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Fungal Flora and Ochratoxin A Associated with Coffee in Cameroon

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NDN, ND, NLT and IM designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors DM and CMFM managed the analyses of the study. 'Author DM managed the literature searches. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aims: 104 samples were collected from the west region and the coastal plain of Cameroon during two coffee campaigns, 2009 and 2010. Two coffee processes were evaluated (wet and dry processes) at different stages from harvesting to storage.
Study Design: Food contaminants.
Place and Duration of Study: Food Microbiology Laboratory, Department of Food Science and Nutrition (ENSAI) University of Ngaoundere; UMR 95 Qualisud, CIRAD of Montpellier, between May 2009 and September 2012.
Methodology: Fungi profile was evaluated by direct plating techniques and identified using morphological and molecular tools. OTA levels were analyzed using HPLC technique after extraction and filtration using an immunoaffinity column.
Results: Results obtained revealed an overall percentage of fungal contamination between 60-92% in 2009 and 70-90% in 2010. There was no ecological difference in the

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composition of ochratoxigenic species present in five sites. Coffee beans sampled in 2009 had a colonization incidence of 18-40% *A. carbonarius*, 12-22% *A. niger*, 3-15% *A. ochraceus* while those of 2010 had a colonization incidence of 15-30% *A. carbonarius*, 35-40% *A. niger*, and 2-7% *A. ochraceus.* Fungal diversity was not correlated with the geographical origin, coffee cultivar and processing method. There was no difference between the processes studied in terms of occurrence of ochratoxigenic fungi. OTA levels were mostly below the recommended standards although some isolated cases of extreme contamination were observed in 2009. A higher level of OTA was detected in the presence of *A. niger*, *A. carbonarius* and *A. ochraceus* than when only *A. niger* was present.
Conclusion: The important fungi with the potential to produce OTA in Cameroonian coffee beans are *A. carbonarius* and *A. niger*. These two species were predominant on each type of coffee beans. It was also observed that once a toxigenic strain was isolated from a coffee sample, the sample contained OTA.

Keywords: Ochratoxin A; Coffee mycoflora; fungi; post-harvest process; Aspergillus carbonarius; Aspergillus niger; Aspergillus ochraceus.

1. INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin which contaminates a wide range of food commodities like cereals, coffee, nuts, dried fruits, wine, beer, grapes and grape juice. OTA has been shown to possess nephrotoxic, carcinogenic, immunosuppressive and teratogenic properties and was classified as carcinogenic for humans (group 2B) [1,2]. This secondary metabolite is produced by filamentous fungi such as *Aspergillus carbonarius*, *Aspergillus niger*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae* and *Aspergillus steynii* as well as *Penicillium verrucosum* and *Penicillium nordicum*. In tropical zones, OTA is mainly produced in coffee beans by *A. ochraceus, A. carbonarius* and *A. westerdijkiae* (section *Circumdati*), which was recently dismembered from *A. ochraceus*, due to their important OTA production and occurrence [3,4,5,6].

The occurrence of OTA in coffee beans can be due to climate, length of storage and transportation and processing conditions (wet, mechanical or dry processes) [7,8]. Briefly, the wet process is a generic term covering several operations (including fermentation) requiring long time and much care. The dry process (natural preparation) requires a natural (sun drying) or artificial (plan or rotary dryer) drying of whole fruit. The occurrence and the formation of OTA in the dry process has been studied by several authors [9,10,11]. OTA was present before storage, indicating the possibility that harvesting and post-harvest handling of coffee cherries could be critical steps leading to contamination [12], [13,14,15]. There is currently little information available on the presence of OTA-producing moulds in coffee beans that undergo wet and mechanical processes and the impact of these processes on the production or presence of OTA. Masoud and Kaltoft [16] published data which indicated that the depulping process significantly reduced the risk of OTA contamination during the subsequent fermentation and drying steps, but mycological studies of these processes are necessary.

The purpose of our study was to evaluate OTA, to screen fungi that colonize coffee beans at different stages of processing and to identify the OTA producing strains, depending on the type of post-harvest process and beans origin.

