rhizopus* produce various enzymes **(Ghosh et Ray, 2011).** Thus Rhizopus sp. would be an antagonist of other molds and capable of hydrolyzing the toxins produced. This work aimed to study the biocontrol of *Aspergillus carbonarius* development and its ochratoxin A production by *Rhizopus* sp. after identifying mycotoxigenic molds by PCR-DGGE according to post-harvest treatments.

2. Material and methods

2.1. Cocoa

The plant material used consisted of cocoa beans from the cultivar ripe pods Amelonado, Ivorian 1st generation of hybrids (Amelonado x West African Trinitario), and open-pollinated progenies harvested at the farmer plantations in January 2014. The experiments were performed at Akoupé, one of the main cocoa regions in Ivory Coast located 140 km, South-East of Abidjan.

2.2. Cocoa bean fermentation

A total of 36 cocoa pod batches (200 pods each) were constituted and the beans of each batch were fermented using the micro-fermentation technique. Two lots of cocoa pods were constituted: healthy pods and decayed or damaged pods. A total of 3600 pods for each lot were harvested. The pods of both lots were divided into three parts (1200 pods/each). The pods of the first sub-lot were opened immediately after harvesting, while the pods of the second sub-lot were opened after 4 days and those of the third sub-lot after 7 days of storage. Just after the pod opening, approximately 300 kg of fresh beans surrounded by mucilage were obtained. This mass of fresh beans was divided into six parts of 50 kg each for fermentation. Three methods of fermentation were studied: fermentation in a wooden box (i), fermentation in a plastic box (ii) where the beans were placed in boxes measuring 50 cm x 50 cm x 50 cm, and fermentation in heaps (iii) where beans were tipped onto banana leaves placed on the ground as previously described (**Kedjebo et al., 2015**). The wooden floor of the wooden boxes and the plastic floor of the plastic boxes had holes to facilitate the drainage of acidic liquid resulting from the liquefaction of mucilaginous pulp covered with plantain leaves. Both the wooden and the plastic floors were raised above ground level, over a drain that

carries away the pulp juices liberated by the degradation of the mucilage. The heap of wet cocoa beans was then covered in the box with other fresh banana leaves to insulate the top of the box before placing the cover. For each fermentation method, fresh cocoa beans of one batch (50 kg) were mixed after 48 and 96 h of processing while the beans of the other batch were processed without mixing. Each type of fermentation lasted 4 or 7 days. All fermentations were carried out simultaneously to exclude the influence of time, weather, and contamination factors (operator hands, banana leaves, knives, etc.).

2.3. Cocoa beans drying

A total of 216 cocoa bean samples were taken from different points in the fermenting cocoa mass and dried in different areas. At the end of each fermentation, three sub-samples of about 9 kg of freshly fermented cocoa beans were taken at the end of different durations of the fermentation process: 4 and 7 days. Fermented beans of each fraction were divided into three fractions (1 kg) and then dried according to natural or sun drying. The natural or sun-drying process consisted of exposing fermented cocoa beans from 9 a.m. until 6 p.m. on an area of about 30-50 cm^2. The beans of three fractions from the same batch were separately spread thinly and dried on a meshed wooden tray, cement floor, and plastic tarpaulin for 5-7 days until they reached a moisture content of about 7%. The beans were mixed to ensure uniformity (**Kedjebo et al., 2015**). A total of 216 samples were aseptically collected and placed in sterile bags then put in a cooler box and sent immediately to Cirad Montpellier (France) by plane. There, all cocoa samples were frozen for seven days before analysis. For analysis, 104 samples of 216 were randomly selected.

2.4. Determination of ochratoxin A level in cocoa beans

OTA was extracted from the entire cocoa beans as previously described by **Kedjeboet al. (2015).** The cocoa beans of each sample were frozen at -80°C, and then ground. Fifty grams of ground beans with 4 g of NaCl were extracted in 200 mL of solvent (acetonitrile/water, 60/40, v/v). The final homogenate solution was filtered using paper Whatman No. 4. Four mL of filtered extract were diluted in 44 mL of phosphate buffer (PBS) with 48 µL of Tween 20. The immunoaffinity column (Ochraprep, R-Biopharm, Darmstadt) was conditioned with 10 mL of PBS and the extract was purified throughout the immunoaffinity column at a flow rate of 1 mL/min. Elution of OTA was performed using two volumes of 1.5 mL of methanol at a flow rate of 1 mL/min and evaporated till dry in a nitrogen stream at 70°C. The residue was resuspended in 1 mL of the mobile phase (water/acetonitrile/acetic acid, 51:48:1, v/v). Quantification was performed using the Shimadzu HPLC system (Shimadzu, Japan) as previously described by **Kedjebo et al. (2015**). A fluorescence detection set at 333 nm

excitation and 460 nm emissions. The mobile phase was acetonitrile/water/acetic acid (51:48:1, v/v/v), and the flow rate was 1 mL/min. An OTA standard was used for the construction of a five-point calibration curve of peak areas versus concentration (ng/mL). The injection volume was 100 µL for both standard solution and sample extracts. The limit of detection was 0.05 µg/kg.

