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Post harvest processing used by farmers impact Ochratoxin A occurrence in coffee cherries in Côte d'Ivoire

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Abstract

In this study, we analysed the occurrence of ochratoxin during post-harvest processing of coffee cherries. The stripping method was used to harvest a mixture of cherries presenting different state of ripening. Cemented, tarpaulin and bamboo surfaces were used to dry the coffee cherries. Ochratoxin produced by fungal strains was extracted and assayed by HPLC using fluorescence detector and C18 column. Moreover, the level of fungal contamination in stored and non-stored coffee cherries was evaluated using DG18 plate agar. The results showed that coffee cherries were mainly contaminated with yeasts and Aspergillii notably ochre, black and green Aspergillii as well as Penicillia and Fusaria. Yeasts were highly present in freshly harvested cherries with a contamination rate (>85 %) and a load of 6.43 ± 1.59 log CFU/g whereas filamentous fungi were very low (<15% and 4.02 ± 0.06 log CFU/g). OTA content yielded in these freshly harvested cherries was 1.5 µg / kg. This trend typically changed in four days-stored cherries in which, yeasts contamination drastically decreased to less than 5 % and fungi particularly black Aspergillii sharply increased to 40-50 %. Hence, OTA production in stored cherries increased 5-folds comparatively to OTA production in non-stored cherries. At the end of the drying process the fungal contamination rate generally increased and the highest contamination rate was observed with black Aspergillii (16 folds), on bamboo drying surface corresponding to the highest OTA production. Overall, this study showed that storing coffee cherries before drying enhanced significantly the occurrence of OTA producing strains during the post-harvest processing and then black Aspergillii might be the main fungal strains involved in OTA production in coffee in Côte d'Ivoire.
Keywords: Ochratoxin, Coffee cherries, storage, drying, fungal strains, Côte d'Ivoire

Résumé

Dans cette étude, nous avons analysé la présence d'ochratoxine dans les cerises de café pendant le processus post-récolte. La méthode de stripping a été utilisée pour récolter les cerises, le niveau de contamination fongique des cerises stockées et non stockées a été évaluées sur milieu DG18, trois différentes aires de séchage notamment aire cimentée, plastique et bambou ont été utilisées. L'Ochratoxine (OTA) produite par les souches fongiques isolées a été d'abord extraite et ensuite dosée par HPLC utilisant un détecteur de fluorescence et une colonne C18. Les résultats ont montré que les cerises étaient essentiellement contaminées par les levures, des moisissures de type aspergillus ochre, noires et verts, de type penicillium et des fusaria. Les levures étaient fortement présentes, avec un taux de contamination >85 % dans les cerises fraiche non stockées, et une charge de 6.43 ± 1.59 log UFC/g tandis que les moisissures avaient un faible taux de contamination <15% et une charge de 4.02 ± 0.06 log UFC/g). La teneur en OTA dans ces cerises fraichement récoltées était de 1.5 µg / kg. Cette tendance s'inverse pratiquement dans les cerise stockées pendant 4 jours, avec une chute drastique de la contamination de levure en dessous de 5 %, et une forte augmentation du taux des moisissures jusqu'à 50%. La contamination en Aspergillus noirs et la teneur en OTA dans les cerises stockées sont respectivement 16 fois et 5 fois plus élevée que dans les cerises fraichement récoltées. Ces aspergillus noirs étaient plus prolifiques sur aire de séchage en bambou correspondant à la plus forte production d'OTA. Au total, le stockage des cerises de café avant le séchage augmente fortement la contamination fongique et la production d'OTA durant le processus post-récolte du café, et les Aspergillus noirs pourraient être les principaux responsable de cette production d'OTA.
Mots clés : Ochratoxine; Cerise de café; Stockage; Séchage, Souches fongiques; Côte d'Ivoire

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Introduction

Ochratoxin A (OTA) is a foodborne mycotoxin found in various agricultural commodities ranging from cereal grains to dried fruits to wine and coffee (Bui-Klimke and Wu, 2015). This toxin is produced by different fungi notably Aspergillus ochraceus, A. carbonarius, A. niger and some Penicillium species such as Petromyces, and Neopetromyces (WHO/FAO, 2001; Larsen et al., 2001). OTA as a naturally occurring toxin, was revealed to be toxic and carcinogenic in animals models with kidney as the main targeted organ (Duarte et al., 2011). Indeed in animals models, OTA was shown to induce a range of toxic effects notably nephrotoxicity and nephrocarcinogenicity, hepatotoxicity, teratogenicity, neurotoxicity, immunotoxicity and genotoxicity (WHO 1999; O'Brien et al., 2005; Pfohl-Leszkowicz and Manderville, 2007) with renal toxicity and carcinogenesis being the key adverse effects. In kidney, OTA accumulate and impairs proximal nephron function and blocks plasma membrane anion conductance (Gekle, et al., 1993) resulting in glucosuria, enzymuria, and a decrease in the transport of para-aminohippuric acid (PAH), a prototypical renal organic anion (Gekle and Silbernagl, 1993; Dahlmann et al., 1998).