2. MATERIALS AND METHODS

2.1 Coffee Samples

In this study, three types of Arabica coffee beans namely parchment, green coffee beans and dried coffee cherries from the west region (Bafoussam and Dschang) of Cameroon, were collected from two selected farms in two different growing sites. Two types of Robusta coffee beans (i.e. dried coffee cherries and green coffee beans) from the west and coastal plain (Bafoussam, Dschang, Santchou, Melong and Bafang) were collected from five selected farms in five different growing sites. A total of 104 samples of coffee (42 Arabica and 62 Robusta) were collected at three stages of the growing season from October-November, November-January, and January-April in 2009, and then repeated in 2010. Investigations on the field were made with farmers every three months for a period of one year. Fig. 1 and Table 1 recapitulates this field work with samples collected at stages I_a, I_b and II of the process. Two types of post-harvest treatment were applied on the coffee: wet processing on Arabica coffee and dry processing technique for Robusta. Coffee samples were drawn from 50kg bags (100g - 10 different sites of the coffee bags). Samples of 1kg of coffee (dry parchment, dry cherries and green coffee beans) (stage I_a, I_b, II) were collected from five different sites of the west region (Bafoussam, Dschang, Santchou and Bafang) and the coastal plain (Melong) in Cameroon. The samples were then placed in sterile plastic bags and taken for laboratory analysis.

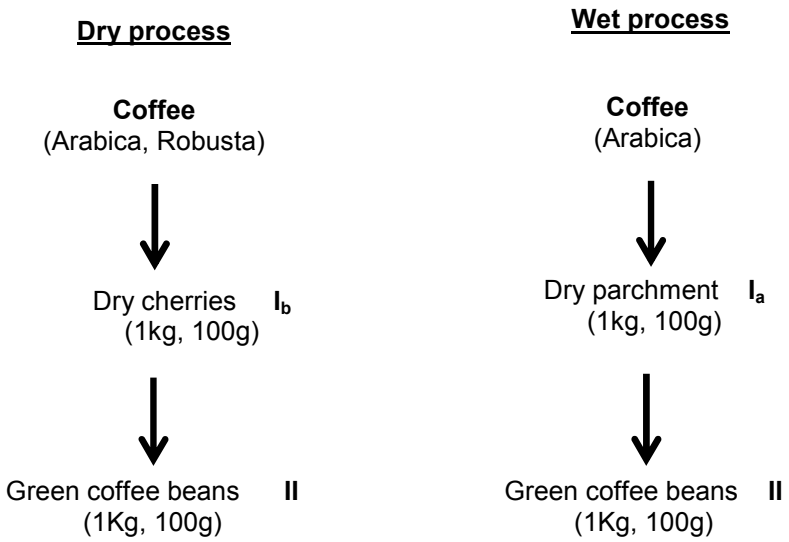

Fig. 1. Short coffee processing steps in Cameroon. I_a, I_b II: sampling steps

Table 1. Summary of coffee processing steps in Cameroon

Sampling period		Site						
		Bfss		Dsch		Baf	San	Mel
October-November								
Processing technique		DP	WP	DP	WP	DP	DP	DP
variety of coffee		R	A	R	A	R	R	R
Number of samples analyzed	year 2009	3	3	3	3	2	3	2
	year 2010	3	3	3	3	2	3	2
Total		38						
November- January								
Processing technique		DP	WP	DP	WP	DP	DP	DP
variety of coffee		R	A	R	A	R	R	R
Number of samples analyzed	year 2009	2	2	3	3	2	2	2
	year 2010	2	2	3	3	2	2	2
Total		32						
January-April								
Processing technique		DP	WP	DP	WP	DP	DP	DP
variety of coffee		R	A	R	A	R	R	R
Number of samples analyzed	year 2009	3	3	3	2	2	2	2
	year 2010	3	3	3	2	2	2	2
Total		34						
Total sampling analyzed		104						

DP: dry process; WP: wet process; R: robusta; A: Arabica; Bfss: Bafoussam; Dsch: Dschang; Baf: Bafang; San: Santchou; Mel: Melong.

2.2 Mycological Analysis

Subsamples of dry parchment coffee, dry cherries or green coffee beans were plated directly onto Petri dishes (100 beans, five beans per plate) containing Dichloran 18% Glycerol agar (DG18) [17,18], without prior superficial disinfection. The plates were incubated at 25ºC for 5–7 days and results expressed as a percentage of infected beans (% infection). The filamentous fungi isolates were selected randomly according to phenotypic criteria. Isolates were identified according to morphological criteria [19]. The identification of *Aspergillus* spp and *Penicillium* spp filamentous fungi was confirmed using molecular techniques by the Fungi and Yeasts Culture Collection at the Catholic University of Leuven in Belgium (BCCM™/MULC Culture Collection). For each sample, the frequency of *A. carbonarius*, *A. ochraceus* and *A. niger* isolates were estimated in relation to total filamentous fungi.

2.3 Fungal DNA Isolation, PCR Amplification and Sequencing

Fungal spores were harvested from plates in aseptic conditions using sterile distilled water with 0.1% Tween 80. Extraction method which combined mechanical/enzymatic/chemical method was applied [20,21].