2.5. Identification of fungal

2.5.1. Morphological characterization of molds according to OTA level in cocoa beans

For fungal identification by conventional microbiology, nine cocoa samples from the 104, whose three (Q, A, and C) contained high OTA levels (10 to 39 µg/kg), three (F, K, and Z) with OTA level between 2 and 10 µg/kg and finally three (G', H and X) contained no detectable OTA level according to the technique used, were selected**(Table 1)**. Three beans per sample were cultured aseptically on PDA-Chloramphenicol with three Petri plates per sample. After incubation for 5 days at 30°C, macroscopic and microscopic identification of each mold was carried out. Isolated molds were identified according to **Guiraud 1998**.

2.5.2. DNA extraction

For each of the 104 samples, 10 g of cocoa beans were aseptically filled into sterile 50 mL falcons containing 10 mL sterile peptone water. The falcons were mixed with a rotating wheel at 12 rpm for 20 min at room temperature. After clarifying for 5 min, the DNA of the molds was extracted with the kit FAST DNA Spin. Then, 0.7 mL of the supernatant was put in the tube lysing Matrix. In this solution, 1 mL of CLS-Y (Cell Lysis Solution) was added. The mixture was homogenized with the homogenizer FAST Prep (BIO101 Savant, MPBIO, USA) for 40 sec and then centrifugated at 14 000 x g for 5 to 10 min. 750 µL of the supernatant were put in 2 mLtube Eppendorf. At this volume, 750 µL of Binding Matrix was added. The mixture was incubated for 5 min at room temperature. Half of this mixture was transferred in a SPIN filter and centrifuged at 14 000 x g for 1 min. The supernatant was eliminated and the rest of the mixture was added to the residue. After another centrifugation, the supernatant was discarded. Five hundred µL of SEWS-M solution were added to the residue and the mixture was centrifuged until the supernatant passed from the filter to the tube Eppendorf. At the residue obtained, 100 µL of DES were added and the mixture was incubated in a water bath at 55°C for 5 min. After the incubation, the solution was centrifuged (14 000 g, 1 min) for eluting the DNA. The filter with the residue was discarded and DNA was recuperated in the solution at the bottom of the tube Eppendorf. The quantity and the quality of extracted DNA were evaluated by UV spectrophotometer (Biospec-Nano), and by

electrophoresis through a 0.8% agarose gel in TAE 1 X buffer (containing 40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2 -EDTA; Eppendorf) with a 1 Kb molecular weight ladder (Promega). After running at 100 V for 30 min, the gels were stained for 30 min with ethidium bromide solution (50 mg/mL in TAE 1X, Promega), rinsed for 20 min in distilled water, then observed and photographed 0on a UV transilluminator, using black and white camera (Scion Co., USA) and Gel Smart 7.3 system software (Clara Vision, Les Ulis, France).

2.5.3. PCR-DGGE analysis

For molds DNA PCR, the 28S regionwasamplifiedusinggc-U1: 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA-3' (Sigma, France) and U2: 5'-GAC TCC TTG GTC CGT GTT-3' (Sigma, France) whichamplify 250 PB **(El Sheikha et Montet, 2011)**. The PCR reaction was performed in a final volume of 50 µL containing 1 µM of each primer, 200µM of deoxyribonucleotide triphosphate (dNTPs, Promega, France), 0,5 mM of $MgCl_2$, 5 µL of Tampon 10 X, 1,25 U of Taq polymerase (Promega, France) and 5 µL of DNA pellets (~100 ng). The amplification was carried out as follows: a denaturation at 94°C for 3 min, followed by 35 cycles including a denaturation at 94°C for 30 sec, a hybridization at 52°C for 30 sec, and an extension at 72°C for 1 min. The final extension was increased at 72°C for 10 min. PCR products (5 µL) were analyzed by using conventional electrophoresis in 2% (W/V) agarose gel with TAE 1X. For that, 2 µL of colorant blue/orange solution was added to 5 µL of DNA amplicons. The mixture, the positive and the negative witness, and the DNA massladder of 100 pb (Invitrogen, USA) were subjected to 100 V for 35 min. The PCR products of each isolated mold were analyzed by DGGE using a Bio-Rad Dcode™ universal mutation detection system (Bio-Rad Laboratories, USA), using the procedure previously described by **Durand et al. (2013)**. PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide/N, N-methylene bisacrylamide, 37.5/1, Promega) in TAE 1X buffer (40 mM Tris-HCl pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2 -EDTA). Electrophoresis was performed at 60°C, using a denaturing gradient in the 30–60% range (100% corresponded to 7 M urea and 40% [v/v] formamide, Promega). The gels were submitted to electrophoresis at 20 V for 10 min and then at 80 V for 12 h. After electrophoresis, the gels were stained for 30 min with Gel Red solution (0.1 µL/mL in TAE 1X) and then photographed on a UV transilluminator with the Gel Smart 7.3 system (Clara Vision, Les Ulis, France).