OTA-contained food is the main source of exposure and constitutes a hazard that should be prevented for food safety. Hence, coffee should retain more attention since it remains a significant source of OTA contamination as resulting from a suboptimal postharvest processing (Ban-Koffi et al., 2015; Viani, 2002). Moreover, as OTA is a chemically stable compound, ordinary food processing treatments fail to significantly reduce its presence in foods. Accordingly, the presence of OTA in roasted coffee sample available on the market has been reported (Tsuboshi et al., 1998). Due to its association with OTA, coffee might be a potentially hazardous food.

Inadequate post-harvest processing of coffee cherries notably the agricultural practices of drying used by farmers, that allow the growth of ochratoxinogen fungi is the essential cause of OTA contamination. Investigations on post-harvest processing of coffee cherries performed by farmers in Cote d'Ivoire are relevant in a context of risk assessment related to coffee contamination with OTA. Previously, we analysed factors influencing the occurrence of fungal contamination of coffee cherries during post-harvest treatment in Côte d'Ivoire (Koffi et al., 2015).

In this paper, we investigated the presence of ochratoxinogen fungi in coffee with regards to the processing technic used on-farm in two main coffee-producing regions.

Materiels and Methods

Plant material, harvesting and ripening rate

Coffee cherries used as plant material in this study belonged to the species Coffea canephora, var. robusta, obtained from two CNRA (Centre National de Recherche Agronomique) experimental plantations notably in the area of Divo (Latitude: 5.83972, Longitude: -5, 36000) and Bingerville (Latitude: 5.35583, Longitude: -3.89000). Cherries were harvested using stripping technique. The ripening rate of freshly harvested Robusta cherries was evaluated using manual count of coloured cherries (yellow, green, red and black) corresponding to different scale of coffee cherries ripening process.

Drying process of coffee cherries

The harvest was divided into two aliquots differing in the storage time. The first was immediately dried after harvest and the second was subjected to four days storage before drying. For storage conditions, about 100 kg of freshly harvested cherries were collected in jute bags, then stored at ambient temperature, protect from rain.

Three drying methods of coffee cherries were used depending on the drying surface notably cemented, bamboo and tarpaulin. Each drying surface was designed to contain 12 units (2 m2, each unit). The units were all uniformly loaded (25 kg/m2) with coffee cherries making a tray with a thickness of 6 cm. The coffee cherries were dried in the different units from 9 am to 5 pm, and regularly mixed at 9 am, 12 am, 2 pm, and 4 pm. Out of the drying period, notably from 5 pm until 9 am of the next day, cherries where protected against moisture by using black plastic cover.

Isolation of filamentous fungi from coffee cherries

Packs of two (2) kg of freshly harvested Robusta coffee cherries were randomly collected from the different units. Then at laboratory, two hundreds coffee cherries were randomly withdrawn and divided into two batches of 100 cherries each. The outer skin (exocarp) and the pulp (mesocarp) of cherries from the first batch were manually removed with scalpel. Then fungal strains were searched on this pulp including outer skin. For this purpose, 10 g of this material were placed in a stomacher bag containing 90 mL peptone water and then mixed to extract the associated microbiota.

The grounded material was used as initial inoculum to make a serial decimal dilution ranging from 10-1 to 10-7. Next, 1 mL of each dilution was plated onto a DG18 agar medium (Dichloran 18% Glycerol Agar) and incubated at 25 ° C 7-10 days.

In the second batch, fungal strains were searched on coffee beans that were obtained by removing the pulp from cherries. Pulp removing consisted in washing the cherries with a brush and 0.1 N sodium hydroxide solution. The de-pulped bean was then disinfected for 10 minutes in a closed jar containing 2% sodium hypochlorite solution. Disinfected coffee beans were drained and plated at a rate of 7 per petri dish on DG18 medium at 25 ° C for a period of 7 to 10 days.