DNA was quantified spectrophotometrically with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA) and kept at 20ºC until used as template for PCR amplification. In addition, the successful application of a eukaryotic universal primer for PCR permitted the amplification and identification of many fungi species in a unique PCR step.

A fragment of the D2 region of the 28S rDNA gene was amplified using eukaryotic universal primers: forward, U1f GC (5' - CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GTG AAA TTG TTG AAA GGG AA – 3'; Sigma); reverse, U2r (5' - GAC TCC TTG GTC CGT GTT – 3'; Sigma), amplifying a 260 bp fragment [20,21,22,23]. A 30 bp GC-clamp (Sigma) was added to the forward primer and PCR was performed in a final volume of 50 µL containing 0.2 µM of each primer, all the deoxyribonucleotide triphosphate (dNTPs) at 200 µM, 1.5 mM of $MgCl_2$, 5 µL of $MgCl_2$-free 10xTaq reaction buffer (Promega), 1.25 Units of Taq DNA polymerase (Promega) and 2 µL of extracted DNA (≈ 30 ng). The amplification was carried out as follows: an initial denaturation at 94ºC for 3 min, 30 cycles of 94ºC for 45 sec, 50ºC for 50 sec, 72ºC for 90 sec and a final extension at 72ºC for 5 min.

PCR products were verified by electrophoresis by loading 5 µL on 2% TAE agarose gels with a 100 pb molecular weight ladder. Gels were stained and photographed as mentioned above. PCR products were purified of DNA from low melting agarose gel, with the Wizard PCR temperature Preps DNA purification system (Promega, France). Then were re-amplified under the same conditions but in this case using primers without GC-clamp and then sent for sequencing at GATC Biotech (Germany). The sequences of the 28S rDNA obtained were compared with those of the database available at NCBI GenBank (National Center for Biotechnology Information databases) using the BLAST program to determine the closest known sequences.

On the other hand, to differenciate *A. ochraceus* from *A. westerdijkiae*, two sets of Internal Transcribed Spacer primers were used [24] and Beta tubuline [25]. Amplification with ITS1-5.8S-ITS2, will make it impossible to differentiate the two strains; but in the case where amplification is effective with Beta tubuline, it indicates that the amplified strain is *A. westerdijkiae*.

2.4 DNA Extraction from Coffee Moulds

8 grams of each coffee beans sample were taken aseptically and filled into sterile 50 mL tubes containing 10 mL of sterile peptone water. The tubes were placed on a rotating wheel for 1 h. 2 mL of supernatant were then collected into Eppendorf tubes of 2 mL containing 0.3 g of glass beads. Extraction was done following the protocol of El Sheikha [21]. DNA extraction samples were verified by electrophoresis and loaded into 0.8% agarose gels in 1 × TAE buffer (40 mMTris–HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2–EDTA;Eppendorf, Germany) with a molecular weight ladder (Supercoiled DNA Ladder 16.21 kb; Invitrogen, USA). After running at 100 V for 30 min, the gels were stained for 30 min in an ethidium bromide solution (50 µg/mL; Promega), rinsed for 20 min in distilled water, then observed and photographed on a UV transilluminator, using a black and white camera (Scion Co., USA) and Gel Smart 7.3 system software (Clara Vision, Les Ulys, France).

2.4.1 Denaturing gradient gel electrophoresis (DGGE) analysis

The PCR products were analyzed by DGGE, by using a Bio-Rad Dcode universal mutation detection system (Bio-Rad, USA), using the procedure first described by El Sheikha [21]. Samples containing approximately equal amounts of PCR amplicons were loaded into 8% w/v polyacrylamide gels (acrylamide: N,N'-methylene bisacrylamide, 37.5 : 1; Promega) in 1 × TAE buffer (40 mMTris–HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na2-EDTA).

All electrophoresis experiments were performed at 60ºC, using a denaturing gradient in the 40–70% range (100% corresponded to 7 M urea and 40% v/v of formamide; Promega). The

gels were electrophoresed at 20 V for 10 min and then at 80 V for 16 h. After electrophoresis, the gels were stained for 30 min with ethidium bromide and rinsed for 20 min in distilled water and then photographed on a UV transilluminator with the Gel Smart 7.3 system (Clara Vision, Les Ulys, France).

2.4.2 Identification of DGGE bands

Detected bands were cut from the DGGE gel with a sterile scalpel. DNA of each band was then eluted in 100 µL TE buffer at 4ºC overnight. 100µL of DNA eluted from each band was purified and sent for sequencing as described above (in section Fungal DNA isolation and PCR amplification) but using U1 primer with no GC clamp, and then sent for sequencing at GATC Biotech (Germany).