2.5.4. Image treatments and DGGE profiles statistical analysis

The pictures were treated with the software ImageQuant TL 7.0 (GE Healthcare, USA) Analysis of data was done using the Dice similarity coefficient (S D), which is calculated according to the formula :

$$RP(X) = \frac{d}{DTe}$$

with :

- *RP*: relative position
- *d*: distance of an X DNA band
- *DTe*: the distance that separated the witness representatives' bands

A numerical classification of the sample bands was made by using the Dice similarity coefficient (S_D), which is calculated according to the formula**(Hamdouche et al., 2015)**:

$$S_{Dice} = \frac{100 \times 2 \times N_{AB}}{N_{AB} + N_{AB}}$$

were :

N_{AB}: represented the number of the samples' communes bands

N_A and N_B represented the numbers of the bands A and B

2.6. *Effect of Rhizopus* sp. *on the production of OTA by Aspergillus carbonarius*

Biocontrol of ochratoxin A production was studied in aco-culture of non-OTA-producing molds (Rhizopus sp.) and those producing (*A. carbonarius*). *A. carbonarius* was chosen because it was the main producer of OTA species funded in the cocoa beans studied. Concerning *Rhizopus* sp., its choice was driven by the fact that in addition to being among the most contaminating mold of cocoa beans, it had a faster growth and appeared more abundant **(Durand, 2013)**. For that, the pre-culture of the two isolated strains was carried out separately on PDA agar at 25°C for 5 days. After purification of the strains by successive subcultures, the conidia of *Aspergillus carbonarius* and *Rhizopus* sp. of the same age were harvested by scraping in a sterile water solution physiological (9‰ of NaCl). After counting the spores with a hemocytometer, suspensions calibrated at 10^6, and 10^4conidia.mL^{-1}were obtained by dilutions of the initial suspensions for inocula preparations. Then 1 mL of each inoculum was injected aseptically using a syringe inside cocoa pods whose shellswere just before pierced

with a nail. Some pods were inoculated only either with spores of *Aspergillus carbonarius*or with spores of *Rhizopus* sp. and other pods underwent a co-inoculation of the spores of the two molds at variable concentrations. The pods thus inoculated were incubated in a sterile climatic chamber at 30°C with 60% relative humidity for periods of 4 to 8 days. At the end of incubation, the pods were opened with a cutlass then the beans were recovered. Then the OTA of the beans was extracted and then assayed by HPLC.

2.7. Statistical analyzes

Statistical analyses based on the correlation were carried out by using the software XLSTAT® 2014.1.01 ANOVA. The correlation coefficients (r) were calculated to identify the incidence of post-harvest treatments on OTA levels in all cocoa samples analyzed. Values were interpreted according to the Fisher test (LSD) with a 95% confidence interval (Tolerance 0.0001).

3. Results

3.1. Morphological characterization of isolated molds and cocoa OTA content

Based on the macroscopic and microscopic characteristics, various fungus species belonging to the genus *Aspergillus, Aureobasidium, Chrysonilia, Mucor, Penicillium,* and *Rhizopus* could be identified (**Table 1, Figure 1**). The genus *Aspergillus* is particularly represented by species such as *A. oryzae, A. parasiticus, A. flavus, A. nigeragg, A. carbonarius, A. tamarii* and *A. fumigatus*.

The results show that the cocoa from damaged pods that had been delayed was contaminated by a wider fungal diversity. This cocoa also has higher levels of OTA, which appear to be linked to the presence of *A. Niger* and or *A. carbonarius*. Cocoa from healthy pods was contaminated only by *Aspergillus niger* while beans from damaged pods were contaminated earlier with *A. carbonarius*.

Cocoa from pods opened immediately after harvest, regardless of pod status, has OTA levels between the non-detectable values (nd) and 2.4 µg/kg. With cocoa from damaged pods, the OTA levels ranged from 4.4 to 39.2 µg/kg. The highest values (28.1 and 39.2 µg/kg) were

observed on fermented cocoa in plastic crates and this cocoa was contaminated with *A. carbonarius*.

Table 1: Genera of moldsidentified according to their macroscopic and microscopiccharacteristics with the identification keys, and ochratoxin A content of merchant cocoa samples having undergone different post-harvest technological treatments

Post-harvest Treatments	**Genera of molds**	**OTA content (µg/kg)**
H'	*Aspergillus* (2 species)	nd
I	*Aspergillus* (2 species), *Chrysonilia, Aureobasidium, Mucor sp*.	nd
X	*Aspergillus* (3 species)	nd
F	*Aspergillus* (1 specie)., *Mucor* sp., *Penicillium sp*.	2.1
H	*Aspergillus* (2 species), *Mucor sp, Rhizopus sp.*	2.4
Z	*Aspergillus* (3 species), *Chrysonilia sp., Mucor sp., Rhizopus sp.*	4.4
X	*Aspergillus* (3 species),*Chrysonilia sp., Mucor sp., Rhizopus sp.*	10.2
A	*Aspergillus* (3 species), *Rhizopus sp, Chrysonilia sp.*	28.1
C	*Aspergillus* (3 species), *Rhizopus sp, Chrysonilia sp., Mucor sp.*	39.2