Filamentous fungi were identified macroscopically by observing the colony features (color, shape, size and hyphae) as described by Klich, (2002) while yeasts were identified microscopically. The identified fungal colonies were then tested for OTA production.

OTA extraction and HPLC assay

This step was to check for OTA production from identified fungal strains analysed. Specifically, this experiment consisted in extracting OTA from the culture medium on which fungal colonies were grown. Hence, using a sterile mini driller with 10 mm diameter, two portions of the culture agar were removed and each placed in an Eppendorf tube and crushed on ice bath. An extraction solution consisting in a mixture of dichloromethane and 1 % acetic acid (1:1, v/v) was added to the crushed material, then mixed and centrifuged at 7,000 rpm for 30 seconds. 500 µl supernatant from each duplicate Eppendorf tubes were pooled and stored at -20°C.

For OTA assay, the filtered extracts were analyzed by high-pressure liquid chromatography (HPLC) with fluorimetric detection (Shimadzu LC-10 ADVP, Japan). The operating conditions were as follows: excitation wavelength of 333 nm and emission wavelength of 460 nm; HPLC column was a C18 Sorbox SB-48 (5 µm, 4.6 × 150 mm) (Agitent, Montpelier, France); 100 µL injection loop, mobile phase was water/acetonitrile/acetic acid, (51:48:1) v/v/v); isocratic flow of 1 mL/min; retention time for the detection of OTA was around 16 min; detection limit was 0.0003 µg·g−1.

Results

Ripening rate of harvested cherries in the different regions

The stripping method used to harvest the cherries is a method that does not screen only the ripen cherries. For this reason, the harvest obtained is a mixture of cherries presenting different levels of ripening (Fig. 1). Hence, we evaluated the rate of each type of cherry present in the cherry mixture. Harvest from Divo contained 51.34 ± 9.56 % of red cherries (fully ripen), whereas the different rates of green cherries (unripen), yellow cherries (fairly ripen) and black cherries were respectively 18.83; 11.59 and 29.45 %. ANOVA showed that the ripen cherries significantly dominated the coffee cherries harvest from Divo. On the other hand, the harvest from Bingerville contained a relatively high rate of green cherries (33.38 %) that co-dominated the mixture with red cherries (31.26 %); yellow and black cherries represented respectively 23.04 and 12.42 %. However, there was no significant difference in the rates of

green, red and yellow cherries, these rates were therefore assessed to be equal.

Aspergillii. The load of this type of fungi was less important with 4.02 ± 0.06 log CFU/g whereas the load of other molds such as Fusaria and Penicillia was 3.72 ± 0.61 log UFC/g (Fig. 2).

Level of fungal contamination and OTA content of the coffee cherries before drying

Yeasts were very abundant in non-stored cherries with a contamination rate of more than 85 % whatever the drying surface (Figure 3A). However, the filamentous fungi namely black and ochre Aspergillii as well as Penicillia and Fusaria were responsible for cherries contaminations at a lower rate, not exceeding 15 % (Figure 3A). Accordingly, at the beginning of the drying process, the OTA content yielded from these non-stored and freshly harvested cherries ranged between 1.1 µg / kg and 1.8 µg / kg depending on the drying surface (Figure 4). On the other hand, in four days-stored cherries, yeasts contamination drastically decreases to a rate of less than 5 %. Meanwhile, cherries contamination from black and other Aspergillii as well as Penicillia recorded a sharp increase reaching a rate up to 50.66, 40.71 and 40.39 %, respectively (Figure 3A). The increase in mold contamination in stored cherries comparatively to non-stored cherries, represented approximately 8-folds. In contrast, the rate of contamination from Fusaria and ochre Aspergillii did not increased in stored cherries (Figure 3A). Moreover, we also observed that the OTA productions from fungal isolates in stored cherries ranged between 5.5 and 6.0 µg / kg, and these productions are around 5-folds higher than OTA yields in freshly harvested cherries (Figure 4). surface (Figures 3A and 3B).