Sequences were finally compared to those in the GenBank database (http://www.ncbi.nlm.nih.gov/ BLAST/) and those of the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) using the BLAST program [26]. Sequences with a percentage identity ≥ 97% or greater were considered to belong to the same species [24 ,27].

2.5 Analyses of OTA in Coffee Beans

Coffee samples were frozen at -80ºC, then ground to pass through a 0.5-mm sieve and analyzed for OTA [28]. The samples were extracted for 30 min with a methanol/3% solution of sodium bicarbonate (50:50 volumes). The extract was centrifuged at 3000g for 30 min and the supernatant diluted with phosphate-buffered saline and filtered using an immunoaffinity column (Ocharaprep^{R}, Rhône Diagnostics, Scotland). OTA was eluted with 3 mL HPLC grade methanol. The eluate was evaporated to dryness under a stream of nitrogen at 70ºC and the residue was dissolved in 1 mL of HPLC mobile phase and then quantified by HPLC (Shimadzu LC-10ADVP, Japan, with a fluorescence detector). The mobile phase consisted of distilled water/acetonitrile/glacial acetic acid (51:48:1). The flow rate was 1 mL min^{-1}. OTA was detected by absorption at 333 nm excitation and 460 nm emissions, and a retention time of 12.3–12.5 min. Standard OTA curves were established with an ochratoxin standard (1000 ng. mL^{-1}; ref P 226 R. Biopharm Rhône Ltd, Scotland); the detection limit was 0.075 µg/kg. The quality assurance data is: recovery 85%; Precision 8%; Quantification limit 0.15 µg/kg; Range of calibration curve 0 µg/kg to 50 µg/kg.

2.6Statistical Analyses

2.6.1 Analysis of variance

Variance analysis had been performed by the software R. Nine parameters of results analysis obtained had been highlighted: year of sample collection, collection period, the site, type of treatment, the deduction stage, and the contamination percentage in *A. niger, A. carbonarius, A. ochraceus,* and the OTA level of coffee beans.

2.6.2 P- value investigation

The p-value is the probability of the formulated hypothesis to be rejected. This test was used to study a correlated of Fungal diversity with the geographical origin, coffee cultivar and processing method. A comparative test (Z-test) was done in order to see the significance of parameters.

Test interpretation:

H0: The difference between the mean is not significantly different from 0
Ha: The difference between the means is significantly different from 0

Where H0 and Ha represent the null hypothesis and the alternative hypothesis respectively at 99% confidence interval.

3. RESULTS AND DISCUSSION

3.1 Evolution of Mould Flora during the Two Processes

According to studies carried by FAO [29] on processed coffee using dry and humid processing technique, it was shown that dry processing poses much OTA contamination problem and that the mode of drying and storage has an impact on the development in coffee. This justified the choice of sampling site performed after drying (dried bean, husk) and after depulping (unripe coffee) in order to confirm or reject the hypothesis that removal of pulp reduces OTA contamination in coffee.

Table 2 shows the moulds isolated and purified from beans collected from the two processes at different stages during the two year period. Direct plating of beans in Petri dishes on DG18 agar for sampling (Table 3) was employed. A high level of infection by moulds was observed in all processes after drying (on dry parchment (Arabica) and dry cherries (Arabica and Robusta)), particularly, *Penicillium, Mucor, Fusarium, Rhizopous, Scopulariopsis and Aspergillus* spp. including well-known potential OTA-producing fungi (*A. ochraceus, A. niger and A. carbonarius*). According to Perrone [30], Leong [31], Taniwaki [32] and Pardo [33], *A. carbonarius* is often found on Robusta, but it can also be found on Arabica. The strain *A. westerdijkiae* was not found on the two coffee types; from the taxonomic point of view it is difficult to effectively differentiate it from *A. ochraceus* [4,5]. Identification via the two sets of primers ITS [34] and Beta tub [25] made it possible to confirm that effectively *A. ochraceus* was isolated, though it produces less OTA than *A.westerdijkiae*. Although not frequent, the only occurrence of *Penicillium citrinum* was in cherries in agreement with its requirement for high water activity (aw) for growth. The presence of *Fusarium* in all stages of maturation and processing with the exception of the green coffee stage where the presence of tannin and lignin may have limited the fungal growth [35]. The presence of some filamentous fungi has relevance for safety of the product. Some of the species identified are related to mycotoxin production, such as *Aspergillus niger, A. carbonarius, A. ochraceus, A. flavus and Penicillium citrinum* [8,36,37].