nd: no detectable

Note: H', I, X, F, H, Z, X, A, and C are defined in figure 2

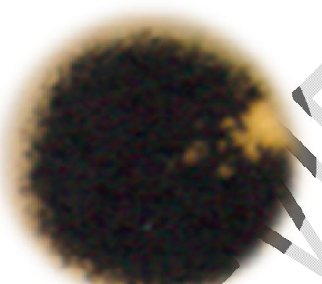
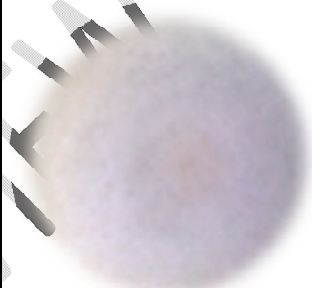
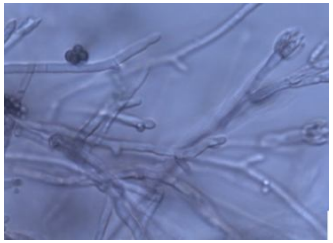
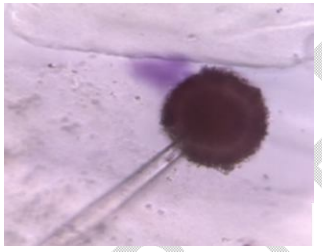
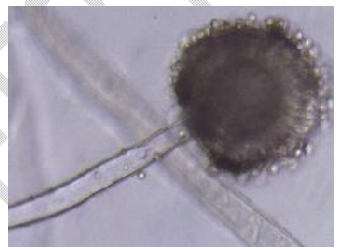
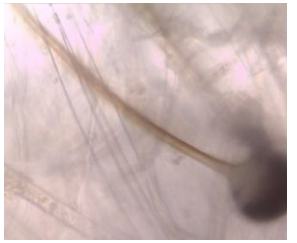
	M1	M2	M3	M4	M5
Macroscopicfeatures					
Microscopicfeatures					

Figure 1: Phenotypic appearance of a few molds isolated from cocoa. **M1)** *Penicillium* sp.; **M2)** *Aspergillus* sp.; **M3:** *Aspergillus* sp; **M4)** *Mucor* spp ;**M5)***Rhizopus* spp.

3.2. Study of fungal diversity by PCR-DGGE and OTA content of merchant cocoa

DNA extracted from 42 cocoa samples that underwent various post-harvest treatments was studied by PCR-DGGE. **Figure 2** presents the gels of cocoa beans fermented in a plastic box (**I**), a wooden box (**II**), and banana leaves (**III**).

The total number of bands, which can be related to the number of species present in the sample, has been recorded with 17 bands on the gel of cocoa beans fermented in the plastic box, 13 for cocoa from the wooden box, and 16 for banana leaves. To identify the species, the study was targeted at the principal ochratoxinogenicmolds contaminating the cocoa analyzed as *Aspergillus carbonarius* and *niger*. The analysis of the gels made it possible to establish **Table 2** which describes, for each sample, the treatment undergone, the presence or absence of the two strong species producing OTA (*A. carbonarius* and *A. niger*), and the OTA content. All the data obtained was statistically treated with the XLSTAT 2015 software (ANOVA). As expected,the number of different species separated on the gels is higher than that obtained on Petri dishes. Five species were found on the DGGE gel versus 2 on a Petri dish for the sample H', 5 instead of 3 for X, 9 instead of 6 for Z, 7 instead of 5 for A, and 16 instead of 6 for C.

First, the analysis of the results shows that the presence of *A.carbonarius* and a lesser degree of *A.niger* have a significant influence (Fisher's ANOVA) on the cocoa OTA content (**Figure 3**). The interactions between the various technological parameters and the presence of ochratoxinogenic strains on the OTA content of the samples have also been studied (ANOVA Fisher test). It appears that significant interactions occur, on the one hand between the presence of *A. Carbonarius* and the method of fermentation in plastic boxes. Indeed, the use of plastic crates as fermentation support in the presence of *A.Carbonarius* promotes a high production of OTA with a high content compared to the boxes of wood and banana leaves (**Figure 4**). On the other, there were interactions between the presence of *A. Carbonarius* and/or *niger* and the fermentation process without missing.

In the absence of ochratoxinogenic strains, missing has little influence on the OTA content of the samples, while their presence leads to high mycotoxin values when there is no missing.

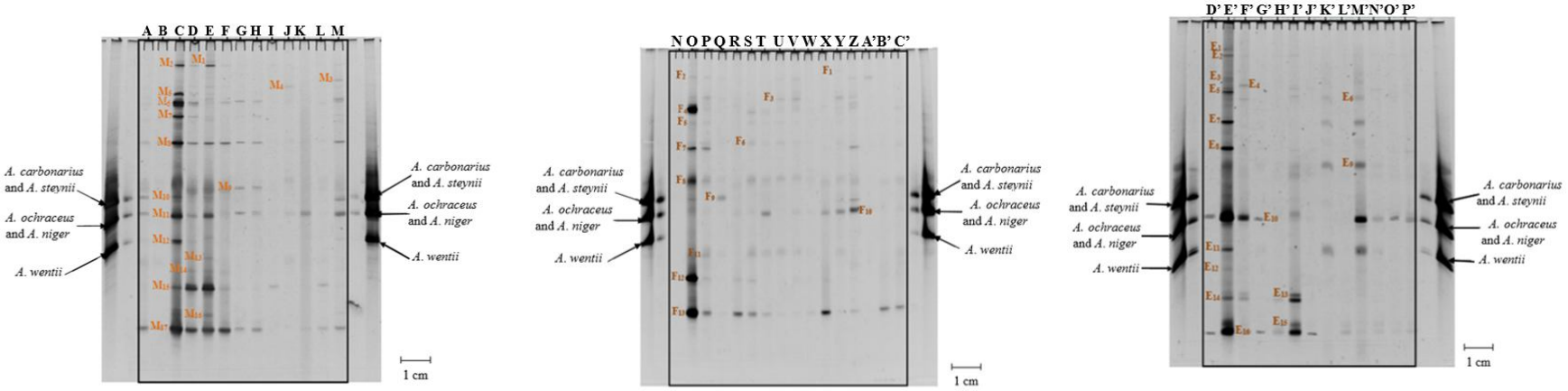


Figure 2: DGGE profile of 28S rDNA of mold species having contaminated the different cocoa samples (**1:** fermentation in the plastic box; **2:** fermentation in wooden box; **3:** fermentation in banana leaves) ;*Aspergillus carbonarius, A.niger, and A.wentii* were used as markers