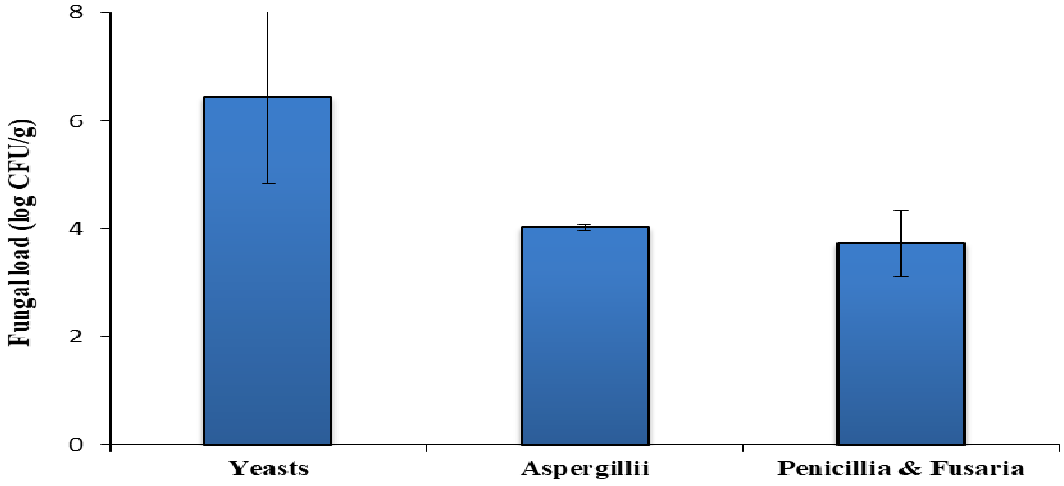

Figure 2. The different groups of fungal strains found in coffee cherries.

Level of fungal contamination and OTA content of the coffee cherries at the end of drying process

At the end of the drying process, in non-stored cherries, the contamination rate due to black Aspergillii strongly increased from less than 7 % at the beginning of the drying process to more than 82 % at the end of the process, thus representing more than 16-folds augmentation (Figures 3A and 3B). However, in stored cherries, the increase of contamination rate due to black Aspergillii at the end of drying was relatively weak, with only 1.5 folds augmentation (Figures 3A and 3B). In contrast, Penicillia and yeasts tend to disappear at the end of drying process since the contamination rates drastically decreased, this effect being more pronounced with Penicillia in stored cherries and yeast in non-stored cherries whatever the drying

On the other hand, the contamination rate of Ochre Aspergillii did not significantly vary from the beginning to the end of the drying process as shown the figures 3A and 3B. The results also showed that the bamboo-drying surface was more favourable for black Aspergillii growth in stored cherries whatever the period of drying surface. Regarding the OTA production in non-stored cherries, OTA yield increased from 1.1 – 1.8 µg / kg at the beginning of the drying process to 3.8 - 4.3 µg / kg at the end, thus representing approximately a 3-folds augmentation. Meanwhile, in stored cherries the OTA yield did not significantly vary from the beginning to the end of drying process (Figure 4) whatever the drying surface, except the bamboo-drying surface which recorded the highest OTA yield from coffee cherries (Figure 4). Yet, the level of OTA production remained significantly more important in stored cherries than in non-