Within the frame work of this study it was not possible to link a fungi profile to a given processing type. Results obtained are similar to those of Noonim [10] which, during analysis of coffee grains obtained identical fungi profile at the dry cherries stage of dried and green coffee beans. However, concerning wet processing, results differ from those cited in literature for microfungi associated with the different coffee processing [10,38]. Noonim [10] found that in course of wet processing the mycoflora on coffee at the parchment stage constituted essentially of *A. westerdijkiae*, *A. niger*, *Penicillium*sp.,*Aspergilli* of sections *Flavi, Terrei* and *Versicolores*, *Cladosporium*sp. whereas the mycoflora in green coffee constituted mainly of *A. westerdijkiae, A. niger, A. steynii, Penicillium* sp., *Eurotium* sp. It thus appears that the biodiversity of fungi strains present during processing of coffee are closely related to the geographical, climatic, ecological and human context [11].

Table 2. Identification of the filamentous fungi isolated during technological treatments

Treatment	Stage of processing	Filamentous fungi isolated
2009		
Dry process	I_b	*A niger; A. tamarii; A. ochraceus; A. carbonarius; P. citrinum; A. flavus; Mucor*sp*; Rhizopus nigricans; P. roqueforti; Scopulariopsis* sp.*; F. lacertarum; Mucorhiemalis; A.* sp.
	II	*A niger; A. ochraceus; A. carbonarius; A. flavus; F. chlamydosprum; Rhizopus nigricans; P. roqueforti; F. lacertarum; A.* sp*; Cladosporium* sp.
Wet process	I_a	*A niger; A. tamarii; A. ochraceus; A. carbonarius; P. citrinum; A. flavus; Mucor* sp*; F. chlamydosprum; Rhizopus nigricans; P. roqueforti; Scopulariopsis* sp.*; F. lacertarum; Rhizopus* sp.*; Mucorhiemalis; A.* sp.
	II	*A niger; A. ochraceus; A. carbonarius; A. flavus; Mucor*sp*; F. chlamydosprum; Rhizopus nigricans; P. roqueforti; Scopulariopsis* sp.*; F. lacertarum; A.* sp*; Cladosporium* sp.
2010		
Dry process	I_b	*A niger; A. tamarii; A. ochraceus; A. carbonarius; P. citrinum; A. flavus; Mucor* sp*; Rhizopus nigricans; P. roqueforti; F. chlamydosprum; Scopulariopsis* sp.*; F. lacertarum; Rhizopus* sp.*; Mucorhiemalis; A.* sp.
	II	*A niger; A. ochraceus; A. carbonarius; A. flavus; Rhizopus nigricans; P. roqueforti; F. lacertarum; A.*sp*; Cladosporium* sp.
Wet process	I_a	*A niger; A. tamarii; A. ochraceus; A. carbonarius; P. citrinum; A. flavus; Mucor* sp*; F. chlamydosprum; Rhizopusnigricans; P. roqueforti; Scopulariopsis* sp.*; F. lacertarum; Rhizopus* sp.*; Mucorhiemalis; A.* sp.
	II	*A niger; A. ochraceus; A. carbonarius; A. flavus; Mucor* sp*; F. chlamydosprum; Rhizopus nigricans; P. roqueforti; F. lacertarum; A.* sp.;*Cladosporium* sp.

I_a: Dry parchment; I_b: Dry cherries; II: Green coffee beans

3.2 DGGE Analysis of Representative Fungal Flora from Coffee

Samples of fungal DNA extracted from coffee beans produced DNA bands on DGGE gels that had sufficient intensities to be analyzed by sequencing (Fig. 2). Two references DNA of *A. carbonarius* and *A. niger* were used as markers and loaded on DGGE gels (bands 1 and 2) to facilitate identification. In Fig. 2, each vertical line represents a coffee beans sample and each band represents a mould species. The PCR-DGGE patterns of duplicate coffee for each stage of processing were similar and revealed the presence of three to twelve bands for

each coffee sample. In addition, the comparative study of strains obtained from both methods suggests a higher diversity in the fungal population obtained by DGGE method.

PCR-DGGE has proven to be a rapid and effective method to describe the fungal communities of coffee [39]. In our case, we have shown that for the same sample analyzed with traditional techniques and with PCR-DGGE, those obtained by DGGE showed, in general, a greater microbial diversity. This confirms the idea put forward by Laforgue [40] who showed that PCR-DGGE was an effective and quick method to follow fungal communities of food products. In fact, it is likely that the detection limit of species in a mixture depends on the level of the total population, the number of species and their specific concentration within the mixture. But in any case, it is important to note that the lack of band detection on a DGGE gel species after direct analysis does not necessarily mean their absence in the sample.