Note: A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, A', B', C', D', E', F', G', H', I', J', K', L', M', N', O', P': Samples of cocoa beans from damaged or healthy pods opened immediately or after 4 or 7 days, fermented either in banana leaves, in plastic or wooden boxes with or without mixing during 4 or 7 days and drying either on black tarpaulin, rack or on a concrete floor; **M_1…M_{17}**: molds observed in beans fermented in plastic boxes; **F_1…F_{13}:**molds observed in beans fermented in wooden boxes; **E_1…E_{16}:**molds observed in beans fermented in banana leaves**.**

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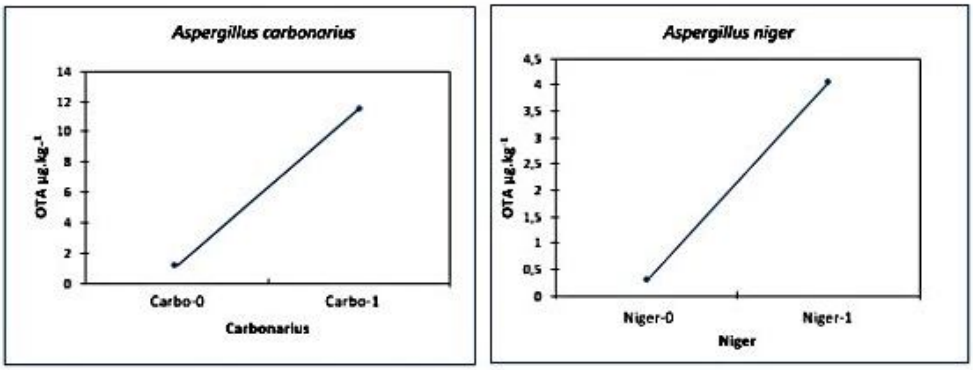
Table 2: Data of each sample concerning: treatment, presence or absence (1 or 0)of *Aspergillus carbonarius*and *A. niger*, number of species, and OTA content.

REF	Podshealthstatus	Podsopeningdelay (days)	Fermentation method	Fermentation process	Fermentation duration	*A. carbonarius*	*A. niger*	OTA ($\mu g.kg^{-1}$)	Speciesnumber
A	D	7	PB	M-	7	1	1	28.1	7
B	D	4	PB	M-	4	0	0	2.3	2
C	D	7	PB	M-	4	1	1	39.2	16
D	H	7	PB	M-	4	0	1	1.2	20
E	H	7	PB	M-	4	0	1	1.2	14
F	H	0	PB	M-	7	0	1	2.1	9
G	D	4	PB	M+	7	0	1	.	6
H	D	0	PB	M-	7	0	1	2.4	6
I	H	0	PB	M+	4	0	1	0.3	2
J	D	0	PB	M+	4	0	1	0	6
K	D	0	PB	M-	7	1	1	4.6	11
L	H	4	PB	M-	7	0	0	0	3
M	D	4	PB	M-	4	1	1	5	11
N	H	0	WB	M-	4	0	0	0	6
O	D	4	WB	M-	4	1	1	1,3	11
P	H	0	WB	M-	7	1	1	6.0	10
Q	D	7	WB	M+	4	1	0	10.2	6
R	H	4	WB	M-	7	0	1	0	4
S	H	7	WB	M-	4	0	1	0.8	8
T	D	4	WB	M+	7	0	1	4.2	6
U	H	0	WB	M-	4	1	1	6	10
V	D	0	WB	M+	4	0	1	0	6
W	H	7	WB	M+	7	0	0	0	3
X	D	0	WB	M-	7	0	1	0	5
Y	D	7	WB	M+	7	1	1	4.1	9
Z	D	7	WB	M-	4	1	1	4.4	9
A'	H	0	WB	M+	4	0	1	0	5
B'	H	7	WB	M+	4	0	0	0	3

C'	D	4	WB	M+	4	1	1	4.5	6
D'	H	7	BL	M+	4	0	1	1.6	2
E'	H	7	BL	M-	4	0	1	2.8	12
F'	H	4	BL	M+	7	1	1	11.2	6
G'	D	0	BL	M-	4	0	1	0.0	5
H'	D	0	BL	M-	7	0	1	1.2	11
I'	H	0	BL	M+	4	0	1	0.1	4
J'	H	7	BL	M-	7	0	1	1.4	6
K'	D	4	BL	M-	4	0	1	5.7	2
L'	D	4	BL	1	7	0	1	1.4	7
M'	D	7	BL	M-	7	0	1	3.7	3
N'	H	0	BL	M-	4	0	1	0.2	5
O'	D	7	BL	M-	7	0	1	3.7	3
P'	D	4	BL	M-	7	0	1	3.4	3

Note:A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, V, W, X, Y, Z, A', B', C', D', E', F', G', H', I', J', K', L', M', N', O', P' are defined in figure 2; D: damaged pods; H: healthy pods ; 0: pods opened immediately; 4: pods stored for 4 days; 7: pods stored for 7 days; WB: wooden boxes; PB: plastic boxes; BL: banana leaves ; M-: fermentation without mixing; M+: fermentation with mixing; 4: fermentation for four days; 7: fermentation for seven days ; Bt: drying on black tarpaulin; R: drying on rack; Cf: drying on concrete flora; 0: absence of *Aspergillus carbonarius*or *A. niger*; 1: presence of *Aspergillus carbonarius*or *A. niger*.