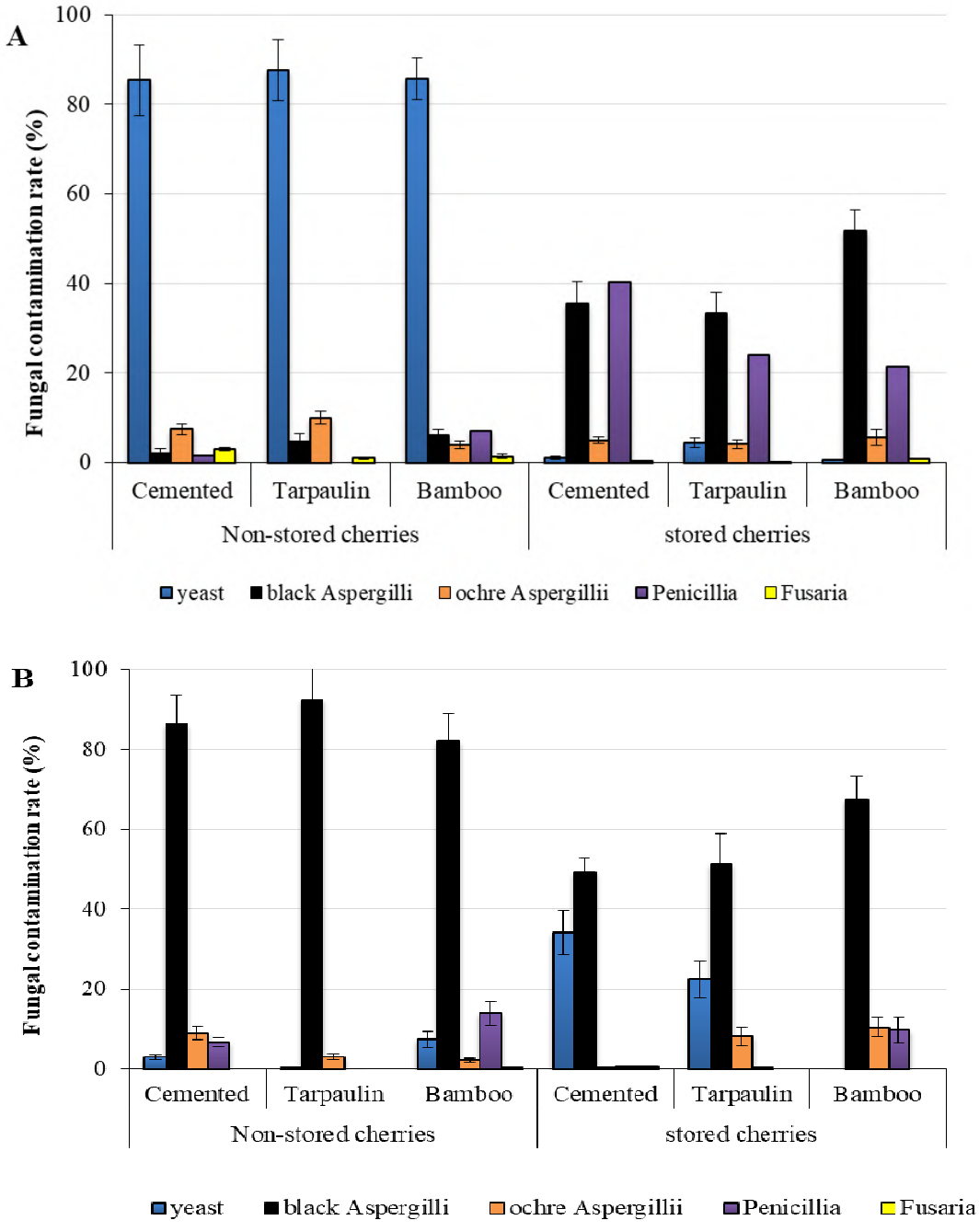

Figure 3. Fungal contamination rates of cherries during the drying process; A. Before drying; B. After drying

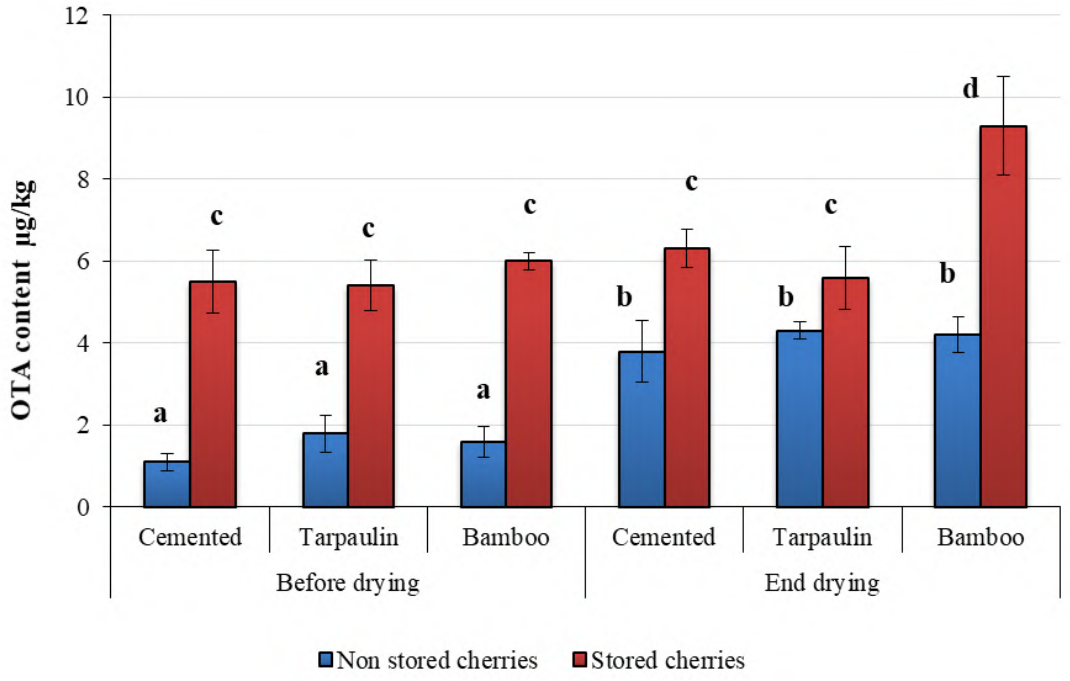

Figure 4. Ochratoxin A content in coffee cherries during the drying process

Discussion

In this study, the occurrence of toxigenic fungal strains associated with coffee cherries in Ivory Coast has been investigated.