Wet processing is applied for Arabica coffee bean and the mycobiota was highly influenced by processing practices, for example, fermentation process (water quality and equipment used) and drying process. Generally, coffee bean is dried on open air and sometimes on bear ground at the site of harvest (time period, cleanliness of equipment and environment). No uniform distribution of filamentous fungi was observed in coffee bean samples from different growing sites in the same region (west and the coastal plain). The harvesting year (2009 and 2010) had an influence on the contamination rate of the coffee beans. These may be as a result of differences in environmental conditions, for example, rain fall (2500-3000mm in Bafoussam, Dschang and Bafang; 3000-6000mm in Santchou and Melong) and the use of recycled bags sometimes not proper for coffee storage, sunlight, temperature (25ºC), water quality etc. from year to year. We found that the samples harvested in 2010 had a higher incidence of *Aspergillus* than those harvested in 2009.

The reasons for the variability in moulds isolated are not well defined. Possible causes could be firstly, due to variation in local conditions that could substantially affect the degree of infection. Such conditions could be, for example, extent and timing of spraying, quality of hygiene and extent of the use of drying surfaces. Secondly, sampling at one point at a time for the different stages of maturation is not the best way to analyze the trend or succession in the microbial flora.

3.3 Rate of Coffee Bean Infection by Moulds

Table 3 shows the rate of coffee bean infection by moulds and percentage occurrence of *Aspergillus* species potentially capable of producing ochratoxin A from different coffee processes. In 2009, it was found that 92% (I_b) and 60% (II) of the beans from the dry process were infected while an infection rate of 70% (I_a) and 50% (II) was observed for the wet process. In 2010 it was found that 90% (I_b) and 70% (II) of the beans from the dry process were infected while 80% (I_a) and 60% (II) of the beans from the wet process were infected. However, the percentage of infection in green coffee was lower than that observed in parchment and in dry cherry coffee, with the detection of three OTA-producing fungi. The percentage of infection, the percentage of occurrence of *A. carbonarius* and the percentage of occurrence of *A. ochraceus* were found to decrease with time (from higher value in 2009 to lower value in 2010) according to the dry process stage I_b; while the percentage of infection increases with time according to the wet process regardless of the different stages. Also, the percentage of occurrence of *A. niger* was found to increase with time in both processes and at both stages.

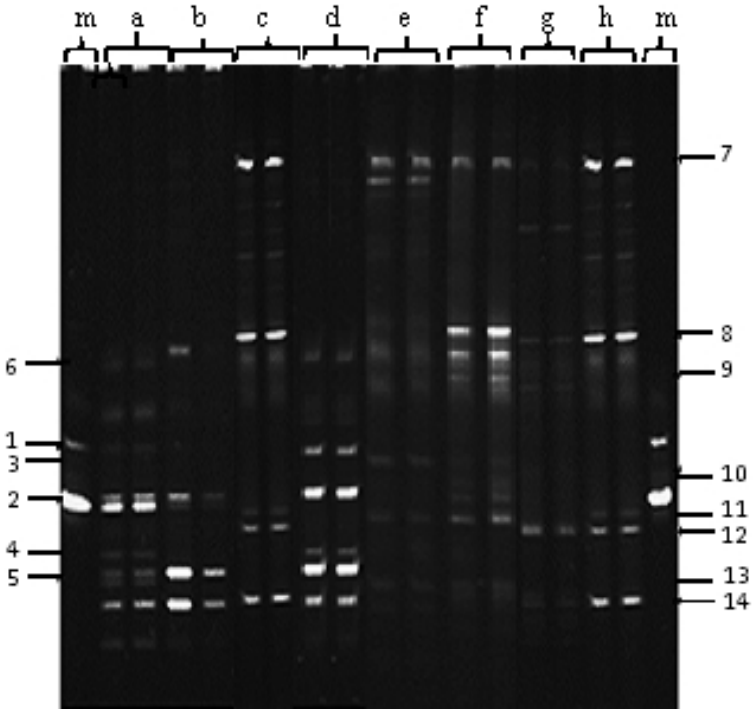

Fig. 2. DGGE profiles of PCR amplicons of the domain D1 of 28S rDNA that represent the fungi biodiversity in samples taken during treatment of coffee (m: marker; a-h: samples of coffee). The position of bands is indicated by numbers that correspond to species of fungi: 1: *A. niger;* 2: *A. carbonarius;* 3: *A. fumigatus;* 4: *P. citrinum;* 5: *P. arenicola;* 6:*Wallemia muriae;* 7:*Mucor sp;* 8: *W. sebi;* 9:*Acremonium murorum;* 10: *A. ochraceus;* 11: *A. nomius;* 12:*Fusarium* sp*;* 13:*Cladosporium musae; P. roqueforti*