Aspergillus carbonarius / Tukey (HSD) / Analysis of the différences between modality with 95% confidence interval

Contrast	Difference	Difference standard	Critical value	Pr>Dff	Significantly
Presence vs Absence	10.373	4.541	2.023	<0.0001	Yes
Critical value of Tukey d					

Modality	Average	Groupes	
Presence	11.482	A	
Absence	1.109		B

Aspergillus niger / Tukey (HSD) / Analysis of the différences between modality with 95% confidence interval

Contrast	Difference	Difference standard	Critical value	Pr>Dff	Significantly
Presence vs Absence	3.761	2.591	2.074	<0.017	Yes
Critical value of Tukey d			2.933		

Modality	Average	Groupes	
Presence	4.048	A	
Absence	0.87		B

Figure 3: Impact of the presence or the absence of *Aspergiluscarbonarius* and *A. niger* on OTA content

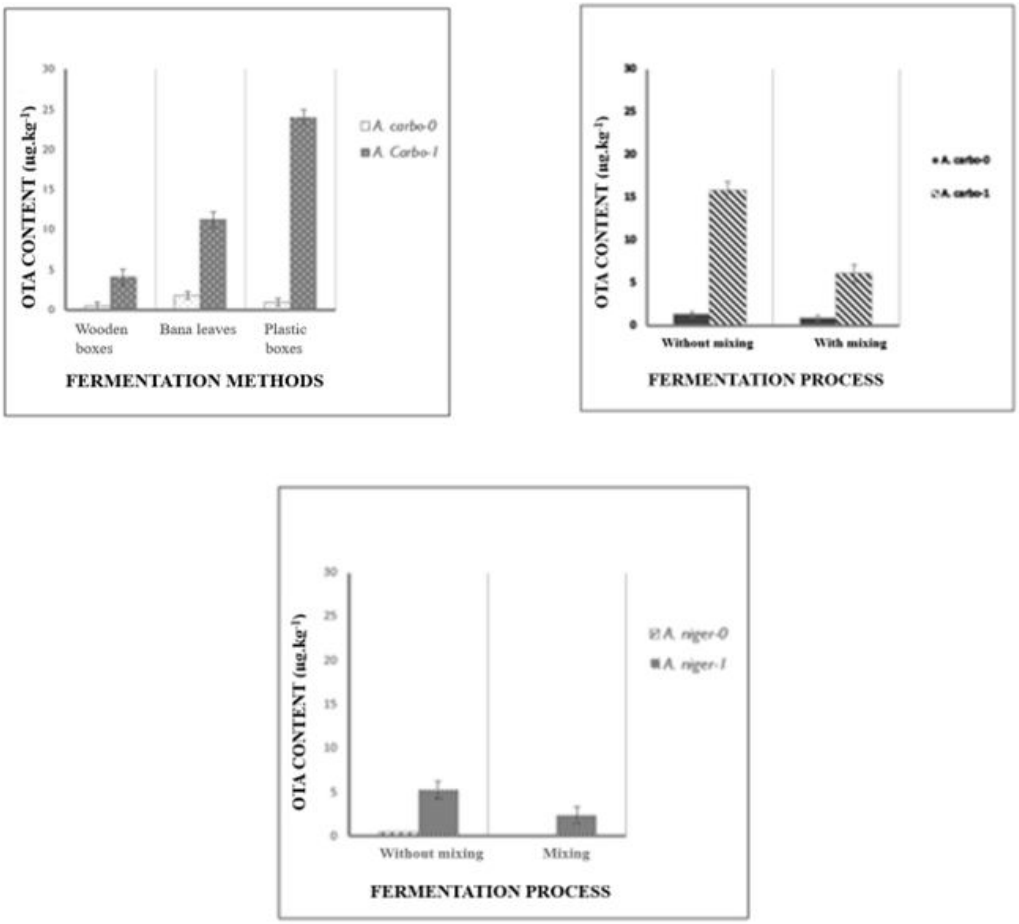
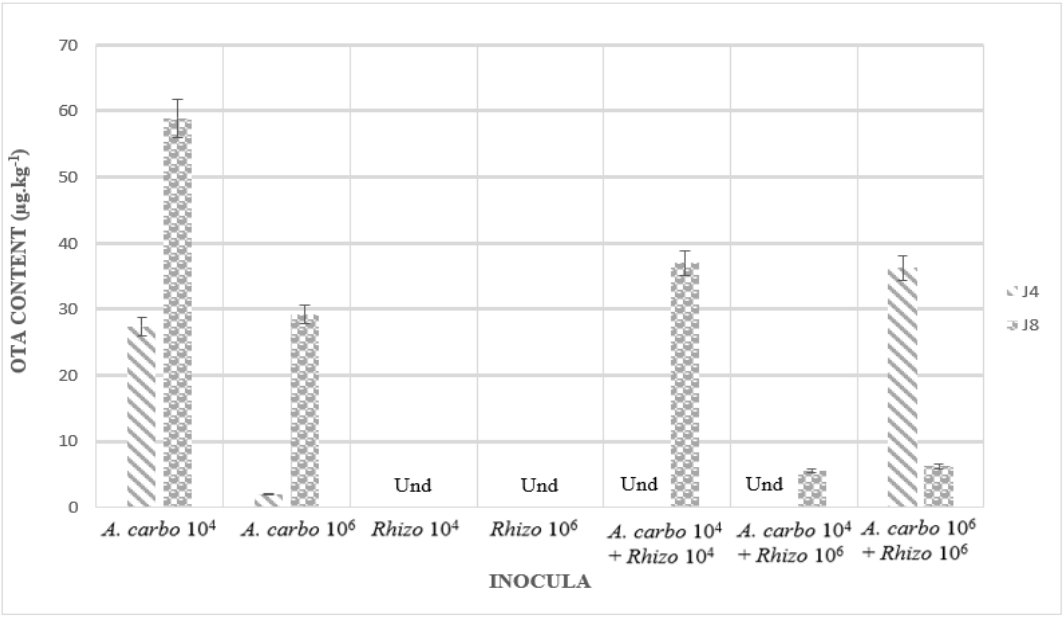