Previously, we reported that some factors related to the drying step during post-harvest processing, notably the drying surface, the load of cherries and the mixing frequency strongly impact the fungal contamination of coffee cherries (Koffi et al., 2015). In the present study, the storage of harvested cherries before drying was also found to significantly increase fungal contamination. The results show that yeasts were dominant in the freshly harvested cherries whereas the presence of filamentous fungi such as black and ochre Aspergillii, Penicillia and Fusaria was weak with approximately 10 % of contamination rate. This trend typically reversed in stored cherries recording a strong increase in fungal contamination up to 51.66 %, particularly with black Aspergillii and Penicillia whiles yeasts decreased drastically. Similar results were obtained by Kouadio, et al., (2014), these found that the storage of coffee cherries after harvest prior to drying, increase the infection of cherries by fungi with a rate varying between 10 and 50 % depending on the storage time.

Although yeasts were not assessed as OTA producing microorganisms, their presence in coffee cherries might be of high importance since they were previously reported as biocontrol agent against toxigenic fungi and OTA production (Souza et al., 2017). Indeed, in this study, a high contamination rate (up to 85 %) of non-stored cherries by yeasts corresponded to a low production of OTA (1.1 – 1.8 µg / kg) in these cherries. Likewise, the 3-folds increase of OTA production up to 5.5 – 6.0 µg / kg in stored cherries corresponded to an absence of yeasts in these cherries.

The fungi potential producers of OTA namely Aspergilli and Penicilli, were found in the analysed coffee cherries with a load ranging from 3.72 ± 0.61 log UFC/g to 4.02 ± 0.06 log CFU/g. This fungal load was very high comparatively to that reported in coffee cherries from Brazil, Timor, Honduras, Angola, Vietnam, Costa Rica, Colombia, Guatemala, Nicaragua, India, and Uganda were the maximum load of fungi did not exceed 3 log UFC/g (Viegas et al., 2017). However, the load of fungal strain

OTA concentration as the OTA synthesis seemed to be an intrinsic strain-dependent mechanism as reported by Marino et al. (2014).

Therefore we checked the capacity of fungal isolates associated with cherries to effectively produce OTA. We observed that the production of OTA was very low (1.1 – 1.8 µg/kg) when the rate of fungal contamination was low, less than 10 %, notably in freshly harvested (non-stored) cherries. Accordingly, a 3-folds increase of OTA concentration (5.5 – 6.0 µg/kg) was found in four days-stored cherries coinciding with an increase of fungal contamination up to 51 %. This strongly supported that the increase of OTA production in stored cherries should probably be due to the increase in fungal contamination particularly black Aspergillii and Penicillia.

However, the other Aspergilli such as ochre Aspergillii that remained consistent in non-stored and stored cherries might be less involved in OTA production. Hence, it appeared that, black Aspergillii and Penicillia might be the most ochratoxinogen fungal strains isolated in this study. These species were widely reported to be involved in the OTA production during post-harvest processing of coffee (Gueule et al., 2007; Moulia et al., 2014).

Ochratoxin production in coffee cherries depends on several factors that impact the growth of fungi and in turn the synthesis of the toxin (Gueule et al., 2017; Wang et al., 2016). This study indicated that the concentration of OTA produced in cherries between 1 and 10 µg/kg was very low comparatively to that reported from Robusta cherries in Philippines, 46.7 µg/kg at the onset of the storage and 120.2 µg/kg of toxin during drying (Barcelo and Barcelo, 2018). On the other hand, lower or similar level of OTA contents (0.1 – 5 µg/kg) were also found in Arabica coffee cherries from Brazil (Batista et al., 2009). This suggests that the post-harvest process used by farmers in Côte d'Ivoire, although using mainly the stripping method, may be less subjected to OTA contamination.

In conclusion, this study showed that storing the coffee cherries before drying enhanced significantly the occurrence of OTA producing strains during the post-harvest processing. The main fungal strains involved in OTA production during this process should be probably black Aspergillii.

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