Table 3 shows the percentage occurrence of *A.carbonarius, A. niger and A. ochraceus* isolated from dry parchment, dry cherries and green coffee beans for each process; this corresponded to the analysis of 1040 beans at each stage of processing during the 2 years period. Through the direct plating technique, several strains of *A. niger, A. carbonarius* and *A. ochraceus* were isolated from beans. It can be seen that the average rate of dry parchment and dry cherries coffee infection by *Aspergillus* fungi species was high (55–73%). This method made it possible to determine the percentage of beans contaminated by moulds, which was high in parchment coffee (70–80%) and decreased later on in the green coffee beans (50–70%). This result shows that, *Aspergillus carbonarius and A. niger* were the predominant group in dried coffee cherry samples and in green coffee beans samples in Cameroon and their occurrence is much higher than reported in the Brazilian or Vietnamese coffee beans which is non-disinfected [8,31,39]. Both *A. carbonarius* and *A. niger* were common and predominant in both types of coffee beans.

Table 3. Rate of coffee bean infection by moulds and percentage occurrence of *Aspergillus* species potentially capable of producing Ochratoxin A from different coffee processes

			% occurrence		
Process	**Stage of processing**	**% infection[a]**	***A. carbonarius***	***A. niger***	***A. ochraceus***
2009					
Dry process	**I_b**	92	40	20	3
	II	60	18	22	5
Wet process	**I_a**	70	40	12	15
	II	50	25	15	2
2010					
Dry process	**I_b**	90	30	35	7
	II	70	20	35	6
Wet process	**I_a**	80	25	30	0
	II	60	15	40	2

[a]Direct planting of 1040 beans on DG18 agar; I_a: Dry parchment; I_b: Dry cherries; II: Green coffee beans

The analysis of stage Ib and II of the dry process in 2009 and 2010 reveals that the p-value (0.439) was greater than the significance level alpha =0.01, one should accept the null hypothesis H0. Hence, there is no significant difference in the % infection between the dry process of year 2009 and 2010.

The analysis of stage Ia and II of the wet process in 2009 and 2010 has revealed that the p-value (0.001) was lower than the significance level alpha= 0.01, one should reject the null hypothesis H0, and accept the alternative hypothesis Ha. Thus there is a significance difference in the % infection between the wet process of 2009 and 2010.

3.4 OTA Quantification in Coffee

Table 4 gives average quantity of OTA as a function of the sampling site, the sampling period, the process applied on the coffee as well as the identity of toxinogenic fungi *(A. carbonarius, A. niger, A. ochraceus*) associated to the coffee grains got from the 2009 and 2010 campaign season. In this framework, treatment by wet processing had shown a much higher contamination in OTA than by the dry process. This difference between the two treatments can be explained by the fact that depulping involves a different decrease depending on the fact that there is fermentation or not.

Table 4. OTA content as a function of processing type, site and toxicogenic strain associated to the coffee campaign season 2009 and 2010

Sampling period	Site	Processing technique	Number of samples analyzed	Average OTA content year 2009	year 2010	Toxicogenic strains associated
SC (October-November)	Bfss	DP	6	4.5	0.47	*A. niger*$^{+}$, *A. carbonarius*$^{++}$, *A. ochraceus*
		WP	6	124.1	0.3	*A. niger*$^{+}$, *A. carbonarius*$^{++}$, *A. ochraceus*
	Dsch	DP	6	1.9	0.4	*A. niger*$^{++}$, *A. carbonarius*$^{++}$
		WP	6	46.81	nd	*A. niger*$^{++}$, *A. carbonarius*$^{+}$
	Baf	DP	4	0.9	10.1	*A. niger*$^{+}$, *A. carbonarius*$^{+}$,
	San	DP	6	0,35	1.5	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	Mel	DP	4	nd	nd	*A. niger*$^{++}$, *A. carbonarius*$^{+}$
MC (November- january)	Bfss	DP	4	nd	0,3	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
		WP	4	0.2	1	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	Dsch	DP	6	0.3	0.11	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
		WP	6	0.12	3.85	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	Baf	DP	4	1.2	0.3	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	San	DP	4	0.6	1.2	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	Mel	DP	4	0.4	1.9	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
EC (January-April)	Bfss	DP	6	0.3	0.25	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
		WP	6	nd	0,9	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	Dsch	DP	6	0.4	0.4	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
		WP	4	nd	nd	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	Baf	DP	4	4.01	2.5	*A. niger*$^{+}$, *A. carbonarius*$^{++}$, *A. ochraceus*
	San	DP	4	0.3	1	*A. niger*$^{+}$, *A. carbonarius*$^{+}$
	Mel	DP	4	5.11	0.3	*A. niger*$^{+}$, *A. carbonarius*$^{++}$, *A. ochraceus*