Figure 4: Impact of fermentation methods and process on cocoa beans' OTA content according to the presence or the absence of *Aspergillus carbonarius* and *A. niger*

3.3. *Interactions between Aspergillus carbonarius and Rhizopus* sp.

Figure 5 presents the OTA levels of cocoa contaminated with inocula based on conidia of *A. carbonarius*and *Rhizopus* separately or in combination after 4 and 8 days of incubation.

After 4 days of incubation of the pods inoculated with conidia of *A. carbonarius* (10^4 spores.mL^{-1}), OTA levels of 27.4 µg.kg^{-1} were measured in cocoa beans. After 8 days of incubation, this OTA concentration has almost doubled to reach 58.9 µg.kg-1. With an inoculum of 10^6 spores.mL^{-1} of *A. carbonarius*, respective OTA contents of 2 and 29.2 µg.kg^{-1} of OTA were measured in cocoa after the same incubation times. On the other hand, cocoa inoculated with *Rhizopus* spores is free of OTA whatever the conidia concentration of the inoculum and the incubation period. Fungal co-inoculation of cocoa with inocula composed of 10^4spores.mL^{-1} of *A. carbonarius*and 10^4 spores.mL^{-1} of *Rhizopus* led to an OTA content of 0.1 µg.kg^{-1} after 4 days of incubation and 37 µg.kg^{-1} after 8 days. The co-inoculation of 10^4spores.mL^{-1} of *A. carbonarius* and 10^6 spores.mL^{-1} of *Rhizopus* led to an OTA content of cocoa of 0.1 µg.kg^{-1} in 4 days of incubation while in 8 days, this concentration reached only 5.6 µg.kg^{-1}. Finally, the co-inoculation of cocoa with 10^6spores.mL^{-1} of *A. carbonarius* and 10^4 spores.mL^{-1} of *Rhizopus* led in 4 and 8 days of incubation to respective OTA contents of 36.3 and 6.2 µg.kg^{-1} of cocoa.



Und: undetectable
A. carbo : *Aspergillus carbonarius*
Rhizo: *Rhizopus* sp.

Figure 5: OTA levels of cocoa contaminated with inocula based on conidia of *A. carbonarius*and *Rhizopus* sp. separately or in combination after 4 and 8 days of incubation.

4. Discussion

The morphological characterization of the molds according to post-harvest treatments has shown different species of molds mainly belong to the genera *Aspergillus, Aureobasidium, Chrysonilia, Mucor, Penicillium* et *Rhizopus***(Mounjouenpou et al., 2012)**. The genera *Aspergillus* was particularly represented with species such as *A. oryzae, A. parasiticus, A. flavus, A.nigeragg, A. carbonarius, A. tamarii*et*A. fumigatus.* The analysis by PCR-DGGE of the fungal microflora contaminating the samples made it possible to provide information that complements what has been described by conventional microbiology techniques. The DGGE has provided more quantitative information on species present in addition to conventional microbiology techniques. Many more species have been demonstrated by the use of molecular techniques compared to techniques using Petri dishes. It should be noted that the identification and enumeration of molds by conventional microbiology techniques have limitations related to the physiological state of the molds, the germination of many fungal spores produced by a mold strain leading to a greater number of mold strains unlike bacteria and yeasts and their subsequent development on Petri dishes, limits whose molecular methods and in particular the PCR-DGGE, make it possible to get rid of in terms of identification **(Hamdouche et al., 2015)**.

The results showed that cocoa from damaged pods with pod-opening delay, in addition to having high OTA levels, has been contaminated by several species of molds. These high OTA levels may be related to the presence of *A. Niger* and/or *A.carbonarius*. Indeed, these two strains are generally considered to be the main potentials producing OTA in cocoa **(Mounjouenpou et al., 2012)**. The unique isolation of *A. Niger* from cocoa healthy pods and the significant contamination of cocoa from the pods damaged by *A. carbonarius* confirms that the fungal diversity of cocoa was not influenced by either the technique or the duration of fermentation as demonstrated by previous studies **(Copetti et al., 2013)**. On the other hand, the health status of the pods and the delay in ecosystems have affected the fungal diversity of cocoa. The influence of pod health status and the delay in the development of molds may be related to the loss of pod integrity due to injuries and rodent activity, to rot due to the over-ripening condition of pods on the cocoa **(Monjouenpou et al., 2012)**. The health status of the pods and the delay in egging affect the "primary" contamination of cocoa by fungal strains. However, it is possible to control the development of toxinogenic strains and thus the

contamination of cocoa in OTA by avoiding these technological practices at risk and no-mixing.

A. niger is present in 87% of the samples analyzed and its presence has little influence on the OTA content. *A. carbonarius* is present in only 23% of the samples. *A. carbonarius* is found in the most contaminated samples, confirming its predominant role in the contamination of cocoa in OTA. *A. carbonarius* was always associated with that of *A. Niger*. Whatever the modalities of the technological treatments used, it is the presence of *A. carbonarius*and to a lesser degree of *A. niger*, which determines the presence of OTA in cocoa. Thus, a relationship was found between the presence of *A. carbonarius*and *A. niger*, the use of plastic crates but also the absence of mixing, and the high levels of OTA. All of these observations could be related to the probable reduction in pressure in plastic boxes and the non-practice of mixing.Indeed, in the absence of oxygen, the development of acetic bacteria is inhibited and thus, as well as the production of acetic acid (decrease in pH) and the increase in temperature in the mass of fermenting beans. All these elements may be favorable for the development of mycotoxinogenic fungal strains. This is followed by a significant production of OTA, which is further enhanced by stress conditions caused by anaerobic conditions on molds**(Durand, 2013)**.

High levels of OTA were recorded in fermented cocoa in plastic crates and this cocoa was contaminated with A. *carbonarius*. In addition, immediate brushing of the pods and, to a lesser extent, avoiding fermentation conducted in plastic crates, limit the contamination of the merchant cocoa in OTA. Thus, and as some authors have shown (**Mounjouenpou et al., 2012)**,these results highlight the importance of agricultural practices before fermentation as well as the method of fermentation.

The duration of fermentation affects the OTA content of the samples but not the presence of the OTA-producing strains. The high content of mycotoxin found after 4 days of fermentation is decreased after 7 days, even in the presence of *A. carbonarius*. This suggests competition with the other strains present that may inhibit the development of *A. carbonarius* and degrading mycotoxin as a source of carbon for energy purposes in a nutrient-depleted fermenting medium **(Dano et al., 2013)**. Moreover, the results of the classical microbiological analyses showed the presence of strains belonging to the genera *Mucor* and/or *Rhizopus* in nearly 80% of the analyzed cocoa samples. These molds, whose rapid growth rapidly leads to an invasion of crops on canisters**(Zheng et al., 2007)**, present themselves as potential candidates for a bio-competition that would reduce the contamination of OTA beans. Exactly, the results of the interaction between *A. carbonarius* and *Rhizopus* sp. showed that the

presence of *Rhizopus* at a concentration equal to that of *A. carbonarius* makes it possible to delay and even greatly reduce contamination by OTA when *Rhizopus* is the majority at inoculation. This situation could be explained by a probable inhibition of the growth of *A. carbonarius* by the growth of *Rhizopus.* Indeed,*Rhizopus* could either develop an invasive growth medium preventing the growth of *A. carbonarius* or synthesize anti-mycotoxigenic substances that block the production of OTA by *A. carbonarius.* In the opposite case when*A. carbonarius* is in the majority during inoculation, the mycotoxin is produced as if the competitor were not present but it seems that the latter consumes it afterward**(Durand, 2013)**. These preliminary results seem promising and tests with other methods or other molds could make it possible to consider biological solutions to limit the contamination of cocoa in OTA.

Conclusion

In this study mold species isolated by microbiology techniques belonged to six genera: *Aspergillus, Aureobasidium, Chrysonilia, Mucor, Penicillium,* and *Rhizopus.* The genus *Aspergillus* is particularly the dominant mycoflora. The beans from damaged pods that have undergone a delay in pod breaking together show a wide fungal diversity and high levels of OTA linked to the presence of *A. niger* and/or *A. carbonarius.* The use of DGGE profiles of mold rDNA amplicons from PCR revealed differences in terms of microbial (fungal) ecology between samples that had undergone different post-harvest treatments. In addition, potentially ochratoxinogenic strains identified by conventional microbiology techniques were found. As expected, the number of different species separated on the gels is higher than that obtained on Petri dishes. Finally, the study of the co-presence of an OTA-producing mold strain and a non-producing mold on cocoa highlighted that the presence of *Rhizopus,* which does not produce OTA, at an equal concentration to that of *A. carbonarius,* an OTA producer, makes it possible to delay and even greatly reduce contamination by OTA when *Rhizopus* is the majority at inoculation. In the opposite case where *A. carbonarius* is the majority, the OTA is produced but *Rhizopus* sp. subsequently consumed it. *Rhizopus* sp. appears to be a promising antifungal agent against *A. carbonarius*and the production of ochratoxin A.

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