SC: start of campaign; MC: Mid- campagn; EC: End of campagn;DP: dry process;WP: wet process; nd:non determined; Bfss: Bafoussam; Dsch: Dschang; Baf: Bafang; San: Santchou; Mel: Melong.

From the table 4, it can be seen that the toxicogenic micro flora associated to the coffee grains contaminated with OTA is in the majority constituted of *A. carbonarius* and *A. niger*. Analysis of variance of the results obtained show that the year of harvest, the period of sampling and the type of processing significantly influence the quantity of OTA in coffee bean samples. However from the analysis, what springs off is that, the stage at which the samples were collected influence much the accrued content of OTA and that the site has no influence on OTA content. The average OTA content of the coffee grains issued via dry processing, fall within the ranges of 0,3 and 5,11 µg/Kg and between 0,11 et 10,1 µg/Kg for the campaign seasons 2009 and 2010 respectively. With regard to the humid processing, the average OTA content of the grains are situated between 0,12 and 124,1 µg/Kg for the campaign season 2009, and between 0,1 and 3,85 µg/Kg for the campaign season 2010. Analysis of variance with R software had shown that the year of collection of samples slightly influences the coffee OTA level; the treatment significantly influences that content; but the deduction stage and the contamination percentage in *A. carbonarius* highly influence the contamination level in OTA of coffee beans.

Moreover the analysis of variance carried on the 104 samples made possible to bring out the incidence of processing on the OTA content in coffee. This result is contrary to that of Noonim [10] who upon analyzing Arabica coffee through all the stages of transformation process were unable to establish a relationship between coffee processing and OTA content. It was suggested that with the elimination of the pulp, a great portion of substrate for the development of OTA producing strains is eliminated [14].

Concerning Robusta coffee processed via dry processing, Noonim [10] observed an increase contamination moving from dried cherries to green coffee; they associated the high contamination level to the fact that Thailand coffee is often in contact with the soil during the drying process. At contrary, it has been shown that the formation of OTA takes place during sun drying of the cherries principally in pulp; on the contrary, the concentration of OTA in green coffee was shown to be 100 times lesser than the shell, which supports the hypothesis of superficial contamination [13] and the reduction of OTA producing strains via the elimination of the external coat [8,41].

Suarez-Quiroz [8] showed the difference in OTA contamination between various processes of coffee processed via dry, traditional humid and ecological humid processing. The differences were explained by the fact that the lowest decrease observed for the mechanical processing was due to the removal of the external layer rich in toxin, whereas the fermentation process « much humid » and of a long duration can favor the penetration of toxins into the parchment. On the contrary, in the course of drying process, the increase in the OTA content can only be explained by a neosynthesis from the toxicogenic contaminating layer.

Within the context of this work samples were collected at the start, middle, and end of the campaign. The high level of contamination observed at the beginning of the 2009 campaign season via humid processing could result from prolonged fermentation in course of the processing, climatic (relative humidity) and environmental conditions or the phytosanitary state of the coffee grains at the times of harvest and transformation [11].

In the course of this work, some samples with heavy contamination of potential toxinogenic strains were obtained. This study is in accordance with observation of other authors who concluded that a significant level of infection does not necessarily imply a contamination risk in OTA [6,41]. Though factors which favor the development of fungi should be taken into

account, it is important to stress that, the presence of moulds does not necessarily imply synthesis of OTA. On the contrary, it is possible to detect mycotoxin in the absence of toxicogenic moulds. The vegetative and spore forms of moulds could be inactivated by chemical or environmental processes whereas the more stable OTA remains unaffected in the substrate [42].

4. CONCLUSION

From this study it can be concluded that the important fungi with the potential to produce OTA in Cameroonian coffee beans are *A. carbonarius* and *A. niger*. These two species were predominant on each type of coffee beans. It was also observed that once a toxigenic strain was isolated from a coffee sample, the sample contained OTA. This shows that the presence of toxigenic strains implicates a great risk of OTA presence. Good manufacturing practices and hygiene throughout the coffee production and processing chain is highly recommended in order to reduce the risk of contamination of processed coffee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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